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Kinetic modelling of naringin hydrolysis using a bitter sweet alfa-rhamnopyranosidase immobilized in k-carrageenan

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Abstract

Naringin, the principal bitter flavonone glycoside and the primary bitter component in grapefruit juice, can be hydrolysed by naringinase (with expressed activity in alfa-L-rhamnosidase and beta-D-glucosidase) into rhamnose and prunin (one-third bitterness ratio to naringin), which can be further hydrolysed into glucose and tasteless naringenin. The main goal of this work was the modelling and optimisation of the enzymatic activity of naringinase entrapped in k-carrageenan beads, in grapefruit juice. In order to attain these goals naringinase activity, naringin conversion and naringenin formation were evaluated. In this study response surface methodology was used for modelling the naringin hydrolysis by naringinase immobilized in k-carrageenan beads as a function of temperature (9–51 °C) and naringinase concentration (0.29–1.7 g L^{-1}) in grapefruit juice. Experiments were carried out following a central composite rotatable design. Naringinase activity could be described by response surfaces that enabled to fit second-order polynomials to experimental data points. The high values of R^2 (0.996) and R^2_{adj} (0.992) of the models show a close agreement between the experimental results and the theoretical values predicted by the models. The higher naringin conversion (95%) was attained with naringinase concentrations higher than 800 mg L^{-1} and temperatures higher than 30 °C. This is a promising result for a future application of enzymatic hydrolysis of naringin immobilized in k-carrageenan, in grapefruit juice industry, maintaining juice properties.

Keywords: Naringin; Hydrolysis; RSM; Naringinase; k-Carrageenan; Grapefruit juice

1. Introduction

Natural products constitute a relevant economic resource for the pharmaceutical, cosmetic and food industries as they are an unsurpassed source of bioactive compounds, useful to improve the well-being and the quality of human life.

The flavonoids from citrus, naringin and naringenin, functional chemicals, have been shown to have anti-oxidant, anti-inflammatory properties, anti-thrombotic and vasodilator, among others. Naringin, the principal bitter flavonone glycoside and the primary bitter component in grapefruit juice, can be hydrolysed by naringinase (with expressed activity in alfa-L-rhamnosidase and beta-D-glucosidase) into rhamnose and prunin (one-third bitterness ratio to naringin), which can be further hydrolysed into glucose and tasteless naringenin (Fig. 1). This product of the enzymatic hydrolysis of naringin may be potentially useful as pharmacological agent, as anticancer, in the treatment or prevention of atherosclerosis, with a number of antiatherogenic activities, such as antithrombotic and vasodilator [1].

This hydrolytic mechanism can considerably reduce the bitterness in citrus juice. Then, the hydrolysis of naringin is still an interesting application. The reduction in bitterness, as a result of the enzymatic process, controlling the quality and improving commercial value of grapefruit and other citrus juices, as the maintenance of health properties, increases the acceptance by the consumer.

Immobilization of biocatalysts has many advantages in large-scale processing. The use of a cheap and simple effective immobilisation method can provide a key asset, if an experimental set-up amenable for scale-up is envisaged.

Entrapment is one of the simplest methods of immobilization and consists of the inclusion of enzymes or cells within polymeric matrices [2]. Despite the problems associated with entrapment within polymers, the method has been extensively used for a wide variety of processes. There are a lot of polymers

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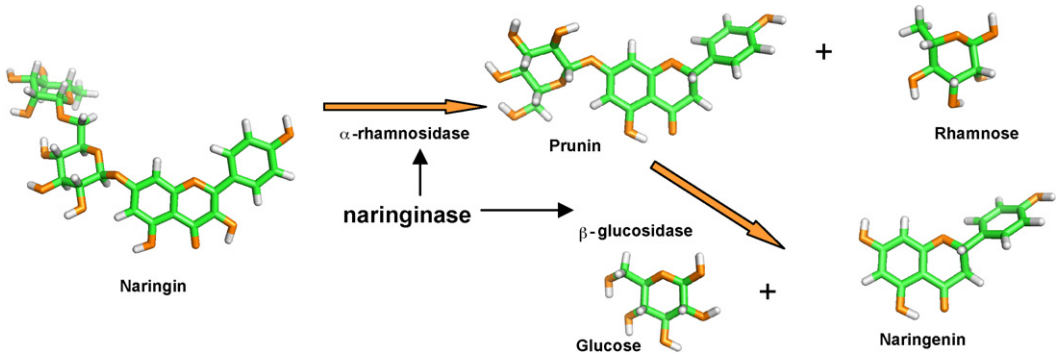

Fig. 1. Hydrolysis of naringin into prunin, rhamnose, naringenin and glucose by naringinase containing α-L-rhamnosidase and β-D-glucosidase activities.

available for enzyme entrapment. Alginate, a naturally occurring polysaccharide that forms gels by ionotropic gelation, is one of the most popular supports. k-Carrageenan another naturally occurring polysaccharide forms gel with K^+ ions, and in some cases, may increase enzyme stability [2].

In addition, the response surface methodology (RSM) was followed as an attempt to model and optimize the naringin hydroysis catalyzed by naringinase, as a function of both the amount of immobilized commercial naringinase and the temperature used.

Response surface methodology (RSM) consists on 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 [3]. With RSM, several variables can be tested simultaneously with a minimum number of trials, according to special experimental designs, which enables to find interactions between variables. This is a non-conventional approach that has proved its utility, among others, in the optimization conditions of fermentation [4,5], food preservation parameters [6], in product recovery and in the field on optimization of enzymatic reactions conditions, as well as in enzyme immobilization techniques. In fact, RSM has been used successfully for modelling the effects and interactions of several factors on the enzyme kinetics and immobilization [7,8]. In this work, central composite rotatable design (CCRD) and RSM were used to compare the effects of naringinase concentration and temperature, on the naringinase activity, naringin conversion and naringenin formation.

The biocatalyst deactivation depends on different factors and is commonly studied for reactor designs to maximize the operational conditions in order to ensure the stability of immobilized enzymes [2].

The catalytic durability of the biocatalyst during continuous operation is called operational stability and is commonly estimated numerically by its apparent half-life, which is the elapsed time at which the catalytic activity is reduced to half value. This parameter is very important for the economic feasibility of the bioprocess concerned and requires an exhaustive analysis.

The main objective of this work was the modelling and optimisation of the activity of naringinase entrapped in k-carrageenan beads, and naringin conversion in grapefruit juice.

2. Materials and methods

2.1. Materials and equipment

Naringin (naringenin-7-rhamnosidoglucosidose) 96.6%; naringenin (4′,5,7-trihydroxyflavanone) 99%; naringinase (CAS number 9068-31-9) were from Sigma (Spain). This commercial naringinase was from *Penicillium decumbens*.

Acetonitrile HPLC grade; sodium acetate trihydrate; glacial acetic acid; absolute ethanol were from Merck (Germany).

High-performance liquid chromatography (HPLC), Waters 2690 Separation Module, a Photo Diodes Array (PDA) detector (Model Waters 996) and the results processed by Millennium®32, Waters software. Separations were performed on a Merck analytical column, Lichrospher® 100, RP18 (5 mm particle size, 250 mm × 4 mm).

2.2. Analytical methods

The analyses of naringin and naringenin were performed using a high-performance liquid chromatographic system (HPLC). The mobile phase consisted on acetonitrile (A)/water (B) and each solvent was filtered through a 0.2 μm pore size hydrophilic polypropylene filter and degassed in an ultrasonic bath before use. Separation was performed using a gradient programme: 0–8 min 23% A; 8–15 min 23–65% A linear; 15–20 min 65–70% A linear; 20–21 min 70–23% A linear; 21–22 min 23% A. The analyses were performed at 25 °C (column oven temperature), with a 1 mL min^{-1} flow rate, the wavelength selected was 280 nm and the injection volume was 20 mL.

Reducing sugars were quantified by the 2,4-dinitrosalicylic acid (DNS) method [9]. Standardization was obtained with different concentrations of an equimolar mixture of D-glucose and D-rhamnose.

Protein (naringinase) concentrations were measured with the Bradford method.

2.3. Naringinase immobilization

k-Carrageenan and alginic acid sodium from brown algae were obtained from Fluka.

Entrapment of naringinase in k-carrageenan beads was carried out as follow, a certain volume of the naringinase solution, in 0.02 M acetate buffer, pH 4.0, was added to a 4% k-carrageenan solution in order to have the desired concentration of naringinase and k-carrageenan. This suspension was prepared by a simple mixing step, then was added, through a 1 mm diameter needle, to a gently stirred, 3.5% potassium chloride solution, except if stated otherwise. The gelling was allowed to proceed for 30 min, at 4 °C. Beads were separated by filtration, rinsed with acetate buffer (0.02 M, pH 4.0) and used for bioconversion trials.

A solution containing 2.4% (w/v) sodium alginate, 0.3% (w/v) k-carrageenan and a certain amount of naringinase solution was mixed and added dropwise to into a solution of calcium chloride 2% (w/v) and potassium chloride 4% (w/v). The droplet forms gel spheres of approximately 3.3 ± 0.2 mm diameters, entrapping the enzyme in a three-dimensional lattice of ionically cross-linked alginate and carrageenan. The beads were maintained in these solutions for 30 min at 4 °C, after they were separated by filtration, rinsed with acetate buffer (0.02 M, pH 4.0) and used for bioconversion trials.

The quantitative of entrapped enzyme per gram of support was calculated by subtracting the protein quantity remained in the calcium chloride 2.0% (w/v) and potassium chloride 4.0% (w/v) solution after immobilization step from the initial protein quantity presented in the hydrocolloid solution before gelling.

Effects of immobilization conditions such as k-carrrageenan concentration, ratio by weight of enzyme to k-carrageenan and bead size on loading efficiency (percentage of total enzyme entrapped) and immobilization yield (specific activity ratio of entrapped naringinase to free naringinase) were investigated.

2.4. Naringin hydrolysis

Naringin bioconversion studies were carried out in standard solutions of naringin (acetate buffer 0.02 M, pH 4.0) and grapefruit juice with immobilized naringinase in k-carrageenan beads. Reaction started by adding a given amount of immobilized naringinase in k-carrageenan beads to the naringin solution, in 0.02 M acetate buffer pH 4.0, in a proportion (v/v) of 4 (reaction media) to 1 (immobilized enzyme). Bioconversion trials, with free and immobilized naringinase, were performed: (i) at different naringinase concentation (200–2000 mg L^{-1}); (ii) in a temperature range of 25–50 °C; (iii) in a naringin concentration range of 250–1000 mg L^{-1}.

The initial rate and other measurements were carried out in triplicate.

2.5. Kinetic parameters

Initial rates of naringin conversion (activity of naringinase) were calculated by linear regression on the first data-points during the initial 20 min reaction time (reducing sugars concentration versus time). A unit of enzyme was defined as the amount of enzyme which liberated 1 μmol of glucose in 1 min at 30 °C and pH 4.0.

The fit of Michaelis–Menten model [$v = ([S]V_{max})/([S] + K_M)$, where v is the initial rate (mg L^{-1} min^{-1}), [S] is the substrate concentration (mg L^{-1}), V_{max} is the maximum initial rate (mg L^{-1} min^{-1}) and K_M is the Michaelis–Menten constant (mg L^{-1})] to experimental data was carried out, by minimizing the residual sum of squares between the experimental data points and the estimated values by the model, using a non-linear curve-fit program in GraphPad, from GraphPad Software Inc., USA. Results were based on triplicate determinations.

2.6. Experimental design

With RSM, several variables were tested simultaneously with a minimum number of trials according to special experimental designs based on factorial designs [10]. This methodology has the advantage of being less expensive and time-consuming than the classical methods.

The best conditions for the bioconversion of naringin in grapefruit juice, with immobilized naringinase in k-carrageenan beads, 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-modeled 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 center point (repeated several times) is frequently added to the design. 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.

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 following a central composite rotatable design (CCRD) [10] as a function of temperature and naringinase concentration (Table 1). With central composite rotatable design, five levels for each factor were used which enabled to fit 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 [11]. 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.

A total of 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 1).

Table 1
Experimental data obtained for the optimization of temperature and naringinase concentration, on naringin hydrolysis

Coded levels	[Naringinase] ($mg\,L^{-1}$)	Temperature (°C)	[Naringenin] ($mg\,L^{-1}$)	Activity ($mg\,L^{-1}\,min$)
(0, 0)	1000	30	72.4	12.67
(0, 0)	1000	30	63.96	19.49
(0, 0)	1000	30	66.36	15.82
$(0, \sqrt{2})$	1000	51	215.08	19.52
$(\sqrt{2}, 0)$	1707	30	124.8	12.53
$(-\sqrt{2}, 0)$	293	30	23.47	3.87
$(0, -\sqrt{2})$	1000	9	11.85	5.42
(1, 1)	1500	45	271.29	24.28
(−1, 1)	500	45	91.52	8.624
(1, −1)	1500	15	16.40	9.88
(−1, −1)	500	15	15.23	4.12

In this study response surface methodology was used for modelling the naringin hydrolysis by naringinase immobilized in k-carrageenan beads as a function of temperature (9–51 °C) and naringinase concentration (0.29–1.7 $g\,L^{-1}$) in grapefruit juice. Experiments were carried out following a central composite rotatable design (Table 1).

2.7. 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 naringenin formation after 1 h reaction and naringin conversion 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})

2.8. Repeated use of naringinase in k-carrageenan beads

In order to test the stability of naringinase entrapped in the k-carrageenan beads, they were used several times for the hydrolytic reaction, in acetate buffer 0.02 M, pH 4.0, with two different naringin concentrations, 250 and 500 $mg\,L^{-1}$, and in grapefruit juice. Each run lasted 24 h after which the beads were separated and washed with acetate buffer pH 4.0. The reaction medium was replaced by fresh medium. The activity of freshly prepared beads in the first run was defined as 100%.

3. Results and discussion

In order to choose the best immobilization matrix, k-carrageenan plus Ca-alginate and k-carrageenan alone, were tested in naringin hydrolysis in acetate buffer solution, pH 4.0.

When naringinase was immobilized by entrapment, in k-carrageenan, 0.3% (w/v) plus Ca-alginate beads 2.4% (w/v) a higher reducing sugars formation (700 $mg\,L^{-1}$) was obtained

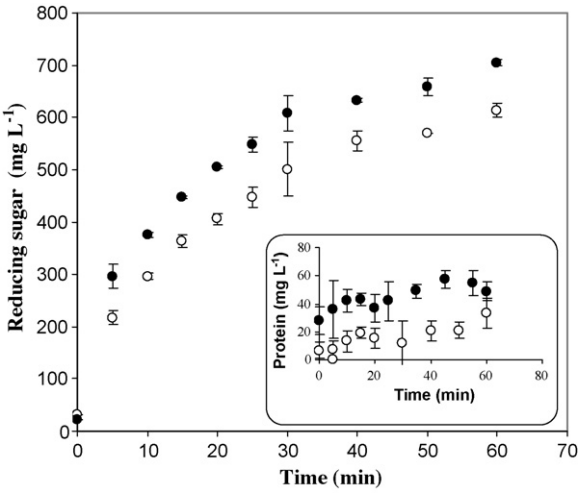

Fig. 2. Reducing sugars formation from naringin with immobilized naringinase in k-carrageenan [2% (w/v)] (◯) and k-carrageenan [0.3% (w/v)] plus calcium alginate [2.4% (w/v)] (●) in model solution of acetate buffer 0.02 M, pH 4.0, 30 °C and 200 rpm, the protein in the reaction media are presented in the small graphic.

compared to the formation (600 $mg\,L^{-1}$) with naringinase immobilized in k-carrageenan beads (2%), but also the protein lost to reaction medium was higher (Fig. 2). In the first case, 40% of protein was lost in the first 10 min reaction time, while with k-carrageenan only 10% was in the reaction media, during the same time.

The loading efficiency (%) was evaluated from the expression $(C_iV_i - C_fV_f)/C_iV_i \times 100$, where C_i is the initial protein concentration, V_i is the initial volume of enzyme solution, C_f is the protein concentration in the total filtrate and V_f is the total volume of filtrate. The amount of protein in the beads was determined from the loading efficiency. For nariniginase immobilized in k-carrageenan plus calcium alginate a loading efficiency of 80% was attained.

The immobilization yield (%) was defined as follows: specific activity of immobilized enzyme (mg/(min $mg_{protein}$))/specific activity of free enzyme (mg/(min $mg_{protein}$)) × 100.

The best results, for naringin hydrolysis were obtained with naringinase immobilized in k-carrageenan beads, 2% (w/v), in a proportion of 4 (reaction media) to 1 (support with the biocatalyst) (v/v), with a loading efficiency of 90% and an immobilization yield of 80%. k-Carrageenan was the matrix used in future experiments.

In order to test the biocompatibility of k-carrageenan beads with the bioconversion media, they were kept in model solution and grapefruit juice, during 1 week in refrigerator (4 °C). No modifications were observed.

The effect of k-carrageenan concentration on naringinase activity was investigated. k-Carrageenan concentration increased from 1 to 4% (w/v), maintaining the ratio of enzyme to carrageenan. The higher naringinase activity was obtained with k-carrageenan 2% (w/v) when naringin was used as substrate (500 $mg\,L^{-1}$), in acetate buffer pH 4.0, 30 °C (Fig. 3). Probably due to conformational changes in the entrapped enzyme and/or limitation of substrate transfer from the bulk phase into the k-

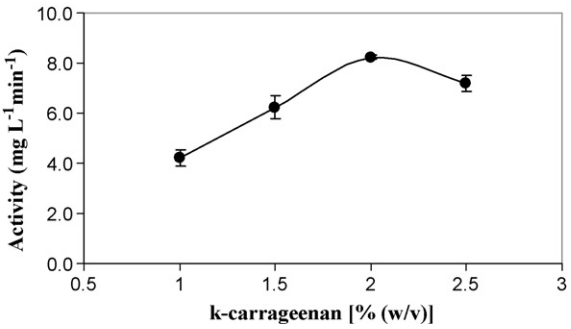

Fig. 3. Effect of k-carrageenan concentration on naringinase activity, in acetate buffer 0.02 M, pH 4.0, 30 °C and 200 rpm.

carrageenan beads as carrageenan concentration increased from 2% (w/v) a decrease in naringinase activity is observed.

As with most heterogeneous catalysts, immobilized enzymes generally present mass transfer limitations. The size of beads in which naringinase is entrapped may be one of the most important parameters of naringinase immobilization. It is expected that enzymes in smaller beads will show higher catalytic activity due to reduced substrate transfer resistance. In this study k-carrageenan beads of four different sizes, where made with a needle through which a mixture of naringinase and k-carrageenan (2%) was dropped into KCl solution (3.5%). The average bead size was 2.5, 3.0, 3.3, 4.0 mm. Changing the bead size from 2.5 to 3.3 mm, almost did not affect naringinase activity (data not shown). The beads diameter of 3.0 mm was chose for the next experiments.

In order to study the effect of external mass transfer limitations the effect of agitation rate in naringinase activity was evaluated. The experiments were conducted in acetate buffer pH 4.0, 30 °C, with naringin, 500 mg L^{-1}, and naringinase (1000 mg L^{-1}) immobilized in k-carrageenan beads 2% (w/v), 3 mm. The agitation speed varied from 100 to 300 rpm, according to Fig. 4. The naringinase activity (12 mg L^{-1} min^{-1}) was almost constant from 200 rpm. This was the agitation rate used in further experiments.

The partition coefficient of naringin (P) between bulk media and k-carrageenan was evaluated, according to the following expression:

$$P = \frac{C_0 - C_{\text{macro}}}{C_{\text{macro}}} \times \frac{V_0}{V - V_0}$$

where C_0 is the initial naringin concentration in reaction media, C_{macro} is the final concentration in the bulk macroenvironment after equilibrium, V_0 is the volume of reaction media and V is the final volume of the system (reaction media plus immobilization matrix).

The estimated partition coefficient with different naringin initial concentrations (75, 282, 394, 459, 576 mg L^{-1}) changed from 0.67 to 0.76 and allowed the estimation of naringin concentration in the microenvironment, according to: $P = (C_{\text{micro}}/C_{\text{macro}})$.

Partition coefficients higher than the unit indicate a favoured migration of substrates to the microenvironment of the biocatalysts. To each concentration tested the partition coefficients where lower than the unit probably due to the hydrophilic characteristics of the support, k-carrageenan and the hydrophobic of the substrate, naringin. However these characteristics can prevent eventual inhibition problems of substrates and/or products.

Characterization of the immobilized system (naringinase in k-carrageenan beads 2%, 3 mm) as related to temperature, substrate concentration, enzyme concentration in model systems and modelling of naringin hydrolysis and biocatalyst reutilization in grapefruit juice were performed.

The influence of temperature in the bioconversion under study was performed, in a range of 25–50 °C. A broader shaped curve was obtained for temperature-activity profile from 30 to 40 °C of the immobilized system, as compared to the free systems. Similar profiles were obtained by Vila-Real et al. [12]. Higher activity (12 mg L^{-1} min^{-1}) was obtained at 30–40 °C, with immobilized naringinase (data not showed). The temperature used in the future experiments was 30 °C.

The influence of naringin concentration was evaluated and the kinetic parameters were estimated for immobilized naringinase in k-carrageenan beads 2% (3 mm) at pH 4 and 30 °C. Michaelis–Menten model fitted by non-linear regression to the experimental data points, yielded a curve that well describes the experimental data over the range of naringin concentration

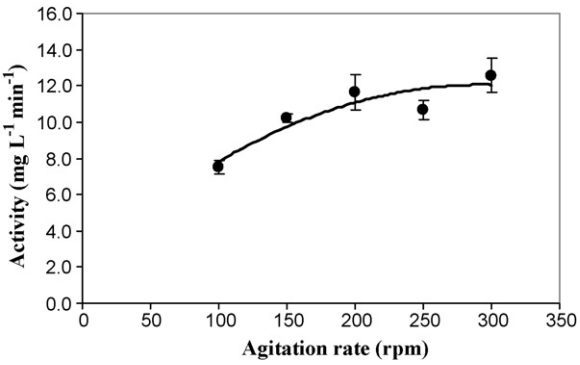

Fig. 4. Effect of agitation rate on naringinase activity, with k-carrageenan beads 2%, 3 mm, in acetate buffer 0.02 M, pH 4.0, 30 °C and 200 rpm.

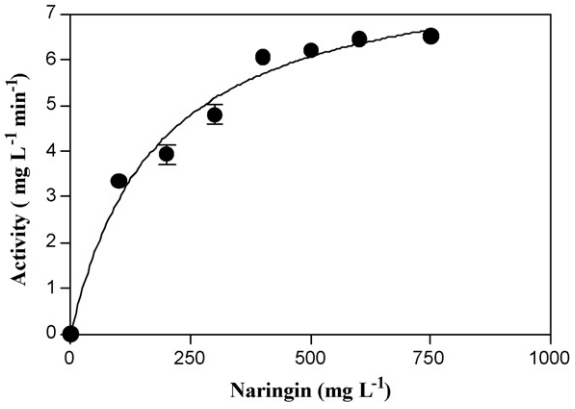

Fig. 5. Effect of naringin concentration on naringinase activity, with k-carrageenan beads 2%, 3 mm, in acetate buffer 0.02 M, pH 4.0, 30 °C and 200 rpm.

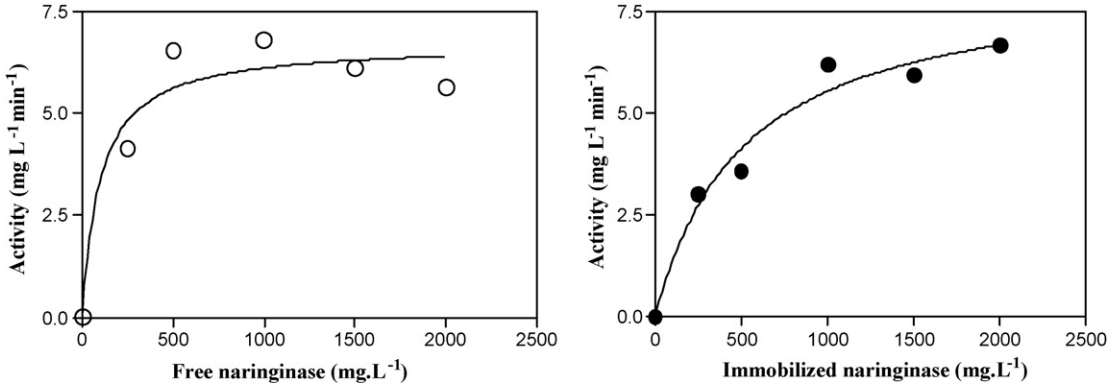

Fig. 6. Effect of naringinase concentration (open circles, free naringinase; full circles, immobilized naringinase in k-carrageenan beads, 2%, 3 mm) on naringinase activity, in acetate buffer 0.02 M, pH 4.0, 30 °C and 200 rpm.

Table 2
Naringin hydrolysis in grapefruit juice with naringinase immobilized in k-carrageenan (2%) beads (3 mm), 30 °C

Time (min)	Naringin		Naringenin
	Concentration (mg L^{-1})	Residual (%)	Concentration (mg L^{-1})
0	900.0	100	–
15	692.5	77	–
60	242.8	50	30.3
120	165.7	29	68.3

(Fig. 5) and showed to be accurate in the estimation of the kinetic parameters. A K_M^{ap} value of 180 mg L^{-1} (0.031 mmol L^{-1}), a V_{max} of 8.3 mg L^{-1} min^{-1} (0.046 mmol L^{-1} min^{-1}) and a k_{cat} of 0.0083 min^{-1} were obtained with immobilized naringinase in k-carrageenan beads, at 30 °C and pH 4.0.

In naringin hydrolysis with naringinase, from *Penicillium* sp., a K_M of 2 mmol L^{-1} was refereed, when the enzyme was used immobilized by covalent binding to wood material [13], while Puri et al. [14] cited a value of 10 mmol L^{-1} for the K_M of naringinase (from *Penicillium* sp.) immobilized in calcium alginate beads. Pedro et al. [15] obtained a K_M of 0.303 mmol L^{-1} and V_{max} of 0.0418 mmol L^{-1} min^{-1} with naringinase immobilized in calcium alginate beads in acetate buffer, pH 4 and 30 °C.

In the experimental conditions used ([naringin] = 500 mg L^{-1} in acetate buffer 0.02 M, pH 4, 30 °C), naringin hydrolysis clearly, depends on the enzyme concentration (Fig. 6).

The experimental relation between initial hydrolysis rate and enzyme concentration, in solution and immobilized in k-carrageenan (2%, 3 mm), is shown in Fig. 6. This rate increases

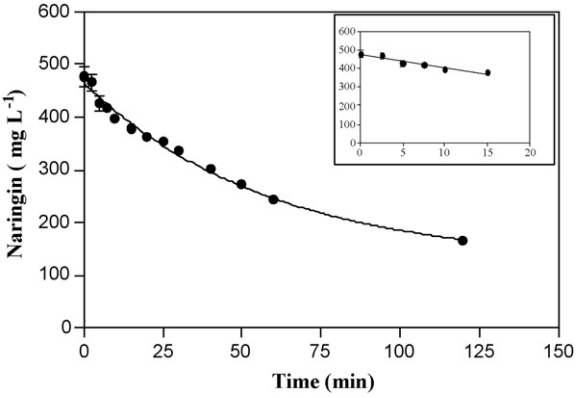

Fig. 7. Naringin hydrolysis in grapefruit juice, at 30 °C, 200 rpm, with naringinase immobilized in k-carrageenan beads, 2%, 3 mm.

with increasing the initial naringinase concentration. However, it is not a linear relation and decreases as the enzyme concentration increases due to saturation of the substrate. For high values of naringinase concentration, the amount of naringin becomes limiting and as it was kept to a constant value, the rate asymptotically approaches to a maximum (immobilized enzyme) and slow decrease when the enzyme was used free in solution (Fig. 6).

Kinetics parameters were determined using a alternative approach of Henri–Michaelis–Menten equation, which suggests that the initial hydrolysis velocity, v_0, is expressed as a function of the enzyme concentration $[E_0]$ [16]. Accordingly, it is convenient to define a maximal velocity, V_{emax}, and a corresponding half-saturation constant, K_e, as the following equation:

$$v_0 = \frac{V_{e\,max} \times [E_0]}{K_e + [E_0]}$$

Table 3
Effects of the tested variables and respective significance levels (*), on the activity of naringinase, on naringin conversion and naringenin formation

Variable	Activity (mg mL^{-1} min^{-1})	Conversion (%)	[Naringenin] (mg mL^{-1})
[Naringinase] (linear term)	8.41^{**}	23.49^{***}	81.09^{***}
[Naringinase] (quadratic term)	-7.08^{*}	-6.12^{*}	8.73
Temperature (linear term)	9.76^{**}	45.05^{***}	155.05^{***}
Temperature (quadratic term)	−2.85	-8.68^{**}	49.08^{**}
[Naringinase] × temperature	4.95	−0.54	89.25^{***}

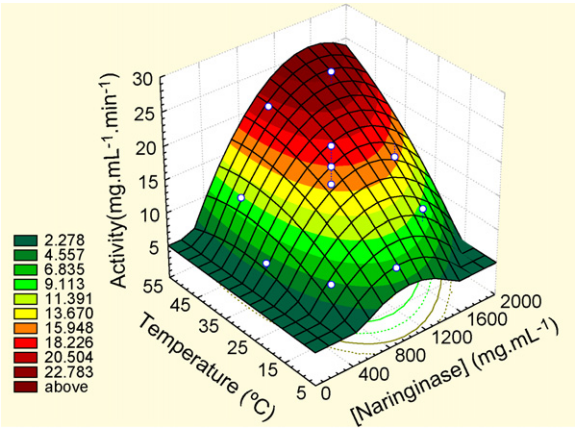

Fig. 8. Response surface, fitted to the experimental data points, corresponding to naringinase activity (mg L^{-1} min^{-1}), as a function of temperature (°C) and naringinase concentration (mg L^{-1}).

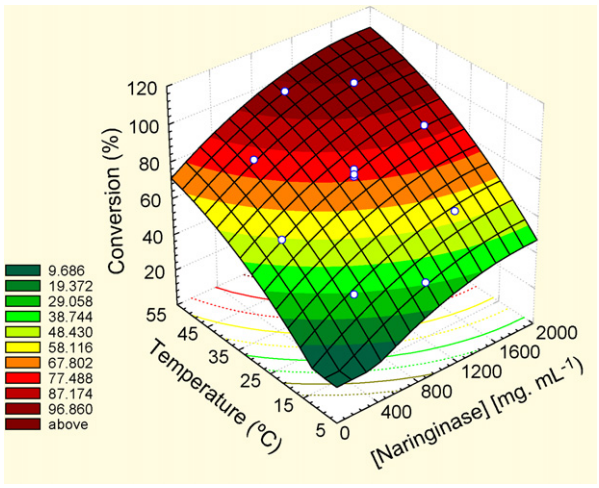

Fig. 9. Response surface, fitted to the experimental data points, corresponding to naringin conversion (%), as a function of temperature (°C) and naringinase concentration (mg L^{-1}).

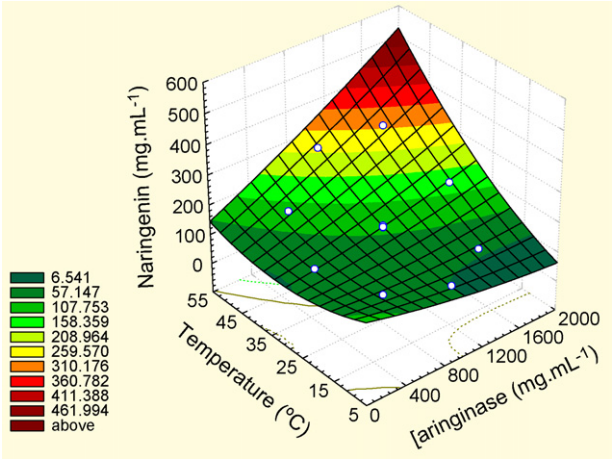

Fig. 10. Response surface, fitted to the experimental data points, corresponding to naringenin formation, as a function of temperature (°C) and naringinase concentration (mg L^{-1}).

A maximum rate, $V_{e\,max}^{ap} = 8.3$ mg L^{-1} min^{-1} and a half saturation constant, $K_e^{ap} = 510$ mg L^{-1} resulted with immobilized naringinase, while with free enzyme a $V_{emax} = 6.7$ mg L^{-1} min^{-1} and $K_e = 98$ mg L^{-1} was obtained. Using these parameters values, the initial rate of hydrolysis was evaluated from the model and compared to the experimental results (dotted curves in Fig. 7). The model fits with the experimental results, with determination coefficients of 0.99.

In subsequent work the biocatalyst concentration was 1000 mg of naringinase per liter of aqueous reaction media.

Naringin hydrolysis was followed in grapefruit juice with immobilized naringinase in k-carrageenan, in a concentration of 1 g $L_{(juice)}^{-1}$, 30 °C (Fig. 6). After 60 min a reduction of 50% in naringin concentration was observed with a formation of 30 mg L^{-1} of naringenin (Table 2).

The decrease in naringin content can be directly correlated with reduction in bitterness. From the amount of residual naringin present, the percentage reduction in bitterness was evaluated. A 70% reduction in naringin concentration in grapefruit juice was observed after 120 min reaction time, with the formation of 68.3 mg L^{-1} of naringenin. A naringin concentration of 243 mg L^{-1} was obtained after 60 min enzymatic reaction time (Fig. 7 and Table 2) which means, according to Soares and Hotchkiss [17], that bitterness in grapefruit juice is not detectable. Some bitterness in grapefruit juice is acceptable to consumers, as it contributes to the characteristic taste and flavour.

Bioconversion of naringin in grapefruit juice by naringinase was carried out for 2 h, according to CCRD as a function of both temperature (T) and naringinase concentration (N). The significant effects of the temperature, naringinase concentration and interaction ($T \times N$) on the activity of naringinase, naringin conversion and naringenin formation are shown in Table 3. Naringinase concentration and temperature showed a significant linear (and/or quadratic) effect on the activity, naringin conversion and naringenin formation.

The response surfaces fitted to the experimental data (Figs. 8–10) can be described by polynomial equations (Table 4).

Table 4
Polynomial model equations for the response surfaces fitted to the experimental data points, of the activity of naringinase, naringin conversion and naringenin formation, as a function of naringinase concentration (mg mL^{-1}) and temperature (°C) and respective R^2 and R_{adj}^2

Model equations	R^2	R_{adj}^2
Activity $= -12.13 - 1.42 \times 10^{-5}[N]^2 + 0.027[N] - 6.3 \times 10^{-3}T^2 + 0.37T + 3.30 \times 10^{-4}(N \times T)$	0.918	0.836
Conversion $= -29.36 - 1.2 \times 10^{-5}[N]^2 + 0.049[N] - 0.0193 \times T^2 + 2.71T - 3.6 \times 10^{-5}(N \times T)$	0.996	0.992
[Naringenin] $= 125.06 + 1.75 \times 10^{-5}[N]^2 - 0.13[N] + 0.11T^2 - 7.31T + 5.95 \times 10^{-3}(N \times T)$	0.994	0.987

N, [Naringinase]; T, temperature.

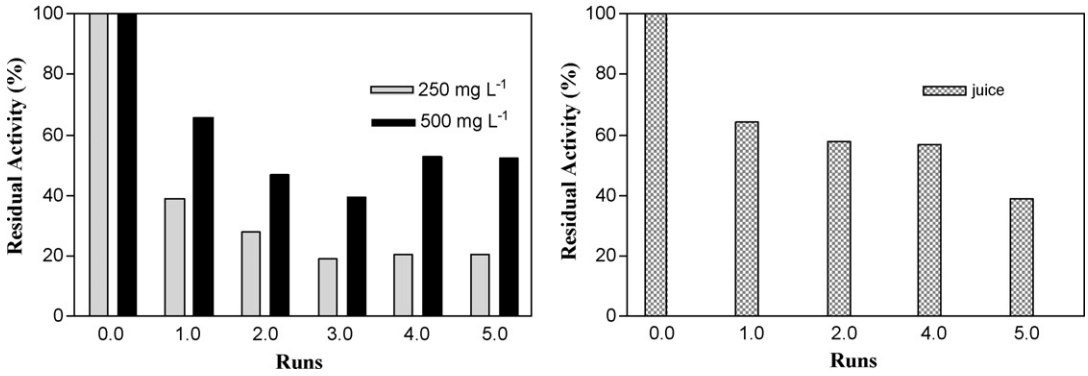

Fig. 11. Residual activity of naringinase immobilized in k-carrageenan beads 2%, 3 mm, used in different runs in model solutions ([naringin] = 250 and 500 mg L^{-1}, acetate buffer 0.02 M, pH 4.0) and in grapefruit juice, at 30 °C, 200 rpm.

The significant effects ($P < 0.05$) and those having a confidence range smaller than the value of the effect or smaller than the standard deviation were included in these model equations. In fact, these later effects have a lower probability, but their values are not small enough to be neglected. 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.

For the activity of naringinase and naringin conversion, convex surfaces (Figs. 8 and 9) were obtained. With respect to the naringin conversion, after 1 h reaction time, an increase with temperature was observed with lower naringinase concentration (Fig. 9). Also a negative interaction $T \times N$ indicates that the highest conversions (>90%) are achieved at high temperature and low naringinase concentration or vice versa. The higher naringenin formation was attained with high temperatures and naringinase concentration. The interaction $T \times [N]$ show a significant effect on naringenin formation.

The immobilization of microbial cells by entrapment in gel such as k-carrageenan, calcium alginate and chitosan is a well-known technique. However, entrapment of enzymes in these gels is not very efficient because the entrapped enzymes can leak out during the course of time as a result of the large pores of the matrix.

The possibilities of reutilization, the ease of separation, a higher stability, are among the advantages of using immobilized biocatalysts. The comparison between quantities of initial and residual active enzyme after each stage showed the influence of enzyme loss. The immobilized naringinase in k-carrageenan beads 2% was effectively reused in six consecutive experiments, although significant activity decay was observed in the first two runs (Fig. 11). An exponential decay model was adjusted to the obtained results, in model solutions ([naringin] = 250 and 500 mg L^{-1}, acetate buffer 0.02 M, pH 4.0) and in grapefruit juice, with determination coefficients of 0.99. The half-life for naringinase immobilized in k-carrageenan used in model solutions was, respectively, 0.50 and 0.53 days, with deactivation constants of 1.4 and 1.3 day^{-1}, respectively, for 250 and 500 mg L^{-1} of naringin. In grapefruit juice a half-life of 0.70 day with a deactivation constant 1.0 day^{-1} was attained.

After four reutilizations of naringinase in k-carrageenan beads (2%), a residual activity of, almost, 60% remains constant. Since no significant protein levels were detected in the bioconversion medium once the reaction was stopped, the decay could be due to loss of bound enzyme during the first run, mainly during washing procedures.

As a conclusion naringin bioconversion may be carried out directly in grapefruit juice, these preliminary results are very encouraging for future implementation on the industrial process.

4. Conclusions

Naringinase activity could be described by response surfaces that enabled to fit second-order polynomials to experimental data points.

The high values of R^2 (0.996) and R^2_{adj} (0.992) of the models show a close agreement between the experimental results and the theoretical values predicted by theses models.

The higher naringin conversion (95%) was attained with naringinase concentrations higher than 800 mg L^{-1} and temperatures higher than 30 °C.

After four reutilizations of naringinase in k-carrageenan beads (2%), a residual activity of, almost, 60% remains constant.

This is a promising result for a future application of enzymatic hydrolysis of naringin with naringinase immobilized in k-carrageenan, in grapefruit juice industry, maintaining juice properties.

References

[1] H. Nishino, M. Ngao, H. Fujili, T. Sugimura, Cancer Lett. 21 (1983) 1.
[2] W. Hartmeier, Immobilized Biocatalysts, An Introduction, Springer Verlag, Berlin, 1986.
[3] M. Giovanni, Food Technol. 37 (1983) 41.
[4] A. Kapat, S.K. Rakshit, T. Panda, Bioprocess. Eng. 15 (1996) 13.
[5] A. Montserrat, R. Inaki, O. François, G. Francesco, C. Carles, Biotechnol. Lett. 15 (1993) 559.
[6] V.A. King, Int. J. Food Sci. Technol. 28 (1993) 519.
[7] S. Ferreira-Dias, A.C. Correia, M.M.R. da Fonseca, J. Mol. Catal. B Enzym. 21 (2003) 71–80.
[8] M.H.L. Ribeiro, D. Silveira, S. Ferreira-Dias, J. Mol. Catal. B Enzym. 21 (2003) 81.

[9] G.L. Miller, Anal. Chem. 31 (1959) 426.
[10] L. Vuataz, in: J.R. Piggott (Ed.), Statistical Procedures in Food Research, Elsevier, London, 1986, p. 100.
[11] S. Weisberg, Applied Linear Regression, Wiley, New York, 1985, p. 217.
[12] H.J. Vila-Real, A.J. Alfaia, A.T. Calado, M.H.L. Ribeiro, Food Chem. 102 (2007) 565.
[13] M. Puri, H. Kaur, J.F. Kennedy, J. Chem. Technol. Biotech. 80 (2005) 1160.
[14] M. Puri, S.S. Marwaha, R.M. Kothari, Enzyme Microb. Technol. 18 (1996) 281.
[15] H.A. Pedro, A.J. Alfaia, J. Marques, H.J. Vila-Real, A.T. Calado, M.H.L. Ribeiro, Enzyme Microb. Technol. 40 (2007) 442.
[16] J. Baley, Biochem. J. 262 (1989) 1001.
[17] N.F.F. Soares, J.H. Hotchkiss, Packaging Technol. Sci. 11 (1998) 9.