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Response surface modelling of the consumption of bitter compounds from orange juice by *Acinetobacter calcoaceticus*

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Abstract

In certain varieties of oranges, an increase in bitterness is currently observed in juices, after extraction, restraining their industrial use. This has been explained by the conversion of the nonbitter precursor, limonoate A-ring lactone, to a bitter compound, limonin, under acidic conditions.

The aim of this study was the modelling of limonin consumption in raw and sterilized orange juices by *Acinetobacter calcoaceticus* isolated from soil. Response Surface Methodology (RSM) was used for modelling bioconversion and optimization reaction conditions, as a function of temperature (23–37 °C) and limonin content (8–16 ppm).

Initial rate of limonin consumption could be described, both in raw and sterilized orange juices, by concave surfaces with a minimum at 26 and 27 °C, respectively.

In raw orange juice, after 7 h reaction time, the amount of converted limonin, increased with temperature. Also, the highest conversions (higher than 33%) were achieved at high temperature (higher than 34 °C) and low initial limonin content. In sterilized juice, a maximum conversion of about 23% is expected at 31 °C, for an initial limonin content of 11 mg l^{-1}. Thus, limonin bioconversion may be carried out directly in raw juice, avoiding juice sterilization. In addition, no significant decrease in reducing sugars was observed.

Keywords: *Acinetobacter calcoaceticus*; Limonin; Orange juice; Response Surface Methodology

1. Introduction

In several citrus juices, bitterness is mainly ascribed to the presence of limonoids namely limonin. In certain varieties of oranges, lemon and grapefruit, an increase in bitterness is observed in juices after extraction ("delayed bitterness"), which has restrained the industrial use of those varieties. Delayed bitterness is caused primarily by limonin. The observed phenomenon has been explained by the conversion of the nonbitter precursor, limonoate A-ring lactone, to limonin, catalysed by limonin D-ring lactone hydrolase, under acidic conditions [1].

Several approaches have been used to reduce the concentration of bitter limonoids in citrus juice, such as (i) adsorption chromatography, (ii) addition of bitterness suppressing agents to juice, (iii) postharvest treatment of fruit with ethylene prior to processing or (iv) biological degradation in a microbial bioreactor [2]. All of these methods for debittering juice have

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Nomenclature

CCRD	central composite rotatable design
L	initial limonin concentration
RSM	Response Surface Methodology
R^2	determination coefficient (quadratic correlation coefficient)
R^2_{adj}	adjusted R^2
T	temperature

several drawbacks since flavour and other components are modified, decreasing the quality of citrus juice. With respect to the debittering process by biological route, the major difficulties are related to the low pH values (3.5–5.0) of citrus juices. In addition there is a risk of oxidation reactions with off-flavours development, under aerobic conditions.

Debittering orange juice by biological processes has been reported by several authors. Hasegawa et al. [3] observed that 81% of limonin was bioconverted from navel orange juice serum with *Corynebacterium fascians* cells immobilized in acrylamide gel, after, after a 24 h reaction time, in a packed bed column. When *Arthrobacter globiformis* cells were used under the same conditions, only a limonin reduction of 73% in navel orange juice was attained [4]. In addition, the enzyme responsible for the bioconversion was unstable at low pH. Also, the use of acrylamide gel as immobilization support is not adequate for food purposes. Cánovas et al. [5] using synthetic juice at pH 4, obtained a limonin conversion of about 70% by *Rhodococcus fascians*, in a batch reactor, after 150 h reaction. When these cells were immobilized in polyurethane foams, 85% limonin conversion was attained, after a 200 h reaction, in a continuous reactor [5].

The aim of this study was the modelling of the consumption of limonin in fresh orange juices by *Acinetobacter calcoaceticus* isolated from soil. In fact, limonoid metabolizing soil bacteria are readily available source of enzymes involved in limonin metabolism. *Acinetobacter* has enzymes for limonin degradation via deoxylimonoid pathway [1]. Response Surface Methodology (RSM) was used for modelling bioconversion and optimization reaction conditions, as a function of temperature (23–37 °C) and limonin content (8–16 ppm) at pH 3.5 and under orbital shaking (200 rpm).

The RSM has been recently used on modelling and optimization of several bioprocesses such as fermentation [6,7], enzymatic reactions [8–12] and product recovery [13], as well as in enzyme immobilization techniques [14].

2. Materials and methods

2.1. Materials

Oranges from Navel variety were harvested in an orange orchard on the South of Portugal, and stored at (−18 °C) until use. *Acinetobacter calcoaceticus anitratus*, isolated from the soil, was from the collection of the Department of Microbiology of the Faculty of Pharmacy of Lisbon. All chemicals were of analytical grade and obtained from various sources.

2.2. Methods

2.2.1. Microbial growth

Acinetobacter calcoaceticus anitratus were grown overnight in Luria Broth medium at 35 °C, in an orbital shaker at 200 rpm, under aerobic conditions. Afterwards, the medium was centrifuged and the recovered cells used as biocatalyst in bioconversion experiments.

2.2.2. Bioconversion studies

After extraction, orange juice was immediately centrifuged and used raw or after sterilization at 121 °C, for 15 min, in bioconversion experiments. The biocatalyst was added to 10 ml of raw or sterilized juice. The experiments were carried out at about pH 3.5 (original pH of the juice) and under orbital shaking (200 rpm) for 24 h.

For both raw and sterilized juices, experiments were carried out as a function of the incubation temperature and of the initial limonin content, according to the experimental design followed (cf. 2.2.4). Different limonin contents were obtained by diluting the original juice. Samples were withdrawn along the bioconversion experiment and residual content of limonin and sugars analyzed. Initial rates of limonin consumption were calculated by linear regression on the first three data points (limonin concentration versus time).

2.2.3. Analytical methods

Biomass was assayed by spectrophotometry at 600 nm. The limonin content in juice was assayed by HPLC at 210 nm in a LC-6 Shimadzu equipped with a RP-C18 column (Merck), a SPD-6A Shimadzu UV spectrophotometric detector and an isocratic mobile phase (45% acetonitrile and 55% water) at 1.0 ml min^{-1} flow rate. Total content of sugars were assayed by DNS method [15].

2.2.4. Experimental design

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 [16,17]. With RSM, several variables are tested simultaneously with a minimum number of trials, according to special experimental designs, which enables to find interactions between variables [16,17]. This is not an option with classical approaches. In addition, RSM has the advantage of being less expensive and time-consuming than the classical methods.

The best conditions for the bioconversion of limonin in crude or sterilized orange juice were established via RSM. In this study, two sets of experiments (for crude or sterilized orange juice, respectively) were carried out following a central composite rotatable design (CCRD), as a function of limonin concentration and temperature. With central composite rotatable designs, five levels for each factor are used which enables to fit 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. the stationary points [16]. However, the identification, for each variable, of the regions corresponding to optimal responses, may be directly achieved by visual examination of the response surfaces and/or contour plots.

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

Coded levels	Temperature (°C)	[Limonin] (mg l^{-1})
(−1)	25	9.2
(+1)	35	14.8
$(-\sqrt{2})$	23	8
$(+\sqrt{2})$	37	16
0	30	12

A total of 11 experiments were carried out in each CCRD: four factorial points (coded levels as (+1) and (−1)); four star points (coded as $(+\sqrt{2})$ and $(-\sqrt{2})$) and three center points (coded as 0). The levels considered in both CCRD are in Table 1.

2.2.5. Statistical analysis

The results of each CCRD were analyzed using the software "Statistica™", version 5, from Statsoft, USA. Both linear and quadratic effects of each of the variables (factors) under study, as well as their interactions, on limonin bioconversion and initial

Table 2
Effects (l: linear; q: quadratic) and respective significance levels (P) of the tested variables (temperature, T; initial limonin concentration, L) and interaction ($T \times L$) on limonin conversion and initial rate of consumption during bioconversion of limonin of raw and sterilized orange juice by *Acinetobacter calcoaceticus anitratus*

Variable	Raw juice		Sterilized juice	
	Initial rate of consumption (mg l^{-1} h^{-1})	Limonin conversion (%)	Initial rate of consumption (mg l^{-1} h^{-1})	Limonin conversion (%)
Temperature (l)	0.42**	13.01*	0.23*	3.96
Temperature (q)	0.30*	−2.30	0.33*	−13.13*
Limonin (l)	−0.12	−3.07	0.034	−3.43
Limonin (q)	0.06	−4.40	−0.01	−6.43
$T \times L$	0.22	−9.90	0.006	0.75

(a) Not significant effect, $P > 0.05$.
* $P \leq 0.05$.
** $P \leq 0.01$.

rate of limonin bioconversion were calculated. Their significance was evaluated by analysis of variance. A surface, described by a first or 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.

3. Results and discussion

Bioconversion of limonin in raw and sterilized orange juice, by *Acinetobacter calcoaceticus*, were carried out for 7 h, according to CCRD as a function of both the temperature (T) and initial limonin (L) content.

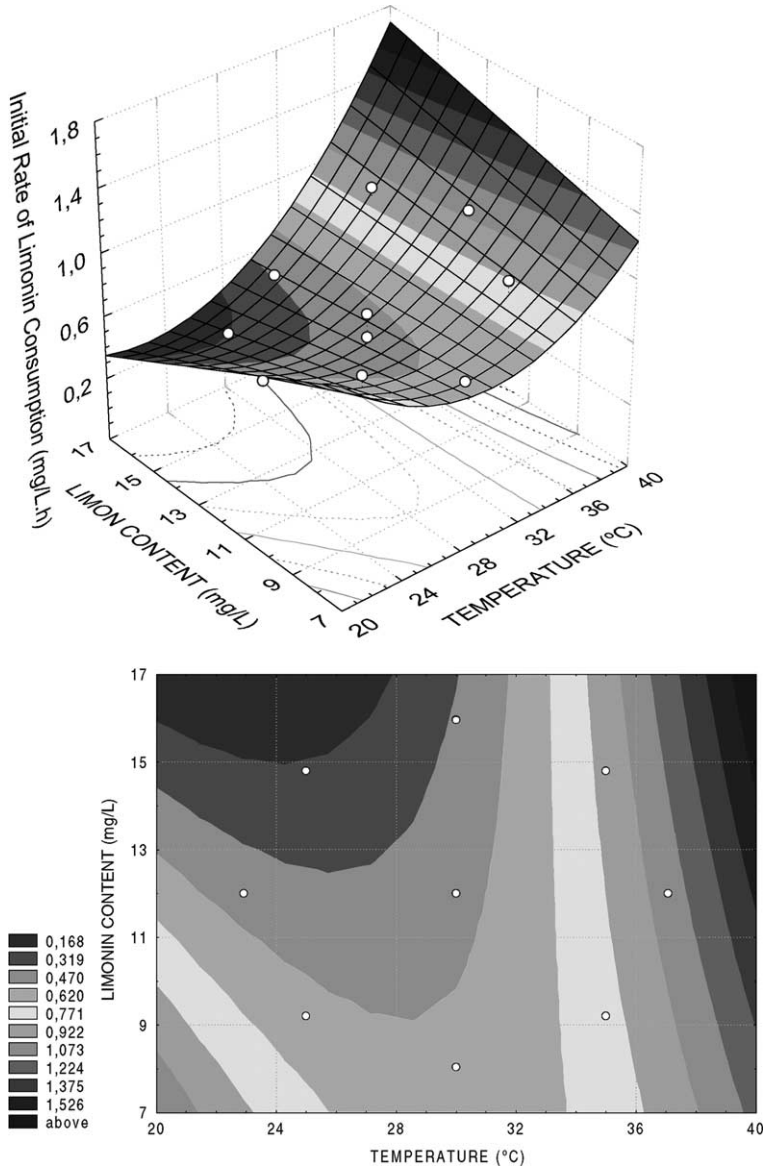

Fig. 1. Response surface and respective contour plot, fitted to the experimental data points corresponding to the initial rates of limonin consumption by *Acinetobacter calcoaceticus anitratus*, from raw orange juice, as a function of temperature and initial limonin content.

The significant effects of the temperature, initial limonin content and interaction ($T \times L$) on the initial rates of limonin consumption and on converted limonin, are shown in Table 2. Temperature showed a significant (linear and/or quadratic) effect on initial rate of limonin consumption and on limonin bioconversion by *Acinetobacter calcoaceticus*, either from raw or sterilized orange juice (Table 2). The initial limonin content had a lower effect on its initial rate of consumption and bioconversion than temperature.

The response surfaces fitted to the experimental data (Figs. 1–4) can be described by polynomial equations (Table 3). 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 (data not shown), 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.

For the initial rate of limonin consumption, concave surfaces (Figs. 1 and 2) were obtained with both raw and sterilized orange juices. The high values of R^2 and R^2_{adj} of these models (Table 3) show a close agreement between the experimental results and the theoretical values predicted by these models [18].

Minimum initial rates of limonin consumption are expected at 26 °C, in raw juice, and at 27 °C in sterilized juice.

With respect to the amount of converted limonin by *Acinetobacter calcoaceticus*, after 7 h reaction time, an increase with temperature was observed when raw

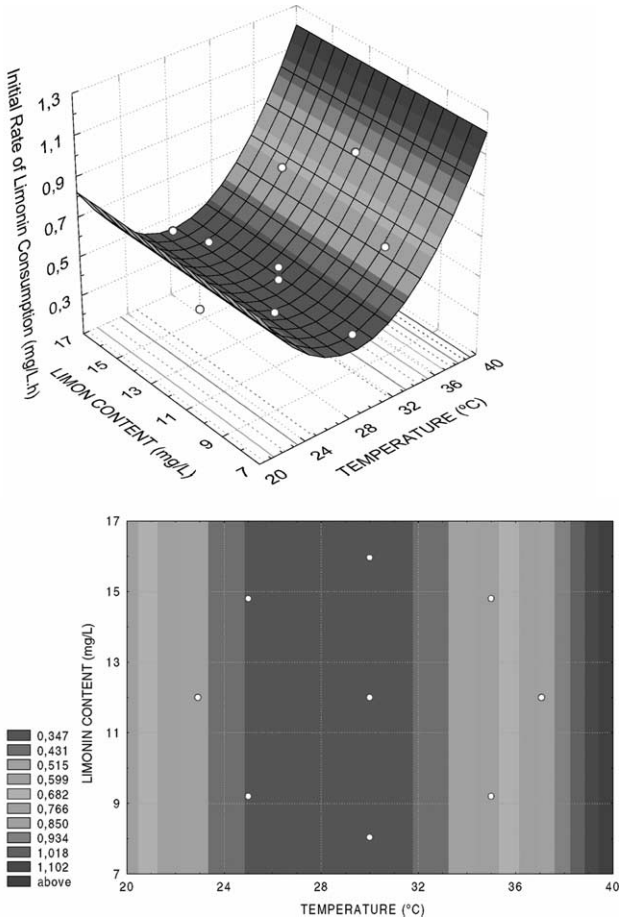

Fig. 2. Response surface and respective contour plot, fitted to the experimental data points corresponding to the initial rates of limonin consumption by *Acinetobacter calcoaceticus anitratus*, from sterilized orange juice, as a function of temperature and initial limonin content.

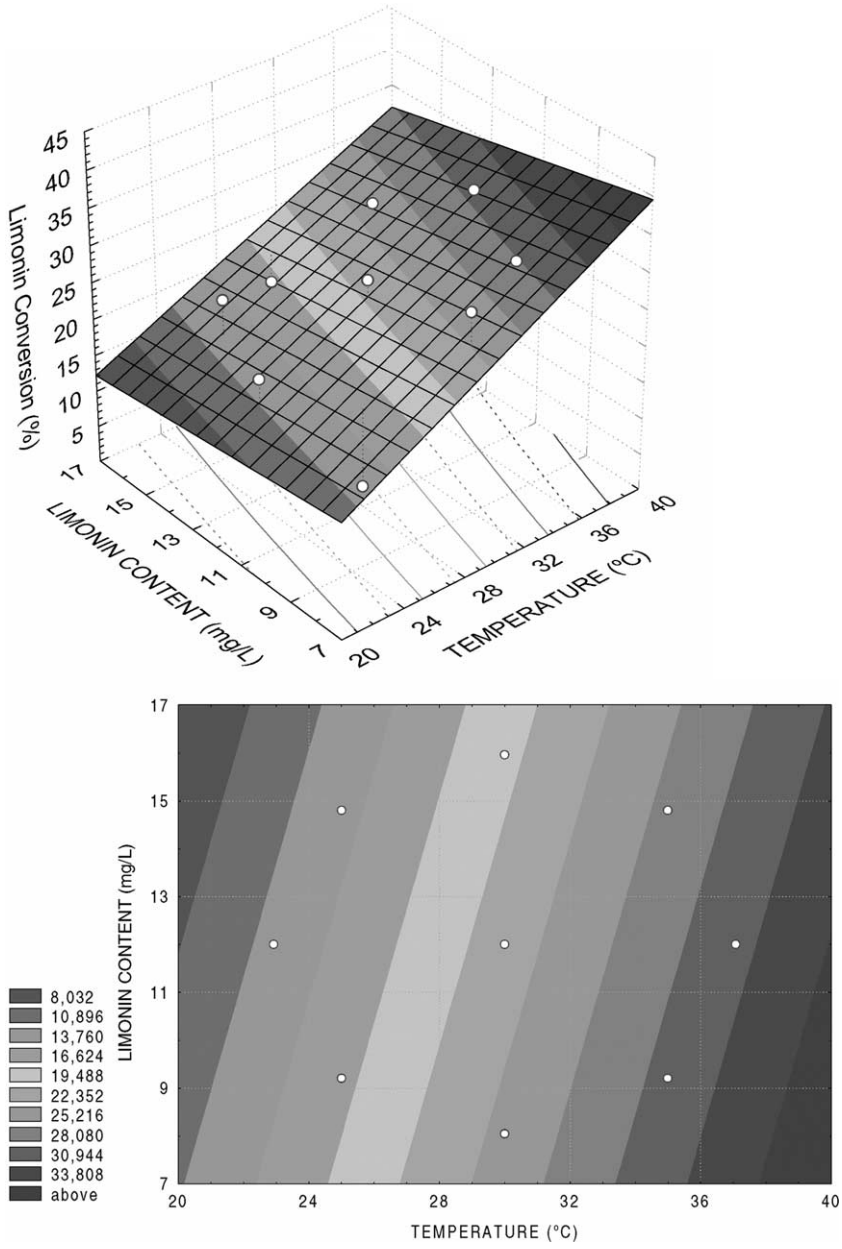

Fig. 3. Response surface and respective contour plot, fitted to the experimental data points corresponding to limonin conversion (%) by *Acinetobacter calcoaceticus anitratus*, from raw orange juice, as a function of temperature and initial limonin content.

orange juice was used (Fig. 3). Also, a negative interaction $T \times L$ indicates that the highest conversions are achieved at high temperature and low initial limonin content. Limonin conversion higher than 33% were obtained at temperatures higher than 34 °C (Fig. 3). The limonin consumption from sterilized juice can be described by a convex surface as a function of temperature and initial limonin concentration (Fig. 4). A maximum conversion of about 23% is expected at 31 °C, for an initial limonin content of 11 mg l^{-1}, in

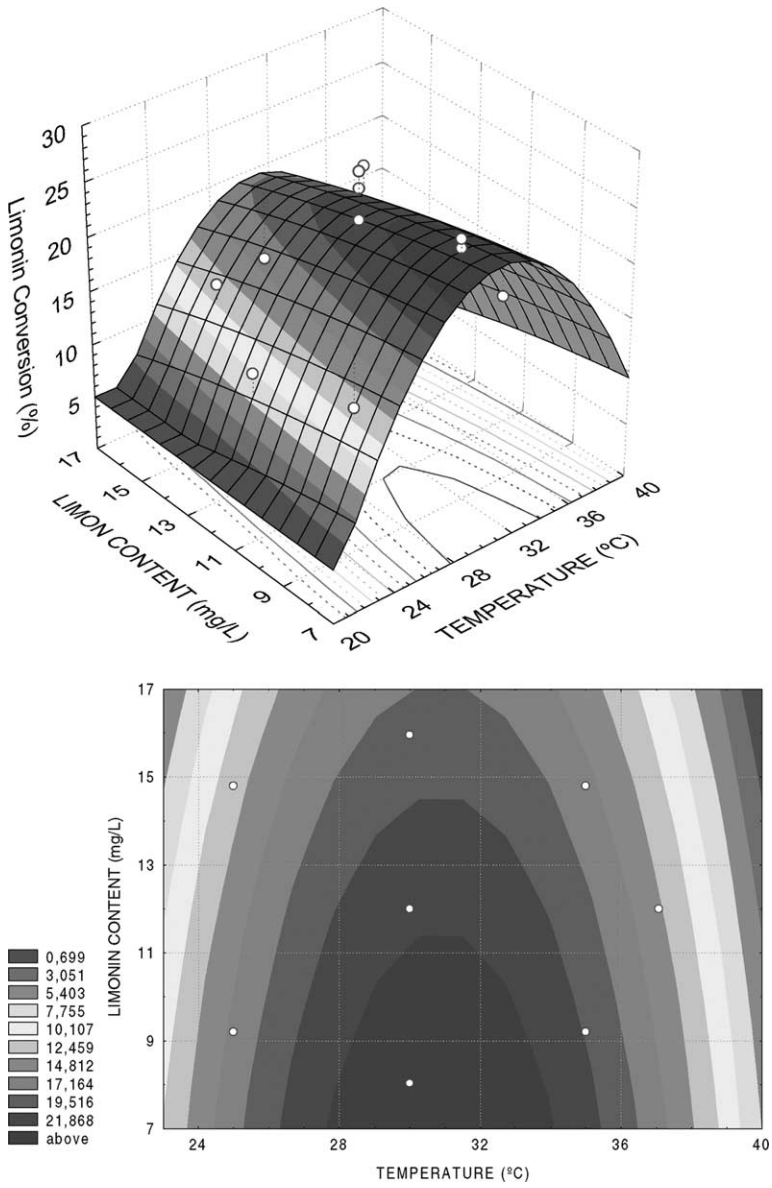

Fig. 4. Response surface and respective contour plot, fitted to the experimental data points corresponding to limonin conversion (%) by *Acinetobacter calcoaceticus anitratus*, from sterilized orange juice, as a function of temperature and initial limonin content.

sterilized juice. Thus, higher conversion of limonin was observed in raw juice than in sterilized juice. Thermal degradation of several compounds (e.g. vitamins and co-factors), eventually important in limonin bioconversion pathway, may explain the observed results with sterilized juice. In addition, in sterilized juices, the highest values of limonin conversion (Fig. 4) were observed under approximately the same reaction conditions corresponding to the lowest initial rates of limonin conversion (Fig. 2). The different behavior exhibited in raw and sterilized juices may be due to different enzymes activities of *Acinetobacter* cells

Table 3
Model equations for the Response Surfaces fitted to the experimental data points (initial rate of limonin consumption (V_0; mg l^{-1} h^{-1}) and limonin conversion (Conv; %)), as a function of the variables (T, temperature, °C; L, initial limonin concentration, mg l^{-1}) and respective R^2 and R^2_{adj}

Juice	Model equation	R^2	R^2_{adj}
Raw	$V_0 = 7.26 - 0.386T + 0.006T^2 - 0.257L + 0.008TL$	0.906	0.831
	$\text{Conv} = -18.12 + 1.575T - 0.023TL$	0.676	0.584
Sterilized	$V_0 = 5.66 - 0.38T + 0.007T^2$	0.778	0.723
	$\text{Conv} = -191.00 + 14.04T - 0.227T^2 - 0.029L^2$	0.638	0.517

grown in different batches. Further experiments have to be carried out for a better understanding of these differences.

In addition, an increase in limonin content after 10 h reaction was observed (data not shown). This may be ascribed to the hydrolysis of the limonin precursor (limonoate A-ring lactone) to limonin [5].

As a conclusion, limonin bioconversion may be carried out directly in raw juice, avoiding juice sterilization. In addition, no significant decrease in reducing sugars was observed. These preliminary results are very encouraging for future implementation on the industrial process. However, since in industrial processes, short reaction times are needed, better conversions are expected if higher biomass concentration is used. Also, microorganism growth conditions should be optimized in order to achieve a higher yield of enzymes responsible for limonin bioconversion.

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References

[1] S. Hasegawa, V.P. Maier, Biochemistry of limonoid citrus juice bitter principles and biochemical debittering processes, in: Bitterness in Food and Beverages, vol. 25, Elsevier, Amsterdam, 1990, p. 293.
[2] S. Hasegawa, V.P. Maier, Food Technol. 37 (1983) 855.
[3] S. Hasegawa, C.E. Vandercook, G.Y. Choi, Z. Herman, P. Ou, J. Food Sci. 50 (1985) 330.
[4] S. Hasegawa, V.A. Pelton, R.D. Bennett, J. Agric. Food Chem. 31 (1983) 1002.
[5] M. Cánovas, L. García-Cases, J.L. Iborra, Enzyme Microb. Technol. 22 (1998) 111.
[6] R.L. Strobel, W. Nakatsukasa, J. Ind. Microbiol. 11 (1993) 121.
[7] R. Sen, J. Chem. Tech. Biotechnol. 68 (1997) 263.
[8] G.K. Robinson, M.J. Alston, C.J. Knowles, P.S.J. Cheetham, K.R. Motion, Enzyme Microb. Technol. 16 (1994) 855.
[9] C.-J. Shieh, C.C. Akoh, P. Koehler, J. Am. Oil Chem. Soc. 72 (1995) 619.
[10] X. Xu, A.R.H. Skands, C.-E. Høy, H. Mu, S. Balchen, J. Adler-Nissen, J. Am. Oil Chem. Soc. 75 (1998) 1179.
[11] N.M. Osório, S. Ferreira-Dias, J.H. Gusmão, M.M.R. da Fonseca, J. Mol. Catal. B: Enzymatic 11 (2001) 677.
[12] S. Ferreira-Dias, A.C. Correia, F.O. Baptista, M.M.R. da Fonseca, J. Mol. Catal. B: Enzymatic 11 (2001) 699.
[13] G. Annadurai, V. Raju, M. Chellapandian, M.R.V. Krishnan, Bioprocess Eng. 16 (1996) 13.
[14] C. Pizarro, M.A. Fernández-Torroba, C. Benito, J.M. González-Sáiz, Biotechnol. Bioeng. 53 (1997) 497.
[15] G.L. Miller, Anal. Chem. 31 (1959) 426.
[16] M.C. Gacula Jr., J. Singh, Response surface designs and analysis, in: Statistical Methods in Food and consumer Research, Food Science and Technology, A Series of Monographs, Academic Press, New York, 1984, p. 214.
[17] D.C. Montgomery, Design and Analysis of Experiments, Wiley, New York, 1991, p. 649.
[18] S. Weisberg, Applied Linear Regression, Wiley, New York, 1985, p. 217.