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Synthesis of the Prospective Anticancer Molecule Perillic Acid from Orange Essential Oil by the Yeast *Yarrowia lipolytica*

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Abstract

The bioconversion of the hydrophobic and volatile limonene to perillic acid, a potential anticancer agent, by the yeast *Yarrowia lipolytica* was studied in two steps. Firstly, experimental design was used for process optimization using high-purity limonene as substrate and secondly orange essential oil containing 89.1% limonene was used as substrate under the previously optimized conditions. Limonene concentration and pH were identified by fractional factorial design as significant factors and were optimized by central composite design. Under optimized process conditions (0.16% (v/v) limonene; pH 6.9), the 24 h biotransformation process resulted in the accumulation of 0.368 $g{\cdot}L^{-1}$ of perillic acid corresponding to a molar yield of 23.1%. A subsequent substrate addition under the same reaction conditions doubled perillic acid concentration to 0.793 $g{\cdot}L^{-1}$ and a molar yield of 24.2%. The use of orange essential oil under the optimized reaction conditions increased both perillic acid accumulation and yield to 0.872 $g{\cdot}L^{-1}$ and 29.7%, respectively. The robustness of *Y. lipolytica* allowed the efficient biotransformation of a crude by-product of the citrus industry into a valuable fine chemical.

Keywords

Yarrowia lipolytica, Bioconversion, Perillic Acid, Limonene, Orange Essential Oil

1. Introduction

The compounds perillyl alcohol, perillaldehyde and perillic acid, derived from the limonene molecule through the oxidation of its exocyclic methyl group, are

known for presenting several biological activities. Perillaldehyde presents antimicrobial property [1] and perillyl alcohol is currently on clinical trials as a multi-target anti-cancer agent [2] [3]. The prospective anticancer activity of perillic acid relates to the accepted mechanism for these monoterpenes as inhibitors of protein prenylation in cancer cells [4] as well as its presence in human plasma after limonene [5] or perillyl alcohol [6] administration. Indeed, perillic acid has demonstrated to be effective in a range of antitumor *in vitro* assays [7] [8] [9] [10].

With rare exceptions [11] volatile monoterpenes in plants present low perillic derivatives content since the O_2/NADPH-dependent biosynthetic step to hydroxylate the limonene moiety in plant cells is known to produce complex mixtures of perillyl, carvyl and menthyl alcohols [12]. Furthermore, perillic derivatives are difficult to obtain by chemical oxidation of limonene, given that the insertion of oxygen into the terminal methyl group is less favourable in comparison to other sites of the molecule. In fact, the allylic hydroxylation of limonene catalyzed by either palladium compounds [13] or metal silicates [14] is highly favored to take place in the cyclic carbons rather than the terminal methyl group of limonene molecule, to generate menthol and carveol isomers.

Nevertheless, several studies on the microbial or enzymatic bio-oxidation of limonene have shown effectiveness to oxyfunctionalize exclusively the exocyclic methyl group for the production of perillic acid [15] [16]. Biotransformation processes using wild type or genetically modified *Pseudomonas putida* cells have shown to be quite promising for the production of perillic acid as a molecule of industrial interest for natural preservation and pharmaceutical application [17] [18] [19]. Studies on this theme included the use of a tubular segmented-flow biofilm reactor in a multistep process with high productivity [19]. Investigations have also been carried out with the bacteria *Escherichia* and *Mycobacterium* [16]. The fungus *Aspergillus cellulosae* [16] and the non-conventional yeasts *Arxula adeninivorans* and *Yarrowia lipolytica* [20] have also been approached to converting limonene to perillic derivatives. Recently, our research group successfully studied the bio-oxidation of *R*-(+)-limonene into *R*-(+)-perillic acid by *Y. lipolytica* ATCC 18942 [21], which is recognized as safe and suitable yeast to be used in processes aiming at products for human consumption [22] [23]. As a continuation of the previous study, the present work aimed to increase the perillic acid yield from high-purity limonene, by applying statistical design of experiments to the bioreaction parameters. Finally, the optimized biotransformation conditions were applied to a crude orange essential oil sample representing an industrial terpenoid mixture containing 83% to 97% of *R*-(+)-limonene.

2. Materials and Methods

2.1. Microorganism, Media, Yeast Cultivation and Bioconversion Assays

Y. lipolytica ATCC 18942 was obtained from the National Institute for Quality Control in Health, Oswaldo Cruz Foundation collection (INCQS 40149, Fiocruz,

Rio de Janeiro, Brazil). Cells were grown in Yeast Malt Broth medium (glucose 10 g·L^{-1}, yeast extract 3 g·L^{-1}, malt extract 3 g·L^{-1} and peptone 5 g·L^{-1}) at 28˚C under stirring (200 rpm) during 48 h. For the bioconversion experiments, cells were separated by centrifugation (3000 × *g*) and re-suspended in phosphate buffer (50 mM) at 20 g·L^{-1}. *R*-(+)-limonene 97% (Sigma) was added and the mixture was incubated under stirring at 200 rpm. Temperature, pH, nutrient addition and limonene concentration were the factors selected for experimental design and their values varied through the experiments.

2.2. Experimental Design

A fractional factorial design [24] was carried out for screening significant factors for limonene bioconversion to perillic acid. Process temperature, process pH, initial *R*-(+)-limonene concentration and nutrient addition were selected at the levels shown in **Table 1**. The experiments were performed in duplicate and perillic acid concentration, measured at 24 h and 48 h, was the response variable.

For the bioconversion optimization, a central composite design [24] was carried out having initial *R*-(+)-limonene concentration and pH as variables at the levels shown in **Table 2**. Nutrients were not added and the temperature was kept at 25˚C. Experiments were performed in non-simultaneous triplicate and aliquots of the bioconversion medium were collected at 24 and 48 h for perillic acid quantification. Two responses (dependent variables) were considered for the statistical analysis: perillic acid concentration and molar bioconversion yield (which considers perillic acid production related to the initial limonene concentration). For each response at each time (24 h and 48 h), the prediction model was calculated such as:

$$\hat{y} = b_0 + b_{Lim}x_{Lim} + b_{pH}x_{pH} + b_{LimpH}x_{Lim}x_{pH} + b_{Lim}^2x_{Lim}^2 + b_{pH}^2x_{pH}^2$$

Table 1. Trial of variables by fractional factorial design: cell mass fixed at 20 g·L^{-1}.

	Variables and levels			
Run	T (˚C)	pH	Initial Limonene concentration (% v/v)	Nutrients * (% v/v)
1	− (20.0)	− (6.2)	− (0.1)	− (0)
2	− (20.0)	− (6.2)	+ (0.5)	+ (10)
3	− (20.0)	+ (7.2)	− (0.1)	+ (10)
4	− (20.0)	+ (7.2)	+ (0.5)	− (0)
5	+ (25.0)	− (6.2)	− (0.1)	+ (10)
6	+ (25.0)	− (6.2)	+ (0.5)	− (0)
7	+ (25.0)	+ (7.2)	− (0.1)	− (0)
8	+ (25.0)	+ (7.2)	+ (0.5)	+ (10)
9	0 (22.5)	0 (6.7)	0 (0.3)	0 (5)
10	0 (22.5)	0 (6.7)	0 (0.3)	0 (5)
11	0 (22.5)	0 (6.7)	0 (0.3)	0 (5)

*Nutrients solution: 3 g·L^{-1} yeast extract, 3 g·L^{-1} malt extract, 5 g·L^{-1} peptone, 10 g·L^{-1} glucose.

Table 2. Optimization by central composite design. Cell mass was fixed at 20 g·L^{-1} and temperature at 25˚C.

	Variables and levels	
Run	Limonene (% v/v)	pH
1	– (0.16)	– (6.35)
2	– (0.16)	+ (7.05)
3	+ (0.44)	– (6.35)
4	+ (0.44)	+ (7.05)
5	a (0.10)	0 (6.7)
6	A (0.50)	0 (6.7)
7	0 (0.30)	a (6.2)
8	0 (0.30)	A (7.2)
9	0 (0.30)	0 (6.7)
10	0 (0.30)	0 (6.7)
11	0 (0.30)	0 (6.7)

where $\hat{y}$ is the predicted response, b_0 is the intercept, b_{Lim} is the linear coefficient of the limonene concentration, x_{Lim} is the codified value of the limonene concentration, b_{pH} is the linear coefficient of the pH, x_{pH} is the codified value of the pH, b_{LimpH} is the coefficient for the interaction between limonene concentration and pH, b^2_{Lim} is the quadratic coefficient of the limonene concentration, b^2_{pH} is the quadratic coefficient of pH value. Design settings and calculations were performed in JMP Statistical Discovery Software version 8.

2.3. Stepwise Addition of Limonene

The conditions optimized by central composite design, pH 6.9 and *R*-(+)-limonene concentration of 0.16% (v/v) were applied in a bioconversion experiment where a fresh equal *R*-(+)-limonene dose was added after 24 h. Aliquots were collected at 24 h and 48 h for perillic acid concentration measurement. Experiment was performed in duplicate.

2.4. Use of Orange Essential Oil for the Production of Perillic Acid

The high-purity limonene optimized process conditions were applied to the bioconversion of the orange essential oil (from Tropfruit Nordeste S. A, Estância, Brazil). This substrate was added to the yeast medium in two portions (2 × 0.16% v/v, pH 6.9) at the beginning and after 24 h of bioconversion. Overall yield was taken after 48 h reaction.

2.5. Analytical Procedures

Cell mass concentration was determined by optical density at 600 nm (OD_{600}). One OD_{600} unit corresponded to a dry cell concentration of 0.444 g·L^{-1}. Glucose concentration was measured using an YSI 2700 Select Biochemistry Analyzer

(Yellow Springs). Perillic acid was determined by gas chromatography (GC) using *(S)*-(-)-perillic acid 95% from Sigma-Aldrich as standard. A sample of the bioconversion supernatant was cleared of debris by centrifuging 15 min at 15000 × *g* and a 1.00 mL supernatant aliquot was transferred to a 1.7 mL microcentrifuge tube (tube 1). Perillic acid was precipitated by adding 100 µl of HCl 0.6 M solution and the resulting suspension was homogenized and centrifuged at 15000 × *g* during 15 minutes. The supernatant was transferred to another 1.7 mL microcentrifuge tube and after repeating the precipitation procedure, the supernatant was discarded. The precipitates from both tubes were dissolved with ethyl acetate and transferred to a 2.00 mL volumetric flask that was filled up with the same solvent. After homogenization the perillic acid solution was analyzed using a GC instrument from Agilent Technologies model 7890 equipped with a 7683 injector and autosampler, split/splitless injector and flame ionization detector (Santa Clara, USA). A J & W Scientific HP-Innowax column with a bonded polyethylene glycol phase, 30 m length, 250 µm internal diameter and 0.250 µm film thickness, was used. All analyses were performed using a temperature program, constant flow, and split injection. The chromatographic conditions were as follows: oven temperature initiated at 50˚C, temperature increasing to 250˚C at 20˚C min^{-1}, held for 5 min; the run time was 15 min. The injector temperature was set at 280˚C, and detector temperature set at 300˚C. Helium was the carrier gas at a flow rate of 1 $mL{\cdot}min^{-1}$. Injection volume was 1.0 µl with the split ratio set at 25:1.

Limonene content in the crude orange essential oil was determined by GC using *R*-(+)-limonene 97% from Sigma as standard. GC-column was a DB-5 30 m length, 250 µm internal diameter and 0.250 µm film thickness. The chromatographic conditions were as follows: the oven temperature was kept at 70˚C for 5 min and then increased at 4˚C min^{-1} up to 170˚C; the run time was 30 min. The injector was set at 250˚C and the detector at 300˚C. Helium was the carrier gas at a flow rate of 0.5 $mL{\cdot}min^{-1}$. Injection volume was 1.0 µl with the split ratio set at 50:1.

3. Results

3.1. Screening of Variables Using Experimental Design

Previous results from our laboratory showed the yeast cell mass and limonene concentrations, pH, temperature and nutrient addition as relevant variables to be assessed for limonene bioconversion to perillic acid, as well as their range values [21]. Cell mass concentration, assayed to sub-levels 5.0 - 12.5 - 20.0 $g{\cdot}L^{-1}$ in a preliminary fractional factorial design experiment, showed a preponderant influence on the response, impairing the assessment of the remaining variables (data not shown). Hence, the screening was designed by fixing the cell mass concentration at 20 $g{\cdot}L^{-1}$ and varying the other factors as shown in **Table 1**.

The statistical analysis (**Figure 1**) showed that the most relevant factors were the limonene concentration followed by the pH. In the range assayed, the temperature was of low significance at 24 h and non-significant at 48 h. Therefore,

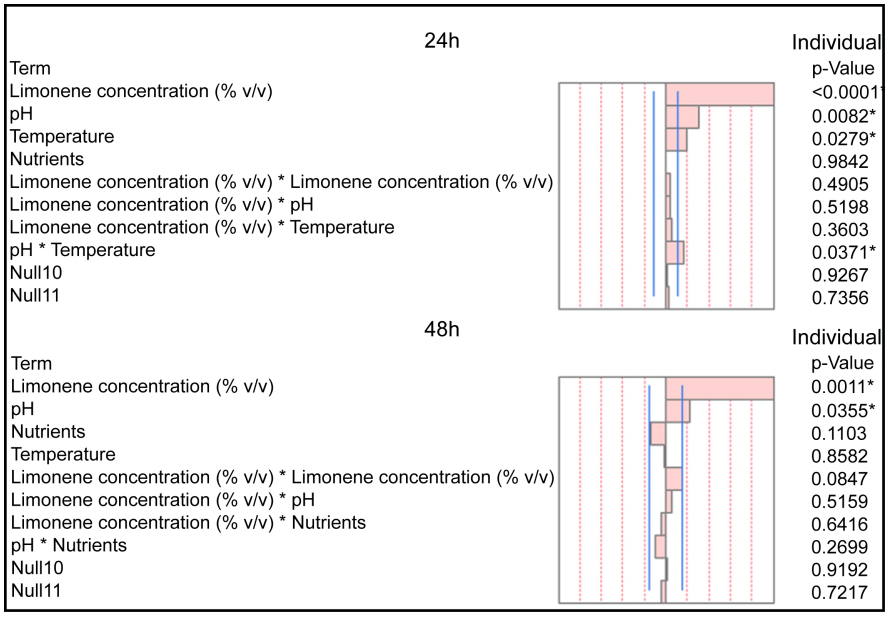

Figure 1. Significance chart for the assessed factors in the fractional factorial design for the bioconversion of limonene to perillic acid.

for the subsequent experiments, the temperature was fixed at 25˚C. The addition of a nutrient solution (containing macro-micronutrients, and growth factors that would be required for cell maintenance and/or enzymatic activity) was not significant for the perillic acid production, even though glucose, when present, was fully metabolized (data not shown). This result indicated that the bioconversion could be carried out without appending extra nutrients to the medium.

3.2. Optimization Using Response Surface Methodology

Process optimization with the two selected variables, limonene concentration and pH, was performed by central composite design according to **Table 2**. **Figure 2** shows the effects of the factors on perillic acid concentration and on the bioconversion molar yield at 24 h and 48 h. The linear and the quadratic effects of limonene concentration were significant for perillic acid concentration at both time intervals. When considering the yield as the response variable, the linear effect of limonene concentration was significant at 24 h while the quadratic effect was significant at 48 h. PH effects in the range assayed were not significant for both responses in this experiment. Predictive models were generated for each response (product concentration and molar bioconversion yield) as shown in **Table 3**. The profiles displayed in **Figure 3** and **Figure 4** showed that the optimum conditions were different according to the type of response evaluated thereof. The overall maximum predicted perillic acid concentration (0.868 ± 0.076 $g{\cdot}L^{-1}$) was reached with 0.44% (v/v) initial limonene concentration and pH 6.94 at 48 h (**Figure 4(a)**). Nevertheless, this value was only 23% higher than the maximum predicted at 24 h (0.706 ± 0.062 $g{\cdot}L^{-1}$) for 0.40% (v/v) limonene concentration and pH 6.96 (**Figure 3(a)**). The predicted bioconversion yields for these conditions were 20.4% ± 2.6% and 18.4% ± 2.2%, respectively. On the other

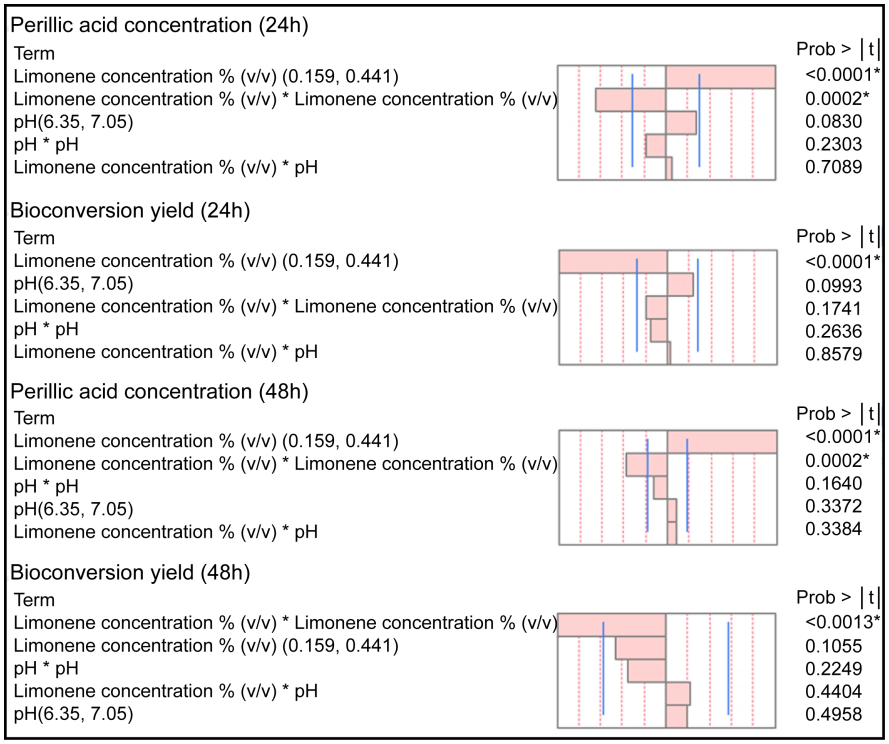

Figure 2. Effects graph for the assessed factors in the central composite design.

Table 3. Prediction models for each response.

Response	Prediction model
Perillic acid concentration ($g \cdot L^{-1}$) 24 h	$\hat{y} = 0.64891438888889 + 0.12883473167398 x_{Lim} + 0.03545373207567 x_{pH} + 0.01050779166667 x_{Lim} x_{pH} - 0.0995462777778\ x_{Lim}^2 - 0.0287720694444\ x_{pH}^2$
Bioconversion yield (%) 24 h	$\hat{y} = 21.0544323390805 - 4.7538065958408 x_{Lim} + 1.11120440122768 x_{pH} + 0.16644282070712 x_{Lim} x_{pH} - 1.0815310302604\ x_{Lim}^2 - 0.8475487296127\ x_{pH}^2$
Perillic acid concentration ($g \cdot L^{-1}$) 48 h	$\hat{y} = 0.72121044444444 + 0.23301431549124 x_{Lim} + 0.019971336804 x_{pH} + 0.02817066666667 x_{Lim} x_{pH} - 0.1028183680556\ x_{Lim}^2 - 0.0348015347222\ x_{pH}^2$
Bioconversion yield (%) 48 h	$\hat{y} = 23.4001229820067 - 1.1416892754269 x_{Lim} + 0.47061378146558 x_{pH} + 0.75476044841651 x_{Lim} x_{pH} - 2.9209782138728\ x_{Lim}^2 - 1.0075914617546\ x_{pH}^2$

hand, the maximum predicted bioconversion yields were 25.7% ± 2.4% (0.428 ± 0.069 $g \cdot L^{-1}$ perillic acid) at 24 h (Figure 3(b)) and 24.3% ± 2.3% (0.681 ± 0.067 $g \cdot L^{-1}$ perillic acid) at 48 h (Figure 4(b)) at much lower limonene concentrations, 0.16% (pH 6.9) and 0.28% (pH 6.76), respectively. The fact that the higher limonene concentrations resulted in lower bioconversion yields could be assigned to the limonene toxicity towards the yeast cells as well as to its volatility. These effects had already been described during previous limonene bio-oxidation studies

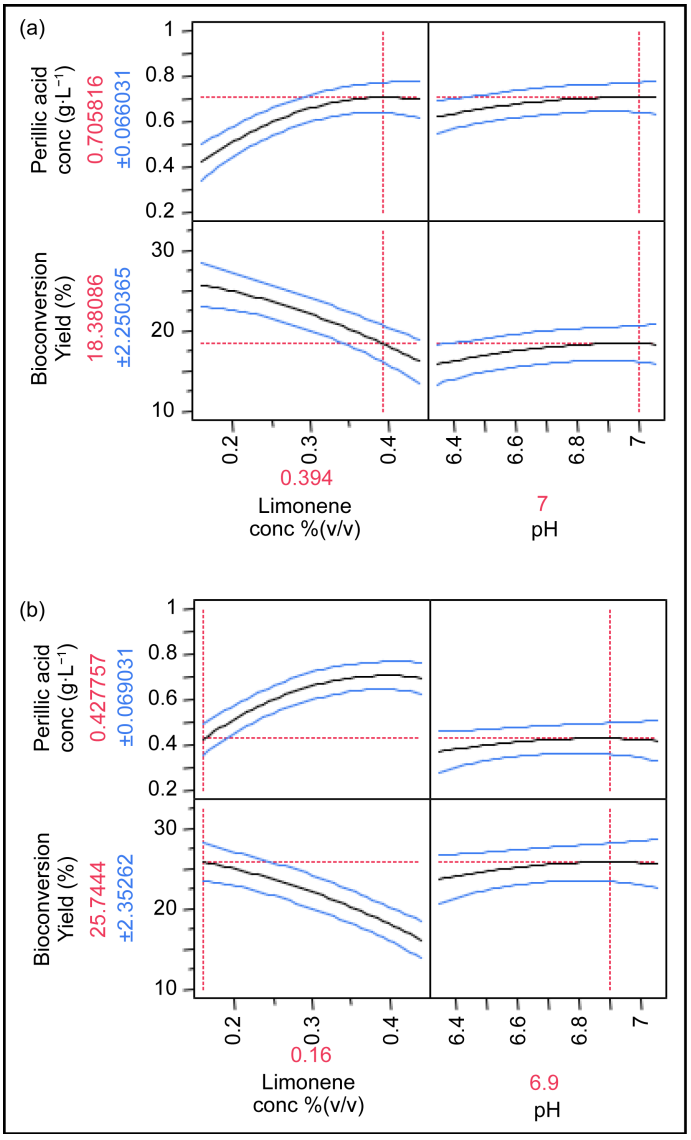

Figure 3. Profiles of the measured responses at 24 h: (a) optimum conditions for perillic acid concentration; (b) optimum conditions for bioconversion yield.

[21]. The pH was not significant for the measured responses at 24 and 48 h. Nevertheless, this parameter was included in the predictive model.

3.3. Stepwise Addition of Limonene

The statistical modeling indicated that the use of 0.16% limonene allowed the maximum molar yield within 24 h bioconversion suggesting that this initial limonene concentration coupled to a fresh limonene addition after 24 h might increase perillic acid concentration while keeping the bioconversion yield. To assess this possibility, a new test was conducted using 0.16% limonene at pH 6.9 followed by an equal limonene addition after 24 h. The product concentration reached 0.368 $g \cdot L^{-1}$ perillic acid (23.1% yield) after 24 h, within the model prediction interval between 0.358 $g \cdot L^{-1}$ and 0.496 $g \cdot L^{-1}$. After the second limonene

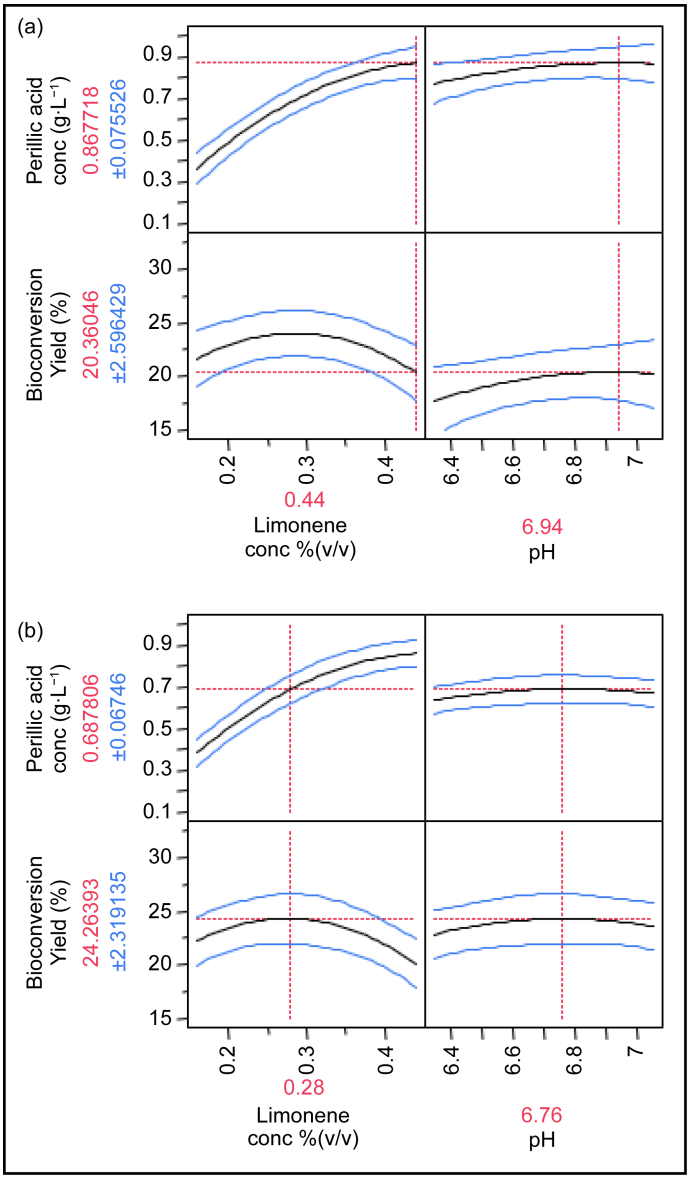

Figure 4. Profiles of the measured responses at 48 h: (a) optimum conditions for perillic acid concentration; (b) optimum conditions for bioconversion yield.

addition, the bioconversion process resumed reaching 0.793 g·L^{-1} of perillic acid (24.2% yield) within 48 h of the total bioconversion process. The two steps limonene addition resulted advantageous either comparing with the intermittent six-portions limonene feeding used in the previous study (0.5% total over 48 h; perillic acid concentration increasing by 50%) or to the total single limonene addition at the beginning of the experiment. Furthermore, the present study improved the reaction molar yield by 1.5 fold in comparison to the previous maximum yield of 16.7% [21].

3.4. Bioconvertion of Orange Essential Oil into Perillic Acid

The optimized conditions found to the bioconversion of high-purity limonene

with *Yarrowia lipolytica* were replicated by using orange essential oil containing 89.1% limonene. Within 48 h bioconversion, an accumulation of 0.872 g·L^{-1} of perillic acid (29.7% molar yield) was achieved; these values being higher than the observed for the use of high-purity limonene. The yield increase could be due to better substrate dispersion, as visually observed. In fact, crude orange oil presents a large variety of components such as oxidized derivatives of other monoterpenes and sesquiterpenes (data not shown) [25] that, nevertheless in minor amounts, would increase the availability of the limonene molecule to the yeast cells.

4. Discussion

Table 4 compares published data from our laboratory [21] and data from the present work regarding the main biotransformation process parameters in terms of reaction conditions, perillic acid final concentration and perillic acid molar yield. The handling of volatile limonene to enhance its bioavailability to the yeast cells in a stirred-process of bioconversion, without using emulsifiers, turned out to be quite challenging. Thus, six limonene additions to the reaction medium had been done over a period of 48 h in our previous work [21]. In the present study however, only two additions were needed, which meant an operational gain, without decreasing the final product concentration. Although the product concentrations obtained by the three reaction conditions presented in Table 4 were very similar, with an average value of 0.85 g·L^{-1}, the molar yields increased 50% and 78% in the optimized process by using high-purity limonene or the orange oil as substrate, respectively. The use of lower substrate amounts of 35% for high-purity limonene and 41% for crude orange oil represented a significant feedstock saving, an important issue regarding cost and process sustainability.

Table 4 also compares the cost of the high-purity limonene or orange oil substrates, for the production of 1 Kg of perillic acid. The biotransformation process using orange oil resulted in an important decrease in the cost from either 552.38

Table 4. Comparison of the main biotransformation process parameters, production data of perillic acid (PA) by *Yarrowia lipolytica* and substrate cost for the use of high-purity limonene (published data and present study) and orange oil (data from the present study).

Measured parameter	Previous process[1]	Optimized process[2]	
	Limonene (96% purity)	Limonene (97% purity)	Orange oil (89% limonene)
Bioconversion conditions	25˚C; pH 7.1; 10 g·L cell mass (dry weight)	25˚C; pH 6.9; 20 g/L cell mass (dry weight)	
Total limonene amount (mM)[3]	29.7 (4.0 g·L^{-1})	19.2 (2.6 g·L^{-1})	17.6 (2.4 g·L^{-1})
Limonene addition mode over 48 h	6 additions	2 additions	2 additions
Final PA concentration	0.855 g·L^{-1}	0.796 g·L^{-1}	0.872 g.L^{-1}
PA molar yield	16.7%	25,0%	29.7%
Cost of limonene substrate to produce 1 Kg PA	552.38 USD[4]	386.67 USD[4]	80.56 USD[4] 12.90 USD[5]

[1]See ref. [21]. [2]Optimized conditions using experimental design (this work). [3]Calculated considering the substrate purity degree. [4]Estimated from prices of (*R*)-(+)-limonene and orange oil from Sigma-Aldrich. [5]Estimated from price of orange oil from the Brazilian Company Tropfruit Nordeste S. A.

USD (non-optimized process) or 386.67 USD (optimized process) for high-purity limonene to 80.56 USD or 12.90 USD for imported or Brazil produced orange oil.

These results are quite valuable due to the present industrial scenario where low-cost industrial feedstocks are particularly at aim for supplying the fine chemical-based industries [26]. Orange essential oil is largely available due to a worldwide orange production of 70 million tons per year [27]. The orange-based industry (28 million tons/year) by itself generates residues amounting 50% of the raw processed fruit whereby the orange oil represents approximately 5% (on dry basis) [28] and can be obtained directly from the cold pressing citrus zests. In this scenario, the large availability of orange essential oil coupled to an environmentally friendly yeast based process to produce perillic acid could be extensively explored to establish a cost-effective synthesis of pharmaceutically valuable molecules. It is also relevant to stress the particular characteristics of *Y. lipolytica* as a robust yeast able to assimilate a great diversity of substrates, being tolerant to pollutants [22] [23] and thus very attractive to industrial use. Besides, the easiness of yeast separation from the biotransformation reaction mixture, as compared to bacteria, is another advantage for the process scale up.

5. Conclusion

The use of statistical design of experiments allowed determining how each reaction condition influenced the selective bio-oxidation of the limonene molecule in order to maximize the formation of perillic acid using a minimum amount of substrate. This approach resulted in 24.2% bioconversion yield on limonene basis, representing an increase of 1.5 fold, as compared to previous reported results. The aforementioned process conditions were applied to orange essential oil, increasing the molar yield even further to 29.7% corresponding to 0.872 $g{\cdot}L^{-1}$ of perillic acid, showing the robustness of *Y. lipolytica* towards this new substrate. The process feasibility using a highly-available industrial feedstock to produce a valuable fine chemical is promising due to the well-known advantages embodied by a green chemistry process as well as to the strategic and cost advantages conveyed by the use of a citrus industry by-product.

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