Palm oil fatty acids and carotenoids extraction with lipase immobilized in magnetic nanoparticles

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Abstract

Magnetite nanoparticles have unique properties including superparamagnetism and low toxicity. They have been used as supports for immobilization of enzymes because of the advantage including easy separation and effective recycle under external magnetic field. Therefore, the present study aimed at developing a new enzymatic biocatalyst from the immobilization of *Yarrowia lipolytica*'s (IMUFRJ 50682) lipase on magnetic nanoparticles for its use in free fatty acids and carotenoids extraction from palm oil (*Elaeis guineenses*). The lipase production conducted in a 4 L benchtop bioreactor generated a crude enzyme extract with hydrolytic p-nitrophenyl laurate activity of 58 U/ml after 24 h. Lipase was immobilized by adsorption on magnetite nanoparticles (Fe_3O_4). The crude enzymatic extract, both free and immobilized, were used in the hydrolysis of palm oil. Temperature, reaction time and substrate ratio (water/palm oil) were evaluated in a central composite experimental design. The initial concentration of fatty acids and carotenoids present in palm oil obtained via traditional extraction (using organic solvents) were 82.45% and 1892.3 mg/kg, respectively. Using crude lipase extract immobilized in nanoparticles, after 120 min, temperature of 24°C and water/oil ratio of 2, it was possible to obtain similar values, with the advantage of easily removing the catalyst from reaction media and reusing it.

Keywords: Lipase, nanoparticle, carotenoids, triacylglycerol, hydrolysis.

Introduction

Magnetic nanoparticles have been greatly used as immobilizing supports for biomolecules because of its easy separation from the reaction medium during processing and large specific surface area enabling high loading of target molecules [1]. Besides it has low toxicity, a chemically modifiable surface and good reusability [2]. Various enzymes have been immobilized on magnetic nanoparticle surfaces, especially lipases [1-3].

Lipases are serine hydrolases defined as triacylglycerol acylhydrolases (E.C. 3.1.1.3), which naturally catalyze the hydrolysis of the ester bond of tri-, di- and mono-glycerides from long-chain fatty acids into fatty acids and glycerol. In thermodynamically favorable conditions, these biocatalysts can catalyze synthesis reactions such as esterification or transesterification [4]. Due to its high production cost, it is necessary to immobilize this enzyme to enable its reutilization.

Lipases have been used successfully for several commercial and industrial purposes and the extraction of carotenoids have been proposed by some authors [5,6].

Palm oil is the richest and cheapest natural source of β-carotene, which is the most important provitamin A source [6]. However, most of the carotenoids in palm oil are destroyed during the refining process and, as a result, various methods of extraction and recovery of palm oil have been developed [6].

The present study describes the use of a lipase from *Yarrowia lipolytica* immobilized in magnetic nanoparticles to extract carotenoids and free fatty acids from palm oil.

Experimental

Materials/ chemicals details

Crude palm oil was obtained by cold extraction performed in Universidade Estadual de Santa Cruz (Ilhéus/Itabuna, Brazil).

A wild strain of *Yarrowia lipolytica* IMUFRJ 50682, isolated from the Guanabara Bay, Rio de Janeiro (Brazil) was used to produce the lipase [7]. Dextrose (Reagen; Rio de Janeiro, Brazil), peptone and yeast extract (Oxoid; Hampshire, UK) were used for culture media.

Lipase production and immobilization

Y. lipolytica lipase production was performed in a 4 L bench fermenter (New Brunswick MF-114, Sci. Inc., USA), using 3 L of YPD medium (Yeast extract: 1%; Peptone: 0.64%; Glucose: 2%), stirring speed of 650 rpm, airflow rate of 1.5 $L.min^{-1}$ and temperature controlled in 28ºC for 24 hours, as described by Brigida et al. [8]. Then, the cells were centrifuged (26,000 *g*) and the cell-free extract was used as crude lipase extract.

The crude lipase extract was immobilized by adsorption on magnetic nanoparticles (Fe_3O_4 magnetite), prepared as described previously [9]. The crude lipase extract (4mL) was mixed with 20mg magnetite nanoparticles. The mixture was agitated in an orbital shaker for 90min, at 200rpm and 20°C. After centrifugation, the supernatant was removed and the immobilized lipase was washed with distilled water. Then, it was freeze-dried and stored at -80°C until its use.

Hydrolysis reaction

Hydrolysis reactions were performed in 125-mL Erlenmeyer flasks with 10 mL reaction volume. This reaction volume was composed by palm oil, distilled water and enzyme, incubated in an incubator shaker at 150 rpm. The enzyme used was the crude lipase extract (4 mL) or 0.02 g of magnetic nanoparticles with lipase immobilized (4 mL of crude lipase extract immobilized in 0.02 g of magnetic nanoparticles). Oil/water ratio, temperature and reaction time were evaluated in an experimental design.

Experimental design

For optimization of carotenoids and fatty acids extraction a two level Box-Behnken design 2^{3-1} with four central points was chosen to evaluate reaction conditions (independent variables): time (x_1), oil/water ratio (x_2) and temperature (x_3) and, as **Table 1** shows.

Response surface regression analyses were used to model the effect of the independent variables on carotenoids and fatty acids concentrations using STATISTCA® 7.0 statistical package. Experimental data were fitted to a quadratic polynomial model. The model proposed for the response (Y) was as follows:

$$Y = b_0 + b_1x_1 + b_2x_2 + b_3x_3 + b_{11}x_{21} + b_{22}x_{22} + b_{33}x_{23} + b_{12}x_1x_2 + b_{13}x_1x_3 + b_{23}x_2x_3$$

where x_1, x_2 and x_3 represented the coded values of the factors according to **Table 1**, and the parameters b_0, b_1,…, b_{23} are constant coefficients.

The adequacy of model was checked based on R^2 and adjusted-R^2 as well as the lack of fit error.

Analytical methods

The free fatty acids (FFA) content was determined based on potentiometric titration [10]. Total carotenoids were measured by spectrophotometry, as described by [11]. Characterization palm oil FFA was performed after treatment with petroleum ether (10 mL of ether for each 5 g of oil) for 2 min and hot ethanol (40 mL).

Results and discussion

The palm oil used presented 82.45 ± 1.45 % of free fatty acids (FFA) and 1892.3 ± 14.1 mg of carotenoids/kg of oil after chemical hydrolysis.

The hydrolysis reactions of palm oil were performed with free lipase (crude lipase extract) and immobilized lipase (crude lipase extract immobilized in magnetic nanoparticles). The results of FFA and carotenoids after this treatment in different conditions of time, temperature and water/oil ratio are present in **Table 1**.

Table 1. Design matrix for the experimental design for free fatty acid (FFA) and Total carotenoid (TC) extraction with crude lipase extract and immobilized crude lipase in magnetic nanoparticles.

Factors			Levels		
			Low (-1)	Central (0)	High (+1)
(x_1) time, min			60	120	180
(x_2) water/oil ratio, v/v			1/1	2/1	3/1
(x_3) Temperature, °C			25	35	45
Crude lipase extract					
Run	(x_1)	(x_2)	(x_3)	FFA (%)	TC (mg/kg)
1	-1	-1	-1	57.32	1457.2
2	-1	-1	1	57.69	1459.1
3	1	-1	-1	57.41	1490.6
4	1	-1	1	57.21	1448.0
5	-1	1	-1	62.34	1508.4
6	-1	1	1	62.45	1406.0
7	1	1	-1	68.91	1458.0
8	1	1	1	63.78	1568.0
9	0	-1	0	57.91	1523.0
10	0	1	0	75.21	1621.9
11	-1	0	0	59.37	1600.2
12	1	0	0	75.64	1589.2
13	0	0	-1	75.71	1556.1
14	0	0	1	57.45	1456.2
15	0	0	0	77.68	1769.6
16	0	0	0	77.52	1773.4
17	0	0	0	77.11	1752.9
18	0	0	0	77.07	1761.7
Immobilized crude lipase extract					
1	-1	-1	-1	62.31	1449.0
2	-1	-1	1	61.85	1483.2
3	1	-1	-1	64.21	1565.8
4	1	-1	1	65.31	1482.3
5	-1	1	-1	72.22	1568.6
6	-1	1	1	71.51	1505.2
7	1	1	-1	75.38	1498.8
8	1	1	1	68.32	1631.2
9	0	-1	0	68.91	1613.2
10	0	1	0	77.67	1753.6
11	-1	0	0	62.84	1764.5
12	1	0	0	77.85	1723.2
13	0	0	-1	79.67	1732.5
14	0	0	1	81.25	1625.4
15	0	0	0	83.45	1856.2
16	0	0	0	83.61	1834.5
17	0	0	0	83.14	1837.2
18	0	0	0	83.75	1829.6

Table 1 shows that in some cases, lipase was even better than the chemical hydrolysis to obtain free fatty acids (Runs 15 to 18 for immobilized crude lipase), since values higher than 82.45 % were detected.

For carotenoids, values close to maximum (1892.3 mg/kg) were obtained also with immobilized lipase (Runs 15 to 18).

The statistical analysis **(Fig. 1)** showed that for FFA extraction with free crude lipase extract, no independent variables were significant at a confidence level of 5% ($p < 0.05$). However, for the immobilized lipase, the quadratic terms of water/oil ratio and time and the linear terms of water/oil ratio and time x water/oil were significant at the same confidence level. Temperature was not significant for FFA extraction, which can be explained by the range of temperature used. Akil et al [3] have shown that this lipase maintains more than 70% of its activity from 20 to 40 °C for 240 h.

For free lipase it was not possible to obtain a statistical model because of the lack of significance. For the immobilized lipase, the model obtained after removing the non-significant terms was:

$$\boldsymbol{FFA} = -0.872 + 0.625\, x_1 - 0.002\, x_1^2 + 42.444\, x_2 - 9.650\, x_2^2 - 0.00001\, x_1 * x_2^2$$

Fig. 2 shows the fitted response surface for the design and depicts the curvature which indicates that a maximum of FFA was achieved. According to the model, the optimum conditions for FFA extraction with lipase immobilized in magnetic nanoparticles are: 117.19 min of contact time and 2.2 mL of water per milliliter of oil, which would attain 82.92% of FFA.

Ribeiro et al. [5] obtained a maximum of 73.6% of FFA with the commercial lipase Lipozyme TL IM from *Thermomyces lanuginosus* (immobilized form) for burity oil after 4 hours of reaction.

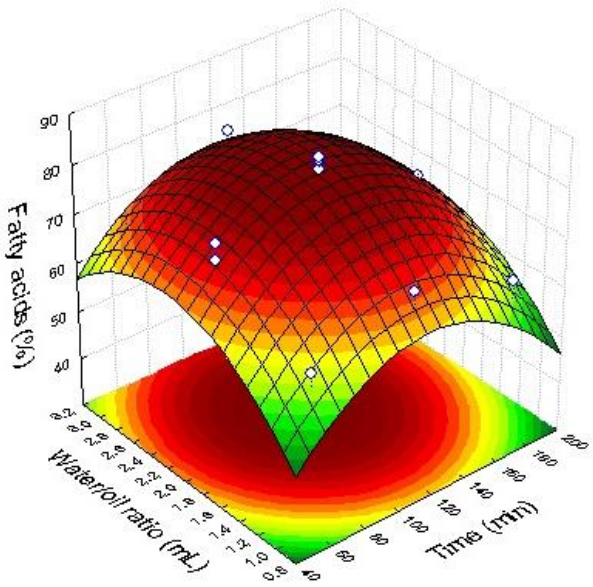

Fig. 2. Response surface for the Box-Behnken design for free fatty acid (FFA) extraction with crude lipase immobilized in magnetic nanoparticles.

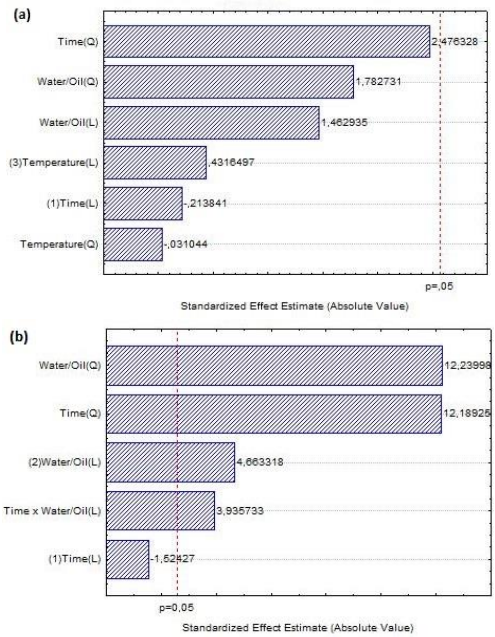

Fig. 1. Pareto chart for the Box-Behnken design for free fatty acid (FFA) extraction with (a) free crude lipase extract and (b) crude lipase immobilized in magnetic nanoparticles.

For total carotenoid extraction some independent variables (reaction conditions) influenced significantly ($p < 0.05$) when free crude lipase was used, as **Fig. 3a** shows. Time, water/oil ratio (quadratic terms) and temperature (linear term) were significant. For the immobilized enzyme, only time and water/oil ratio (quadradic terms) were significant **(Fig. 3b),** as observed for FFA extraction (Fig. 1b). Akil *et al* [3] showed that the immobilized form of this lipase is more stable in relation to temperature than the free form, which explains why temperature was only significant for carotenoid extraction with the free form.

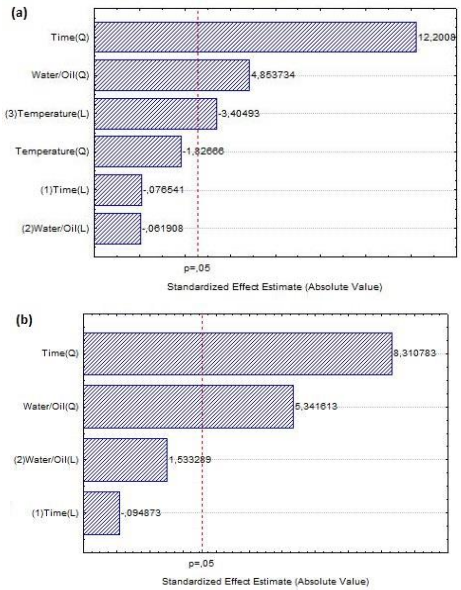

Fig. 3. Pareto chart for the Box-Behnken design for carotenoid extraction with (a) free crude lipase extract and (b) crude lipase immobilized in magnetic nanoparticles.

The model obtained after removing the non-significant terms, for free lipase, was:

$$TC = 116.008 + 1.221\ x_1 - 0.005\ x_1^2 + 29.124\ x_2 - 7.2983\ x_2^2 - 2.3008\ x_3 + 0.0275\ x_3^2$$

and for immobilized lipase was:

$$TC = 116.008 + 1.222\ x_1 - 0.005\ x_1^2 + 29.125\ x_2 - 7.298\ x_2^2$$

Fig. 4 shows the fitted response surfaces for the design and depicts the curvature for free **(Fig. 4a)** and immobilized **(Fig. 4b)** lipase, which indicates that a maximum of carotenoid was achieved. According to the model, the optimum conditions for carotenoid extraction with free lipase are: 119.86 min of contact time, 2.0 mL of water per milliliter of oil and temperature of 41.88°C which would attain 1700.9 mg of total carotenoid per kg of oil and for lipase immobilized in magnetic nanoparticles are: 119.74 min of contact time and 2.1 mL of water per milliliter of oil, which would attain 1836.7 mg of total carotenoid per kg of oil.

The best conditions for carotenoid were similar to fatty acid extraction. Therefore, any of those conditions could be used with high values as can be seen in **Fig. 3** and **4**. Similar results of carotenoid extraction for burity oil were obtained previously with lipase from *Y. lipolytica* after 4 h of reaction [5]. However, a maximum of 35% of fatty acid was achieved in this case.

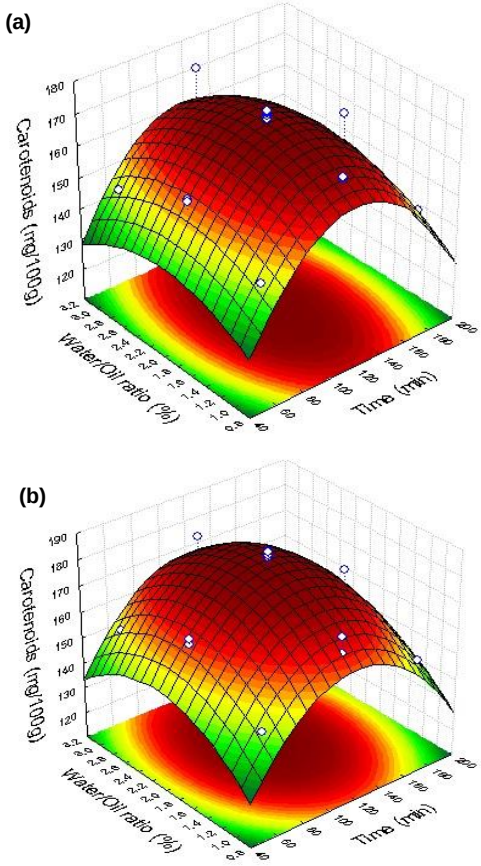

Fig. 4. Response surface for the Box-Behnken design for carotenoid extraction with (a) free crude lipase extract and (b) crude lipase immobilized in magnetic nanoparticles.

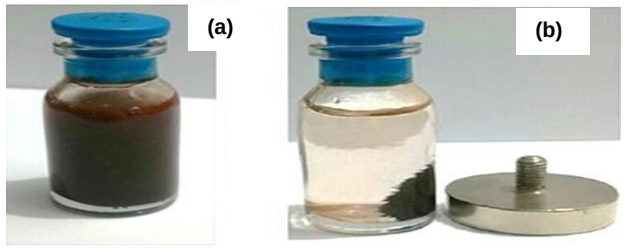

Fig. 5. Suspension of iron oxide nanoparticles in aqueous medium: (a) suspended nanoparticles; (b) action of magnetic field on nanoparticles.

Better results were obtained with the immobilized form of the enzyme, which adds the advantage of the possibility of being reused after the application of a magnetic field, as can be seen in **Fig. 5.**

Conclusion

High values of fatty acid (>80%) and carotenoid (approximately 1800 mg/kg) were achieved after a 2 h hydrolysis of palm oil at a proportion of 2 mL of water per milliliter of oil with lipase from *Y. lipolytica* immobilized in magnetic nanoparticles. The easy separation of the enzyme from the reaction media adds an advantage for this process.

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Author's contributions

Conceived the plan: Tamires Carvalho, Priscilla V. Finotelli, Priscilla F. F. Amaral; Performed the expeirments: Tamires Carvalho; Data analysis: Tamires Carvalho; Wrote the paper: Tamires Carvalho, Priscilla V. Finotelli, Priscilla F. F. Amaral. Authors have no competing financial interests.

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