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RESEARCH PAPER

# Efficient and low-cost alternative of lipase concentration aiming at the application in the treatment of waste cooking oils

**Karina P. Preczeski[1] · Angela B. Kamanski[1] · Thamarys Scapini[1] · Aline F. Camargo[1] · Tatiani A. Modkoski[1] · Vanusa Rossetto[1] · Bruno Venturin[1] · Jéssica Mulinari[2] · Simone M. Golunski[1] · Altemir J. Mossi[1] · Helen Treichel[1]**

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## Abstract

In this study, we evaluated the concentration of lipases from *Aspergillus niger* using efficient and low-cost methods aiming at application in the treatment of waste cooking oils. The change in ionic strength of the medium by the addition of salt and precipitation with ethanol increased the specific activity from 2.90 to 28.50 U/mg, resulting in a purification factor of 9.82-fold. The use of acetone resulted in a specific activity of 33.63 U/mg, resulting in a purification factor of 11.60-fold. After that, the concentrated lipase was used in the hydrolysis of waste cooking oil and 753.07 and 421.60 µmol/mL of free fatty acids were obtained for the enzyme precipitated with ethanol and acetone, respectively. The hydrolysis of waste cooking oil catalyzed by homemade purified lipase in ultrasonic media can be considered a pretreatment of oil by converting a significant amount of triglycerides into free fatty acids.

**Keywords** Homemade lipases · Precipitation · Waste cooking oil

## Introduction

Lipases belong to a group of interesting enzymes, not only for the ability to act on water insoluble substrates, but also for the ability to catalyze different reactions, such as hydrolysis, esterification, interesterification, alcoholysis, acidolysis, and aminolysis. The interest in using these biocatalysts in the structural modification of oils and fats has received a lot of attention due to their specificity in relation with the substrate [1]. The diversity of lipase properties allows the use of these enzymes in different fields of application [2].

Production of enzymes using biotechnological processes, as in the case of lipases, generates a crude extract composed of an aqueous mixture of cells, extra and intracellular products, substrates, and unconverted components. After fermentation, depending on the application, enzymatic purification operations are required, which must be included in the final cost of the product.

The development of techniques of concentration, purification, and immobilization of enzymes is essential for obtaining enzymes with high catalytic efficiency. Pure and stable enzymes with high specificity for substrates can be used in industrial processes similar to chemical catalysts, and their recovery and reuse can facilitate cost-effectiveness [3]. Most of the published studies involving lipase purification use chromatographic processes [4, 5]. However, this process is difficult to scale and has a high cost. To minimize these costs, the optimization of fast and effective methodologies for the purification process is of great importance and extremely desirable [6–11].

Some fungal and bacterial lipases have been exploited as cheap and versatile catalysts in various industries, such as foodstuffs, dairy products, detergents, and pharmaceuticals, as well as the degradation of greasy residues and the production of biodiesel [12, 13]. In view of the efficiency of the degradation of greasy residues and the growing concern with the consequences of human activities on natural resources, the search for alternative techniques of treatment of residues, such as waste cooking oil, is extremely important.

The waste cooking oil, when thrown in the sinks, causes clogging of the pipes, and when discarded in the soil, forms

✉ Helen Treichel
helentreichel@gmail.com

[1] Universidade Federal da Fronteira Sul-UFFS, Rodovia ERS 135, km 72, no 200, Erechim, RS, Brazil

[2] Departamento de Engenharia Química e de Alimentos, Universidade Federal de Santa Catarina, Campus Universitário, Florianópolis, SC 88800-000, Brazil

a waterproofing layer which hinders the drainage and the gas exchanges with the atmosphere, causing the death of the fauna and flora present in the soil [14]. The negative impacts of fat and oil residues could be minimized by the use of various hydrolytic microbial enzymes. Lipolytic pre-hydrolysis of oil and fat residues by enzyme such as purified lipases can improve the removal efficiency and accelerate the degradation process. Therefore, the objective of this study was to develop an efficient and low-cost alternative of lipases concentration aiming at the application in the treatment of waste cooking oils.

## Experimental

### Lipase production and biochemical characterization

The enzyme was produced by solid-state fermentation of canola cake by *Aspergillus niger*. The solid substrate was supplemented with 2% (w/w) nitrogen and 60% (w/w) moisture. For lipase extraction, it was used a 100 mM sodium phosphate buffer pH 8 as extraction solution and temperature of 50 °C for 35 min in an orbital shaker at 165 rpm [15].

The experimental design showed that the maximum hydrolytic activity was obtained at pH 6.0 and 35 °C. Furthermore, the enzymes does not catalyze reactions with palmitic acid and saturated chains, but its hydrolytic activity is higher when oil containing medium chains (C12:0) and long chains (C18:0), for example, canola, soybean, and coconut oil. The enzyme also showed good stability in low temperatures (− 1 °C and 10 °C) [15].

### Precipitation with salt and organic solvents

First, the change of the ionic strength of the crude extract was carried out by the addition of salt (NaCl) followed by the addition of organic solvents (acetone or ethanol). The effects of salt amount (0–1.0 mol/L), organic solvent concentration (10–90%), and feed rate (0.76–19.24 mL/min) were evaluated by a central composite rotational design (CCRD) $2^3$. These levels were defined based on the previous work of our research group [16, 17]. All determinations were carried out in triplicates.

Aliquots of salt were added to the crude extract with the purpose of altering the ionic strength of the medium and promoting precipitation. The solvents (ethanol and acetone) were cooled and added dropwise in an aliquot of the crude extract (10 mL) at a given feed rate using a peristaltic pump (MsTecnopon). The enzyme extract solution was maintained at 5 °C under constant stirring throughout the process. After addition of the solvent, the precipitate was centrifuged at 9000×*g* for 15 min at 4 °C. The precipitate was collected and dissolved in 100 mM sodium phosphate buffer, pH 8 [17].

### Analytical methods

#### Enzymatic activity

For the determination of lipase activity, the method established by Treichel et al. [15] was used. An emulsion was prepared using olive oil 10% (w/v) and gum arabic 5% (w/v) diluted in 90% (v/v) of 100 mM sodium phosphate buffer pH 6. To the hydrolysis reaction process, 1 mL of the enzyme extract was added to 18 mL of emulsion and the solution was incubated for 32 min at 35 °C with agitation of 165 rpm. After this period, the reaction was stopped by adding 20 mL of acetone–ethanol solution (1:1, v/v). The fatty acids released during the reaction were titrated to pH 11 with NaOH 0.05 M. All determinations were carried out in triplicates. For comparison between the initial amount of free fatty acids (FFA) and the quantity after the hydrolysis reaction, a control sample was used, in which acetone–ethanol solution was added before adding the enzyme to the emulsion. Thereby, the hydrolytic activity of the lipase was obtained by Eq. (1). A hydrolytic activity unit (U) is defined as the amount of enzyme capable of releasing 1 µmol of fatty acids per minute in reaction conditions [18]:

$$\mathrm{HA} = \left( \frac{(V_a - V_b) * M * 1000}{t * V_c} \right) * \mathrm{WF} \quad (1)$$

where HA = hydrolytic activity of the lipase (U/g), $V_a$ = volume of NaOH solution used in the titration of the samples (mL), $V_b$ = volume of NaOH used in the titration of the control sample (mL), $M$ = NaOH molarity, $t$ = reaction time (min), $V_c$ = volume of crude enzymatic extract used in the reaction (mL), and WF = weight factor obtained by dividing the amount of extraction solution (mL) by the average weight of dry cake (g) added to each fermentation flask.

#### Total protein determination

The concentration of total protein was determined by the Bradford method using bovine serum albumin (BSA) as standard [19]. All determinations were carried out in triplicates.

### Hydrolytic reaction of the waste cooking oil

The waste cooking oil was obtained from a local restaurant and was placed in a bottle at 10 °C until use. The characterization of oil was done in terms of percentage of FFA by gas chromatography carried out in partnership with the Oils and Greases Laboratory of the University of the Republic (UdelaR) of Montevideo, Uruguay [20]. The hydrolysis of the oil was evaluated in terms of the release of free fatty acids in

reaction catalyzed by the purified lipase in an ultrasonic system (Unique Inc., USC-1800A model, frequency US 40 kHz and maximum power of 132 W–0.42 W/cm$^2$) and shaker (45 °C, 170 rpm, 12 h). The conditions used in the ultrasonic bath were 45 °C, 50% power (66 W), and a reaction time of 12 h [20]. Distilled water was used as solvent, since the ultrasound promotes a greater homogeneity of the medium, and a mechanical stirrer at 170 rpm was used to promote the mixing of the solution. A ratio of 1:3 (v/v) oil:solvent was used in a proportion of 15% purified enzyme [20].

### Determination of free fatty acids

To determine the quantity of free fatty acids produced after each hydrolysis reaction of the waste cooking oil, 10 mL were removed from the reaction medium, to which was added 10 mL of acetone ethanol solution (1:1) to stop the reaction. Thus, the free fatty acids were titrated with NaOH 0.02 M to pH 11 [21]. All determinations were carried out in triplicates. To determine only the amount of free fatty acids liberated during the reaction excluding those already in the oil, a control sample was done to each condition of the experimental design. From this, the amount of produced free fatty acids was calculated by Eq. (2) and it is expressed in μmol/mL:

$$\mathrm{FFA} = \left((V_a - V_b) * M * 1000\right)/V_c \quad (2)$$

where $V_a$ = volume of NaOH spent in the sample (mL), $V_b$ = volume of NaOH spent in the control sample (mL), $M$ = NaOH molarity, and $V_c$ = volume of sample used in the reaction (mL) [20].

## Results and discussion

### Purification of enzymatic extract by precipitation with salt and solvents

In this work, the precipitation was evaluated using a central composite rotational design (CCRD) $2^3$, which matrix with the actual and coded values for the independent variables (salt concentration, solvent concentration, and solvent flow) and the respective responses in terms of specific activity, enzyme yield, and purification factor are shown in Table 1.

The specific activity of the crude enzyme extract was 2.90 U/mg for both tests performed. Enzymatic activity

**Table 1** Matrix of the experimental design (coded and real values) and the response in terms of specific activity, purification factor, and activity yield

| Run | Flow rate (mL/min) | Conc. of solvent[a] (%) | Conc. of NaCl[a] (mol/L) | Ethanol[b] | | | Acetone[b] | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | Specific activity (U/mg) | Activity yield (%) | Purification factor (PF) | Specific activity (U/mg) | Activity yield (%) | Purification factor (PF) |
| Crude enzyme | – | – | – | 2.90 | 100 | 1.00 | 2.90 | 100 | 1.00 |
| 1 | − 1 (4.5) | − 1 (26) | − 1 (0.2) | 15.27 | 157.31 | 5.26 | 12.21 | 115.10 | 4.21 |
| 2 | 1 (15.5) | − 1 (26) | − 1 (0.2) | 13.95 | 131.41 | 4.81 | 28.31 | 263.77 | 9.76 |
| 3 | − 1 (4.5) | 1 (74) | − 1 (0.2) | 0.00 | 0.00 | 0.00 | 9.06 | 63.31 | 3.12 |
| 4 | 1 (15.5) | 1 (74) | − 1 (0.2) | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| 5 | − 1 (4.5) | − 1 (26) | 1 (0.8) | 6.73 | 66.18 | 2.32 | 14.80 | 152.51 | 5.10 |
| 6 | 1 (15.5) | − 1 (26) | 1 (0.8) | 10.21 | 98.80 | 3.52 | 18.69 | 192.00 | 6.44 |
| 7 | − 1 (4.5) | 1 (74) | 1 (0.8) | 0.00 | 0.00 | 0.00 | 5.36 | 11.75 | 1.87 |
| 8 | 1 (15.5) | 1 (74) | 1 (0.8) | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| 9 | − 1.68 (0.76) | 0 (50) | 0 (0.5) | 28.50 | 156.73 | 9.82 | 21.02 | 167.51 | 7.25 |
| 10 | 1.68 (19.24) | 0 (50) | 0 (0.5) | 7.03 | 40.16 | 2.42 | 33.63 | 221.39 | 11.60 |
| 11 | 0 (10) | − 1.68 (10) | 0 (0.5) | 5.18 | 61.71 | 1.79 | 4.13 | 56.82 | 1.42 |
| 12 | 0 (10) | 1.68 (90) | 0 (0.5) | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| 13 | 0 (10) | 0 (50) | − 1.68 (0) | 14.35 | 100.96 | 4.95 | 5.75 | 40.16 | 1.98 |
| 14 | 0 (10) | 0 (50) | 1.68 (1.0) | 14.13 | 100.96 | 4.87 | 19.23 | 143.63 | 6.63 |
| 15 | 0 (10) | 0 (50) | 0 (0.5) | 19.21 | 128.53 | 6.62 | 30.70 | 173.39 | 10.58 |
| 16 | 0 (10) | 0 (50) | 0 (0.5) | 16.42 | 89.20 | 5.66 | 30.46 | 164.57 | 10.50 |
| 17 | 0 (10) | 0 (50) | 0 (0.5) | 19.57 | 104.55 | 6.75 | 28.64 | 164.57 | 9.87 |

[a] *Conc* concentration

[b] Standard deviations were all less than 10%

and total protein were determined in the supernatant and the precipitate and the results showed that the enzyme was in the supernatant. This fact demonstrates that the salt and the solvents used possibly had a greater affinity for the interferents than for the enzyme. This fact has also been observed by Barbosa et al. [22] who studied purification of the *Bacillus* lipase by precipitation with the ammonium sulfate $(NH4)_2SO_4$ salt. The authors observed during the study that the enzyme lipase remained in the fermented broth (supernatant), while the contaminants precipitated.

After precipitation, an increase in specific activity is generally observed. For the tests carried out with the ethanol solvent, run 9 showed a higher specific activity (28.50 U/mg), using a saline concentration of 0.5 mol/L, and a solvent concentration of 50% added at a flow rate of 0.76 mL/min. For the tests carried out with acetone as precipitating agent, the run 10 presented the highest specific activity in relation with the crude extract, where a specific activity of 33.63 U/mg was obtained with the same concentrations used for ethanol, but with an addition rate to the crude extract of 19.24 mL/min. This difference between solvents can be attributed to the fact that ethanol precipitates the interferents in smaller addition flows, necessitating a longer time of addition to the crude extract, unlike acetone, which with larger flows and a shorter time presented similar results.

The highest yields were found at concentrations of 26% solvent (263.78% for acetone and 157.31% for ethanol) for both solvents used. Relevant yields were also found in run 9 and 2, followed by the center point for the tests performed with the ethanol solvent. For the tests performed with acetone, the yields that were relevant beyond run 2 were run 10, 9 and the center point. This increase in the yield of the activity may be related to a favoring of interferent precipitation by the addition of the salt, where a greater aggregation of inhibitors may occur. It should be noted that the yield of the enzyme was greater than 100% in most assays for both acetone and ethanol. These results suggest a removal of enzymatic inhibitors, such as heavy metals, commonly present in fermentation media [23].

The results also show that changing the ionic strength of the medium with the addition of salt followed by precipitation with organic solvents can lead to different degrees of purification. The highest purification factor found for the assays performed with ethanol was 9.82-fold (run 9) using a salt concentration of 0.5 mol/L; a concentration of 50% ethanol added at a flow rate of 0,76 mL/min. For the tests performed with acetone, the highest purification factor was 11.60-fold (run 10), where the concentrations used were the same as ethanol, changing only the flow rate, which was 19.24 mL/min.

The statistical analysis of the experimental data allowed the validation of empirical models for the purification factor as a function of salt concentration, solvent concentration, and flow rate. Equations (3) and (4) present the coded models for the purification factor for the solvents (ethanol and acetone) used. For ethanol, the experimental model was not validated by the variance analysis (ANOVA), but, for acetone, the model was validated by analysis of variance (ANOVA) with correlation coefficient of 0.93 and calculated $F$ test higher than the tabulated one, turning the model valid with 95% of confidence, only for the acetone solvent:

$$\begin{aligned} Y_{\mathrm{CALCethanol}} = {} & 6.46 - 0.86 * V - 0.49 * V^2 - 1.39 * C \\ & - 2.34 * C^2 - 0.32 * S - 0.92 * S^2 \\ & - 0.09 * V * C + 0.21 * V * S \\ & + 0.53 * C * S \end{aligned} \tag{3}$$

$$\begin{aligned} Y_{\mathrm{CALCacetone}} = {} & 10.36 + 0.67 * V - 0.46 * V^2 - 1.68 * C \\ & - 3.55 * C^2 + 0.30 * S - 2.28 * S^2 \\ & - 1.48 * V * C - 0.37 * V * S \\ & + 0.14 * C * S \end{aligned} \tag{4}$$

where $Y_{\mathrm{CALC}}$ is the activity yield, $C$ and $V$ are the concentration and flow rate of the solvent used, respectively, and $S$ is the amount of salt.

Analysis of the equations showed that the purification factor is dependent on the concentration and flow rate of the organic solvents used and the amount of salt used. The parameters of the equations showed that the salt concentration of both the organic solvents used affects the purification factor positively or negatively, demonstrating that an increase in salt concentration may cause an increase in the enzyme purification factor. However, it should be noted that after a certain level, high salt concentrations may cause loss of enzyme activity due to denaturation.

The results of the statistical analysis for the purification performance are shown in Fig. 1. It can be seen that the solvent concentration was the only variable that influences the process. The use of the purified lipase resulted in favorable enzyme yields both for precipitation with the acetone solvent and for the ethanol. This suggests that the precipitation removes enzyme inhibitors. This behavior can be attributed to two reasons: (1) there is the removal of secondary metabolites or metabolites during purification that inhibits enzymatic activity and (2) a high concentration of salt and/or protein helps to maintain the protein conformation for the protein active [23, 24]. The presence of organic solvents may allow the lipase to maintain its flexibility and the active site in an open conformation, thus facilitating the diffusion of the substrate to the active site [25]. Therefore, conducting the reactions in organic solvents can improve the dissolution of the substrates and increase the availability of the substrate, as well as assist in the easy separation of enzymes from substrates or products [26]. It is thus understood that salt precipitation is a promising method for the purification

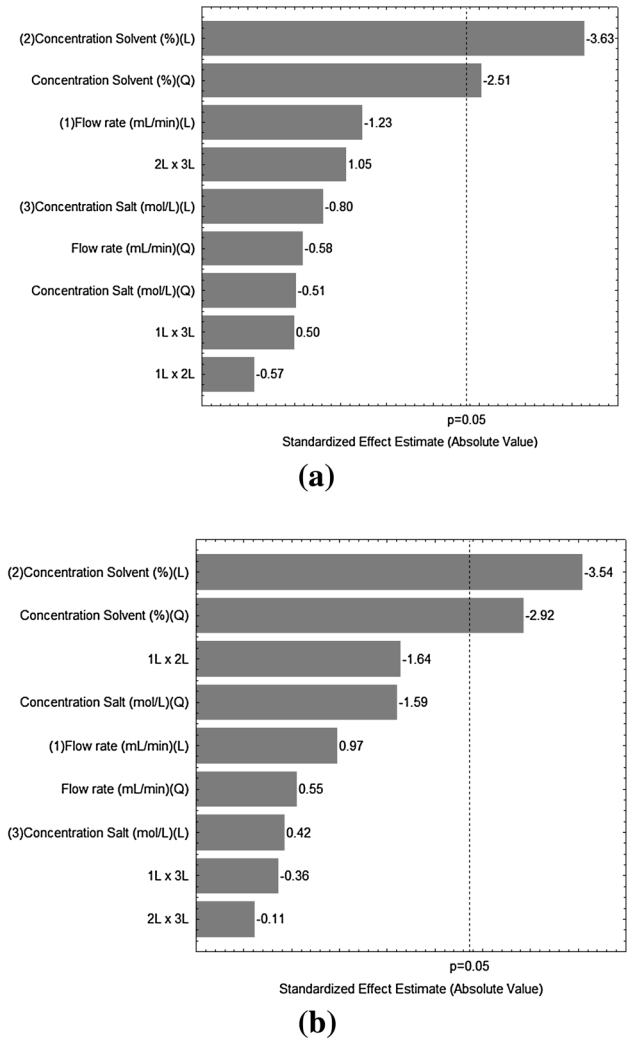


**Fig. 1** Pareto chart of effects for activity yield using **a** ethanol and **b** acetone as precipitation agents

of enzymes, separating proteins from other interfering compounds from the environment that are harmless to the enzyme [27].

Based on the results found in this study, the concentration of salt of 0.5 mol/L NaCl, an organic solvent concentration (acetone or ethanol) of 50%, and a rate of addition to the enzyme extract of 19.24 mL/min for acetone and 0.76 mL/min for ethanol were used to continue the work. These concentrations were defined, since in these conditions, the best purification factors were obtained for both solvents; in addition, considering the process costs, these are the most feasible.

The purification factors and yields found in the present study are comparable and even superior to those reported in the literature. Yong et al. [28] studied the lipase purification of *Botryococcus sudeticus* UTEX 2629 by precipitation with ammonium sulfate resulting in a purification factor of

**Table 2** Evaluation of the enzymatic hydrolysis of the waste cooking oil in shaker and ultrasound and their results in free fatty acids

| Run | Free fatty acids (FFA) (μmol/mL) |
|---|---|
| Ultrasound-lipase precipitated with acetone | 421.60 |
| Ultrasound-lipase precipitated with ethanol | 753.07 |
| Shaker-lipase precipitated with acetone | 0.00 |
| Shaker-lipase precipitated with ethanol | 0.00 |

1.36-fold. Dialyzed filtrate with lipase activity was further purified using size exclusion column chromatography resulting in a 1.52-fold purification factor. Saxena et al. [29] evaluated the purification of *Bacillus stearothermophilus* lipase by CM-Sepharose and DEAE-Sepharose chromatography with a recovery of 62.2% of the enzymatic activity and an 11.6-fold purification factor, a result similar to that found in this work. These comparisons show that the present study presents a purification strategy that is simple and even more effective than some chromatographic techniques.

## Characterization of the waste cooking oil

The characterization of WCO showed that it is composed mostly by 46.69% oleic acid (C18:1 cis), 20.72% elaidic acid (C18:1 trans), and 11.55% palmitic acid (C16:0). Studies conducted by our research group have shown that *Aspergillus niger* lipases have high affinity for these carbon chains. Thus, the presence of these fatty acids makes this oil an excellent substrate for the performance of lipase enzymes [20].

## Enzymatic hydrolysis of the waste cooking oil

The results obtained for the hydrolysis of the waste cooking oil in orbital shaker and ultrasound are presented in Table 2. For the tests carried out in shaker, the release of fatty acids was not observed; this was possibly due to the fact that in a mechanically agitated bioreactor, the total free interfacial area is limited. Therefore, increasing the stirring speed may increase the interfacial area [30]. However, high speeds may result in increased shear rate on lipase molecules and lead to denaturation of the enzyme, resulting in reduced activity [31].

The condition that released the largest amount of free fatty acids on ultrasound (753.07 μmol/mL) was with the enzyme precipitated with the organic solvent ethanol. The enzyme precipitated with acetone showed 421.60 μmol/mL of free fatty acids. Evaluating the results, we can conclude that these are satisfactory when compared with the literature, since Mulinari et al. [20] using the same enzyme and the same conditions used in this work, obtained 62.67 μmol/mL for the crude enzyme. The differential of this work is the

purified enzyme, where a higher concentration of enzymes was used, thus presenting a better catalytic performance.

The fact that the tests performed present better results using the ultrasound can be justified by Gonçalves et al. [32]. According to it, the ultrasound causes the area of the oil–water interface to increase, which results in high conversion rates of triglycerides to free fatty acids even in environments with less water. This is because the lipase acts through the oil–water interface and must penetrate it to catalyze the desired reaction; thus, the total free interfacial area is an important factor to increase the reaction performance. In addition, the use of ultrasound does not require the addition of emulsifying agents, which makes the technology more economically attractive for the industry.

## Conclusions

The use of salt to change the ionic strength of the medium followed by precipitation with organic solvents (ethanol and/or acetone) caused activation of the enzyme, possibly due to the removal of inhibitors. The results showed that a purification of 9.8-fold was obtained in a yield of 156.73% after ionic strength change of the medium followed by ethanol solvent precipitation and 11.60-fold purification in a yield of 221.39%, after ionic strength change of the medium followed by precipitation with acetone solvent. The results obtained are promising when compared to the literature, proving that a purification with simple and low-cost techniques may be more effective than some chromatographic techniques.

In the hydrolysis of the waste cooking oil by the purified lipase, a release of 753.07 μmol/mL of free fatty acids was verified for the enzyme precipitated with ethanol and 421.60 μmol/mL for the enzyme precipitated with acetone. Thus, the hydrolysis of waste cooking oil catalyzed by *Aspergillus niger* purified lipase in ultrasonic media can be considered a pretreatment of oil by converting a significant amount of triglycerides into free fatty acids. This work resulted in a promising sustainable alternative for the purification of microbial lipase applied in the pretreatment of waste cooking oil, which can minimize the environmental damage caused by oil residues.

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## References

1. Castro HF, Mendes AA, Santos JC, Aguiar CL (2004) Modificação de óleos e gorduras por biotransformação. Quím Nova 27:146–156
2. Pandey A, Benjamin S, Soccol CR, Nigam P, Krieger N, Thomaz-Soccol V (1999) The realm of microbial lipases in biotechnology. Biotechnol App Biochem 65:252–254
3. Cunha AG, Besteti M, Manoel EA, Silva AAT, Almeida RV, Simas AB, Fernandez-Lafuente R, Pinto JC (2014) Preparation of core–shell polymer supports to immobilize lipase B from *Candida antarctica* effect of the support nature on catalytic properties. J Mol Catal B-Enzym 100:59–67
4. Ayaz B, Ugur A, Boran R (2015) Purification and characterization of organic solvent tolerant lipases from *Streptomyces* sp. OC119-7 for biodiesel production. Biocatal Agric Biotechnol 4:103–108
5. Gururaj SR, Ganesan ND, Pennathur G (2016) Process optimization for production and purification of a thermostable, organic solvent tolerant lipase from *Acinetobacter* sp. AU07 P. Braz J Microbiol 47:647–657
6. Ishihara T, Yamamoto S (2005) Optimization of monoclonal antibody purification by ion-exchange chromatography. Application of simple methods with linear gradient elution experimental data. J Chromatogr A 1069:99–106
7. Moraes CC, Kalil SJ (2009) Strategy for a protein purification design using C-phycocyanin extract. Bioresour Technol 100:5312–5317
8. Gill PK, Manhas RK, Singh P (2006) Purification and properties of a heat-stable exo-inulinase isoform from *Aspergillus fumigatus*. Bioresour Technol 97:894–902
9. Cui L, Du G, Zhang D, Liu H, Chen J (2007) Purification and characterization of transglutaminase from a newly isolated *Streptomyces hygroscopicus*. Food Chem 105:612–618
10. Martins TS (2001) Produção e purificação de lipases de *Yarrowia lipolytica* (IMUFRJ 50682), Dissertação de Mestrado. Centro de Ciências da Saúde, Universidade Federal de Rio de Janeiro, Rio de Janeiro
11. Cortez EV, Pessoa AJR (1999) Xylanase and β-xylosidase separation by fractional precipitation. Process Biochem 35:277–283
12. Gupta R, Beg O, Lorenz P (2012) Bacterial alkaline proteases: molecular approaches and industrial applications. Appl Microbiol Biotechnol 59:15–32
13. Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685
14. Pinto-Coelho RM (2009) Reciclagem e desenvolvimento sustentável no Brasil. Recóleo Coleta e Reciclagem de Óleos, Belo Horizonte, pp 241–282
15. Treichel H, Sbardelotto M, Venturin B, Dall Agnol A, Mulinari J, Golunski SM, Baldoni DB, Bevilacqua CB, Jacques RJS, Vargas GDLP, Mossi AJ (2016) Lipase production from a newly isolated *Aspergillus niger* by solid state fermentation. Curr Biotechnol 5:1–7
16. Golunski S, Astolfi V, Carniel N, Oliveira D, DI Luccio M, Mazutti MA, Treichel H (2011) Ethanol precipitation and ultrafiltration of inulinases from *Kluyveromyces marxianus*. Sep Purif Technol 78:261–265
17. Golunski SM, Silva MF, Torbes C, Rosseto V, Perin RK, Mossi AJ, Rigo D, Dallago R, Di Luccio M, Treichel H (2017) Purification of inulinases by changing the ionic strength of the medium and precipitation with alcohols. An Acad Bras Ciênc 89:57–63
18. Cavalcanti EAC, Gutarra MLE, Freire DMG, Castilho LR, Sant'Anna GL (2005) Lipase production by solid-state fermentation in fixed-bed bioreactors. Braz Arch Biol Technol 48:9–84
19. Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254
20. Mulinari J, Venturin B, Sbardelotto M, Dall Agnol A, Scapini T, Camargo AF, Baldissarelli DP, Modkovski TA, Rossetto V, Dalla Rosa C, Reichert FW Jr, Golunski SM, Vieitez I, Vargas GDLP., Mossi AJ, Treichel H (2017) Ultrasound-assisted hydrolysis of

waste cooking oil catalyzed by homemade lipases. Ultrason Sonochem 35:313–318
21. Freire DM, Teles EM, Bom EP, Sant'Anna GL Jr (1997) Lipase production by *Peniillium restrictum* in a bench-scale fermenter: effect of carbon and nitrogen nutrition, agitation, and aeration. Appl Biochem Biotech 63:63–65
22. Barbosa JMP, Souza RL, Fricks AT, Zanin GM, Soares CMF, Lima ÁS (2011) Purification of lipase produced by a new source of *Bacillus* in submerged fermentation using an aqueous two-phase system. J Chromatogr B 879:3853–3858
23. Porto TS, Silva GM, Porto CS, Cavalcanti MTH, Neto BB, Lima-Filho JL, Converti A, Porto ALF, Pessoa A Jr (2008) Liquid–liquid extraction of proteases from fermented broth by PEG/citrate aqueous two-phase system. Chem Eng Process 47:716–721
24. Pan IH, Yao HJ, Li YK (2001) Effective extraction and purification of β-xylosidase from *Trichodermakoningii* fermentation culture by aqueous two-phase partitioning. Enzym Microb Tech 28:196–201
25. Balaji L, Jayaraman G (2014) Metal ion activated lipase from halo tolerant *Bacillus* sp. VITL8 displays broader operational range. Int J Biol Macromol 67:380–386
26. Park S, Kazlauskas RJ (2003) Biocatalysis in ionic liquids—advantages beyond green technology. Curr Opin Biotechnol 14:432–437
27. Amid M, Manap MY (2014) Purification and characterization of a novel amylase enzyme from red pitaya (*Hylocereus polyhizus*) peel. Food Chem 165:412–418
28. Yong SY, Lim BH, Saleh S, Lai-Hock T (2016) Optimisation, purification and characterisation of extracellular lipase from *Botryococcus sudeticus* (UTEX 2629). J Mol Catal B: Enzym 126:99–105
29. Saxena RK, Sheoran A, Giri B, Davidson WS (2003) Purification strategies for microbial lipases. J Microbiol Methods 52:1–18
30. Al-Zuhair S, Ramachandran KB, Hasan M (2004) Investigation of the specific interfacial area of a palm oil–water system. J Chem Technol Biotechnol 79:706–710
31. Shamel MM, Ramachandran KB, Hasan M (2005) Operational stability of lipase enzyme: effect of temperature and shear. Dev Chem Eng Min Process 13:599–604
32. Gonçalves KM, Sutili FK, Leite SGF, De Roma S, Leal ICR, (2012) Palm oil hydrolysis catalyzed by lipases under ultrasound irradiation: the use of experimental design as a tool for variable evaluation. Ultrason Sonochem 19:232–236