

## LIPASE PRODUCTION BY *ASPERGILLUS IBERICUS* USING OIL CAKES AND ITS APPLICATION IN ESTERIFICATION REACTIONS

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**Keywords:** Palm kernel oil cake (PKOC), sesame oil cake (SOC), solid-state fermentation (SSF), *A. ibericus*, lipase production, aroma esters production

### Abstract

Agro-industrial residues are a good nutrient source which may be applied in biotechnological processes. The aim of this work was to optimize lipase production using *Aspergillus ibericus* MUM 03.49 in different oil cakes (OC) produced in Brazil, by solid-state fermentation (SSF) process; and to apply produced lipase in esterification reactions for aroma esters production.

SSFs were performed in 250 mL Erlenmeyer's flasks containing 15 g of OC. After lipase extraction, lipase activity was determined by spectrophotometric method, using *p*-nitrophenyl butyrate as substrate. Esterification reactions were performed in falcon tubes with 5 mL volume reaction. Conversion yield was determined by the titration of the residual acid content, using 0.1 M NaOH.

Highest lipase production of  $127 \pm 17$  U/g was obtained using palm kernel oil cake (PKOC) and the combination of PKOC with sesame oil cake (SOC) improved lipase production to  $328 \pm 6$  U/g. Optimization process led to  $460 \pm 38$  U/g of lipase, after 6 days of fermentation, at a ratio of 1:1.2 PKOC:SOC, at 57% moisture content (MC) and 1% (w/w) NH<sub>4</sub>Cl. The obtained lipase was used in esterification reactions, in a solvent-free system. Maximum conversion yield (100%) to aroma ester was observed using decanoic acid with butanol and 5% (w/v) of biocatalyst at pH 7 or 8, at 200 rpm and 37 °C for 24 h.

SSF of mixture of PKOC and SOC by *A. ibericus* is an interesting strategy that allows considerable amounts of lipase production that can be used in aroma esters production.

## 1- INTRODUCTION

Agricultural residues are produced in large amounts in many countries, being mainly used as animal feed or simply as landfills [1]. Oil cakes (OC) are agro-industrial by-products obtained after oil extraction from seeds [2]. OC have been reported to be good substrates for microbial enzymes production by solid-state fermentation (SSF), due to the residual nutritional content as carbon and nitrogen sources [2]. Particularly, OC can be used for lipase production, due to their residual oil content [3].

Solid-state fermentation (SSF) is a fermentation process involving the cultivation of microorganisms on moist solid substrate [5]. SSF is a technique of special economic interest for countries with abundant biomass and agro-industrial residues, as these can be used as cheap raw materials. *Aspergillus* species are filamentous fungi with relevant importance in biotechnological processes. *Aspergillus ibericus* is a fungus isolated from wine grapes from Portugal and Spain [6]. Previous studies reported its ability to produce lipase by submerged fermentation using olive mill wastewater [7], and by SSF using olive pomace [8–11].

Lipases (triacylglycerol acylhydrolases, EC 3.1.1.3) are efficient biocatalysts for the hydrolysis of water-insoluble fatty-acid esters. Lipases are widely used at industry with applications in food, fine chemicals, detergent, waste water treatment, cosmetics, pharmaceuticals, leather processing and biomedical assays. In addition, lipases also have important application in the field of bioenergy, especially in biodiesel production [1]. In recent years, increasing attention has been paid to the agro-industrial residues processing by SSF to obtain lipase with different applications, such as in aroma esters production, in a solvent-free system, that is of much commercial interest with the increasing demand of consumers for natural products [12].

This work deals with the optimization of lipase production by SSF of different OC from Brazil, using *Aspergillus ibericus* MUM 03.49. Also, lipase produced was used in enzymatic esterification reactions, in a solvent-free system, for aroma esters production.

## 2- MATERIALS AND METHODS

### Biological material

*Aspergillus ibericus* MUM 03.49 (MUM culture collection, Braga, Portugal) was used. *A. ibericus* was revived on malt extract agar (MEA) plates (2% (w/v) malt extract, 2% (w/v) glucose, 0.1% (w/v) peptone and 2% (w/v) agar) from a frozen glycerol stock. Spore suspension of the inoculum was prepared by adding peptone solution (0.1% (w/v) peptone and 0.001% (w/v) Tween 80) to seven-day-grown culture plates at 30 °C. The spore concentration of the suspension was adjusted to 10<sup>7</sup> spores/mL using a Neubauer counting chamber.

### OC used as substrates

Agro-industrial residues obtained from oil extraction, known as oil cakes (OC) were used. Andiroba oil cake (AOC), canola oil cake (CaOC), crambe oil cake (CrOC), cupuassu oil cake (CuOC), green coffee oil cake (GCOC), macauba oil cake (MOC), palm kernel oil cake (PKOC) and sesame oil cake (SOC), obtained from companies from Rio de Janeiro and São Paulo, Brazil, during the season 2013/2014, were used. Also, soy bean meal (SBM), from Rio de Janeiro, was used. Residues were ground and sieved to provide particle size  $\leq 1.19$  mm.

### SSF of OC for lipase production

SSF were carried out using cotton-plugged 250 mL Erlenmeyer flasks containing 15 g of substrate. The initial moisture content (MC) was adjusted to 60% (wet basis) by adding distilled water. Flasks were autoclaved at 121 °C for 15 min, cooled and inoculated with 0.5 mL of inoculum suspension. Flasks were incubated at 30 °C for 7 days of fermentation. All fermentations were performed in duplicate.

### SSF with combination of OC

Initially, the effect of each residue on lipase production was determined. Therefore, the best residue for lipase production, PKOC, was combined in a ratio of 1:1 (w/w) with CrOC, CuOC and SOC. Fermentations were carried out using 7.5 g of each OC mixed with 7.5 g of PKOC, at MC adjusted to 60%.

### SSF optimization for lipase production

A central composite design, with two factors and two levels with additional star points, from Statistica 7 software (StatSoft, Tulsa, USA) was used. MC ranged from 50%, 60% and 70%, and ratio of substrates PKOC:SOC ranged from 0.75:0.25, 0.5:0.5, and 0.25:0.75 (w/w). The central point was performed in triplicate, as presented in Table 2.

Polynomial equation was fitted to the experimental values of lipase activity using Statistica 7 software and best levels of MC and ratio PKOC:SOC which originated the maximum lipase activity were determined using Solver tool from Microsoft Excel 2010. The relationship between the dependent (lipase activity) and independent (MC and ratio PKOC:SOC) variables was established by the polynomial equation 1, as follows:

$$y = a_0 + a_1x_1 + a_2x_2 + a_{11}x_1^2 + a_{22}x_2^2 + a_{12}x_{12} \quad (1)$$

Where  $y$  is the predicted response,  $x_1$  and  $x_2$  are independent variables,  $a_0$  is the intercept,  $a_1$  and  $a_2$  are linear coefficients,  $a_{11}$  and  $a_{22}$  are the quadratic coefficients and  $a_{12}$  is the interaction coefficient.

Selection of nitrogen source ( $\text{NH}_4\text{Cl}$ ) concentration was studied in the range of 0% to 5% (w/w). And the kinetics of lipase production was followed by the time course profile monitoring over a period of 20 days of lipase production and productivity under the optimum SSF conditions determined.

### Lipase extraction and determination

At the end of the incubation period, enzymes were extracted by adding 112.5 mL of 1% (w/v) Triton X-100 (7.5 mL/g dried solid substrate) to the fermented substrates. They were homogenized at 250 rpm and 25 °C for 30 min, using a shaker. Homogenates were then centrifuged (10,000 x  $g$  and 10 min at 4 °C) and filtered using Whatman No. 1 filter paper. The resulting enzymatic extracts were immediately used for lipase determination. Lipase activity was determined by a spectrophotometric method, using a reaction mixture composed by 5  $\mu\text{L}$  of enzymatic extract and 300  $\mu\text{L}$  of 2 mM *p*-nitrophenyl butyrate in potassium phosphate 50 mM at pH 7.0. The absorbance was measured at 405 nm after reaction during 15 min at 37 °C. One unit of lipase activity (U) was expressed as the amount of enzyme which produced 1  $\mu\text{mol}$  of *p*-nitrophenol per minute, under the assay conditions. The analyses were performed in triplicate. Lipase activity obtained was expressed as units per gram of dry solid substrate (U/g).

### Esterification reactions catalysed by lipase

Esterification reactions were performed using different organic acids and alcohols. The mixture used was in a ratio 1:1 (molar) organic acid:alcohol in a total volume of 5 mL. Lipase used was obtained from SSF at optimum conditions achieved for 7 days of fermentation, without performing the extraction from the fermented solid substrate. Therefore, the biocatalyst used (the fermented substrate containing lipase) was frozen and lyophilized. Reactions occurred in 15 mL falcons with 5 mL of organic acid mixed with alcohol in a ratio 1:1 (molar), and 20% (w/v) (0.2 g/mL) of biocatalyst was added to the mixture. Falcons were incubated in a shaker at 200 rpm and 37 °C for 24 h. At the end of reaction time, 5 mL of 1:1 (v/v) acetone with absolute ethanol was added. Reaction yield (%) was determined by titration of the remaining organic acid with 0.1 M NaOH until achieving the pKa of the organic acid used. Esterification reactions were performed in duplicate.

Initially, different organic acids and alcohols were used. Since decanoic acid with butanol presented higher conversion yield (100%), this combination was used in further studies. The minimum lipase concentration to achieve 100% of conversion yield was determined, for a 24 h time reaction. For that, 20% (w/v), 10%, 5% and 1% of biocatalyst was used. Using 10% (w/v) of biocatalyst (minimum lipase concentration), a time course profile of conversion yield over reaction time was performed. Conversion yield was determined after 2 h, 4 h, 8 h, 16 h and 24 h of reaction time.

Finally, the effect of buffering during lyophilization on reaction yield was studied. For that, a low amount of lyophilized biocatalyst, 5% (w/v), was added to the eppendorf tubes and resuspended in 1 mL Britton Robinson universal buffer (0.04 M H<sub>3</sub>BO<sub>3</sub>, 0.04 M H<sub>3</sub>PO<sub>4</sub>, 0.04 M CH<sub>3</sub>COOH and 0.2 M NaOH) at pH of 5, 6, 7, 8 and 9. Eppendorf tubes were frozen and lyophilized; and after, the content was transferred to the mixture of decanoic acid with butanol to start the reaction.

### Statistical treatment

The data obtained were statistically analysed using SPSS (IBM SPSS Statistics, Version 22.0. Armonk, NY: IBM Corp.) to study the effect of variables on lipase production and on reaction yield. Data were tested for homogeneity, submitted to one-way analysis of variance (ANOVA) and a pair-wise multiple comparison procedure (Tukey test) at a confidence level of 95%.

## 3- RESULTS AND DISCUSSION

### SSF of OC for lipase production

Different OC were used for SSF to produce *A. ibericus* lipase. Results are presented in Table 1. Lipase produced was significantly affected ( $p < 0.0001$ ) by OC used. PKOC presented higher lipase production ( $127 \pm 17$  U/g), following by SOC ( $78 \pm 2$  U/g).

A screening of the combination of PKOC with three OC was performed. Combinations of three substrates presented significant effect ( $p < 0.001$ ) on lipase, where the use of PKOC+SOC led to a maximum production of  $328 \pm 6$  U/g, corresponding to a 2.6-fold increase. Also, the mixture PKOC+CrOC presented significant yields ( $272 \pm 23$  U/g). The combination of PKOC+CuOC led to a lipase production of  $88 \pm 3$  U/g. The combination of residues is attractive for the growth of microorganisms on SSF, since residues may act differently as support matrix and nutrient source for the production of enzymes [3].

Oil cakes	Lipase activity $\pm$ SD/(U/g)
AOC	1 $\pm$ 0 <sup>a</sup>
CaOC	47 $\pm$ 11 <sup>c</sup>
CrOC	44 $\pm$ 3 <sup>bc</sup>
CuOC	11 $\pm$ 1 <sup>a</sup>
GCOC	4 $\pm$ 0 <sup>a</sup>
MOC	1 $\pm$ 0 <sup>a</sup>
PKOC	127 $\pm$ 17 <sup>e</sup>
SOC	78 $\pm$ 2 <sup>d</sup>
SBM	19 $\pm$ 0 <sup>ab</sup>

Table 1: Results of lipase activity obtained using different oil cakes. Values are the mean of two individual fermentations  $\pm$  standard deviation (SD). Means with the same letter do not differ significantly at  $p > 0.05$  (T-test).

Using the best combination of OC, a study of MC and ratio of PKOC:SOC (RPKOC), represented by g of PKOC per g of total substrate, was performed to maximize lipase. A central composite design was used and results are presented in Table 2. Lipase production was significantly influenced ( $p < 0.05$ ) by MC and ratio PKOC:SOC. In fact, higher or lower levels of MC may affect significantly the biosynthesis of the enzymes.

Run	MC/(g/g)	RPKOC/(g/g)	Experimental LA $\pm$ SD/(U/g)
1	0.5	0.25	313 $\pm$ 16
2	0.5	0.75	122 $\pm$ 22
3	0.7	0.25	55 $\pm$ 7
4	0.7	0.75	69 $\pm$ 25
5	0.4586	0.5	208 $\pm$ 31
6	0.7414	0.5	69 $\pm$ 37
7	0.6	0.1464	172 $\pm$ 27
8	0.6	0.8536	198 $\pm$ 16
9	0.6	0.5	377 $\pm$ 31
10	0.6	0.5	330 $\pm$ 15
11	0.6	0.5	336 $\pm$ 11

Table 2: Factors and assigned levels in a central composite design. Experimental values of lipase activity (LA). Values are the mean of triplicate analysis  $\pm$  standard deviation (SD).

Polynomial equation as a function of moisture content (MC) and ratio of palm kernel oil cake: sesame oil cake (RPKOC), represented by g of PKOC per g of total substrate, was fitted to the experimental values of lipase activity (LA, U/g), as follows:

$$LA = -2929.5 + 11547.0MC - 11007.0MC^2 + 85.4RPKOC - 1387.7RPKOC^2 + 2052.3MC.RPKOC \quad (2)$$

The analysis of variance indicated a satisfactory fitting of the equation to the experimental data, presenting a coefficient of determination ( $R^2$ ) of 0.932, which explains 93.2% of the data variability. To set optimum SSF conditions, Solver tool was used to maximize lipase production through equation 1. A MC of 0.57 (57%) and a ratio of 1:1.2 PKOC:SOC (45% and 55% (w/w), respectively) were obtained, predicting a lipase production of 360 U/g. These results of MC and substrates ratio were similar to that found by Oliveira et al. [8] and [9], respectively, using *A. ibericus* to produce lipase by SSF of olive pomace with wheat bran.

At optimum SSF conditions obtained, a study of the supplementation with  $\text{NH}_4\text{Cl}$  on SSF was performed. In the range of values tested an optimum concentration of 1%  $\text{NH}_4\text{Cl}$  was obtained, yielding  $482 \pm 19$  U/g.

Finally, the time course profile of lipase and its productivity was monitored at optimized conditions (Fig. 1). After 6 days, a lipase production of  $460 \pm 38$  U/g was obtained, and it was slightly increasing till 20 days of fermentation, reaching  $578 \pm 20$  U/g. However, maximum productivity was obtained on the 6<sup>th</sup> day ( $3.2 \pm 0.3$  U/(g h)) and it was decreasing after that till  $1.2 \pm 0$  U/(gh). Time course profile of lipase and productivity was similar to the obtained by Oliveira et al. [9], using *A. ibericus* on olive pomace with wheat bran.

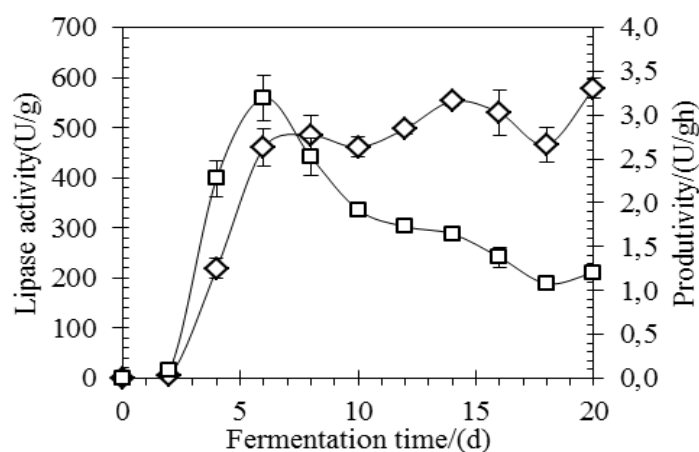


Figure 1: Profiles of lipase activity (—◇—) and its productivity (—□—) over fermentation time. Values are the mean of two individual fermentations  $\pm$  standard deviation.

#### Esterification reactions catalyzed by lipase

The high stability of lipase in organic solvents may allow its use in esterification reactions. Parameters conditions optimization such as substrate used, enzyme load, reaction time and buffering may increase aroma esters production catalyzed by lipase. Enzymatic esterification reactions were performed using 20% (w/v) of biocatalyst in the mixture of 5 mL 1:1 (molar) organic acid:alcohol, for 24 h. Maximum conversion yield ( $100 \pm 0\%$ ) was observed for the combination of decanoic acid with butanol. Also, butyric acid with menthol presented high conversion yield ( $93 \pm 11\%$ ). The polarity of the reaction mixture may affect lipase activity. Substrates with high polarity may inhibit enzyme activity [13]. Decanoic acid and menthol presents high  $\log P$  (between 3 and 4), contributing to lower polarity of the reaction mixture, increasing reaction yield. Therefore, the mixture of decanoic acid with butanol in esterification reactions was used in further studies, to obtain butyl decanoate ester. Butyl decanoate ester is a colorless liquid with a characteristic odor to whiskey, and can be applied in food industry, to various formulations requiring a dairy product/creamy note [14].

From an economic perspective, achieving high conversion yield using a low amount of enzyme in the reaction is important. Therefore, the effect of the biocatalyst load on esterification reaction of decanoic acid with butanol, for a 24 h reaction time was studied. Results showed that a minimum of 10% (w/v) of biocatalyst was enough to obtain the total conversion yield ( $100 \pm 0\%$ ) (Table 3a). A time course profile of conversion yield over reaction time was performed, using 10% (w/v) of biocatalyst in the mixture of 5 mL 1:1 (molar) of decanoic acid with butanol. The conversion yield increased steadily, obtaining maximum conversion (100%) after 24 h reaction.

It has been reported the positive effect of the buffering in the stability and activity of the biocatalyst, improving substantially the conversion yield of the reaction. The biocatalyst lyophilized in universal buffer at pH of 7 and 8 led to the maximum conversion yield (100%), using 5% (w/v) of biocatalyst (Table 3c).

Conditions/ Conversion yield $\pm$ SD/(%)						
a) Biocatalyst load/(%)	1	5	10	20	-	-
Conversion yield $\pm$ SD/(%)	$2 \pm 1^a$	$22 \pm 1^b$	$100 \pm 0^c$	$100 \pm 0^c$	-	-
b) Time reaction/(h)	0	2	4	8	16	24
Conversion yield $\pm$ SD/(%)	$0^a$	$28 \pm 11^b$	$25 \pm 7^b$	$58 \pm 4^c$	$68 \pm 4^c$	$100 \pm 0^d$
c) Universal buffer pH	5	6	7	8	9	-
Conversion yield $\pm$ SD/(%)	$64 \pm 6^a$	$71 \pm 6^{ab}$	$100 \pm 0^c$	$100 \pm 0^c$	$82 \pm 2^b$	-

Table 3: Conversion yields of esterification reaction of decanoic acid with butanol at 200 rpm and 37 °C a) using different biocatalyst load, after 24 h of reaction; b) over reaction time using 10% (w/v) biocatalyst; and c) using 5% (w/v) biocatalyst lyophilized in universal buffer at different pH, after 24 h of reaction. Values are the mean of two individual experiments  $\pm$  standard deviation (SD). Means with the same letter do not differ significantly at  $p > 0.05$  (T-test).

#### 4- CONCLUSIONS

This study presented an interesting alternative to produce lipase using agro-industrial residues. Different combinations of agro-industrial residues led to different lipase yields. Mixing PKOC with SOC is a promising strategy to obtain higher lipase yields while treating wastes. Optimizing SSF conditions led to a final lipase production of  $460 \pm 38$  U/g, and maximum productivity of  $3.2 \pm 0.3$  U/(g h) after 6 days of fermentation, using a ratio of 1:1.2 PKOC:SOC, at 57% MC and 1% (w/w)  $\text{NH}_4\text{Cl}$ . Lipase produced was able to produce butyl decanoate ester in a solvent-free system. The optimization process led to the use of 5% (w/v) of biocatalyst lyophilized in universal buffer at pH 7 or 8 for 24 h in the esterification reaction of decanoic acid with butanol, in a solvent-free system.

#### ACKNOWLEDGMENTS

Felisbela Oliveira acknowledges the financial support from FAPERJ N° 43/2013 – Programa de Apoio ao Doutorado-Sanduiche Reverso – 2013, from Rio de Janeiro, Brazil and from Fundação para a Ciência e Tecnologia (FCT) of Portugal through the grant SFRH/BD/87953/2012. The authors thank the FCT Strategic Project of UID/BIO/04469/2013 unit, the project RECI/BBB-EBI/0179/2012 (FCOMP-01-0124-FEDER-027462) and the



project “BioInd - Biotechnology and Bioengineering for improved Industrial and Agro-Food processes”, REF. NORTE-07-0124-FEDER-000028, co-funded by the Programa Operacional Regional do Norte (ON.2 – O Novo Norte), QREN, FEDER. Carlos E. Souza and Verônica R.O. Lopes thank the financial support given by CNPq and CAPES from Brazil, respectively. Maria A.Z. Coelho thanks the financial support given by CNPq and FAPERJ from Brazil.

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