I COBBIND
I CONGRESSO BRASILEIRO DE BIOTECNOLOGIA INDUSTRIAL
XXIV SINAFERM ·· XV SHEB ·· XV ENZITEC FLORIANÓPOLIS ·· 2024
Creating connections between biotechnology and industrial sustainability
August 25 to 28, 2024
Costão do Santinho Resort, Florianópolis, SC, Brazil
INDUSTRIAL ENZYMOLOGY

PRODUCTION OF NATURALLY IMMOBILIZED LIPASE ON CELLULAR DEBRIS OF *Yarrowia lipolytica* USING RESIDUAL FRYING OILS (RFO)

Rafaelle Franson[1], Adejanildo S. Pereira[2] & Priscilla F.F. Amaral[2]*

[1] *Programa de pós-graduação em Ciência de Alimentos/ Chemistry Institute/ Federal University of Rio de Janeiro, RJ, Brazil.*
[2] *Biochemical Engineering Department, School of Chemistry, Federal University of Rio de Janeiro, Rio de Janeiro, Brazil.*
** Corresponding author's email address: pamaral@eq.ufrj.br*

ABSTRACT

Residual frying oils from different sources (olive, cottonseed and soybean oils) were tested for the production of lipases from *Y. lipolytica* in comparison to its original oils (non-fried oils). The naturally immobilized lipase attached to cell debris of *Y. lipolytica*, referred to as LipImDebris, reached its maximum activity after 15 h of cultivation with all the oils tested as carbon source. The activity was higher for non-fried oils, with cottonseed oil exhibiting the highest production (718.40 U/g). This production was five times greater than the lipolytic activity of whole cells, justifying the ultrasonication treatment to obtain cell debris. No significant difference in LipImDebris production was identified among the residual frying oils (RFO), which is important since residual oils are usually mixed when collected from restaurant and houses. These characteristics highlight that RFOs serve as effective inducers for LipImDebris production and underscore the potential of these biocatalysts for industrial applications.

Keywords: Cell debris; Lipase; *Yarrowia lipolytica*; Residual frying oils

1 INTRODUCTION

Yarrowia lipolytica has been widely studied due to its high capacity to produce intracellular, membrane-bound, and extracellular lipases, which are very effective in the degradation of various substrates[1]. Enzymes linked to the cell membrane, also considered naturally immobilized, are advantageous for industrial use, as they do not require support or additional steps for immobilization, which reduces process costs[2]. The industrial use of lipases is still limited due to their high production cost, which means that these biocatalysts have a high commercial value. Cost reduction strategies usually involve using agro-industrial waste as raw material in the production stage[3], with residual frying oil being a successful strategy to produce lipases from *Y. lipolytica*. Residual frying oils (RFO) are generated from edible vegetable oils after consecutive uses under heating at high temperatures (160 °C to 200 °C), releasing toxic chemical substances. RFOs are composed of triacylglycerols (TAGs) that undergo many physical and chemical modifications, and these toxic compounds are formed through oxidation, hydrolysis, and polymerization reactions of TAGs, making them unsuitable for consumption and leading to improper disposal by the population. This is a major global environmental problem because RFOs are pollutants that damage land and water resources[4]. The use of RFO has been a successful strategy in the production of lipases from *Y. lipolytica*[2], but the influence of the vegetable oil type has not been evaluated yet.

Therefore, the objective of this work was to explore the production of natural immobilized *Y. lipolytica* lipases - lipases immobilized in cellular debris (LipImDebris) - in media containing different residual vegetable oils (cotton, soy, or olive) obtained after frying foods under controlled conditions in comparison to the non-fried vegetable oils.

2 MATERIAL & METHODS

Residual frying oils (RFO): RFOs were obtained after frying (200°C) frozen potatoes (McCain®) five times with vegetable oils (cotton, soy, or olive) for 4 min.

Lipase production: A strain of *Yarrowia lipolytica* IMUFRJ 50682 isolated from Guanabara Bay was used and stored in YPD medium containing agar (1% yeast extract, 2% peptone, 2% glucose) at a temperature of 4 ° C. For the pre-inoculum, the cells were cultivated at 28 °C and 160 rpm, in 500 mL Erlenmeyer flasks containing 200 mL of YPD medium with 1% vegetable oil for 48 h or 72 h. The pre-inoculum cells were used to inoculate (1 g/L of cells, in dry mass) 100 mL of YP* medium (1% yeast extract and 0.64% peptone) with 1% virgin oil (olive, soy, or cotton) or residual frying oils (olive, soy, or cotton), in 500 mL-Erlenmeyer flasks. These flasks were incubated in shakers at 28°C, 250 rpm, for 72 h.

Lipase fractions: Extracellular lipase - Samples (20 mL) of the production medium were centrifuged (4ºC, 3000 rpm for 5 minutes) to obtain the crude extract rich in lipase in the supernatant. **Cellular debris lipase (LipImDebris) and intracellular lipase**: The cell precipitate from the extracellular lipase samples (20 mL) was washed with distilled water, centrifuged (4ºC, 3000 rpm for 5 minutes), washed with MOPS (3-(N-morpholino) propanesulfonic acid) buffer pH 7 and centrifuged in the same conditions. After washing, the cells are resuspended in 20 mL of MOPS buffer and sonicated in an Ultrasonic Cleaner (2 cycles of 9 min in an ice bath at 30% power). After this procedure, a new centrifugation is performed, resulting in the intracellular extract (supernatant) and LipImDebris, which is the precipitate. **Whole-cell lipase and cell wash lipase:** Samples (1.5 mL) from the production medium were centrifuged (4ºC, 3000 rpm for 5 minutes), and the cells were washed 3 times with 0.5 mL of phosphate

buffer (50 mM and pH 7). The buffer from the three washes is kept in a single tube to determine the activity of the cell wash, and the cells are resuspended in phosphate buffer to measure whole-cell lipase[5].

Determination of cell growth: Cell growth was monitored through absorbance measurements on a spectrophotometer (Shimadzu Model UV-1800) at 570 nm using a previously established conversion factor to obtain the dry cell weight per liter (g dcw/L).

Determination of lipase hydrolysis activity: The hydrolysis of p-nitrophenyl laurate (560 µM) dissolved in 50 mM phosphate buffer (pH 7.0) at 37 °C by the enzyme is monitored for 100 s at 410 nm in a spectrophotometer (Shimadzu Model UV-1800). The activity unit (U) is defined as the amount of enzyme that releases 1 µmol of p-nitrophenol (pNP) per minute at pH 7.0 and 37 °C[2].

3 RESULTS & DISCUSSION

Initially, it was investigated whether the pre-inoculum cultivation time would influence cell growth and LipImDebris production. Although the LipImDebris values obtained in the cultivation with olive oil with cells from a 72-h pre-inoculum were slightly higher than those of a 48-h pre-inoculum, there were no significant differences. Extracellular and whole-cell lipase fractions benefited from the shorter periods of pre-inoculum. However, a cultivation for 72 h was established for the pre-inoculum for practical purposes.

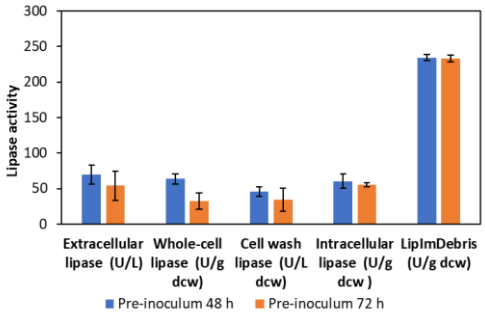

Figure 1. Production of lipases by *Y. lipolytica* grown for 16 h in a medium with virgin olive oil at 250 rpm, 28 °C from a pre-inoculum of 48 h or 72 h. The values represent the mean and standard deviation between three cell growth cultures. There was no significant difference between the two pre-inoculum times (p=0.07).

Cell growth kinetics were performed to identify the best vegetable oil and the best cultivation time to produce LipImDebris, with non-fried oils and with RFO's (Figures 2 and 3). Similar kinetic profiles were observed for non-fried oils: cell growth or lipase fractions (Figure 2). Higher lipase activity values were found after 15 hours of cultivation in all media. There is a reduction in lipase activity with the use of RFO's (Figure 3), compared to their respective vegetable oils (Figure 2). For LipImDebris, activity is reduced by half for olive and soybean oils and 80% for cottonseed oil. Even with the reduction in activity, very relevant activity values were found (224.9 U/g dcw for olive RFO; 158.9 U/g dcw for cotton RFO and 314.6 U/g dcw for soy RFO) and can be justified by the reduction in process costs. Nunes et al.[2] produced 210 U/g dcw for lipase associated to cell debris of *Y. lipolytica* with residual soybean oil from a restaurant.

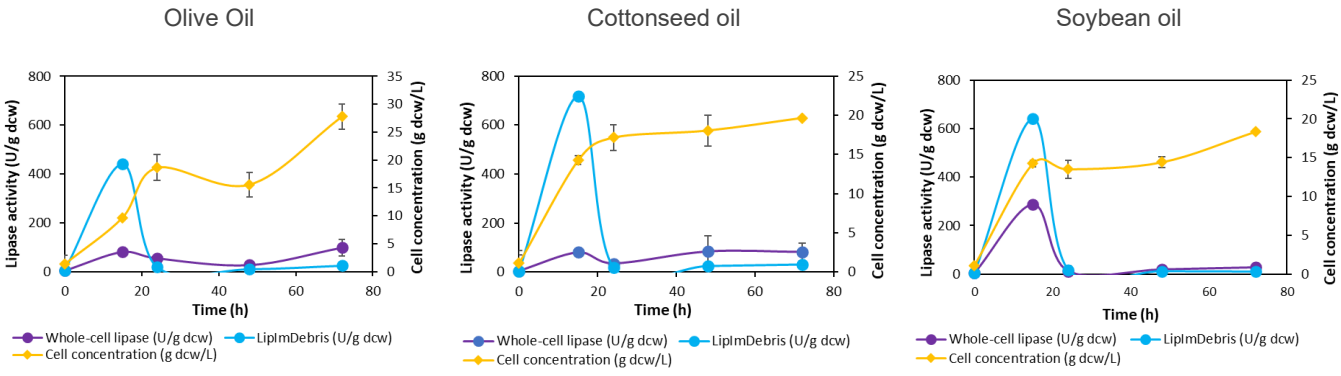

Figure 2 Kinetics of cell growth and lipases production by *Y. lipolytica* in media with non fryed oils: olive, cotton, or soybean, incubated at 250 rpm, 28 °C, for 72h. The values represent the mean and standard deviation between three cell growth cultives.

For all oils tested (fried and non-fried) it is possible to observe that LipImDebris activity is higher than the whole cell activity (Figures 2 and 3). Althought whole-cell lipase would have a reduced cost for its production since no ultrasound treatment would

be needed, the presence of viable cells during the biocatalyst application could have a negative impact in the reaction medium, with side reactions and by-products from microbial metabolism. Fraga et al.[6] have shown that non-viable cells are found in LipImDebris.

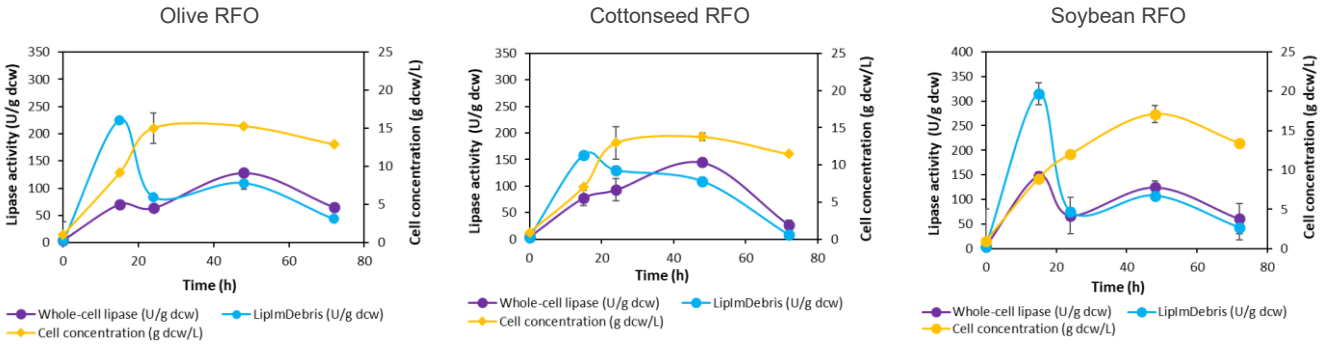

Figure 3 Kinetics of cell growth and lipases production by *Y. lipolytica* in media with residual frying oils (RFOs) olive, cotton, or soybean, incubated at 250 rpm, 28 °C, for 72 h. The values represent the mean and standard deviation between three cell growth cultives.

Y. lipolytica is generally isolated from media containing lipid carbon source and is therefore particularly adapted to hydrophobic substrates. The production of lipases is intense in the presence of hydrophobic substrates, such as oils, fatty acids, and methyl esters[1]. Fatty acids with 16 to 22 carbons, generally present in vegetable oils (olive, soybean, sunflower, among others) are excellent inducers of the production of these enzymes, as they are compounds of oleic and linolenic acid that generate high levels of lipase activity when used as a source carbon[7].

4 CONCLUSION

Based on the results obtained, it can be concluded that the production of LipImDebris by *Y. lipolytica* in a medium containing olive oil as an inducer is not influenced by the pre-inoculum age. The type of vegetable oil does not seem to influence cell growth or lipases production, but the fried version of these oils reduced LipImDebris production. The maximum production of this fraction occurs after 15 h of cultivation. The activity of LipImDebri was up to five times greater than that of whole-cell lipase, when cultivated with olive RFO or soy RFO, which justifies the ultrasound treatment to obtain it. RFOs from different vegetable oils are excellent inducers for LipImDebris production by *Y. lipolytica*.

REFERENCES

1 BRÍGIDA, A. I., AMARAL, P. F., COELHO, M. A., & GONCALVES, L. R. Lipase from Yarrowia lipolytica: Production, characterization and application as an industrial biocatalyst. Journal of Molecular Catalysis B: Enzymatic, 101, 148-158, 2014.

2 NUNES, PATRÍCIA M. B.; FRAGA, JULLY L.; RATIER, RAFAEL B.; ROCHA-LEÃO, MARIA HELENA M.; BRÍGIDA, ANA I. S.; FICKERS, PATRICK; AMARAL, PRISCILLA F. F. Waste soybean frying oil for the production, extraction, and characterization of cell-wall-associated lipases from Yarrowia lipolytica. BIOPROCESS AND BIOSYSTEMS ENGINEERING. , v.44, p.809 - 818, 2021.

3 FRAGA, JL, SOUZA, CPL, PEREIRA, AS ET AL. Palm oil waste as raw material for lipase production by Yarrowia lipolytica and application/reuse of biocatalysts. 3 Biotechnologies 11 , 191 (2021). https://doi.org/10.1007/s13205-021-02748-1

4 GUIRRA, P. D. DOS S.., & BARBOSA, A. A. (2023). Environmental awareness through the reuse of frying oil in soap production. Brazilian Journal of Environmental Education *(RevBEA)*, *18*(7), 403–416. https://doi.org/10.34024/revbea.2023.v18.15041

5 FICKERS, P., FUDALEJ, F., LE DALL, M. T., CASAREGOLA, S., GAILLARDIN, C., THONART, P., & NICAUD, J. M. Identification, and characterization of LIP7 and LIP8 genes encoding two extracellular triacylglycerol lipases in the yeast Yarrowia lipolytica. Fungal genetics and biology, 42(3), 264-274, 2005a.

6 FRAGA JL, DA PENHA ACB, AKIL E, SILVA KA, AMARAL PFF. Catalytic and physical features of a naturally immobilized *Yarrowia lipolytic* lipase in cell debris (LipImDebri) displaying high thermostability. 3 Biotech. 2020 Oct;10(10):454. doi: 10.1007/s13205-020-02444-6.

7 TAN, T.; ZHANG, M.; WANG, B.; YING, C.; DENG, L. Screening of high lipase producing Candida sp. and production of lipase by fermentation. Proc. Biochem., v. 39: 459-465, 2003.

ACKNOWLEDGEMENTS

We would like to thank the funding agencies: CNPQ and CAPES.