Synergistic action of Penicillium camemberti and Yarrowia lipolytica lipases in milk fat hydrolysis.

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ABSTRACT

Lipolyzed Milk Fat (LMF) is a very important ingredient in food industry by acting as an additive (taste and aroma) in bakery, dairy and candy products. LMF can be produced by enzymatic route using lipases. Lipases from microorganisms are capable of promoting the hydrolysis of milk fat and can be obtained by fermenting processes with yeasts, such as *Yarrowia lipolytica*. In the present work, a commercial lipase obtained from *Penicillium camemberti* was tested for LMF production, as well as the enzyme extract produced in a 3L-bioreactor by *Y. lipolytica*. The highest lipase activity detected during *Y. lipolytica* growth was 6.278 U/L in 51 h. Different esters chain length were hydrolyzed by the lipases of *Y. lipolytica* (LipYl) and P. camemberti (LipPc) in two distinct type-emulsions (Arabic gum or Tween 20). Higher activity (5.890 U/L) was observed in the hydrolysis promoted by *Y. lipolytica* in milk fat emulsion with Arabic gum. The combination of both enzymes was effective in promoting hydrolysis in Arabic gum (1.77 Mol of LMF). The aim of this study was to evaluate a possible synergistic action between the lipases of *P. Camemberti* and *Y. lipolytica* in the hydrolysis of milk fat, in order to generate a LMF with a greater variety of fatty acids short chain. Thus, through this work it was possible to estimate a possible complementary action of lipases that can generate a greater range of fatty acids during the production of LMF.

Keywords: *Enzymes. Lipolytic activity. Lipolyzed Milk Fat. Biotechnology. Microorganisms.*

RESUMO

A gordura de leite lipolisada (GLL) é um ingrediente importante na indústria de alimentos atuando como um aditivo (intensificador de aroma e sabor) em produtos de panificação, laticínios e doces. A GLL pode ser produzido por via enzimática usando lipases produzidas por microrganismos, estas enzimas são capazes de promover a hidrólise da gordura do leite. As lipases podem ser obtidas através de processos fermentativos com leveduras como a Yarrowia lipolytica. *Neste trabalho uma lipase comercial de* Penicillium camemberti *foi usada na produção de GLL, bem como o extrato enzimático produzido em biorreator por* Y. lipolytica. *A maior atividade enzimática detectada durante o crescimento de* Y. lipolytica *foi de 6.278 U/L em 51 h. Diferentes comprimentos de cadeia de ésteres foram hidrolisados pelas lipases de* Y. lipolytica *(LipYl) e* Penicillium camemberti *(LipPc) em duas emulsões distintas (goma arábica ou Tween 20). A maior atividade (5.890 U/L) foi observada na hidrólise com LipYl na emulsão de gordura de leite com goma arábica. A combinação das enzimas foi eficaz na hidrólise em goma arábica (1,77 mol de LMF). O objetivo deste estudo foi avaliar uma possível ação de sinergismo entre as lipases de* P. Camemberti *e* Y. lipolytica *na hidrólise da gordura de leite anidra afim de gerar um GLL com uma maior variedade de ácidos graxos de cadeia curta. Sendo assim, através deste trabalho foi possível estimar uma possível ação complementar das lipases que podem gerar uma gama maior de ácidos graxos durante a produção de GLL.*

Palavras-chave: Enzimas. Atividade lipolítica. Gordura de Leite Lipolisada. Biotecnologia. Microrganismos.

1 Introduction

Milk and dairy products in general are traditional elements in the human diet. Milk is composed of a balanced amount of fats, proteins, sugars, vitamins and minerals (BALCÃO & MALCATA, 2002; HUPPERTZ *et al.*, 2009). Milk fat can be found in cow's milk in concentrations of 3.5-5% (m/m) (MACEDO & XAVIER, 2015). However, there is a great rejection in the use of milk fat by consumers, due to the difficulty of handling this material, which has low spreading in refrigeration temperature (it becomes rigid) and the high concentration of saturated fat, something that is commonly associated with cardiovascular diseases (LUBARY *et al.*, 2011). Scientists have promoted a global research effort to find an alternative way to use milk fat that has great nutritional value, (LUBARY *et al.*, 2011), which has a good and rich chemical composition, including a range of bioactive compounds and substances beneficial to health. Milk fat has huge potential for the food industry, about 160 million tons of milk fat has been thrown away every year around the world (FAO, 2012). One way to utilize this lipid fraction of milk is by performing chemical or enzymatic modifications by interesterification or hydrolysis using chemical or enzymatic catalysis.

Lipolyzed Milk Fat (LMF) has a great applicability as a vehicle for the aroma and flavor of cheese, and this is a convenient ingredient for some processed foods, for several types of preparations being able to intensify the flavor in low concentration incorporations (2% w/w) (REGADO *et al*, 2007). LMF was one of the first flavor produced with milk fat by lipase in large-scale microbiological process (fermentation). This process may be performed by enzymatic hydrolysis of milk fat using lipase.

Enzymatic reactions in milk fat to obtain LMF can be used to create a range of characteristic cheese aromas that can be incorporated into a variety of products such as ice cream and industrial cheese (DE GREYT, 1995; ALLUE *et al.*, 2008). Enzymes from different sources have been widely used commercially to promote enzymatic catalysis of LMF. This reaction is capable of chemically redesigning milk fat in order to generate chemical, physical and / or nutritional changes in its properties. Lipase-catalyzed reactions have smoother conditions than chemical pathways, so they are more likely to be successful in not promoting drastic changes in aromatic fatty acids (DE GREYT, 1995).

Lipases (glycerol ester hydrolases E.C. 3.1.1.3.) have emerged as one of the leading biocatalysts because of the possibility to catalyze ester bond, hydrolysis reactions of triacylglycerols as well as synthesis reactions. Additionally, it stands out for its ability to remain stable at more extreme temperatures, pH, organic solvents and still possess chemo-, regio- and enantioselectivity (VERMA *et al.*, 2012). The choice of the enzyme is a critical step, as it will determine the profile of the fatty acids generated and consequently the taste and aroma of the product obtained. These enzymes catalyze the partial and selective hydrolysis of fats in a mixture of free fatty acids, generating aromatic compounds or precursors to them.

Microorganisms such as fungi and bacteria have a great potential for extracellular lipase production (TREICHEL *et al.*, 2010). *Yarrowia lipolytica* presents an excellent performance in fermentative processes and has great ability to degrade hydrophobic substrates very efficiently secreting metabolites of industrial interest in the extracellular medium (RUIZ-HERRERA & SENTRANDEU, 2002). Lipases can be produced by *Y. lipolytica* in different ways, including intracellular, cell-bound, and extracellular enzymes (NUNES *et al.*, 2014). The production of these enzymes is dependent of medium composition and environment condition (PEREIRA-MEIRELLES *et al.* 2000; LOPES *et al.* 2008).

Residual frying oil is produced in large quantities both in homes and in restaurants and fast-food chains, creating a major environmental problem due to disposal in river networks (LUO *et al.*, 2013). Residual oils of industrial origin have already been widely used as inducers or alternative carbon sources in the production of metabolites of microbial origin (LUO *et al.*, 2013). One of the examples of oil residues that can be reused in fermentative processes are: oil mill effluents (MERCADAME *et al.*, 1993), oil refinery residues (BEDNARSKI *et al.*, 2004), whey (DUBEY *et al.*, 2001), effluents from the processing of potatoes, soap (BENINCASA *et al.*, 2002) and even oils used in food frying (RAZA & KHAN, 2006) and the olive residual frying oil (NUNES *et al.*, 2015). All these residual oils previously mentioned have already been used in the production of biosurfactants by microorganisms. In this study, residual frying oil was used to induce lipase production in the fermentation process with *Yarrowia lipolytica*. This has been a viable way to use oils that have passed through the successive frying process and can no longer be used.

Other microorganisms that are known for their ability to produce lipase are those of the Penicillium species. The lipolytic activity of *P. camemberti* can be clearly seen through the white surface formed on Brie and Camembert cheeses. (MEGHWANSHI & VASHISHTHA, 2018). Lipases from *P. camemberti* are specific for mono- and diacylglycerols (RIVERA-MUÑOZ *et al.*, 1991).

Thus, the objective of this study was to evaluate *P. camemberti* and *Y. lipolytica* lipases for milk fat hydrolysis separately and in cooperation and also to characterize these lipases for substrate specificity by hydrolysis of short (C2 and C4), medium (C8 and C12) and long (C16 and C18) chains p-nitrophenyl esters.

2 Materials and methods

2.1 Materials

Residual Frying oil (RFO) was used as an inducer in the production of lipase and was donated by Brazil Fast Food Corporation, from a fast food restaurant.

Preparation of anhydrous milk fat - Pasteurized unsalted non-fermented butter was pre-treated according Kalo *et al*. (1990) with some modifications (BALCÃO *et al.*, 1997).

Penicillium camemberti lipase was obtained from Sigma Aldrich and used to hydrolyze milk fat (this enzyme was chosen for use in comparison with *Y. lipolytica* lipase performance according Regado *et al.*, 2007). *Yarrowia lipolytica* IMUFRJ 50682 was used to produce lipase (HAGLER and MENDONÇA-HAGLER, 1981). Cells were stored at 4 °C after 24h growth in test tubes with YPD (Yeast Extract, Peptone, Dextrose) medium containing (w/v): 1% yeast extract, 2% peptone, 2% glucose and, 2% Agar (Amaral, 2006).

2.2 Lipase production in bioreactor

Lipase production was performed in a New Brunswick MF-114 Microferm reactor, with 3 L of medium (6.4 g / L peptone and 10g / L yeast extract), 2 mL of antifoam and 75 mL of crude RFO. *Y. lipolytica* cells were inoculated in sufficient amount to achieve 1 g dry weight (d.w) of cells/L (Nunes, 2014). Fermentation was conducted with Rushton type stirrers, aeration at 1.5 L/min, with mechanical agitation (650 rpm, 28 °C) for 72 h. Samples were collected at 0, 3, 6, 24, 27, 30, 48, 51, 54 and 72 h and centrifuged at room temperature for 10 min at 2016 g. The enzymes used in this study were a crude extracellular extract of *Yarrowia lipolytica* (LipYl) and a 10mg/mL solution of a commercial *Penicilium Camemberti* lipase (LipPc).

2.3 Lipase activity

Spectrophotometer method - Hydrolysis of p-nitrophenyl laurate (pNF-laurate) with absorbance change at 410 nm in a spectrophotometer (Shimadzu Model UV-1800) was performed with 1.9 mL of 560 µM pNP-laurate dissolved in 50 mM phosphate buffer (pH 7.0) containing 1% (v/v) dimethylsulfoxide (DMSO) mixed with 0.1 ml of enzyme at 37 °C (NUNES *et al.*, 2014). One unit of lipase activity is defined as the amount of enzyme that produce 1 mol of fat acid per minute.

Titrimetric method - Lipase activity was performed using 5 ml of 20% (v /v) olive oil emulsion in 5% (w/v) gum arabic and 2 ml of 50 mM potassium phosphate buffer, pH 7.0, at 37°C (DELLMORA-ORTIZ *et al.* 1997). Fifteen minutes after adding 3 ml of the enzyme preparation, the reaction is stopped by adding 10 ml of an ethanol and acetone mixture (1: 1). The fatty acids formed are quantified by titration against 0.025 N NaOH, using phenolphthalein as an indicator. The reaction blank was made with the enzyme inactivated by ethanol-acetone mixture. One unit of enzyme activity (1U) corresponds to the amount of enzyme that produces 1 µmol of fatty acid per minute, under the test conditions.

2.4 Specificity of Yarrowia lipolytica and Penicillium camemberti lipases in different substrates

In order to evaluate the affinity of lipases for different substrates the hydrolysis reactions of p-nitophenyl (pNF) esters with different chain lengths were performed. The ester used were pNF acetate (C2), pNF butyrate (C4), pNF caprylate (C8), pNF laurate (C12), pNF palmitate (C16) and pNF stearate (C18). Substrates (p-NFL) were prepared by solubilizing 0.018 g of each ester in 1 mL of Dimethylsulfoxide (DMSO) (560 µM) then diluted in 100 mL of potassium phosphate buffer (50mM) pH 7.0, pH 7 at 37 °C. Then the enzymatic activity obtained for the different esters was evaluated the hydrolyse of different pNF esters.

2.5 Milk fat hydrolysis reaction by *Y. lipolytica* and *P. camemberti*

The hydrolysis reactions of milk fat with *Y. lipolytica* and *P. camembert* lipases was performed at 37 °C in a 250-rpm stirring shaker for 5 hours with 5 mL of emulsion of anhydrous milk fat 20% (v / v) and 3 mL of enzymes (LipPc and/or LipYl) for production of lipolyzed milk fat (LMF) according to the method adapted from Regado *et al.* 2007.

In order to establish the percentage of hydrolysis obtained using the two enzymes, the titration method was performed as follows: using 5 mL of 20% (v / v) anhydrous butterfat in 5% (w / v) Arabic gum (AG) or Tween 20 (T20) and 2 mL of 50 mM potassium phosphate buffer, pH 7.0, at 37 °C (DELLAMORA-ORTIZ *et al.*, 1997). The amount of free fatty acids (Mol) generated during the lipolysis of anhydrous milk fat by enzymes was termed as the LMF formed, according to equation 1.

$$A=\frac{[(V_a-V_b)*M*D*1000000]}{\Delta_t*V_e} \quad (1)$$

A = enzyme activity (U / L), where 1 U is the amount of enzyme capable of releasing 1 µmol of fatty acid per minute of reaction.

Va = Volume of NaOH used in L to titrate the reaction system after a given reaction time.

Vb = Volume of NaOH used in L to titrate the reaction system before the reaction - blank;

M = molar concentration of NaOH used, mol / L;

D = Sample dilution factor;

t = reaction time in minutes;

Ve = Volume of enzyme used, in L;

3 Results and discussions

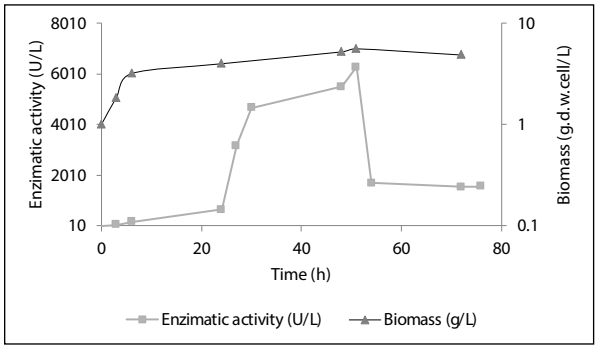
3.1 Cultivation of *Yarrowia lipolytica* with residual frying oil

Y. lipolytica was cultivated in 0.5% (v/v) RFO as an inducer, which showed no toxicity to cells, since growth was observed as well as lipase production (figure 1).

Figure 1 shows an exponential growth phase in the first 10 h of culture. The results obtained in this study are similar to those found by Botelho (2014). This author also performed the fermentation with *Y. lipolytica*, in YP medium (Peptone and yeast extract) containing olive oil and residual frying oil (1%) as inducers in the production of lipase.

The exponential growth phase is probably related to the consumption of long chain fatty acids (mainly C18) from enzymatic hydrolysis of triglycerides as described by Pereira-Meirelles *et al.* (2000). Low extracellular activity was reported by Nunes (2014) which might be related to the use of an RFO from soybean oil. In the present study, RFO was originated from a palm oil.

Figure 1 – *Yarrowia lipolytica* growth profile and lipase production with 0.5% (v/v) residual frying oil (OFR) in a 4 L Bioreactor with 3.5 L of YP + OFR medium at 500 rpm and 28 ° C.



Diauxia was observed in the growth curve using Bob's OFR as an inducer (19 to 24h) and in the study by Nunes (2015). This probably occurred due to the sequential consumption of fatty acids and glycerol obtained from the hydrolysis of triglycerides present in this residue by *Y. lipolytica*. This phenomenon of diauxia is intrinsically linked to the composition of the oil, since Kamzolova *et al.* (2011) who carried out the fermentation with *Y. lipolytica* using canola oil as an inducer did not report diauxic growth, in this study oleic acid and glycerol were consumed simultaneously.

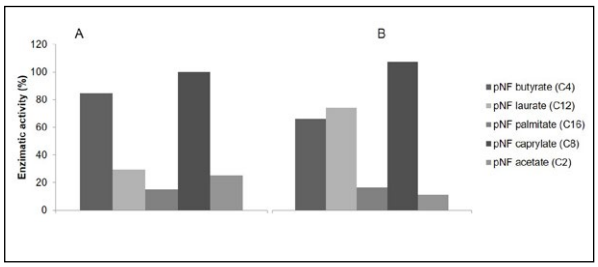
Highest cell concentration was observed at 48 h (5.65 g.d.w.cell/ L), which is lower than commonly found in previous studies in shake flasks (data not shown). It was probably due to cell adhesion to oil, which caused the migration of the cells to the oil fraction. In Amaral *et al.*, (2006) study was observed the same behavior for the *Y. lipolytica* cells that tends to remain attached to hydrophobic surfaces. Extracellular lipase production occurred at the stationary phase of growth, that probably coincides with the carbon source depletion (PEREIRA-MEIRELLES *et al.* 2000).

3.2 Specificity of Yarrowia lipolytica and Penicillium camemberti lipases towards different substrates

According to Balcão and Malcata's (1998) *Penicillium camemberti* lipase has higher specificity towards short chain fatty acids, which is good for LMF production.

Specificity of substrate and enzyme is so important for estimate the type of fat acid formed after hydrolysis, for this the quantification of enzymatic activity by the spectrophotometric method was made for the pNF-acetate (C2), pNF-butyrate (C4), pNF-caprylate (C8), pNF-laurate (C12) and pNF-palmitate (C16) substrates. This study evaluated the specificity of enzymes for carbon chain lengths of different esters (figure 3). LipPc showed higher enzymatic activity towards pNF-butyrate (4703.9 U/L) and pNF-caprylate (5569.1 U/L) indicating the greatest potential of this enzyme to catalyze the hydrolysis of C4 to C8 substrates (figure 2).

Figure 2 – Hydrolytic activity determined by the spectrophotometric method with different esters (C2-C16) for lipase *P. camemberti* (A) and *Y. lipolytica* (B) produced in OFR medium.

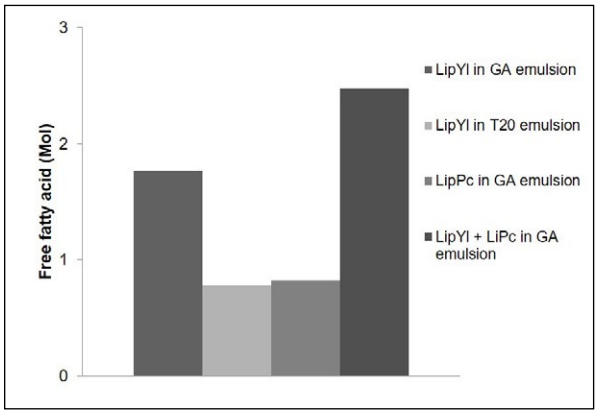


In the hydrolysis of different substrates by LipYl a higher enzymatic activity (around 231 U/L) was detected for pNF-caprylate, similar to LipPc. However, LipYl a better choice for the hydrolysis of pNF-laurate, which is an intermediate chain size ester (C12) with an activity of 159.4 U/L (Figure 2). LipYl has been shown to be effective in promoting hydrolysis of 4-12 carbon chains and therefore the two lipases can act in a complementary form, being able to generate short chain and some other long and median chains fatty acids to obtain aroma (lipolyzed milk fat - LMF) (AKIL *et al.*, 2016).

3.3 Hydrolysis of anhydrous milk fat by *Y. lipolytica* and *P. camemberti* lipases in different emulsions

P. camemberti lipase was used by Regado *et al* (2007) for lipolysis of milk fat from different sources (cattle, sheep and goats) and good results were obtained regarding the release of short chain fatty acids. In that study, *C. lipolytica* lipase showed better results for long chain fatty acids. Therefore, in the present work milk fat lipolysis was performed with *P. camemberti* (LipPc) and compared to hydrolysis by *Y. lipolytica* (LipYl) lipase for a possible synergistic use (Figure 3).

Figure 3 – LMF production (Free fatty acids) in hydrolysis reaction with P. camemberti (LipPc) and Y. lipolytica (LipYl) lipases in Arabica gum (GA) and Tween 20 (T20) anhydrous milk fat emulsion by titration method.



Titration method was used to measure lipase activities of LipPc and LipYl using two different emulsifying agents (Arabic gum and Tween 20). These emulsifiers were tested with anhydrous milk fat to assess the influence of these substances in the hydrolysis reaction (Figure 3). Tween 20 apparently inhibits LipYl since an approximately 50% reduction in the activity of this enzyme was observed in comparison to Arabic gum. It is possible that the interaction between the aqueous and organic phase (fat) promoted by Tween 20 is so intense that it hinders the action of the enzyme (substrate interaction with the active site). As provided in the Handbook (2009), this substance is composed of polyoxyethylene sorbitan esters (sorbitol and its anhydrides copolymerized with 20 moles of ethylene oxide) partially esterified with higher fatty acids and it is a hydrophilic nonionic surfactant. It has

broad and intense emulsifying and suspending power, giving rise to external aqueous phase emulsions (oil/water).

LipPc lipase showed lipase activity inferior to that of LipYl with the same surfactant (GA). The (1:1) mixture of LipY1 and LipPc proved to be effective because the activity obtained with both enzymes was increased in relation to that obtained with the enzymes individually (Figure 3). This may indicate synergistic action of the two enzymes, as LipYl is better for the hydrolysis of triglycerides with medium to long chain fatty acids, while LipPc has greater affinity for short and medium chain fatty acids (Regado *et al.*, 2007).

4 Conclusions

Residual frying oil (RFO) was a good lipase inducer for *Y. lipolytica* cultivation in bioreactor. The use of RFO for lipase production may be a way to reduce process costs and can also contribute to reducing a little the environmental impact generated with oil disposal. LipYl was better for the hydrolysis of long and medium chain esters, while LipPc primarily promoted hydrolysis of short and medium chain esters. It was possible to observe that the emulsifying agent used interferes with the enzymatic activity obtained by the titrimetric method. LipYl and LipPc may have a synergy during triglyceride hydrolysis, because of the increased enzymatic activity in milk fat generated by the combination of the two enzymes.

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