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Stability of Lipases Produced by *Yarrowia lipolytica* in the Presence of Cheese Whey

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Countries traditionally importers of bioproducts such as Brazil, India and China have been strong growth in research, production and market expansion of enzymes that moved around U$ $6x10^{12}$ in 2011. There are predicting an estimate of market growth of 6.3 % in 2013. Among the hydrolytic enzymes, lipases are of industrial interest and occupy a prominent position in global trade. These enzymes have been produced by microorganisms in the presence of industrial waste. The cheese whey is a by-product that retains approximately 55 % of the nutrients in milk that can be metabolized by *Yarrowia lipolytica* in bioproducts of commercial value. This study aimed to investigate the production and stability of metabolic liquid with lipolytic activity. Lipases were produced by *Yarrowia lipolytica* in the presence of cheese whey, olive oil and glycerol. A 2^4 factorial planning with four replications at the central point was proposed to investigate the best condition for producing these enzymes. Erlenmeyer's flasks (250 mL) were filled with 100 mL of liquid medium in the presence of the inoculum to 10 % v/v (10^6 CFU/mL). The flasks were incubated at 28 °C with shaking at 150 rpm. The samples were collected, centrifuged and the supernatant used to determine pH and enzyme activity during 60 days of storage. The production reached maximum lipase 132 IU/mL at 120 h of cultivation at pH 7.3 in the presence of whey at 10 %, glycerol 6 % and olive oil 0.2 %. The bioproduct with maximum activity showed enzymatic stability (156 IU/mL) for 60 days of storage in both refrigerated at 4 °C, as in a room temperature, at 28 °C. The presence of glycerol in the culture medium improved the stability of lipases for such chemical agent is a polyol which inhibits both the oxidation reaction and avoids protein deamidation

1. Introduction

The world market for enzymes reached around US $ $6x10^{12}$ in 2011 and expected to grow around 6.3 % in 2013 (Nigam et al., 2012). According to David et al., (2009), the demand for special enzymes in various industrial sectors has favored developing countries as Brazil, India and China. These countries have produced bioproducts which technologies have been dominated by Western Europe, North America and Japan. Lipases and proteases of microbial sources represent 40 % of the enzymes in all over the world (Jdhaav et al., 2013).

The Brazil has large quantity and variety of agro-industrial waste that can be transformed by microbial metabolisms. The nutrients of the wastes are metabolized to produce enzymes with low cost of the process whose reduction is approximately of 40 % (Coelho et al., 2008).

The cheese whey is a waste of the dairy industry that represents approximately 90 % of the volume of milk in the cheese manufacture; contains approximately 5 % of lactose, 0.5 % of protein and minerals. This residue has the potential to produce microbial metabolites by submerged culture. Furthermore, the reuse of waste reduces the environmental impact caused by its discharge (Chaves et al., 2010; Oliveira et al., 2012).

The yeasts are capable of producing metabolites of commercial interest in the presence industrial residue as substrates. The *Yarrowia lipolytica* is an aerobic yeast recognized by the ability to produce enzymes,

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mainly lipases. These biocatalysts have wide application in industry: pharmaceuticals, detergents, food, textiles and in wastewater treatment (Jdhaav et al., 2013; Sirisha et al., 2010).
Lipases are hydrolases which catalyze the conversion of triglycerides to free fatty acids and glycerol. These enzymes have significant potential as biocatalysts in organic synthesis reactions in non-aqueous media with high yields (Medeiros et al., 2013). The lipase production has been carried out by submerged fermentation process, however, the process in the solid phase is also promising.
The aim of this study was to investigate the stability of lipases produced by submerged culture of *Yarrowia lipolytica* in cheese whey, glycerol and olive oil as inducer.

2. Material and methods

2.1 Microorganism

Yarrowia lipolytica (*Candida lipolytica* URM-1120) from the laboratory culture collections (Universidade Federal de Pernambuco, Brazil) was cultured in Sabouraud medium, incubated at 28 °C for 48 h and stored at 4 °C.

2.2 Industrial effluent

The cheese whey was collected, packed in plastic container and stored frozen. It was donated by the cheese factory Campo da Serra (Northeast of Brazil).

2.3 Inoculum

Y. lipolytica was inoculated into 500 mL of nutrient broth in Erlenmeyer flask. This culture was incubated at 150 rpm, 28 °C and 48 h. The viable cells of the inoculum (10^6 CFU/mL) were determined in Sabouraud medium at 28 °C/48 h.

2.4 Enzyme production

The culture medium was investigated in accordance to the 24 factorial design with four replications at the central point (Table 1).

Table 1: Factors and levels proposed in the 2^4 factorial design

Factors	Levels		
	− 1	0	+ 1
Cheese whey	4.0	7,0	10
Glicerol	0.0	3,0	6,0
Olive oil	0.0	0,1	0,2
pH	6,0	7,5	9,0

2.5 Lipase assay

The substrate was prepared by the emulsion (1:1) of olive oil and gum arabic 7 %. The reaction system was: substrate 5 mL, sodium phosphate buffer (0.1 M, pH 7.0) 2 mL and the metabolic liquid 1 mL. The reaction at 37 °C/10 minutes under agitation was stopped by the addition of acetone:ethanol (1:1) 2 mL. The released fatty acids were titrated with 0.025 M of KOH solution, in the presence of phenolphthalein (Soares et al., 1999). One international unit of activity (IU/mL) was defined as the amount of enzyme which liberates 1 µmol of fatty acid per minute of the reaction under the conditions of the assay.

3. Results and discussion

Knowledge of the chemical composition of whey is essential for the development of new ingredients or products. In this context, the chemical composition of cheese whey medium used in this work is: 94 % of water, 30 % of organic carbon and 3,000 mg/L of oil and lipids at pH 5.0, which justifies the use of this residue for the metabolism of microorganisms for obtaining bioproducts.
Being an industrial waste with high content of organic matter , is an agent of environmental pollution include biochemical oxygen demand (BOD) in the range 30,000-50,000 mg/L which, when discarded in soil and water sources without proper treatment , cause environmental degradation (Von Sperling, 2005).
Brazil is a major producer of milk and cheese, and thus generates a large volume of whey. On the other hand, imports significant amounts of whey powder, because it is not feasible to reuse the liquid whey and therefore no waste of BLP (Almeida et al., 2001). To present in its chemical composition, functional properties with various substances (proteins) that can be converted into bioactive peptides, this waste has

been reused in food production. Another application of this serum is its use in research in biotechnology as an alternative substrate for low cost in the formulation of culture media . Reuse cheese whey have reduced the problems caused by the disposal of such waste in the environment (Antunes, 2003; Chaves et al., 2010; Macedo and Macedo, 2011; Oliveira et al., 2012; Richards, 2002; Smith, 2003). Table 2 shows the lipase activities produced by *Y. lipolytica* under submerged culture. The culture of *Y. lipolytica* produced lipolytic activities in the conditions investigated. A atividade enzimática aumentou com o tempo de cultivo em mais de 50 % dos assays. The highest lipase activity was 132 IU/mL for 120 h of culture in the presence of cheese whey at 10 %, glycerol at 6.0 % and olive oil at 0.2 % as inductor (assay 8).

Table 2: Matrix decoded with lipolytic activities of the full factorial design 2^4

Assay	Factors				Lipolytic activity (IU/mL)			
	Cheese whey (%)	Glycerol (%)	Olive oil (%)	Initial pH	48 h	72 h	96 h	120 h
1	-1	-1	-1	-1	0	4	12	12
2	+1	-1	-1	-1	0	0	4	8
3	-1	+1	-1	-1	32	36	80	92
4	+1	+1	-1	-1	32	32	52	40
5	-1	-1	+1	-1	32	20	12	8
6	+1	-1	+1	-1	0	8	4	0
7	-1	+1	+1	-1	28	40	60	72
8	+1	+1	+1	-1	24	60	120	132
9	-1	-1	-1	+1	0	4	4	0
10	+1	-1	-1	+1	0	0	6	8
11	-1	+1	-1	+1	28	24	16	12
12	+1	+1	-1	+1	12	24	32	32
13	-1	-1	+1	+1	4	0	0	0
14	+1	-1	+1	+1	12	8	12	12
15	-1	+1	+1	+1	36	80	100	104
16	+1	+1	+1	+1	28	28	60	56
17	0	0	0	0	32	40	60	64
18	0	0	0	0	48	60	72	80
19	0	0	0	0	32	64	76	80
20	0	0	0	0	32	64	80	96

Moftah et al. (2013) studied the lipase production by *Y. lipolytica* in culture media alternative. These authors medium supplemented with oil, ammonium sulfate (0.6 %), yeast extract (0.1 %) , maltose (0.5 %) , olive oil (0.3 %) and peptone (0; 1 %). This yeast yielded maximum lipase activity of 850 UI/dm^3 after 4 days under submerged culture .

Kebabci and Cihangi (2012) compared the lipase production for three different strains of *Y. lipolytica* in production medium in the presence of olive oil (1 %) supplemented with ammonium sulfate (1 %). The highest lipase activity (16 U/mL) was produced by *Y. lipolytica* NBRC 1658, at a temperature of 30 °C under orbital shaking at 200 rpm after 48 h of cultivation.

Riaz et al. (2010) whey used in the composition of the production medium. According to these authors, the higher enzyme activity produced by *Bacillus sp*. FH5 was determined in the presence of 0.1 % serum (v/v),

corn steep liquor 0.2 % (v/v) molasses and cane sugar 0.5 %. The maximum activity of 95.12 U/mL, determined after 48 h of culture can be compared to that produced by *Y. lipolytica* this work also in presence of whey.

The values of pH during the cultivation of *Y. lipolytica* after 96 and 120 h are illustrated in the figures 1 and 2, according to the full factorial design 2^4. Initially, the culture media were adjusted to pH 6.0 in the assays 1-8, at pH 9.0 in the assays 9-16 and at pH 7.5 in the assays 17-20 (the central point). The pH was acid during the first 72 h; the minimum and the maximum values were pH 3.9 and pH 5.7, respectively. The pH increased and reached the neutrality in some cultures after 72 h. The pH value increased during the cultivation, pH 4.8 to 7.3 in the assay 8, when the greatest activity was determined at 120 h of culture. The pH is a physicochemical parameter that influences the enzymes activity and the adaptation of the microorganisms in the medium. Significant changes in pH value can alter the conformation of the protein and inhibit its catalytic action.

Figure 1

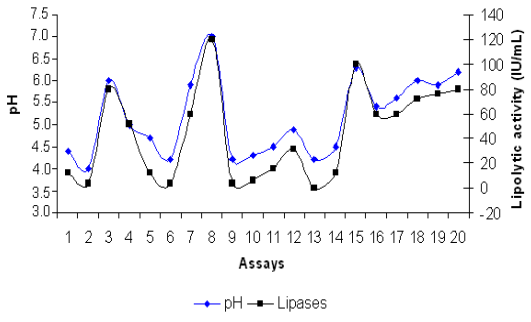
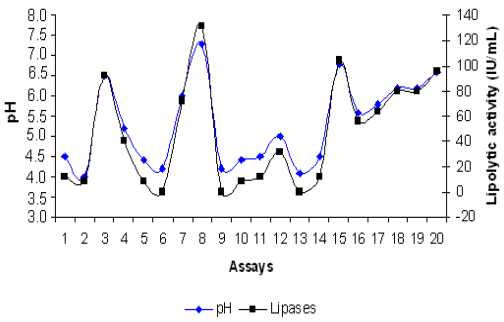
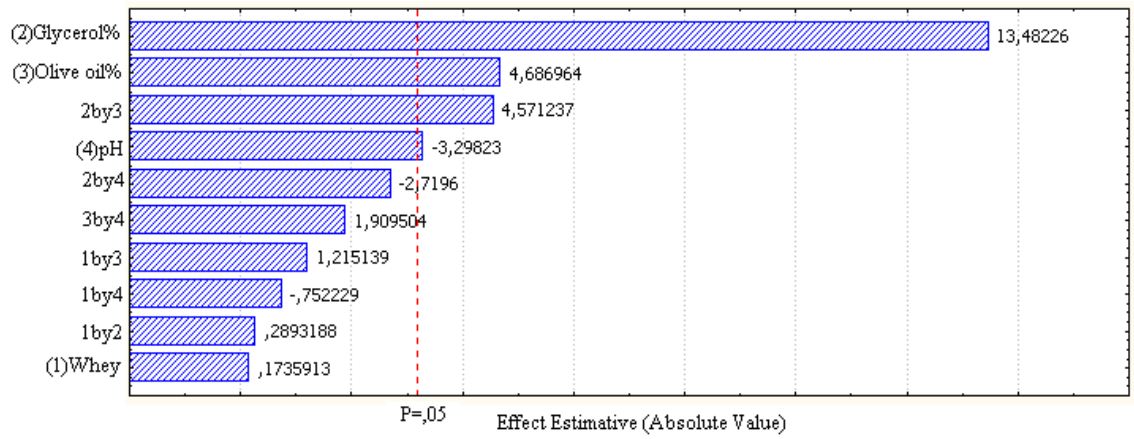


Figure 2



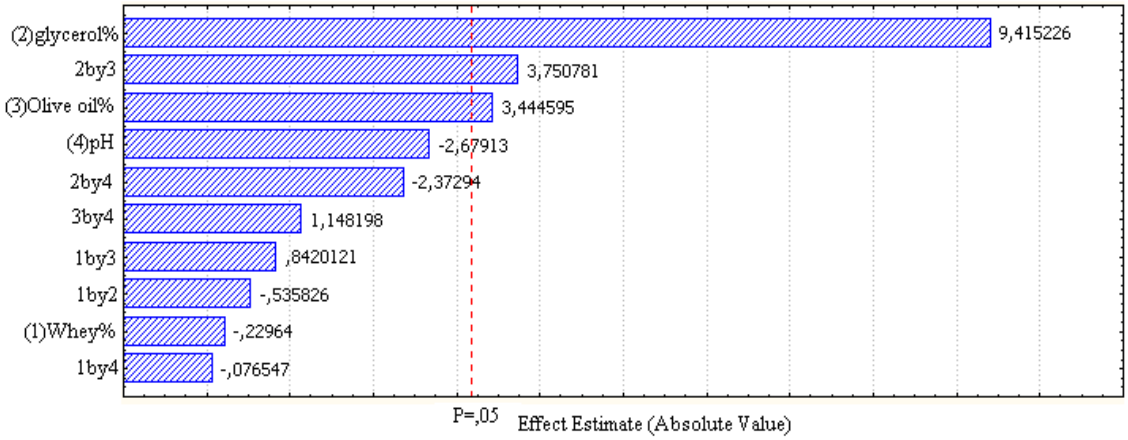
Figures 1 and 2 show the influence of pH and the lipolytic activity after 96 and 120 hours, respectively, by submerged culture of Y. lipolytica.

The effects caused by the independent variables cheese whey, glycerol, olive oil and pH on the response variable lipase production by *Y. lipolytica* was evaluated by Pareto illustrated in Figures 3 and 4 in the times of 96 and 120 h respectively submerged cultivation. The variable with the most positive influence on lipase production was glycerol. This means that the greater the amount of glycerol in the composition of the production medium, increased enzymatic activity. Therefore, the aim of optimizing the production medium, subsequent experiments should be performed by increasing the amount of this component. Olive oil and its interaction with the glycerol also showed statistically significant positive effects on lipase activity. Moreover the variable pH had a negative effect, namely, increasing the value of this parameter, the enzyme activity decreases.



*The vertical dashed line indicates where the estimated effects were statistically significant extent.

Figure 3 Pareto of lipase production by Y. lipolytica 96 h submerged cultivation, according to the independent variables: cheese whey (1), glycerol (2), olive oil (3) e pH (4).



*Vertical dotted line indicates where the estimated effects were statistically significant extent.

Figure 4 Pareto of lipase production by Y. lipolytica 120 h submerged cultivation, according to the independent variables: cheese whey (1), glycerol (2), olive oil (3) and pH (4).

Glycerol is part of the composition of various lipids, besides being a residue generated in large quantities by the soap industry and the production of biodiesel. This polyol may be used as a sole carbon source for microorganisms in the production of various metabolites with enzyme activity and storage stability (Silva et al., 2009.

Figure 5 shows the results of enzymatic activity produced by *Y. lipolytica* in submerged cultivation determined in cell free metabolic liquid containing 0.5% sodium sorbate. A byproduct of this increased lipase activity during storage at 28 ºC. The zero hour, the specific activity was 132 IU/mL value increased to 160 IU/mL at 60 days. The stability of lipase can be justified by the presence of glycerol byproduct which in the end is a polyol tends to form hydrogen bonds and the "shell-type" mechanism that protects the reaction of amino acids deamidation and inhibits oxidation of the protein structure (Buxbaum, 2011).

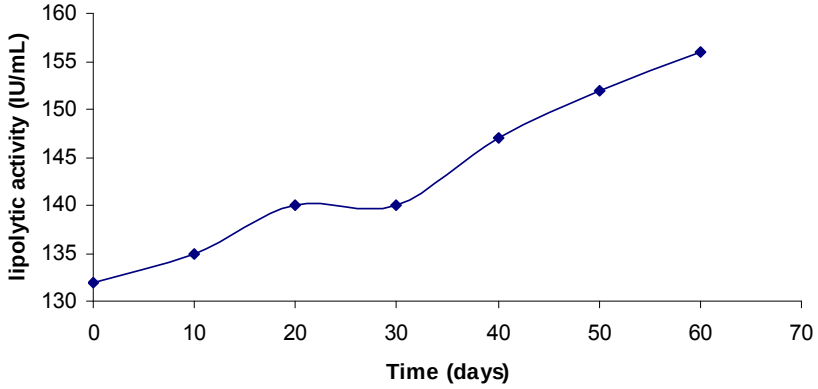


Figure 5 Stability of byproduct lipase activity with storage at 28 ° C for a time 60 days

The lipase activity produced by *Y. lipolytica* grown in cheese whey (10 %), glycerol (6.0 %) and olive oil (0.2 %) showed significant stability during storage under refrigeration at 4 ^{0}C and incubated at 28 °C, with an increase of around 18 % in the enzymatic activity of metabolic liquid after 60 days.

4. **Conclusion**

In this study, *Yarrowia lipolytica* showed adaptation to the culture medium consisting of whey as the principal carbon source and glycerol probably due to their high nutritional value and complete. The peak of enzyme lipase concentration occurred after 120 hours indicating that this was the time required under the conditions studied in this work, for the production of lipase. The glycerol can be combined or used as a

sole carbon source for microorganisms in the production of various metabolites with enzyme activity and storage stability.

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