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Reuse of Whey Cheese for Lipase Production by *Candida lipolytica*

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This work evaluated the use of cheese whey for lipase production by *Candida lipolytica* under submerged cultivation. A 2^3 factorial design with four replications at the center point was carried out to investigate the production of the enzyme. Assays were performed in Erlenmeyer flasks at 10 % v / v inoculum (standardized to 106 CFU / mL), useful volume of 100 mL, 150 rpm and at 28 °C in the presence of cheese whey, olive oil and Tween - 80, initial pH 5.0. The centrifuged samples were used to determine pH and enzyme activity. The maximum lipase activity reached 118 U/mL at 24 h of culture with different concentrations of the production medium in the presence of the whey at 100 to 99.8 %, independent of olive oil at 0.2 % and Tween-80, 0.2 % and pH 5.0-5.3. The micro-organism studied produced lipase in first 24 h of cultivation, using whey cheese as substrate, with great environmental potential for be applied in effluent treatment of this type

1. Introduction

The demand for different types of enzymes in the industries for a wide range of applications has direct research for the development of more efficient and cost-effective technological processes and environmental sustainability (Hasan, Shah, Hameed, 2009; Lima et al., 2014; Nigan, 2013).
The world market for enzymes reached around US $ 6 billion 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. The yeasts are capable of producing metabolites of commercial interest in the presence industrial residue as substrates (Chaves, Callegaro, Silva, 2010 and Oliveira, Bravo, Tonial, 2012). The *Candida lipolytica* is an aerobic yeast that has been investigated due to their ability to produce commercially valuable metabolites (organic acids, extracellular proteases, lipases, esterases, phosphatases), and other byproducts wide industrial application (Szabo and Stofanikova, 2002). The lipase produced by *Candida lipolytica* is a byproduct of great importance in the industry. Due to its versatility of this enzyme may be applied in the production of detergents, pharmaceuticals, foodstuffs, textiles and in wastewater treatment. Microbial lipases have several advantages compared the lipases produced by animal and plant cells (Lima et al., 2014; Seth et al., 2014), ranging from a high conversion efficiency of substrate to product, and the weather conditions are not susceptible, not need a lot of area for production,

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have large capacity adjust to environmental conditions, are capable of performing catalytic reactions under extreme conditions of temperature and pH in the presence of organic solvents and (Lima et al., 2014; Messias et al, 2011; Nagar, Dwivedi, Shrivastava, 2013; Nigam, 2013; Sharma, Kanwar, 2014).
The chemical structure and the kinetic characteristics of microbial lipases depend on the microorganism used, genus, species and the medium composition used (Fickers, Nicaud, 2013; Massadeh, Sabra, 2011; Lima et al., 2014). The culture medium for lipase production shall be constituted of long chain fatty acids, or esters triple bonds. Natural substrates of lipase used in the composition producing means are oils and fats containing triglycerides made up of long chain fatty acids (Hult, Berglund, 2007; Ray, 2012).
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 (Jdhaav et al., 2013; Medeiros et al., 2013; Messias et al., 2011; Sirisha et al., 2010). 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 evaluate the use of cheese whey for lipase production by *Candida lipolytica* under submerged cultivation.

2. Material and Methods

2.1 Microorganism

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

C. 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 2^3 factorial design with four replications at the central point (Table 1).

Table 1: Factors and levels proposed in the 2^3 factorial design

Fatores	Levels		
	− 1	0	+ 1
Cheese whey	40	70	100
Olive Oil	0.0	0.1	0.2
Tween-80	0.0	0.1	0.2

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).

2.6 pH

The pH measurement was carried out in digital potentiometer.

3. Results and discussion

The reuse of whey in the presence of olive oil and Tween-80 during the *Candida lipolytica* growing URM-1120 for 120 h produced metabolic liquid with significant lipase activity with 24 and 48 h of submerged cultivation. The whey was chosen for this research because of the wide availability of the effluent associated with its nutritional value.

Table 2 shows the values of the lipolytic activity produced by *C. lipolytica* the presence of whey with 24, 48 and 72 h submerged cultures, demonstrating that the culture produced no enzymatic activity at 96 and 120 h. *C. lipolytica* in submerged culture produced enzyme activity as proposed in the planning. The maximum activity occurred at 24 h of fermentation. The highest lipase activity was 118 IU / mL, pH 5.0 to 5.3 and temperature of 28 ° C in the presence of cheese whey (+1), independently of the presence of olive oil (+1) and Tween-80 (+1).

Table 2: Lipase activity of cell-free broth produced by *Candida lipolytica* in submerged culturesAssays

	Factors			Lipases (UI/mL)		
	Cheese whey	Olive Oil	Tween-80	24h h	48 h	72 h
1	-1	-1	-1	44	16	0
2	+1	-1	-1	118	56	0
3	-1	+1	-1	60	68	0
4	+1	+1	-1	118	32	4
5	-1	-1	+1	36	32	0
6	+1	-1	+1	118	92	12
7	-1	+1	+1	44	8	4
8	+1	+1	+1	72	0	0
9	0	0	0	76	48	12
10	0	0	0	72	28	16
11	0	0	0	76	44	16
12	0	0	0	68	44	12

The enzymatic activity of results obtained in this study were higher than those found by Kebabci and Cihangir (2012) in reporting the enzymatic activity of 10.67 IU / mL in medium containing ammonium sulfate as nitrogen source and absence of olive oil in similar conditions those described in this work. On the other hand, differ from the results obtained by Corzo et al. (1999) to cite that the production of lipase by *Candida lipolytica* is dependent on the concentration of Tween-80 (0.5-2g / mL) and high substrate concentration olive oil used. Olive oil is one of the most used inductors in lipase production by *C. lipolytica* investigations (Fiametti et al, 2011; Iftikhar et al, 2012; Kumar et al, 2012; Nunes et al., 2011). However in some published studies, were determined maximum activities of lipases produced in the absence of olive oil, which confirms the results obtained in this work (Imandi et al, 2010; Kebabci and Cihangir, 2012). The behavior of micro-organisms is directly connected with the conditions of the medium and depend on factors such as pH to adapt to the environment and produce the metabolite of interest. The conformation, stability and consequently the catalytic action of the proteins are modified by sudden changes in pH, temperature or concentration of the medium (Nolting, 2006; Nelson and Cox, 2011).

The pH during the cultivation of *Candida lipolytica* is illustrated in Figure 1. At the beginning of the experiment the pH of all runs were adjusted to 5.0. The pH was maintained with acid characteristic in the range from 5.0 to 6.6 in all tests with exception of assay 1 to 48 and 72 h of culture and assay 6 to 72 h of culture where the pH was near neutrality in the range of 6.8 to 7.2. The highest lipase activity was produced between pH 5.0 and 5.3. The results of this work were similar to those obtained by Corzo et al., (1999) who described the

extracellular production of lipase produced by *C. lipolytica* strain in medium containing optimal pH and temperature with values between 4.7 and 29,5 ºC, respectively.

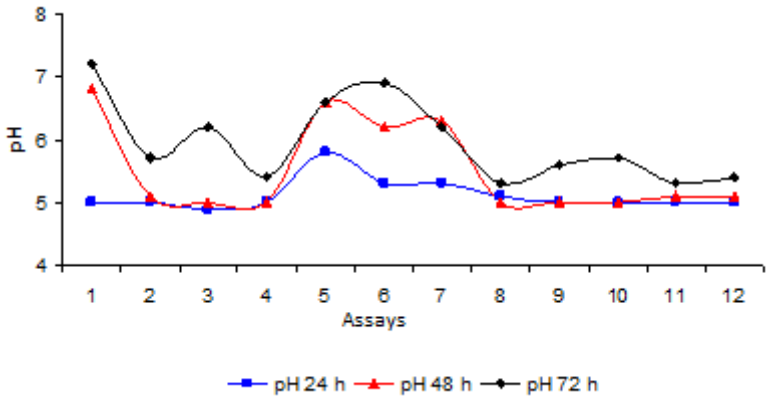

Figure 1: Values of pH during the cultivation of Candida lipolytica

Figure 2 illustrates a Pareto chart of the 2^3 factorial design with four center point replicates in on lipase production by *Candida lipolytica* at 24 h of fermentation in the submerged function of the independent variables cheese whey, olive oil and Tween-80. In accordance with the Pareto chart, standardized to 95 % confidence level, the whey was independent statistically most significant variable to help increase the lipase production by *Candida lipolytica*. The association between the independent variables cheese whey and olive oil, olive oil and Tween-80 and Tween-80 alone were all representative with values above the confidence dashed line (p), but did not induced the increase of lipase production. The statistical tool (Statistica 7.0 software) was presented as a useful and effective tool in determining the appropriate concentrations of the medium for maximum lipase production under the conditions studied in this work.

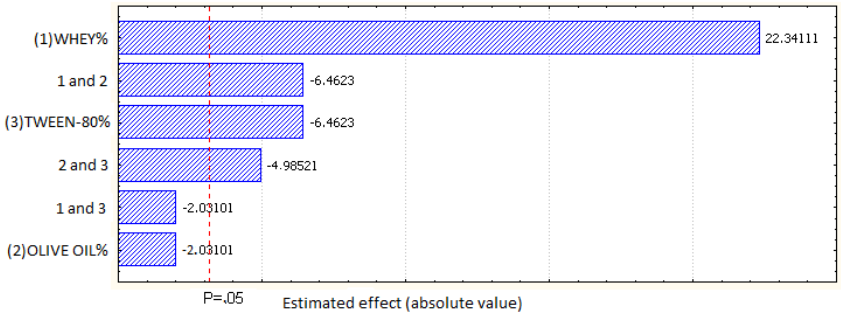

Figure 2: Pareto chart of lipase production by Candida lipolytica after 24 hours in submerged culture, in function of the independent variables: whey (1), olive (2) and Tween-80 (3)

Olive oil is an inducer of lipase production. *Candida cylindracea* cultivated by Brozzoli et al., (2009) in waste water from the production of olive oil industry in the cultivation of *C. cylindracea* lipase activity produced equal to 20.4 U / mL. The presence of oil in the culture medium favoring cell growth and enzyme production. According to Vieira et al., (2006), the assay of enzyme activity using Tween 20 and Tween 80 has proven effective for the selection of bacteria with the ability to degrade the biodiesel from palm oil, to determine that 64% of the isolates had at least one activity lipase and / or esterase enzyme. Dartora et al., (2003) isolated microorganisms using a liquid culture medium based on whey under conditions of 30 °C for 48 hours. Of the 12 strains investigated, three strains of yeast, two strains of bacteria and filamentous fungi presented lipolytic activity through the enzymatic index (EI).

4. Conclusions

The results of this research indicate that *Candida lipolytica* produces cell-free broth with enzyme activity in the presence of whey, regardless of the presence of the inductor olive oil and Tween-80, pH remaining in the acidic range (5.0-5.3) from the beginning of cultivation.

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