Mycelium-bound lipase production of immobilized whole cell from a wild-type *Penicilium citrinum* strain

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***Abstract*—** *Pencillium citrinum whole cell was cultivated and immobilized in a low cost support aiming to be used as an active and stable biocatalyst for modification of oil and fats. PUF (polyurethane foam) coated in 6mm was used as support and the immobilization occurred as a natural consequence of cell growth. Olive oil was the mycelium-bound lipase inductor and the production was optimized by statistical analysis of pH and temperature effects in culture broth. Immobilized whole cell was characterized as a natural immobilized lipase and all the assays were made using olive oil hydrolysis. The mycelium-bound lipase production was improved by adjustment the culture broth to pH 7.5 and 35°C of incubation temperature. The SEM micrographs showed the entangled cells morphology and a high adhesion in support matrix. Biochemical characterization revealed maximum values of lipase activity in pH 8 and 40°C and a half-life time at 60°C was 2.2h. Results from kinetics study indicated the biocatalyst follow the Michaelis-Menten kinetic. The potential catalytic of immobilized whole cells was assessed in soybean oil hydrolysis and 55.7% of degree of hydrolysis was attained in 12h.*

***Keywords*— whole cell, mycelium-bound lipase, immobilization, hydrolysis, oil.**

I. INTRODUCTION

Generally, enzymes associated with the mycelia are referred to as mycelium-bound enzymes. Mycelium-bound enzymes are economically attractive because they can be produced at low cost and considered as naturally immobilized enzymes that may be used without the laborious operations of isolation, purification and addition of co-factors, *etc* [1]. The whole-cell biocatalyst includes the cell wall- or the cell membrane-bound lipase (i.e., intracellular enzyme), which can be used instead of extracellular lipase [2]. Over the past decades, mycelium-bound lipase of filamentous fungus has been extensively studied as an alternative biocatalyst in biotransformation processes [3].

Mycelium-bound lipase can be used directly as suspended free cells or immobilized within biomass support particles as whole cells biocatalyst [4]. Immobilization of whole cells provides stability as well as improves reusability [5]. The concept of immobilized whole cells is different for those applied in fermentation process, since whole cells keep the enzymes in active state, without cell replication. Whole cell immobilization requires the same techniques applied to conventional enzyme immobilization, such as adsorption, covalent attachment, cross-link and entrapment, although the most useful for filamentous fungi is the entrapment in porous support, due to its morphology [3].

The literature has shown that whole cells immobilization in reticulated polyurethane foam (PUF) is a convenient way to spontaneously obtain immobilized whole cells. So far, a few strains were studied as potential mycelium-bound lipase producers, such as *Rhizopus* sp. [6, 7, 8], *Aspergillus* sp. [9, 10, 11] and *Mucor* sp. [12, 13, 14]. The genus *Penicillium* is also a known fungal producer of lipases which are employed in the dairy industry and in a number of bioconversions of industrial importance [15], nevertheless its potential as whole cells biocatalyst were not enough explored.

In this context the present work aims to investigate the potential of a wild-type *Penicillium citrinum* strain as a mycelium-bound lipase producer and its immobilization in PUF. The objective was to evaluate the catalytic potential of the immobilized whole cells in enzymatic modification of fats and oils, such as hydrolysis, transesterification and interesterification processes. For this, firstly, the physicochemical parameters were optimized to enhance the production of mycelium-bound lipase and then the immobilized whole cells produced were characterized as a conventional immobilized lipase. Additionally, the efficiency biocatalytic process of soybean hydrolysis with immobilized whole cells was evaluated on batch reaction.

II. MATERIALS AND METHODS

2.1 Materials

PUF (Esfrebon^{MR}) with an average porosity of 0.76±0.11mm and a density of 0.02±0.01 g cm^{-3} was purchased from a local market, cut into 6mm cubes [13], and treated with nitric acid solution (0.1mol L^{-1}) to remove impurities. The PUF was then

washed twice with distilled water and dried in an oven at 60 ◦C for 24h. Olive and soybean oils were purchased locally. Arabic gum (Synth), ethanol (Synth) and acetone (Synth) were of analytical grade.

2.2 Microorganism and growth media

Fungal strain *P. citrinum* URM 4216 was purchased from culture collection URM (University Recife Mycologia) at Federal University of Pernambuco (Pernambuco, Brazil). PDA (Potato Dextrose Agar—Difco) was used as solid culture medium for fungi propagation. The culture medium used for cell growth contained soy peptone (Himedia) 70 g L^{-1}, $NaNO_3$ (Vetec) 1.0 g L^{-1}, KH_2PO_4 (Synth) g L^{-1}, $MgSO_4 \cdot 7H_2O$ (Vetec) 0.5 g L^{-1}, and olive oil (Carbonell) 30 g L^{-1}.

2.3 Preparation of immobilized whole cells biocatalyst

Whole cells immobilized were prepared by inoculating a suspension of fungal cells spores (10 – 50 mL L^{-1}) into 250 mL conical flasks containing 100 mL of culture medium (pH adjusted) and 0.6 g of cuboidal PUF sterilized previously. The system was incubated at controlled temperature for a maximum period of 120 h. The immobilized whole cell biocatalyst was separated from the liquid medium by filtration, washed twice with distilled water and acetone, and dried under vacuum for 24h.

2.4 Experimental design

A 2^2 full experimental design with three replicates at the center point was used to evaluate the effect of physic-chemical parameters pH (X_1) and temperature (X_2) in the mycelium-bound lipase production. The lipase activities were the response variable of the design experiment and assayed by the hydrolysis of olive oil emulsion [13]. The experimental design results were analyzed using Statistica version 7 (StatSoft Inc., USA).

2.5 Determination of morphological, biochemical and kinetics properties

Surface morphology of immobilized whole cells was observed by Scanning Electron Microscopy (SEM) (LEO 440i Oxford). The pH and temperature optimal of the immobilized whole cell lipase was studied within the 6.0 – 8.5 and 30–55°C range, respectively. The influence of substrate concentration (olive oil) on the hydrolytic activities was also analyzed in the hydrolysis assay varying the proportion of oil in the emulsion from 5 to 70% wt. Michaelis-Menten constant (K_m) and the maximum velocity (V_{max}) were calculated using OriginPro version 8 (Originlab Corporation). The thermal stability of lipase was determined by incubating the whole cells at 60◦C for 150 min with periodical withdrawals every 30 min to quantification the residual lipase activity and calculation the denaturation rate constant (k_D) and half-life time ($t_{1/2}$). In all the experiments, the lipase activities were assayed by the hydrolysis of olive oil emulsion 10 % wt [13].

2.6 Soybean oil hydrolysis

Batch hydrolytic reactions were performed in 250mL conical flasks in an orbital shaker (170 rpm) at 37°C and atmospheric pressure for 24 h, containing 50 g emulsion soybean oil (oil/water ratio = 1/4; 2.5% Arabic gum). After addition of 10% wt. of immobilized whole cells, small samples were removed from the reactor periodically to quantify the free fatty acids formed. The degree of hydrolysis was calculated according to Equation (1) [16].

$$\%\, Hidrólise = \frac{(V_a - V_b) * M * 10^{-3} * MWm}{Wt * f} * 100 \quad (1)$$

where Va is the volume of potassium hydroxide solution (KOH) required during titration of sample; Vb is the volume of potassium hydroxide solution (KOH) required during titration of control; M is the KOH molarity (0.02 M); MWm is the average molecular weight of fatty acids (278.6 $gmol^{-1}$); Wt is the weight of the sample taken and f is the fraction of oil at start of reaction.

III. RESULTS AND DISCUSSION

3.1 Mycelium-bound lipase immobilization

P. citrinum whole cell was prepared and immobilized in PUF in a medium containing olive oil at 30°C for 120h of incubation time. The biomass growth kinetic and lipase activity of mycelium and extract broth are showed in Fig.1. As observed, there was no expressive growth up to 48 h of incubation time. After that, biomass growth curve followed typical microorganism growth and the highest biomass concentration (49.3 ± 1.5 g L^{-1}) was attained in 96 h of incubation. Mycelium-bound lipase production was confirmed by the high lipase activity reached, in order to 105.5 ± 3.0 U g^{-1}, in

comparison with low value obtained by extract broth. In addition, it was also verified a proportional relation between the fungal growth and mycelium-bound lipase activity.

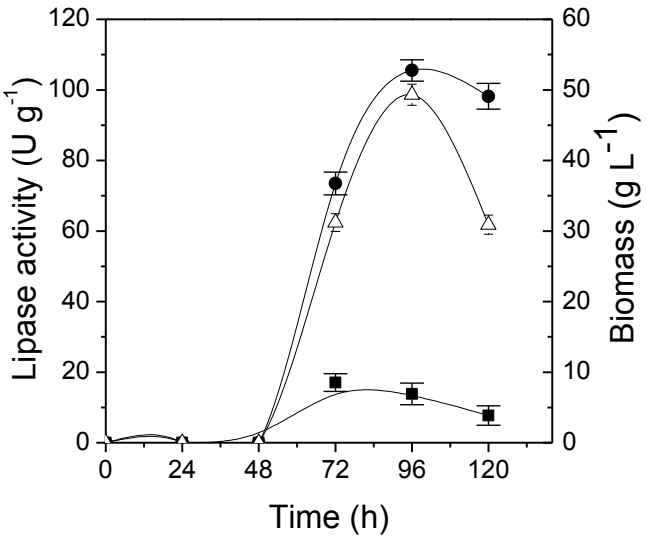

FIGURE 1: PROFILE OF BIOMASS GROWTH (-△-), MYCELIUM-BOUND LIPASE ACTIVITY (-●-) AND EXTRACT BROTH LIPASE ACTIVITY (-■-). CULTIVATION CONDITIONS: PH 6.8 (WITHOUT ADJUSTMENT), 30 °C, 0.3 L INOCULUM

Previous work [12] reported an unsatisfactory mycelium-bound lipase activity using *P. citrinum* whole cells immobilized in PUF, with only 10.53 ± 0.67 U g^{-1} lipase activity. However, a study about the profile of biomass growth was not performed, adopting 72h of incubation time. As can be observed in Fig. 1, 72h is considered to be insufficient since the biomass growth was still at the lag phase. Therefore, the incubation time of 96h was adopted in all followed experiments.

3.2 Experimental design

Optimization of culture conditions is very important as they affect the enzyme production and the ratio between mycelium-bound and extracellular lipases produced by the microorganism [3]. Studies of temperature and pH effects on growth and metabolite production are scare with respect to filamentous fungi, mainly *P. citrinum* cells. In this work, a full factorial 2^2 design with three replicates in the center points was used to study the combined effect of the pH and temperature in the culture medium to enhance the mycelium-bound lipase production by *P. citrinum* immobilized whole cells. In all runs olive oil was used as a lipase inductor and an incubation time of 96 h was assumed. The experimental design is shown in Table 1 together with the experimental results in terms of lipase activity.

TABLE 1

EXPERIMENTAL DESIGN AND RESULTS ACCORDING TO A FULL 2^2 FACTORIAL DESIGN TO EVALUATE THE INFLUENCE OF THE VARIABLES PH AND TEMPERATURE IN MYCELIUM-BOUND LIPASE PRODUCTION BY *P. CITRINUM* IMMOBILIZED WHOLE CELLS

Runs	Independent variables (coded values in parenthesis)		Response variable
	pH (X_1)	Temperature (°C) (X_2)	Lipase activity (U g^{-1})
1	6.5 (-1)	25 (-1)	67.92
2	6.5 (-1)	35 (+1)	100.83
3	7.5 (+1)	25 (-1)	130.16
4	7.5 (+1)	35 (+1)	162.78
5	7.0 (0)	30 (0)	71.10
6	7.0 (0)	30 (0)	64.53
7	7.0 (0)	30 (0)	67.33

Results in Table 1 showed the strong influence of pH and temperature in mycelium-bound lipase production. Lipase activity were varied about 67 – 163 U g^{-1} and the maximum value was attained at the highest pH level (7.5) and temperature (35°C) as shown in run 4. Much lower values were found at center points (pH 7.0 and 30°C) and at the lowest level (pH 6.5 and 25°C), indicating the positive effects of pH and temperature on the mycelium-bound activity. Similar results are found in literature, since most of the lipases from *Penicillia* are reported to be most active and stable in neutral to alkaline pH range and high temperatures [15, 17].

This hypothesis was confirmed by the statistical analysis of these results that showed significant and positive effects for both studied variables at a 95 % confidence level, unlike interaction, as described in Table 2.

TABLE 2
ESTIMATED EFFECTS, STANDARD ERRORS AND STUDENT'S T TEST FOR MYCELIUM-BOUND LIPASE PRODUCTION BY *P. CITRINUM* IMMOBILIZED WHOLE CELLS USING A FULL 2^2 FACTORIAL DESIGN

Variable	Effects	Standard error	p
Mean	119.27	± 1.70	0.0002*
X1	64.16	± 3.41	0.0028*
X2	33.86	± 3.41	0.0099*
X1.X2	-0.15	± 3.41	0.9645

**** Significant at 95 % confidence level. X_1 and X_2 represent the variables pH and temperature, respectively.***

The main effects (Table 2) were fitted by multiple regression analysis to a linear model and the best fitting response function can be written by Equation (2), in which A= Lipase activity (U g^{-1}); X_1= coded value of temperature (°C) and X_2 = coded value of pH.

$$A = 119.27 + 32.08X_1 + 16.93X_2 \quad (2)$$

The statistical significance of this model was evaluated by the F test (Table 3), which revealed that this regression is statistically significant at 95 % probability level. The model did not show lack of fit and the determination coefficient (R^2 = 0.9975) indicates that the model can explain 99.75 % of the variability. Thus, the fitted equation displayed was considered to be suitable for describing the lipase activity as a function of the studied variables and was used to plot the response surfaces as showed in Figure 2.

TABLE 3
ANALYSIS OF VARIANCE (ANOVA) FOR THE REGRESSION OF THE MODEL THAT REPRESENTS MYCELIUM-BOUND LIPASE PRODUCTION BY *P. CITRINUM* IMMOBILIZED WHOLE CELLS USING A FULL 2^2 FACTORIAL DESIGN

Variables	Sum of squares	Degree of freedom	Mean square	F	*p*
X_1	4116.79	1	4116.79	354.75	0.0028*
X_2	1146.48	1	1146.48	98.80	0.0099*
$X_1.X_2$	0.022	1	0.022	0.002	0.9694
Pure error	23.21	2	11.61		
R^2	0.9975				

**** Significant at 95 % confidence level. X_1 and X_2 represent the variables pH and temperature, respectively.***

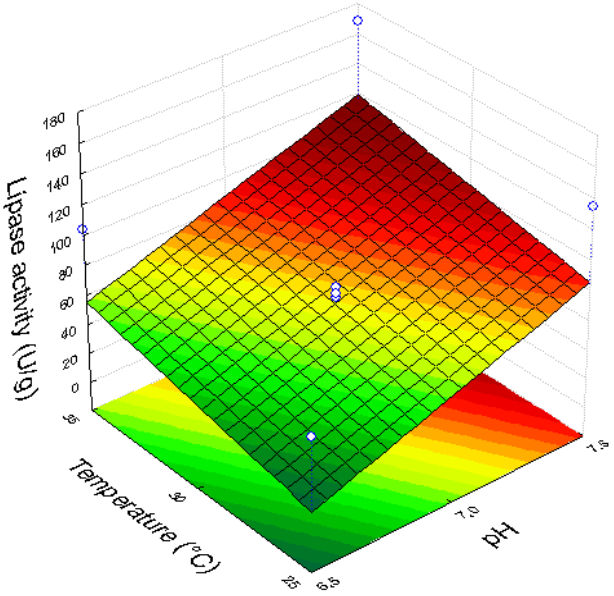

FIGURE 2. SURFACE RESPONSES FOR THE MYCELIUM-BOUND LIPASE PRODUCTION BY *P. CITRINUM* IMMOBILIZED WHOLE CELLS AS A FUNCTION OF pH AND TEMPERATURE ACCORDING TO THE FITTED MATHEMATICAL MODEL

Data showed the best conditions to enhance mycelium-bound lipase production is at pH 7.5 and temperature of 35°C. To validate the model (Equation 2), cultivation runs were performed under the optimal predicted conditions and the differences between the experimental and theoretical values were lower than 2%.

The spore concentration of the inoculum appears to be a critical factor for the process outcome and also in the immobilized cells and lipase activity [13]. Under the establishment conditions in terms of pH and temperature, the effect of inoculum in mycelium-bound lipase production was evaluated and illustrated by Figure 3.

Figure 3 shows, except 10 mL L^{-1}, there were no expressive difference between mycelium-bound lipase production in range of 20 to 50 mL L^{-1} of inoculum concentration. The highest lipase activity value was attained with 30 mL L^{-1} that corresponds to about $1x10^6$ spores mL^{-1} medium concentration. Similar behavior was obtained in a previous study using *Mucor circinelloides* whole cells immobilized in PUF, in which high lipase activity was found with the same spore concentration [13].

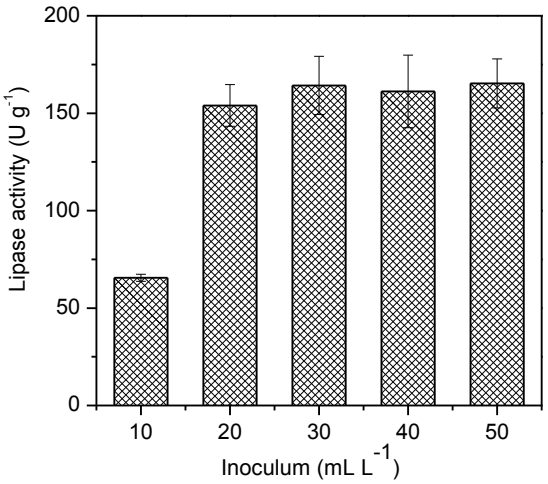

FIGURE 3. THE EFFECT OF INOCULUM SPORES IN THE MYCELIUM-BOUND LIPASE PRODUCTION BY *P. CITRINUM* IMMOBILIZED WHOLE CELLS

Wolski et al. [17] evaluate the effect of inoculum concentration in lipase activity from *Penicillium* sp. and found that 40 mL L^{-1} was the best inoculum concentration. Some reports related the effect of inoculum in fungal morphology, since the biomass growth in fully entangled filaments are obtained with lower inoculum concentration, thus enhancing the lipase production [18].

3.3 Morphological, biochemical and kinetics properties

Figure 4 (a-b) shows the SEM images of a cross sectional of whole cells immobilized in 6-mm cubic PUF particle. As can be observed in Fig. (4a), the cells have formed a dense film inside the reticulated fiber of PUF, which means the strong adhesion of cells into the support. This is very important and indicated that the cells would not release from the support even under vigorous agitation. With 5 times magnification (Fig. 4b), it is possible to see fully entangled morphology of cells around the fiber of PUF.

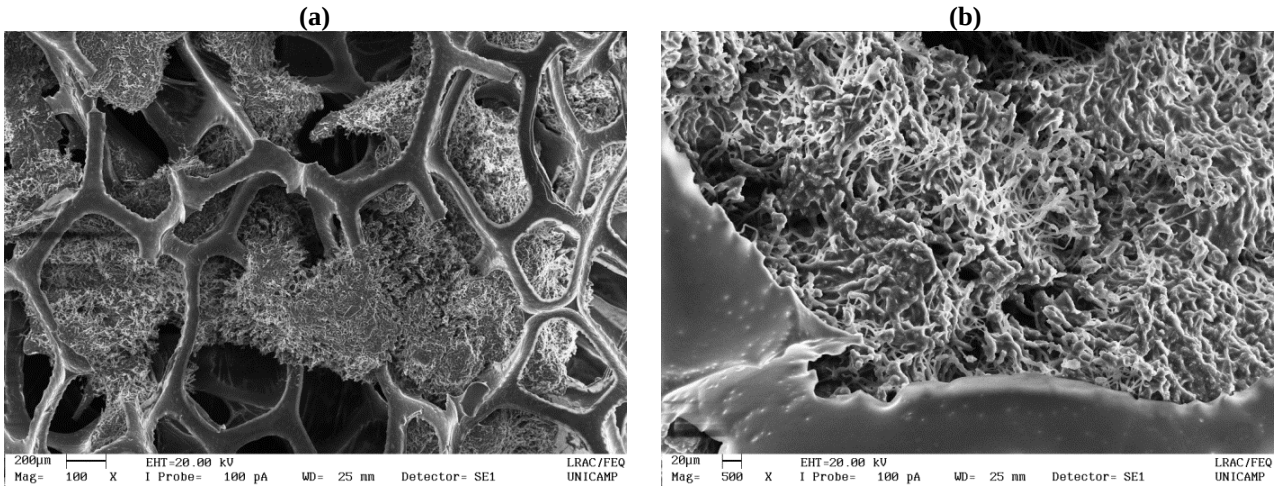

FIGURE 4. SEM IMAGES OF *P. citrinum* WHOLE CELLS IMMOBILIZED IN PUF WITH MAG OF (A) 100 X (B) 500X

The effect of the pH and temperature on mycelium-bound lipase activity was investigated by varying the buffer pH from 6.0 to 8.5 and temperature from 30 to 55 °C. The higher values of lipase activity was on average 166.8 ± 3.2 U g^{-1} and, as illustrated in Fig. 5a, the lipase activity increase proportionally with pH, achieving the maximum value in pH 8.0. For temperature, the optimal lipase activity was attained at 40 °C (Fig. 5b).

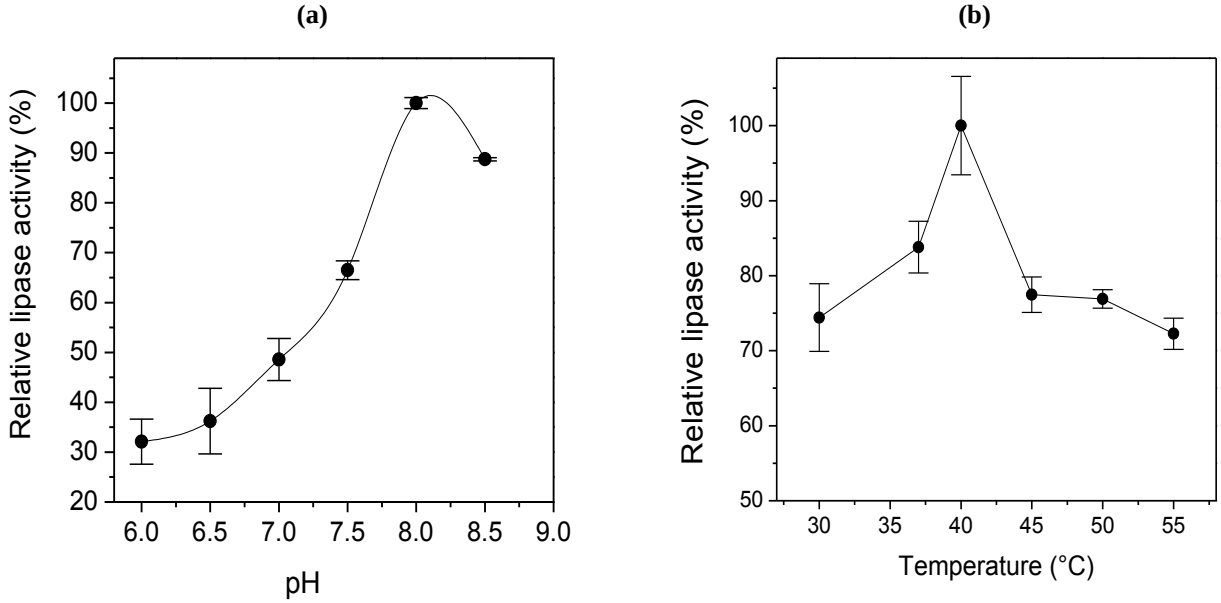

FIGURA 5. PH (A) AND TEMPERATURE (B) OPTIMAL OF *P. CITRINUM* IMMOBILIZED WHOLE CELLS

The thermal stability of mycelium-bound lipase from immobilized whole cells was determined aiming to evaluate the irreversible loss of enzymatic activity on exposure to high temperature. Figure 6 shows the denaturation kinetic of lipase activity at 60 °C for 150 min. After this period, the mycelium-bound lipase remained lower than 50% of its original activity. Based on these results, by fitting a model of first order deactivation, the thermal deactivation constant (k_d) and half-life time ($t_{1/2}$) of immobilized whole cells were determined and the values achieved were $8.62x10^{-5}$ h^{-1} and 2.23h, respectively. The half-life time is the time which takes for the activity to reduce to a half of the original activity and inversely proportional to the rate of deactivation.

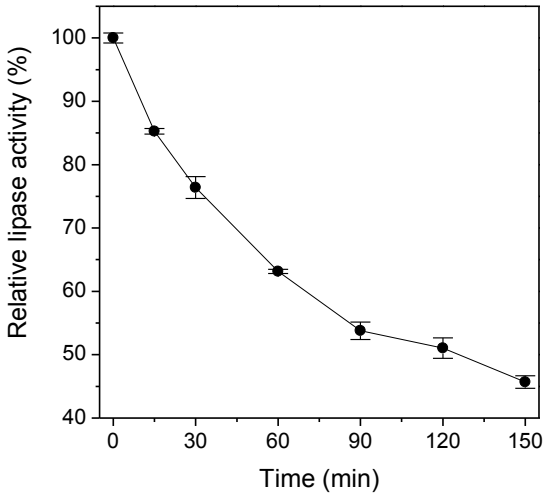

FIGURE 6. RESIDUAL LIPASE ACTIVITY OF *P. CITRINUM* IMMOBILIZED WHOLE CELLS AT 60°C

The enzyme kinetics parameters K_m and V_{max} were measured from nonlinear regression technique aiming to determine the catalytic effectiveness of the immobilized whole cells. The apparent V_{max} value indicated the maximum reaction rate and the K_m value indicated the substrate affinity and enzyme conformational changes, while the catalytic efficiency (V_{max}/K_m) indicated the overall cumulative effect of V_{max} and K_m on enzyme activity [19]. The lipase activity of immobilized whole cells at different substrate concentrations was determined, as illustrated in Figure 7, and the maximum reaction rate obtained (V_{max}) was 123.2 U g^{-1}. The lower affinity of the mycelium-bound lipase-substrate was revealed by its elevated K_m value of 158.10 mM, confirmed by catalytic efficiency of 0.78. This behavior can be due to the presence of support matrix associated to mycelium, which would cause diffusional effects in mass transfer between lipase and substrate [8].

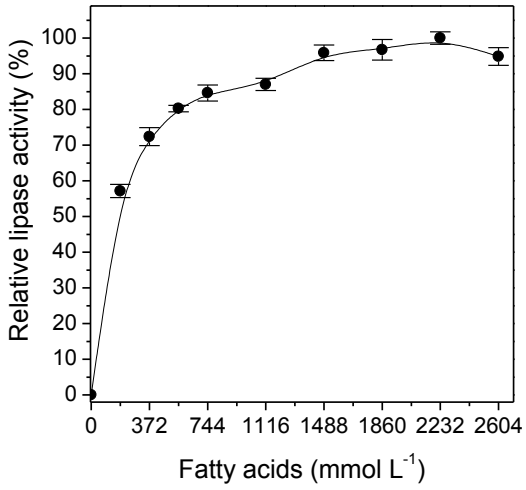

FIGURE 7. EFFECT OF SUBSTRATE CONCENTRATION IN LIPASE ACTIVITY OF *P. CITRINUM* IMMOBILIZED WHOLE CELLS (EXPRESSED BY TOTAL FATTY ACIDS INTO OIL EMULSION)

3.4 Soybean oil hydrolysis

The catalytic potential of immobilized whole cells was evaluated in the hydrolysis of vegetable oil with long-chain polyunsaturated fatty acids such as soybean. Soybean oil hydrolysis was performed in a stirred-tank reactor for 24 h at 37 ◦C with Arabic gum as emulsifier in oil emulsion (25% wt.) at pH 7.0 buffer phosphate 0.1mol L^{-1}. The kinetic profile of the soybean oil hydrolysis is displayed in Figure 8.

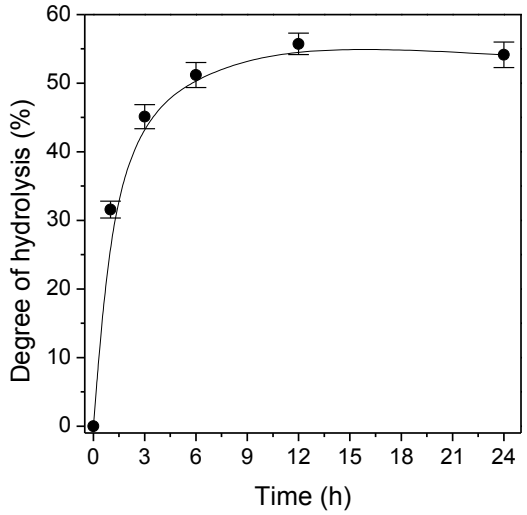

FIGURE 8- KINETIC OF SOYBEAN OIL HYDROLYSIS CATALYZED BY *P. CITRINUM* IMMOBILIZED WHOLE CELLS (T=37 °C, PH 7.0)

Under these conditions, soybean oil was efficiently hydrolyzed by immobilized whole cells achieving almost 50% of hydrolysis degree in 3h of reaction time. Maximum hydrolysis degree of 55.7 ± 1.6% was attained in 12h of reaction time, which remained stable for a further 12 h. These results evidenced the potential of immobilized whole cells as biocatalyst in reaction of hydrolysis, since the literature reports similar results employing commercial preparations lipase. Aarthy et al. [20] investigate the hydrolysis of cod liver, salmon, sardine and shark oils using *Cryptococcus* sp. lipase and found in 24h hydrolysis degree in a range of 28.6 to 36.4 %. After an optimization of reaction conditions such as time, emulsifier and loading lipase, the hydrolysis ratio was enhanced to 83.7%, but in 72h of reaction time. Avelar et al. [21] tested soybean, canola and olive oils in the hydrolysis reactions employing lipase from dormant castor bean seed and found in 3h of reaction about 88.2% degree of hydrolysis for canola oil. They optimized the parameters mass ratio oil:buffer, temperature and $CaCl_2$, and achieved full hydrolysis of canola oil in 3h of reaction time.

IV. CONCLUSION

P. citrinum whole cells were successfully immobilized in PUF and suitable conditions to enhance the mycelium-bound lipase production were determined. Maximum lipase activity was achieved in 96 h incubation time in medium at pH=7.5 at 30 °C using inoculum size of 30 mL L^{-1}. SEM images showed strong adhesion of cells into the support matrix under the establishment conditions. The immobilized whole cells were characterized and revealed optimum lipase performance in pH 8.0 at 37 °C, with V_{max} 123.24 U g^{-1} and satisfactory thermal stability at 60° C (half-life time =2.23 h). Enzymatic soybean hydrolysis was carried out by immobilized whole cells, which obtained maximum hydrolysis degree of 55.7± 1.56% in 12h of reaction time. These results suggest the use of immobilized whole cells as biocatalyst are promising and economically attractive in oils and fats industry.

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