Immobilization of commercial lipase onto different supports: characterization and application in esterification reaction

Willian de Souza Matias Reis[1], Renata Deda Mendonça Ferreira[2], Ernandes Benedito Pereira[3*]

[1]Bioprocess Laboratory, Federal University of Alfenas, BR
[2]Chemical Department, Federal Technological University of Paraná, Campus PatoBranco, BR
[3*]Pharmaceutical Sciences Department, Federal University of Alfenas, BR
*Correspondence to: Ernandes Benedito Pereira

Abstract— *The current preference of costumers for natural and healthy products is increasing the employment of biotechnological processes that use enzymes, and the synthesis of esters is an example of this change. However, enzymes are high-cost product, which stimulates research in finding solutions that make them more economically attractive, like immobilization. This work aimed to use different protocols for immobilizing lipase and its application in ester synthesis. The results showed that Pseudomonas fluorences lipase (AKL) was the most efficient for immobilization among other studied lipases (Pseudomonas fluorences lipase (AKL), Pseudomonas cepacia lipase (PSL), Hog Pancreas lipase (PHL), Pancreas Porcinas lipase (PPL), and Mucor Javanicus lipase (MJL)), with hydrolytic activity of 3323.6 U/g. Both immobilization methods (physical adsorption and entrapment) showed promising results towards hydrolytic activity. The best immobilization by adsorption was obtained using AKL onto PHB (polyhydroxybutyrate), with 698.61 U/g of hydrolytic activity. For entrapment, AKL also presented the best result, with 247.30 U/g of hydrolytic activity. For the synthesis of ester, after a 60 h-reaction using the immobilized derivatives by physical adsorption, the esterification yield was 74.26 %. In terms of hydrolytic activity, the employed protocols were very promising and encourage the continuity of this study towards the optimization of processes using industrial lipases.*

Keywords— Adsorption, Entrapment, Lipase, Ester, Hydrolysis.

I. INTRODUCTION

In the drive for alternative solutions for production, reduction of costs, and application of environmental friendly production, the replacement of chemical catalysts for biological catalysts has been growing over the time due to the attractive characteristics of enzyme for industries, such as: high specificity an selectivity, low toxicity, purity of the product, reduced environmental impact, easy handling, reducing energy cost by lowering the process temperature, use of mild pH values, among others [1,2,3]. Even if the enzyme demand is high, not all types of enzymes are produced in industrial scale because of the difficulty in finding high yield, activity, and stability enzymes [4].

In order to bypass the difficulties of using enzymes industrially, the development of immobilization techniques revolutionized and expanded the employment of enzymes, because the immobilization process allows them to be reused many times [5]. The most known and applied immobilization methods are: covalent binding [6], physical adsorption [7], entrapment (or encapsulation) in polymeric matrix [8], reticulation [9], and bio selective adsorption [10].

The entrapment of enzymes in porous matrices promotes the creation of a protector microenvironment for the enzymatic structure, which improves its operational stability [11]. The pores formed in the enzyme-support complex allow small molecules to diffuse easily and reach the active site of the entrapment enzyme, which enables it to keep its activity even after immobilization [8]. In physical adsorption, the molecules of enzyme adhere to the support surface through hydrophobic interactions, and, because of that, the adsorbed enzymes usually are resistant to proteolysis and aggregation [12, 13].

Lipases, formally named as triacylglycerol hydrolases (E. C. 3.1.1.3), consist in a class of hydrolytic enzymes of great applicability as biocatalyst in various reactions, such as esterification and transesterification [14]. They act on hydrolysis of oils and fats, releasing fatty acids and glycerides in the presence of water. Lipases can be obtained from vegetal or animal tissues, or from microorganism cultivation [15]. They present attractive aspects for industrial application such as: easy modification of catalytic properties that could be shaped through changes in its genetic structure, or reaction conditions; high specificity; and high enantioselectivity, which makes it to be used in organic synthesis [16, 17, 18].

Among many applications for lipase, the synthesis of esters via enzymatic reaction is growing due to the increase of interest *in natural* products in food industry. Esters (RCOOR') are organic compounds well distributed in nature. Glycerin esters are called glycerides, and they form the main oils and fats. A lot of simple esters are liquids with pleasant scent, responsible for fruits and flowers aromas. Those short chain esters have great industrial application as flavoring agents, besides their presence in fermented beverages [19]. Some aromatic esters of short chain fatty acid and alcohols are great value compounds for fragrance in food industry. That is why some authors study the direct esterification of fatty acids and alcohols in a non-polar media using free or immobilized lipase as biocatalysts [20, 21, 19].

In this work, different supports were used to immobilize commercial lipases from microbial and animal sources by physical adsorption and entrapment, applied in hydrolysis and esterification reactions.

II. MATERIALS AND METHODS

2.1 Lipases, supports and reagents

The commercial lipases used in this work (*Pseudomonas fluorences* (AKL), *Pseudomonas cepacia* (PSL), *Hog Pancreas* (PHL), *Porcine Pancreas* (PPL), and *Mucorjavanicus* (MJL)) were acquired from Sigma Co, USA. Chitosan (chit) *in natura*, polyhydroxybutyrate (PHB), and sodium alginate were commercially obtained and used as support for immobilization.

Other reagents used were: solvents (acetone, ethanol); salts (dehydrate calcium chloride, sodium chloride, dibasic potassium phosphate, monobasic potassium phosphate); emulsifiers (gum arabic); alkali (sodium hydroxide). The reagents used were of analytical grade, and purchased from Synth. Specific reagents, such as: bovine serum albumin (BSA) and Coomassie Brilliant Blue G-250, were acquired from Sigma Co, USA.

2.2 Support treatment for physical adsorption

At first, chitosan and PHB *in natura* were soaked in ethanol 95% w/w, and kept at rest at room temperature for 2 h(hours)[22]. Thereafter, the support was vacuum filtered and washed with distilled water.

2.3 Lipase immobilization by physical adsorption

An amount of 10 g of pre-treated support was added to 190 mL of enzymatic solution with initial protein loading of 5 and 10 $mg/g_{support}$. This system was incubated for 8 h, at 125 rpm, and room temperature (around 25 °C). At the end of the process, the immobilized derivative was vacuum filtered, washed with distilled water, and stored for drying.

2.4 Lipase immobilization by entrapment (hydrogel)

Two lipase solution was prepared (5 and 10 mg/mL), and also two $CaCl_2$ solution (0.1 and 0.2 mol/L). 0.5 g of sodium alginate was dissolved in 10 mL of each lipase preparation, and afterwards, lipase solution was slowly trickled into sodium alginate solution. By doing this, the lipase solution is converted into sodium alginate small spheres. Those spheres were vacuum filtered and washed with a known volume of distilled water. This filtered was analyzed for protein content and hydrolytic activity as well as the washing water.

For both immobilization protocols, the evaluation of immobilization procedure was measured by immobilization yield (IY) according to Eq. (1).

$$IY(\%) = \frac{P_t}{P_0} * 100 \quad (1)$$

Where P_t is the concentration of residual protein after an incubation time (mg/mL), P_0 is the initial concentration of protein (mg/mL).

2.5 Protein Determination

The concentration of protein from commercial enzymes preparation was evaluated in duplicate by Bradford method [23], which is based on the binding of Coomassie Brilliant Blue G-250 dye to the protein chain. Bovine serum albumin was used as a standard to construct the calibration curve between 0 and 0.9 mg/mL. The reading was obtained in spectrophotometer at 595nm.

2.6 Hydrolytic Activity

The hydrolytic activity of the immobilized enzyme was measured by titration method through hydrolysis of olive oil [24]. The substrate was an emulsion of Arabic Gum at 7% (w/v) and olive oil in a proportion of 50% (v/v), and the reaction mixture was composed by 5 mL of substrate, 4.9 mL of sodium phosphate buffer (0.1 mol/L, pH 7.0), and 100 µL of enzyme solution or 100 mg of immobilized enzyme (dry weight) placed in a 125 mL erlenmeyer flasks. The flasks were incubated in a water bath shaker with controlled temperature at 37 °C under continuous agitation at 200 rpm for 5 min. After this time, the reaction was stopped by adding 10 mL of acetone and ethanol mixture 1:1 (v/v), and the free fatty acids were titrated with NaOH 0.02 mol/L standard solution using phenolphthalein as indicator. According to the consumed volume in titration, it was possible to calculate the hydrolytic activity of free and immobilized lipase from Eq. (2), considering that one unit of activity corresponds to the amount of enzyme required to release 1 µmol of fatty acid per minute of reaction at test conditions. The results were expressed as U/g.

$$HA = \frac{(Vb - Va) * 1000 * M}{t * m \text{ or } v} \quad (2)$$

Where HA is hydrolytic activity (U/g); Vb is the volume of sodium hydroxide used for blank sample (mL); Va is the volume of sodium hydroxide used for the sample (mL); M is the molar concentration of sodium hydroxide (mol/L); t is the time reaction (min); m or v is the mass (mg) or volume (mL), respectively, of enzyme.

2.7 Biochemical Properties: pH and temperature influence

For determining the influence of pH and temperature over the enzyme, the previous methodology was used, varying the sodium phosphate buffer pH (4.0, 5.0, 6.0, 7.0, 8.0, 9.0) and preserving the temperature at 37 °C (pH influence), or varying the temperature of water bath (40, 45, 50, 55, 60 °C) and preserving the buffer pH in 7.0 (temperature influence).

The optimum pH and temperature of the lipases used in this work according to the manufacturer were: AKL (pH 8.0, 55 °C); PCL (pH 8.0, 37 °C); HPL (pH 7.5, 40 °C); PPL (pH 7.5, 40 °C); MJL (pH 8.0, 50 °C).

2.8 Biochemical Properties: substrate concentration influence

The influence of initial concentration of substrate over the reaction rate, and the occurrence of some type of enzyme inhibition were studied according to previous methodology of hydrolytic activity using AKL, and varying the olive oil proportion on substrate (5 to 50% (v/v)). The assays were carried out at 37 °C and pH 7.0, and k_m and V_{max} values were calculated using Line weaver-Burk equation.

2.9 Thermal Stability

The thermal stability of immobilized enzyme was evaluated by incubation of samples (100 mg) at 40, 50, and 60 °C in aqueous solution of sodium phosphate buffer (0.1 mol/L, pH 7.0) for 60 min. After thermal treatment, the samples were immediately cooled in ice bath in order to stop the enzymatic inactivation reaction, and the residual activity was measured. The inactivation constant (k_d) for immobilized lipase was calculated by Eq. (3), where A_{in} is the residual activity after thermal treatment (U/g), A_{in0} is the residual activity before thermal treatment (U/g), and t is the time of incubation (h).

$$ln\left(\frac{A_{in}}{A_{ino}}\right) = K_d * t \quad (3)$$

The half-life time ($t_{1/2}$ (h)) was obtained according to Eq. (4).

$$t_{1/2} = \frac{0{,}693}{K_d} \quad (4)$$

2.10 Esterification Activity

Firstly, the hexane was treated to decrease water percentage in order to avoid any interference in esters formation. A 500 ml volume of hexane was treated with 4 g of magnesium sulfate and left to rest at room temperature for 2 hours in order to decrease water percentage and avoid any interference in esters formation [25]. The esterification activity of immobilized lipase was determined through the reaction, at equimolar ratio, of isoamyl alcohol (0.06 mol/L) with acetic acid (0.06 mol/L)

in hexane, producing the aromatic ester isoamyl acetate. The reactions were carried out in a 100 mL-closed flasks at 40 °C by adding 0.250 g of dry weight enzyme (free and immobilized) to 40 mL of reaction media in rotational shaker at 200 rpm. A blank reaction was also conducted in order to evaluate the spontaneous formation of esters. Aliquots of 1 mL were removed from the reaction after 6, 12, 24, 36, 42, 48, 54, 68, 80, and 92 hours, and the amount of esters produced was quantified by titration. One unit of esterification activity was defined as the amount of enzyme necessary for formation of 1 µmol of ester per minute of reaction at assay conditions [25].

2.11 Residual Acid Determination

The residual acid concentration was determined by titration of aliquots dissolved in 15 mL of acetone-ethanol solution 1:2 with 0.02 mol/L sodium hydroxide and 1% phenolphthalein as indicator. The math was done by equation (5), where C is the percentage of acid (%), V is the volume of sodium hydroxide used (mL), N is the concentration of sodium hydroxide (mol/L), M is the molar weight of acid (g/mol), and W is the weight that corresponds to 1 mL of aliquot used for titration.

$$C = \frac{V*N*M}{10*W} \tag{5}$$

The esterification percentage corresponds to the consumed acid percentage, according to Eq. (6), where C_i is the initial concentration of free residual acid (mol/L), and C is the concentration of free residual acid at a time (mol/L).

$$\%\ esterification = \frac{(C_i - C)}{C_i} * 100 \tag{6}$$

III. RESULTS AND DISCUSSION

3.1 Hydrolytic Activity of free lipases

In a first step, the selection of commercial lipases that presented potential and satisfactory results for immobilization was performed. Table 1 presents the preliminary results obtained in this first stage of selection of commercial lipases.

TABLE 1
HYDROLYTIC ACTIVITY OF COMMERCIAL LIPASES

Lipase	Hydrolytic Activity (U/g)
AKL	3323.6
PSL	2646
PHL	1839.6
PPL	868
MJL	725.2

From the results presented in Table 1, the best ones were obtained from *Pseudomonas fluorences* (AKL) and *Pseudomonas cepacia* (PSL) lipases, with hydrolytic activity of 3323.6 U/g and 2646 U/g, respectively. Then, the enzymes AKL and PSL were selected to proceed with immobilization processes.

3.2 Initial protein load and immobilization protocol selection

Both previously selected lipases were immobilized in PHB and chitosan by physical adsorption and entrapment with initial protein load of 5 and 10 mg/g. Table 2 shows the results obtained for physical adsorption.

The immobilization of lipase by physical adsorption proved to be efficient, providing a significant increase in almost all protocols used, which implies that the active site of the enzymes was not blocked. The highest activity for immobilization by physical adsorption was obtained for AKL in PHB with 5 mg/g of initial protein loading (698.60 ± 5.53 U/g), with immobilization yield of 87.30 ± 1.909 %.

By evaluating the performance of the supports used, PHB presented higher final hydrolytic activities for both enzymes and initial protein loading compared to the results from chitosan. This one was more efficient in terms of hydrolytic activity in PSL immobilization only, resulting in 510.46 ± 0.46 U/g and 99.63 ± 0.454 % of immobilization yield for PSL with 10 mg/g of initial protein loading.

TABLE 2
HYDROLYTIC ACTIVITY OF IMMOBILIZED ENZYME; INITIAL AND FINAL PROTEIN CONCENTRATION; YIELD OF IMMOBILIZATION, AND IMMOBILIZED PROTEIN FOR PHYSICAL ADSORPTION PROTOCOL

Lipase IPL* + Support	Immobilized Enzyme Activity (U/g)	Initial Protein (mg/g)	Final protein (mg/g)	IY (%)
AKL 5 mg/g + Chit	289.59 ± 0.46	0.303 ± 0.007	0.293 ± 0.008	96.57 ± 0.493
AKL 10 mg/g + Chit	61.83 ± 0.45	0.163 ± 0.008	0.132 ± 0.011	80.63 ± 0.382
AKL 5 mg/g + PHB	698.60 ± 5.53	0.300 ± 0.0124	0.262 ± 0.005	87.30 ± 1.909
AKL 10 mg/g + PHB	683.57 ± 5.48	0.140 ± 0.0109	0.1287 ± 0.002	92.10 ± 5.644
PSL 5 mg/g + Chit	406.37 ± 0.44	0.0389 ± 0.0005	0.0372 ± 0.015	95.95 ± 5.155
PSL 10 mg/g + Chit	510.46 ± 0.46	0.0238 ± 0.0087	0.0237 ± 0.009	99.63 ± 0.454
PSL 5 mg/g + PHB	677.09 ± 5.70	0.0419 ± 0.0096	0.0393 ± 0.013	92.44 ± 10.107
PSL 10 mg/g + PHB	672.61 ± 5.28	0.029 ± 0.0056	0.0284 ± 0.005	98.20 ± 1.507

IPL: initial protein load

Analyzing the influence of the initial protein loading, it can be inferred that the increase from 5 to 10 mg/g did not affect the immobilization of PSL, whereas the immobilization of AKL seemed to have been affected when using chitosan as support, decreasing from 289.59 U/g to 61.83 U/g. The size of enzymes and pores of supports may have influence on that result.

Dabaja et al. [26] utilized PHB as immobilization support, and the same initial protein loading as the present work. They were able to achieve 291.03 U/g for 5 mg/g of initial protein loading, which shows the potential of the present work that, with the same conditions, obtained 698.60 U/g.

The protocol of immobilization was chosen according to the highest hydrolytic activity obtained, which turned out to be AKL, 5mg/g in PHB, with 698.60 ± 5.53 U/g for physical adsorption. The second immobilization method evaluated was sodium alginate encapsulation. AKL and PSL lipases with initial concentration of 5 and 10 mg/mL, and different $CaCl_2$ concentrations (0.1 and 0.2 mol/L) were tested for alginate bead formation. The results are presented in Table 3.

TABLE 3
HYDROLYTIC ACTIVITY OF IMMOBILIZED ENZYME; INITIAL AND FINAL PROTEIN CONCENTRATION; YIELD OF IMMOBILIZATION, AND IMMOBILIZED PROTEIN FOR ENTRAPMENT PROTOCOL.

Lipase IPL* + [$CaCl_2$]	Immobilized Enzyme Activity (U/g)	InitialProtein (mg/g)	Final protein (mg/g)	IY (%)
PSL 5 mg/g + 0.1 mol/L	102.67 ± 0.69	0.857 ± 0.004	0.822 ± 0.0025	95.85 ± 0.173
PSL 5 mg/g + 0.2 mol/L	81.35 ± 1.17	0.844 ± 0.007	0.819 ± 0.0107	96.91 ± 2.128
PSL 10 mg/g + 0.1 mol/L	182.46 ± 1.80	0.867 ± 0.0005	0.807 ± 0.0008	93.09 ± 0.042
PSL 10 mg/g + 0.2 mol/L	111.14 ± 1.32	0.857 ± 0.0016	0.815 ± 0.0008	95.10 ± 0.087
AKL 5 mg/g + 0.1 mol/L	247.30 ± 1.13	0.920 ± 0.0016	0.817 ± 0.0041	88.82 ± 0.609
AKL 5 mg/g + 0.2 mol/L	144.92 ± 1.43	0.897 ± 0.0207	0.816 ± 0.0041	90.97 ± 1.261
AKL 10 mg/g + 0.1 mol/L	176.51 ± 1.63	0.883 ± 0.0058	0.816 ± 0.0058	92.40 ± 1.261
AKL 10 mg/g + 0.2 mol/L	152.93 ± 1.18	0.882 ± 0.0016	0.807 ± 0.0058	91.52 ± 0.485

IPL: initial protein load

From the results presented in Table 3, the encapsulation immobilization protocol was efficient in increasing the hydrolytic activity of the enzymes in all tested protocols. However, when compared to the results of physical adsorption, the

encapsulation was less efficient in preserving the hydrolytic properties. The decrease in HA may have been caused by diffusional limitations faced by the pores formation in the alginate spheres, reducing the formation of enzyme-substrate complex and hence the product formation.

The catalytic properties of immobilized enzymes can be influenced by three forms of interaction: the binding of the enzyme in the matrix may cause conformational changes that affect the tridimensional structure of the enzyme; substrate access to the active site may be affected by steric hindrance of the support; and the support properties can affect the mode of action of the enzyme [27]. The influence of initial protein loading resulted in an increase of HA with the increase in initial protein provided, except for the combination of AKL immobilized in 0.1 mol/L sodium alginate. The concentration of sodium alginate, in turn, had a negative influence on HA, which implies that the increase in sodium alginate concentration resulted in spheres with smaller pore diameters, so a steric hindrance occurs and the substrate does not reach the active site of enzyme properly.

Teixeira et al. [28] used calcium alginate and the same initial protein loading in their study, and they obtained lower HA for the immobilized enzymes than these work shows, which confirms the efficiency of sodium alginate in entrapment immobilization.

Lipase immobilization by entrapment had its best result when using AKL 5 mg/mL, 0.1 mol/L $CaCl_2$, with 247.30 ± 1.13 U/gof HA.

In order to proceed with the work, the best result from physical adsorption and entrapment immobilization were used to study the influence of pH, temperature, substrate concentration, thermal stability, and ester synthesis.

3.3 Influence of pH

The medium pH can influence the structure of enzymes, and, consequently, their activity, in a manner that the enzyme presents an optimal pH value. Immobilized enzymes may follow the typical behavior of free enzymes, but the pH profile of immobilized and free enzymes is not always the same, as the immobilization process may induce conformational changes [15]. The results for physical adsorption and entrapment are shown in Fig. 1.

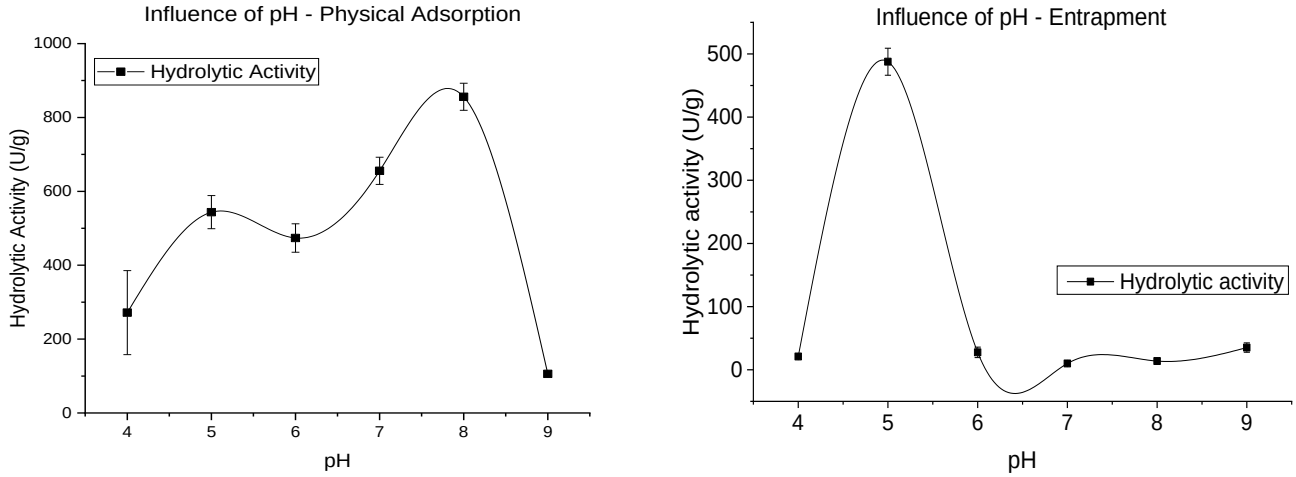

FIGURE 1: Influence of pH on immobilization of AKL by physical adsorption (a) and entrapment (b)

For physical adsorption (Fig. 1a), the optimum pH was 8.0 (856.10 ± 36.65 U/g), which is the same as presented by the manufacturer for free lipase. Below or above this pH value, there are changes in enzyme 3D structure that harm its activity.

For entrapment (Fig. 1b), the immobilized enzyme presented an optimal pH of 5, with HA of 487.69 ± 21.36 U/g. In this case, a shift from 8 to 5 of optimum pH is observed upon immobilization.

Optimum pH of 8.0 was also obtained by Dabaja et al. [26] and Tintor et al. [29].

3.4 Influence of Temperature

The rate of enzymatic reactions is extremely temperature dependent. Increasing the temperature gives rise to the energy of the molecules and thereby increases the enzymatic activity. However, at very high temperatures, the enzyme may be

denatured and thus lose its activity [15]. Fig. 2 presents the influence of temperature on immobilization of AKL by physical adsorption (Fig. 2a) and entrapment (Fig. 2b).

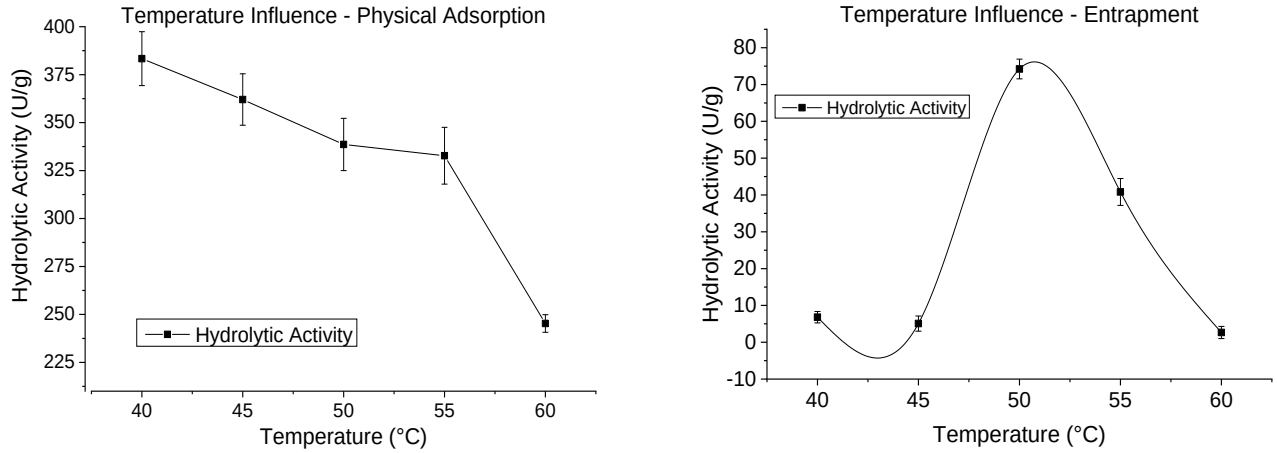

FIGURE 2: Influence of temperature on immobilization of AKL by physical adsorption (a) and entrapment (b).

From Fig. 2a, it was found that the best temperature for immobilized AKL by physical adsorption was 40 °C, with 383.37 ± 13.76 U/g, which is lower that the optimum temperature presented by the manufacturer for free lipase (55 °C). For some industrial processes, the products are heat sensible, so the decrease in optimum temperature becomes an advantage for those applications. For immobilization by entrapment, Fig. 2b shows that there was not a great shift of optimum temperature, since the immobilized lipase showed its higher activity at 50°C. Nevertheless, unlike the physical adsorption, the hydrolytic activity decreased considerably at higher temperatures, suggesting a poor thermal stability.

3.5 Influence of substrate concentration

The analysis of kinetic parameters such as K_m and V_{max} is important because they reveal the specificity changes in relation to the substrates that the enzymes undergo after immobilization process [30]. Fig. 3 presents the influence of substrate concentration (olive oil) on reaction rate using immobilized AKL by physical adsorption (Fig. 3a) and entrapment (Fig. 3b).

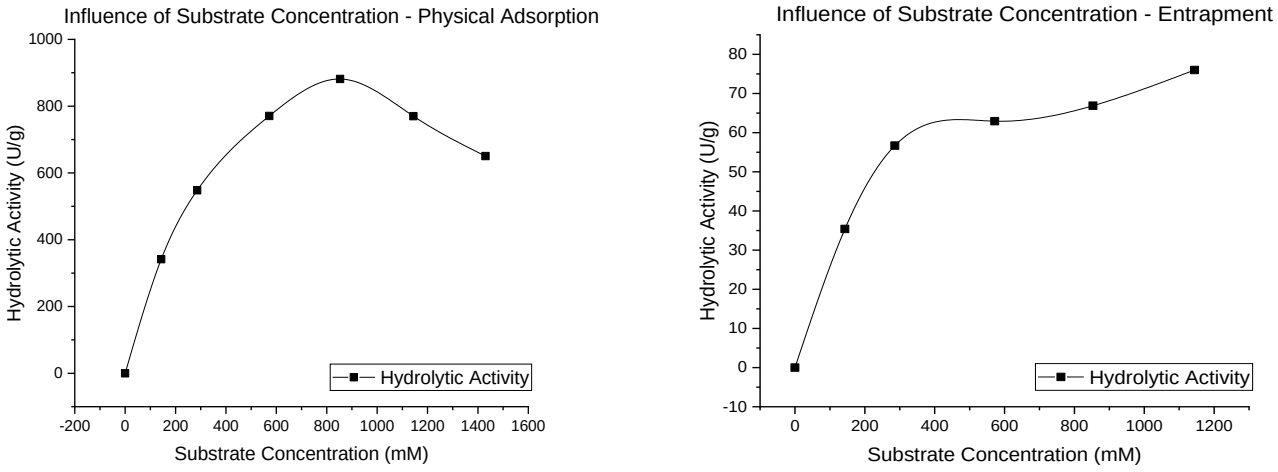

FIGURE 3: Influence of substrate concentration (olive oil) on reaction rate using immobilized AKL by physical adsorption (a) and entrapment (b).

Analyzing the results from Fig. 3a, it can be noted that for the physical adsorption procedure, he ratio that presented the highest activity was 30 % of olive oil and 70% of water, with an activity of 881.30 U/g and the profile obtained follows the Michaelis-Menten model. For immobilization by entrapment (Fig. 3b), the best substrate concentration was 40%, with activity of 75.99U/g, and it also followed the Michaelis-Menten model profile.

From the obtained data, it was possible to use Lineweaver-Burk linearization tool in a graph of 1/HA versus 1/S, where HA is the hydrolytic activity, and S is the substrate concentration in order to calculate the affinity constant (K_m) and the

maximum reaction rate (V_{max}). For the adsorption protocol, K_m and V_{max} were 0.05 mM and 1000 µmol/min.g, respectively, and for the entrapment protocol K_m was 0.03 mM, and V_{max} was 81.97 µmol/min.g.

AKL immobilized by physical adsorption in PHB showed an apparent V_{max} value of 1000 U/g and K_m of 0.05 mM, while when immobilized by entrapment in sodium alginate, it presented much lower V_{max} (81.97 U/g), and similar, but lower K_m (0.03 mM), which confirms the diffusional problems presented by the entrapment method, since the reaction catalyzed by AKL in PHB is much faster than the reaction catalyzed by AKL in alginate.

3.6 Thermal stability

The results for thermal stability for both immobilization methods are presented in Table 4.

TABLE 4
RESIDUAL ACTIVITY OF IMMOBILIZED LIPASE WITHIN AFTER 60 min-incubation.

PhysicalAdsorption		Entrapment	
Temperature(ºC)	**Residual Activity (U/g)**	**Temperature(ºC)**	**Residual Activity (U/g)**
40	610.44 ± 25.64	40	74.92 ± 16.26
50	613.63 ± 78.02	50	172.36 ± 12.47
60	655.69 ± 6.59	60	221.36 ± 44.17

It can be observed that for all temperatures applied, AKL immobilized in both methods presented good thermal stability since it did not lose activity with the increase in incubation temperature, avoiding enzyme denaturation and damage to the structure of sodium alginate beads. From the data presented in Table 4, it was possible to calculate de inactivation constant (k_d) and the half-time life ($t_{1/2}$) for immobilized lipases, and the results are shown in Table 5.

TABLE 5
INACTIVATION CONSTANT AND HALF-LIFE TIME FOR IMMOBILIZED LIPASE.

	Physical Adsorption		Entrapment	
Temperature (ºC)	**K_d (h^{-1})**	**$t_{1/2}$ (h)**	**K_d(h^{-1})**	**$t_{1/2}$ (h)**
40	0.1349	5.137	1.1942	0.5803
50	0.1297	5.344	0.3610	1.9195
60	0.0634	10.931	0.1108	6.2540

For the immobilization by physical adsorption, the temperature of 60 °C presented the longest half-time life (10.931 h), and for immobilization by entrapment, the longer time was 6.2540 h, which means that the enzyme immobilized by physical adsorption lasts more time to reduce its activity to 50 % of the initial value.

For both immobilizations methods, it was observed that the half-life time increased with the increment in temperature. From the results obtained in Table 5, indicate that for both immobilization methods, the enzyme immobilized derivative can be used at higher temperatures, without significant loss of its activity and such result is of great importance for future industrial application.

3.7 Application: Esterification reaction (Synthesis of esters and flavorings)

The synthesis of esters via enzymatic route involves a complex mechanism dependent on the type of substrate, enzyme, organic solvent, and concentration of the reaction medium. The aromatic ester selected was isoamyl acetate, characterized by the aroma of banana and pear.

A comparison of the performance of free and immobilized lipases was carried out alongside a control (a reaction without the presence of enzyme). Acid consumption and ester formation were quantified by titration method through the disappearance of fatty acid. The residual acid content was calculated from Eq. 6 and the results are shown in Table 6.

TABLE 6
ISOAMYL ACETATE YIELD PERCENTAGE WITHIN 78 H OF REACTION.

Time(h)	Adsorption	Entrapment	Free lipase
6	31.187	28.147	17.146
12	29.686	29.745	26.204
24	23.951	19.112	34.403
36	31.338	26.459	35.008
48	41.456	30.179	34.977
54	9.558	32.850	54.147
60	74.264	26.989	36.823
66	16.898	32.048	50.400
72	34.425	37.843	40.052
78	7.079	41.992	40.033

From Table 6, the formation of isoamyl acetate was higher when using immobilized lipase by physical adsorption, with 74.264% after 60 h of reaction, whereas immobilized lipase by entrapment achieved 41.992% after 78 h, and free lipase achieved 54.15% after 54 h. Although the immobilized lipase by physical adsorption presented a better esterification yield, its profile was not linear, increasing exponentially within 54 h of reaction and remaining below 50% after 60 h of reaction. On the other hand, the immobilized lipase by entrapment kept a linear profile, achieving the maximum percentage yield after 78 h of reaction.

IV. CONCLUSION

At the end of this work it is possible to consider that both immobilization methods presented satisfactory results as the enzymes were able to increase their hydrolytic activity under several conditions. The lipase immobilized by physical adsorption demonstrates the potential of PHB *in natura* to be used as support for lipase immobilization. Free and immobilized lipases had similar operating conditions, and the the PHB immobilized AKL showed the best percentage yield of isoamyl acetate formation after 60h-reaction (74.26%).

ACKNOWLEDGEMENTS

The authors are grateful to UNIFAL-MG for their technical and financial support.

REFERENCES

[1] F. M. S. Santos, R. M. C. Dos Santos, M. N. Melo, Á. S. Lima, H. M. Alvarez, C. M. F. Soares, A. T. Fricks, "Imobilização de peroxidase de raiz forte por encapsulamento em microesferas de alginate na presença de líquidos iônicos imidazólios", BlucherChemicalEngineeringProceedings, São Paulo, vol. 1, n. 2, pp. 2011-2016, 2015.

[2] B. H. De Oliveira, "Produção e purificação da lipase de *Burkholderia lata* LBBIO-BL02, sua caracterização cinética e aplicação em reações de biocatálise de interesse farmacológico e industrial", PhD Thesis, Universidade Estadual Paulista, Rio Claro, São Paulo, 2017.

[3] S. Sanchez, A. L. Demain, "Chapter 1 - Useful Microbial Enzymes: An Introduction", Biotechnology of Microbial Enzymes, pp. 1-11, 2017.

[4] C. H. Tan, P. L. Show, C. W. Ooi, E.-P. Ng, J. C.-W. Lan, T. C. Ling, "Novel lipase purification methods – a review of the latest developments", Biotechnology Journal, vol. 10, n. 1, pp. 31-44, 2015.

[5] K. Khaldi, S. Sam, A. Lounas, C. Yaddaden, N.-E. Gabouze, "Comparative investigation of two methods for Acetylcholinesterase enzyme immobilization on modified porous silicon", Applied Surface Science, vol. 421, pp. 148-154, 2017.

[6] K. Pashangeh, M. Akhond, H. R. Karbalaei-Heidari, G. Absalan, "Biochemical characterization and stability assessment of *Rhizopusoryzae* lipase covalently immobilized on amino-functionalized magnetic nanoparticles", International journal of biological macromolecules, vol. 105, n. 1, pp. 300-307, 2017.

[7] M. Koenig, E. Bittrich, U. König, B. L. Rajeev, M. Müller, K. J. Eichhorn, S. Thomas, M. Stamm, P. Uhlmann, "Adsorption of enzymes to stimuli-responsive polymer brushes: influence of brush conformation on adsorbed amount and biocatalytic activity", Colloids and Surfaces B: Biointerfaces, vol. 146, pp. 737-745, 2016.

[8] Z. Zhang, R. Zhang, D. J. Mcclements, "Lactase (β-galactosidase) encapsulation in hydrogel beads with controlled internal pH microenvironments: Impact of bead characteristics on enzyme activity", Food Hydrocolloids, vol. 67, pp. 85-93, 2017.

[9] S. L. Martins, B. F. Albuquerque, M. A. P. Nunes, M. H. L. Ribeiro, "Exploring magnetic and imprinted cross-linked enzyme aggregates of rhamnopyranosidase in microbioreactors", Bioresource Technology, vol. 249, pp. 704-712, 2018.

[10] C. L. Cardoso, M. C. De Moraes, Q. B. Cass, "Imobilização de enzimas em suportes cromatográficos: uma ferramenta na busca por substâncias bioativas", Química Nova, vol. 32, n. 1, pp. 175-187, 2009.

[11] J. Kim, J. W. Grate, P. Wang, "Nanostructures for enzyme stabilization", Chemical Engineering Science, vol. 61, n. 3, pp. 1017-1026, 2006.

[12] R. M. Pereira, "Técnicas de imobilização e estabilização de lipases obtidas a partir de diferentes fontes microbianas", Final WorkofUndergraduation (ChemicalEngineering), Universidade Federal de Alfenas, Campus de Poços de Caldas, 2014.

[13] V. L. Sirisha, A. Jain, A. Jain, "Enzyme Immobilization: An Overview on Methods, Support Material, and Applications of Immobilized Enzymes", Advances in Food and Nutrition Research, vol. 79, pp. 179-211, 2016.

[14] S. Menoncin, N. M. Domingues, D. M. G. Freire, J. V. Oliveira, M. Di Luccio, H. Treichel, D. De Oliveira, "Imobilização de lipases produzidas por fermentação em estado sólido utilizando *Penicilliumverrucosum* em suportes hidrofóbicos", Ciência e Tecnologia de Alimentos, vol. 29, n. 2, pp. 440-443, 2009.

[15] S. Liu, Bioprocess engineering: Kinetics, Sustainability, and Reactor Design. 2nd, Elsevier, NY, USA, 2017, pp. 297-373.

[16] G. Da S. Padilha, "Caracterização, purificação e encapsulamento de lipase de *Burkholderiacepacia*", PhD Thesis, Universidade Estadual de Campinas, Campinas, São Paulo, 2010.

[17] N. B. Carvalho, A. S. Lima, C. M. F. Soares, "Uso de sílicas modificadas para imobilização de lipases", Química Nova, vol. 38, n. 3, pp. 399-409, 2015.

[18] K. B. Dias, "Acúmulo de lipídios intracelulares e imobilização de lipase por *Candidaviswanathii*: potencial para hidrólise de gordura de frango", Master dissertation, Universidade Federal do Tocantins, Gurupi, Tocantins, 2016.

[19] A. Pimentel, M.G. Nascimento, D. Sebrão, "Síntese de Ésteres Catalisada por Lipases Imobilizadas em Filmes de PVA", Final WorkofUndergraduation (Chemistry). Universidade Federal de Santa Catarina, Florianópolis, 2006.

[20] J. Chen, "Production of ethyl butyrate using gel-entrapped *Candida cylindracea* lipase", Journal of fermentation and bioengineering, vol. 82, n. 4, pp. 404-407, 1996.

[21] P. O. Carvalho, P. R. B. Campos, M. D. Noffs, J. G. De Oliveira, M. T. Shimizu, D. M. Da Silva, "Aplicação de lipases microbianas na obtenção de concentrados de ácidos graxos poli-insaturados", Química Nova, vol. 26, n. 1, pp. 75-80, 2003.

[22] A. A. Mendes, P. C. Oliveira, A. M. Vélez, R. C. Giordano, R. de L. C. Giordano, H. F. De Castro, "Evaluation of immobilized lipases on poly-hydroxybutyrate beads to catalyze biodiesel synthesis", International Journal of Biological Macromolecules, vol. 50, n. 3, pp. 503-511, 2012.

[23] M. M. Bradford, "A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principles of protein-dye binding", Analytical Biochemistry, vol. 72, pp. 248-254, 1976.

[24] C. M. F. Soares, H. F. De Castro, F. M. De Moraes, G. M. Zanin, "Characterization and utilization of *Candida rugosa* lipase immobilized on controlled pore silica", Applied Biochemistry and Biotechnology, vol. 79, n. 1–3, pp. 745-757, 1999.

[25] V. C. Aragão, A. Anschau, B. D. A. Porciuncula, C. Thiesen, S. J. Kalil, C. A. V. Burkert, J. F. de M. Burkert, "Síntese enzimática de butirato de isoamila empregando lipases microbianas comerciais", Química Nova, vol. 32, n. 9, pp. 2268-2272, 2009.

[26] M. Z. Dabaja, B. De M. Bizzo, E. B. Pereira, "Síntese de biodiesel a partir do óleo de açaí empregando lipase comercial imobilizada em suporte de baixo custo", Revista da Universidade Vale do Rio Verde, vol. 16, n. 2, pp. 1-9, 2014.

[27] J. Tu, S. Bolla, J. Barr, J. Miedema, X. Li, B. Jasti, "Alginate microparticles prepared by spray-coagulation method: Preparation, drug loading and release characterization", International Journal of Pharmaceutics, vol. 303, n. 1-2, pp. 171-181, 2005.

[28] V. F. T. Teixeira, "Estudo da obtenção de biocatalisadores com matrizes de alginato de cálcio visando a produção de biodiesel", Master Dissertation, Universidade Estadual do Norte Fluminense Darcy Ribeiro, Campos dos Goytacazes, Rio de Janeiro, 2011.

[29] C. B. Tintor, B. M. Bizzo, A. A. Mendes, D. B. Hirata, E. B. Pereira, "Avaliação da lipase AK de *Pseudomonasfluorescens* imobilizada em suporte PHB na catálise da reação de transesterificação", BlucherChemicalEngineeringProceedings, São Paulo, vol. 1, n. 2, pp. 1271-1279, 2015.

[30] S. M. Villela, "Imobilização de Lacase e seu uso na biotransformação de Efluentes de indústrias papeleiras", Master Dissertation, Universidade Federal de Santa Catarina, Florianópolis, 2006.