Lipase and Glucoamylase Immobilization on The New Calcium Polyphosphate Support Obtained by Sol-Gel Method

César Milton Baratto*[1], Elen Rossi[1], Jane Mary Lafayette Neves Gelinski[1], Vera Lucia Pissaia[2], Willian Kopp[3]

[1]Universidade do Oeste de Santa Catarina – Molecular Biology Laboratory. 198, Paese Street. CEP. 89560-000 Videira-SC-Brazil.

[2]Dublin Institute of Technology, Campus Planning Office, Grangegorman Lower, Dublin 7, Ireland.

[3]Departamento de Engenharia Química, Universidade Federal de São Carlos, São Carlos, SP 13565-905, Brazil.

*[1]Correponding author E-mail: cesar.baratto@unoesc.edu.br
Phone number: +55 49 35334479 Fax: +55 49 35334422

Abstract—**Enzymes immobilization onto insoluble supports is an alternative to enhance thermal and operational stabilities besides providing the reuse of the enzymes. The interesting potential of the enzyme immobilization has stimulated the search for new effective supports to immobilization and economically viable for industrial application. This study aimed to analyze the efficiency of the support obtained from calcium polyphosphate on immobilization by adsorption of commercial enzymes. The xerogel-S support was produced by sol-gel method, heat-treated and used for the immobilization of Lipolase and Glucoamylase AG, were tested different times and protein concentration offered on process. The parameters used to analyze the immobilization effectiveness were immobilization yield, retention activity and protein yield. Furthermore, were evaluated the optimum temperature and pH and thermal stability for free and immobilized enzymes. According to results the optimal immobilization times were 1 hour for lipase and 1-2 hours for glucoamylase and the immobilization parameters indicated that a support had a greater efficiency for the glucoamylase immobilization than the lipase. Optimal activity temperature for free and immobilized lipase was found to be 60°C and 30°C, the optimal activity pH was around 5,0 for both free and immobilized lipase and the immobilized lipase has increased thermal stability in the temperature of 70°C. While the optimal conditions of temperature and pH for maximum glucoamylase activity as well as thermal stability were not statistically different between free and immobilized enzyme. In conclusion, the xerogel-S show potential use for immobilization by adsorption of commercial enzymes.**

Keywords - adsorption; calcium polyphosphate; enzyme immobilization; phosphate gel; heat treatment.

I. INTRODUCTION

In various production stages, processing and exploitation of raw materials in industries, enzymatic bioconversion processes have been widely used. Among the many advantages, the demand for enzymatic processes has occurred due to the search for cleaner technologies and less harmful to the environment [1]. Furthermore, enzymes are characterized by having a high degree of substrate specificity, much larger than any known chemical catalyst, representing a considerable saving of time, energy and investment in equipment corrosion resistant and high pressures or temperatures [2]. However due to the huge cost of the purification, as well its instability in solution, water or organic solvents, there are difficulties of reuse in consecutive processes [2, 3].

On the other hand, this problem can be reduced by enzyme immobilization on a solid support, such techniques usually increase the enzyme stability and enable their use in many processes, since the immobilized system can be used by multiple reaction cycles without renewal of the catalyst [1, 4, 5].

The enzyme immobilization besides providing the biocatalyst reuse, making the process less costly, it also benefits by raising the thermal stability and resistance to change in pH of the reaction, offers the possibility of using the enzyme in high concentrations with subsequent reduction of the reaction volume, and the easy purification of the product without contamination by the catalyst, whereas the immobilized enzyme is not soluble in the reaction medium [6].

The enzymes can be immobilized in several ways as well as immersed microcapsules in gel, adsorbed on insoluble supports such as silica and resins [5, 7, 8] or be attached by covalent bond in these insoluble polymeric matrix [4].

Regarding the selection of the support for the immobilization of enzymes, is necessary to evaluate some characteristics that are important for application on industrial processes, which stand out the mechanical and microbial resistance, thermal stability, chemical functionality, hydrophobic or hydrophilic character, facility in regeneration, the cost, beyond size, shape and the density [1, 2].

The supports commonly used for immobilization may be divided into two categories, hydrophobic and hydrophilic matrix, but they can also be classified according to their morphology and structure, as porous materials, non-porous, and gel structure [4, 9]. On the other hand, the support need to own good mechanical strength, since the immobilization process and its use may require operations such as filtration, centrifugation and agitation, in addition display thermal stability, chemical resistance on the immobilization or reaction conditions, and possess chemical groups that can be activated or modified, allowing the enzyme binding without its denaturation [1, 10].

Therefore, novel materials have been researched and developed for the immobilization using technology of nano and microparticles, however a major drawback is the high cost for their production, which prevents its application to large-scale [11, 12]. Thus, are sought materials with low cost and the great potential for immobilization which enables the use in industrial processes [5, 13].

Within the materials chemistry one of most interesting processes being used to obtain supports for immobilization is the sol-gel method [3, 14]. This method allows to obtain materials with special characteristics at low temperatures, enabling control of the material characteristics, generate materials with high purity and high values of porosity and specific surface [3,14]. The sodium polyphosphate is a compound of low cost which is soluble in water in the presence of electrolyte has the potential for this purpose [15].

The sodium polyphosphate is constituted by linear chains of phosphates, and depending on the origin may contain, in small amounts, cyclic phosphate [16]. Polyphosphate in high concentration and under suitable conditions in the presence of electrolyte an interesting step of the phase separation occurs, this coacervation phenomenon, in which two liquid phases with different densities and viscosities become apparent [17]. The viscous phase, rich in colloidal particles, is called the coacervate (xerogel), and the less viscous phase, poor in colloidal particles, is the equilibrium liquid. The coacervate contains water, polyphosphate chains and cations, including sodium, coming from the Graham salt [18].

Calcium polyphosphate has potential application as a replacement of bone, drug delivery systems, coating materials, optical devices, adsorption of heavy metals, among others [15, 16, 19], however, the latest suggested possibility is its use as a support for enzyme immobilization [18].

The possible use of calcium polyphosphate coacervates for the enzymes immobilization needs to be verified. This work aims to determine the performance of polyphosphate calcium-based material (xerogel-S), obtained by a sol-gel method and heat-treated for the immobilization of commercial lipase and glucoamylase.

II. MATERIALS AND METHODS

A. Materials

Were used for preparing of the support calcium chloride (Vetec) and sodium hexametaphosphate (Nuclear). For measurements of hydrolytic activity of enzymes were used as substrate p-NPP (p-nitrophenol palmitate; Sigma), for the lipase, and DNS (3,5-dinitrosalicylic acid; Vetec), for glucoamylase. The enzymes immobilized were the Lipolase 100L *(triacylglycerol hydrolase* - E.C. 3.1.1.3*)* obtained of *Thermomyces lanuginosa* and *Amylase™ AG (glucan 1,4-alpha-glucosidase* **–** E.C. 3.2.1.3*)* obtained of *Aspergillus niger*, both acquired from Novozymes Latin America (Araucaria, PR).

B. Production of the polyphosphate matrix – Xerogel-S

The polyphosphate-based materials were prepared according to recommendations of Kopp et al. [18] with some modifications. The preparation occurred by slow addition of a $CaCl_2$ solution (2 $mol.L^{-1}$) on $NaPO_3$ solution (1 $mol.L^{-1}$) under stirring. The supernatant was discarded and the gel obtained was dried at 60°C in a greenhouse (Odontobras – Ind. e Com. Equip. Med. Odont. Ltda, São Paulo, Brazil) for 24 hours. The xerogel was thermally treated, 200°C for 24 hours, 300°C for 24 hours and 500°C for 24 hours in a muffle furnace (Fornitec – Ind. e Com. Ltda, Riberão Preto, Brazil), resulting in a sintered xerogel (xerogel-S). This matrix was macerated in a porcelain crucible and sieved to ensure uniform particle size varying between 0.1 and 0.5 mm, using ABNT sieves and subsequently stored in a desiccator until the use.

C. Enzymes immobilization

Lipase and glucoamylase immobilization on xerogel-S was performed using 1.0g of support, 10 mL of respective buffer and 1 $mg.mL^{-1}$ of each enzyme. Was used 100mM phosphate buffer (pH 8.0) and 100mM

acetate buffer (pH 5.0) for lipase and glicoamylase, respectively. They were kept under mild agitation at 25°C for 1 hour and the support was washed three times with 10 mL of distilled water.

The optimization of the enzymes immobilization by adsorption method on the support was considered varying the immobilization time (1, 2, 6, 12, 18 and 24 hours). Regarding the analysis of the immobilization efficiency (immobilization yield, retention activity and protein yield) of the support (xerogel-S), was analyzed the varying of the enzymes concentration (2, 5, 10 and 20 $mg.mL^{-1}$ of protein) during immobilization, using 1 hour of immobilization time course.

D. Properties of immobilized enzymes

In order to determine the biochemical characteristics of the immobilized enzymes, a curve of optimum temperature (20-80°C) and optimum pH (3-9) curve was performed using citrate buffers (pH 3.0-6.0) and phosphate buffer (pH 7.0-9.0). It was also analyzed the thermal stability of the immobilized with treatment at 70°C for different times (5, 10, 15, 20, 25, 30, 40 and 60 minutes) and the residual activity was determined. For comparison the free enzymes were also evaluated. Aliquots were removed of the immobilization solution at the beginning and at the end of the procedure to determination of protein concentration and catalytic activity measurement, well as was determined the activity of the support (immobilized derivative) for respective enzymes. All experiments were performed in triplicate.

E. Analysis of the immobilization efficiency

The parameters used to analyze the effectiveness of the polyphosphate-based support (xerogel-S) for the enzymes immobilization were calculated according to Branco et al. [7] end Adriano et al. [10], as described in the following equations:

Immobilization Yield (IY%):

$$IY(\%) = \frac{A0 - At}{A0} \times 100 \quad (1)$$

Retention Activity (RA%):

$$RA(\%) = \frac{AI}{A0 - At} \times 10^{0} \quad (2)$$

Were *A*0 is the enzyme activities of the supernatant (free enzyme) before immobilization (U/mL); *At* is the enzyme activities of the supernatant after immobilization (U/mL); *AI* is the units of enzyme activity of the immobilized derivative (U/g).

Protein yield (PY%):

$$PY(\%) = \frac{P0 - Pt}{P0} \times 10^{0} \quad (3)$$

Were *P*0 is the protein offered for immobilization (mg/mL); and *Pt* is the protein present in solution after immobilization (mg/mL).

F. Activity assays

Catalytic activity of the lipase was determined by using p-NPP. The enzymatic reaction mixture contained 0.5 mL of p-NPP (15 mM in absolute ethanol; Vetec), 0.5 mL of phosphate buffer (100mM pH 8.0) and 10 mg of xerogel-S with immobilized enzyme or 50 µL of immobilization supernatant (free enzyme). The reactions were at 30°C for 5 minutes with agitation of 130 rpm. The reaction was stopped by addition of 1 mL of 0.5 N sodium carbonate and centrifuged for 10 minutes at 10,000 rpm. The centrifuged supernatant was subjected to spectrophotometric analysis (Biospectro SP-22) of absorbance at 410nm against a blank without enzyme [20]. One unit of lipase (U) was defined as the amount of enzyme that releases 1 *µ*mol *p*-nitrophenol per minute in the assay conditions. A standard curve was prepared using *p*-nitrophenol according to Pencreac'h and Baratti [21].

Catalytic activity of the glucoamylase was determined by DNS (3,5-dinitrosalicylic) colorimetric method. The enzymatic reaction mixture contained 200 µL of 50 mM acetate buffer (pH 4,7), 100 µL of substrate (1.0% starch solution) and 10 mg of xerogel-S (immobilized enzyme) or 50 µL of free enzyme. The reactions were at 37°C for 10 minutes with agitation. After was added 200 µL of DNS, boiling by 5 minutes, and cooled at room temperature. Finally was added 1 mL of distilled water and analyzed by spectrophotometer in absorbance at 540

nm against a reaction blank. One glucoamylase unit (U) was expressed as the amount of enzyme that release 1 μmol of reducing group per minute under assay conditions. A standard curve of reducing sugar with D-glucose was prepared according to Miller [22].

The protein measuring was performed by Bradford method [23]. The calibration curve was constructed using bovine serum albumin (BSA) as the standard.

G. Statistical analysis

All the data obtained from the different immobilization parameters were statistically evaluated by variance analysis (ANOVA) and Tukey's multiple comparison test. The statistical differences between averages were tested with $p < 0.05$ using the StatSoft South America software. All determinations were performed in triplicate.

III. RESULTS AND DISCUSSION

During the support preparing process, occurred the formation of small insoluble portions of the polyphosphate, generating the coacervate (a gel) provided by the concentration of colloids, which is stabilized by calcium ions [16]. The stability of gel portions is increased with the addition of calcium solution on the sodium polyphosphate solution $(NaPO_3)n$ until finally the small portions of polyphosphate stay aggregated and began to precipitate, characterizing the sol-gel process by the formation of two phase separation, where the lower layer is a gel, and the supernatant is liquid [18].

The utilization of the sol-gel method allows obtaining materials with special characteristics at low temperatures and mild pH conditions [14] The method are commonly used with alkoxides as precursors of supports, especially silica, that despite being widely studied on the enzymes immobilization [3, 4, 24, 25], these materials obtained by the hydrolysis and condensation are expensive, their preparation can be difficult and time consuming, requiring suitable equipment [26], thereby stimulating the study alternative precursors.

Was observed in ours results that the gel obtained after being dried at 60°C, yielded an extremely hygroscopic and very crumbly material (xerogel). Meantime, the thermal treatment up to 500°C, generated a sintered matrix (xerogel-S), decreasing significantly 55% (± 2%) of original volume, but it increases the material resistance, which can be applied in numerous processes that requires high strength, including protein immobilization [18].

Aiming to analyze the potential of the xerogel-S for enzymes immobilization by the adsorption method the enzymes were put in contact with supports for different times, utilizing the enzyme activity of the immobilized derivative as response variable (Fig. 1).

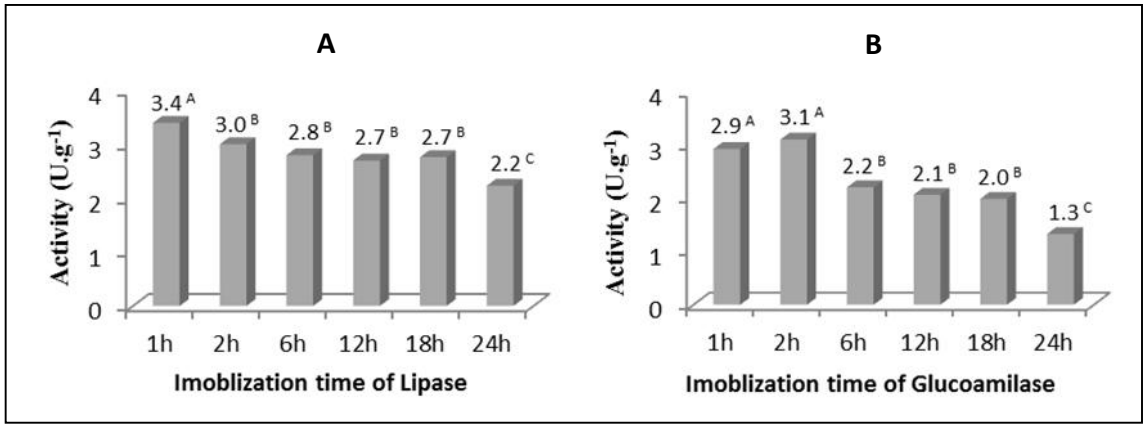

Figure 1. Effect of the immobilization time on lipase (A) and glucoamilase (B) activity onto the xerogel-S derivate. Immobilization of 1mg.mL^{-1} of enzymes with constant stirring at 25°C. Enzyme activity means followed by different letters are statistically different with Tukey' test ($p<0.05$).

The results indicated that smaller immobilization times showed better efficiency, once that one hour of lipase immobilization was significantly more efficient than other times, and the amylase immobilization after two hours was more efficient, but this was not statistically different from 1 hour, so this was the time of immobilization used for the remaining analyzes. Similarly, Reshmi et al. [27] used 1 ½ hours for lipase immobilization on alumina support and Ghamgui et al. [28] used 30 minutes of immobilization on hydrophobic supports, suggesting that shorter times are more suitable for immobilization by the adsorption method. These

date are very interesting because, despite efficiency not be high compared with other supports [7, 27, 28], the xerogel of thermally treated calcium polyphosphate, was capable to immobilize both enzymes, lipase and glucoamylase. Moreover the xerogel-S such as other inorganic materials including silica gels, alumina, and layered double hydroxides are known to be thermally and mechanically stable and highly resistant against microbial attacks and organic solvents [27].

It was also tested the treatment of the support with glutaraldehyde at different concentrations, however this did not improve the efficiency of immobilization (data not shown), since the original surface of the xerogel-S does not possess amino groups, thus not realizing the activation neither covalent binding, as occurs with other matrices [10].

For a better characterization of the xerogel-S regarding the immobilization efficiency were performed variations in the amount of enzymatic protein (mg/mL) offered in the process. The response variables analyzed were enzyme activity of the immobilized derivative and the parameters of immobilization efficiency determined were immobilization yield, retention activity and protein yield, according with Branco et al. [7] and Adriano et al. [10].

It is observed that the increase of the enzyme concentration reflects an increase in the activity of the enzymes immobilized on support (Fig. 2) though the amylase immobilization showed a greater response than lipase. The greater efficiency in glucoamylase immobilization is also observed when it is considered the immobilization yield (IY) and the immobilized protein (PY), because even with higher throughput compared with glucoamylase, the lipase showed a steeper decay of the immobilization yield in higher concentrations of protein (Fig. 3). This result turns out to be evident when analyzed the retention activity (RA), once in this parameter the glucoamylase has a yield increase with the highest concentration of protein, indicating a cooperative effect in immobilization, while the lipase displays an inversely proportional response. However, the use of lower concentrations of lipase on immobilization, this will present interesting result and with the possibility of successful use in various biotechnological process.

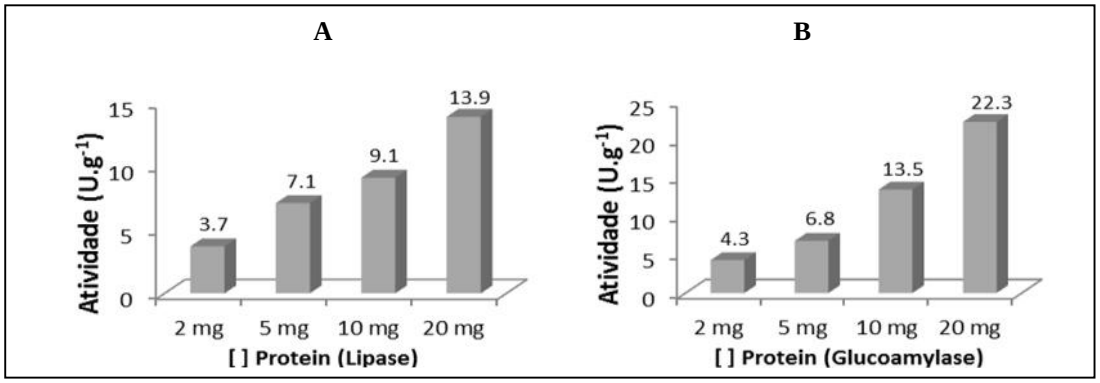

Figure 2. Effect of the protein concentration used for immobilization on lipase (A) and glucoamylase (B) activity onto the xerogel-S. [ ] protein concentration offered during immobilization.

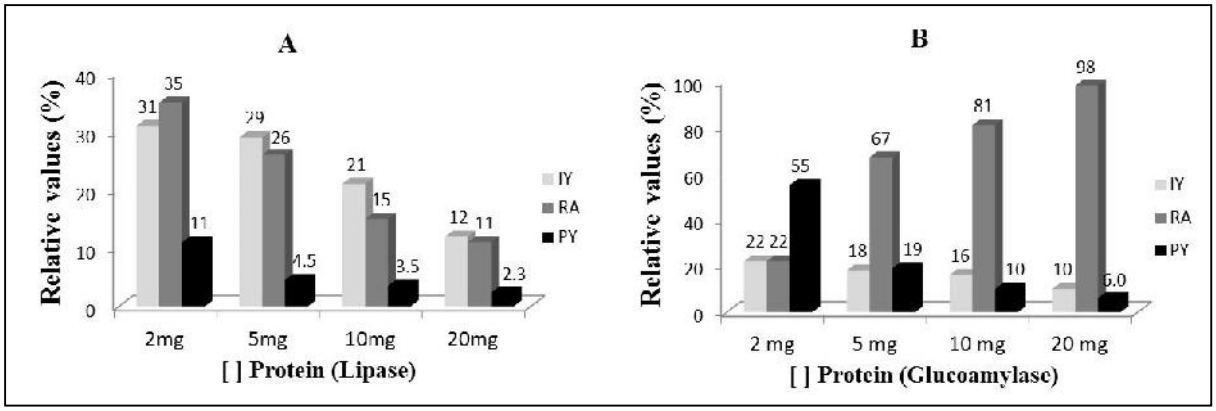

Figure 3. Effect of protein concentration used for immobilization on the efficiency parameters of lipase (A) and glucoamylase (B) immobilization. [ ] protein concentration offered during immobilization. Parameters analyzed: retention activity (*RA*), immobilization yield (*IY*) and immobilized protein (*PY*).

The data show that even using the highest concentration of glucoamylase (20 mg of protein) for immobilization has not occurred saturation of the support, while the lipase presented a marked decaying in the analyzed parameters, indicating reaching thresholds near of saturation.

This results is due primarily the low values of surface area and pore volume providing a smaller area for the immobilization [18] resulting in greater difficulty of diffusion of the substrate and its contact with the enzyme [7]. However, it is possible to provide a large surface area even at low porosity media, treating the material to obtain granules smaller [13], so the greater support maceration easily increasing the surface area, once were used on the work beads ranging from 0.1 to 0.5 mm.

Another aspect is related to the nature of charged surface of the support, once the xerogel-S having its surface charged (P- and Ca+), especially with free hydroxyl groups, may occur the denaturation of the lipase [18]. Several studies indicate that more hydrophobic support suitable for the immobilization of lipase by adsorption because these supports mimic the enzymes' natural medium [5, 7, 28], besides favoring the adsorption of lipase to promote environment with low ionic strength [29]. On the other hand, this problem does not occur with amylases immobilized on supports with the charged surface [27].

Free and immobilized enzymes were characterized since the support and the immobilization method can change the stability and the optimal conditions for maximum enzymes activity [5]. So were determined the activity curves face the variation of pH and temperature of both enzymes.

On analysis of temperature and pH effects on lipase activity (Fig. 4) was perceptible the pronounced changes in the optimal conditions of free and immobilized enzyme, the average ranged from 60°C to 30°C, for free and immobilized enzyme, respectively.

This change type seems recurrent in some studies of immobilization by adsorption. Lipase of *Pseudozyma hubeiensis* immobilized by adsorption onto a polystyrene-divinylbenzene hydrophobic support showed high activity for temperatures from 68 to 52°C [5]. While lipase of *Rhizopus cohnii* was immobilized by adsorption on porous chitosan polyphosphate beads and the optimum activity temperature of free and immobilized lipase was 37°C and 30°C, respectively [30]. Meantime immobilized cross-linked lipase has an increase in optimal temperature compared to the free enzyme [31].

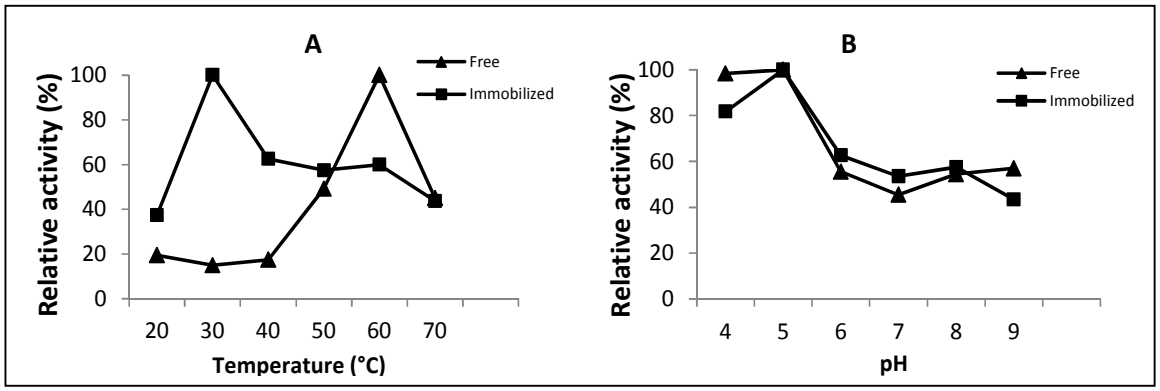

Figure 4. Optimal temperature and pH for free (*triangle*) and immobilized lipase (*square*).

For the purpose of industrial applications of the lipases such as in the synthesis reactions, esterifications and transesterifications this characteristic of the immobilized enzyme by adsorption is interesting because it allows the use of the enzyme with greater efficiency in milder temperature [20].

However on analysis of pH effects on activity of the lipase immobilized, this did not suffer significant changes when compared with the free enzyme (optimum pH 4.0 and 5.0), maintaining the optimum activity at pH 5.0, presenting only a small decaying of activity in pH 4.0. Similarly Krakowiak et al. [30] and Bussamara et al. [5] showed that there was little or no change in optimal pH of the immobilized lipases, even on different supports and immobilization types.

When it was performed the analysis of optimal pH and temperature of immobilized glucoamylase activity (Fig. 5), again there was a change in the behavior of the results when compared with the immobilized lipase. Because when glucoamylase was immobilized in xerogel-S did not change in optimum temperature activity,

which was 60°C, while the optimal pH of activity changed from pH 6.0 to 5.0, compared to free and immobilized enzyme, respectively.

These results were similar when this same enzyme, glucoamilase (GA), was covalently coupled onto silica mesoporous by George et al. [8]. However, according with theirs results the free enzyme showed optimum pH of 5.5 whereas in the case of support, pH shifts towards 6.0. The optimum pH shifts in alkaline direction occur because when the enzyme is covalently coupled polyanionic carrier [8]. On the another hand, Reshmi et al. [27] immobilizing α-amylase onto alumina support obtained that free enzyme exhibits maximum activity in the pH range 5.0-7.0 and immobilized enzyme exhibit high activity in the pH range 6.0-8.0.

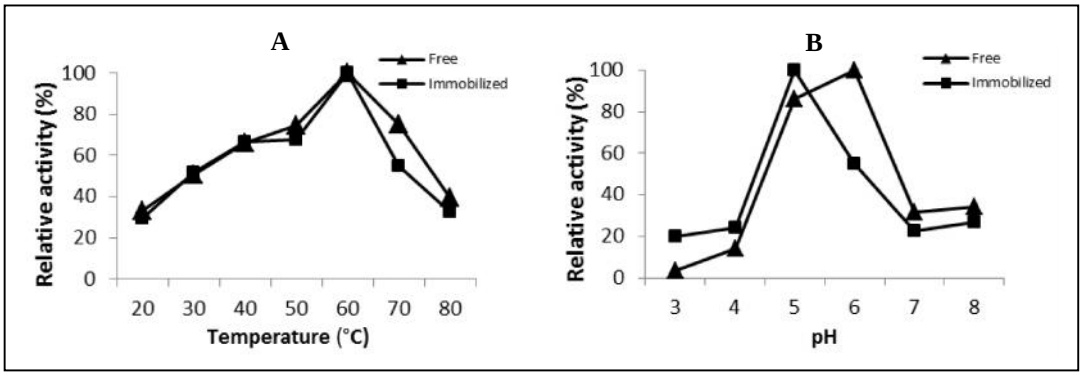

Figure 5. Optimal temperature (A) and pH (B) for free (*triangle*) and immobilized glucoamilase (*square*).

Both glucoamylase and lipase were analyzed regarding the thermal stability front the exposure to a temperature of 70°C (Fig. 6). On the results the immobilized lipase had a shorter decay compared to the free enzyme over the treatment periods, indicating the immobilization on the support contribute to enzyme stability in high temperatures. The values were statistically differ from immobilized and free enzyme by the ANOVA Tukey test (P = 0.05). The increased thermal stability for lipase with immobilization may be the result of a common increase in rigidity of the protein structure [2].

Moreover, the immobilized glucoamylase showed a slight decrease in stability compared to the free enzyme, these differences were not statistically significant (P = 0.05). When studying new materials, seeks mainly supports to increase the relative activity, the optimum pH range for activity and thermal stability after immobilization [5, 6, 13].

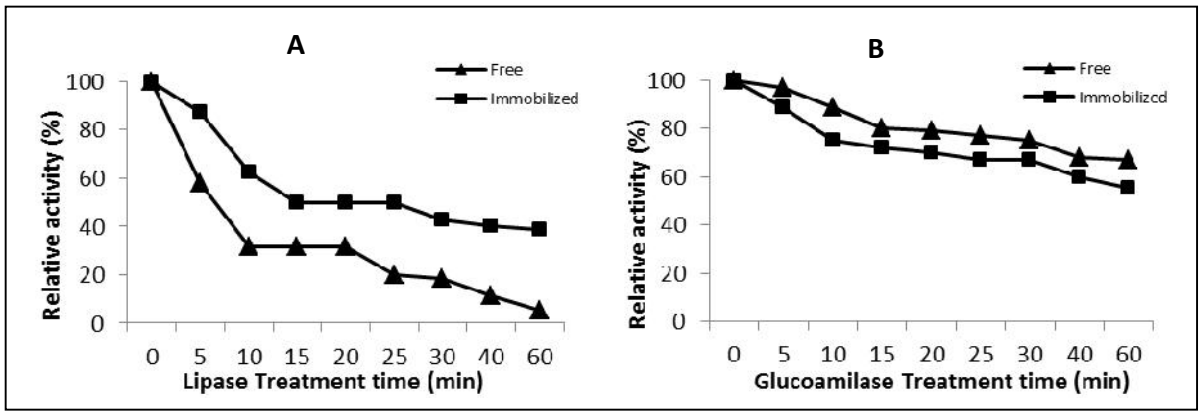

Figure 6. Thermal stability at 70°C of the lipase (a) and glicoamylase (b) immobilized on the calcium polyphosphate support thermally treated. Free (*triangle*) and immobilized enzymes (*square*).

The process of immobilization by adsorption is still quite common, because it presents low cost, few deleterious effects on the enzyme and may have selective power, however, the support´s characteristic may be important depending on the type of enzyme to be immobilized [2, 9]. This way the polyphosphate-based support

heat treated, has great potential for use in the enzymes immobilization which prefer the hydrophilic environments, such as glucoamylases [8, 27].

However, during the lipolase immobilization, even not having high performance, the support showed great potential for providing thermal stability and interesting conditions of optimal activity of temperature and pH, stimulating further studies to optimize the efficiency of immobilization, which can be emphasized modifications on the methodology of obtaining xerogel, modifying its surface and especially with the use of other immobilization methods.

IV. CONCLUSION

The present investigation shows that xerogel obtained of calcium polyphosphate and thermally treated up to 500°C, besides to show interesting features for industrial application due to its low cost and great resistance, have potential use as support for immobilization by adsorption of commercial enzymes such as glucoamylase AG and Lipase 100L. The best conditions for enzymes immobilization onto support were 1 hour and 1-2 hours for lipolase and glucoamylase immobilization, respectively. The immobilization parameters as well as immobilization yield, immobilized protein, and specially, the retention activity indicate a better efficiency of the process for the glucoamylase immobilization than amylase. Optimal temperature conditions for maximum lipase activity ranged from 60°C to 30°C for free and immobilized enzyme, while the optimal pH of the free and immobilized lipase remained at low pH around 5,0. The immobilized lipase has increased thermal stability in the temperature of 70°C when compared to the free enzyme. Already the optimal conditions of temperature and pH for maximum glucoamylase activity as well as thermal stability were not statistically different between free and immobilized enzyme as demonstrated in some studies with amylase immobilized by adsorption. The parameters yield of enzyme immobilization onto support of the calcium polyphosphate coacervates obtained by sol-gel method and heat-treated (xerogel-S) is more suitable for the immobilization of glucoamylase, however these immobilization results, even for lipase, can be improved from its structural modification, surface or immobilization method.

ACKNOWLEDGEMENTS

This work was supported by CNPq (Conselho Nacional de Desenvolvimento Cientifico e Tecnologico) and FAPESC (Fundação de Amparo a Pesquisa e Inovação do Estado de Santa Catarina) grants.

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