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Optimization and characterization of the immobilization process of trypsin in calcium alginate beads

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Abstract

The aim of this study was to optimize a procedure for the immobilization of trypsin on calcium alginate (Ca-alginate) and to compare the structure of the Ca-alginate beads based on their stability, porosity and enzymatic activity using bovine serum albumin (BSA) as a source of hydrolyzable protein. The optimal reagent concentrations used in the immobilization process were determined using a central composite routable design. The sodium alginate ($C_6H_7O_6Na$) and calcium chloride ($CaCl_2$) concentrations that yielded the highest enzymatic activity and immobilization rate were 2.92% (w/v) and 1.28 M, respectively. The beads generated under these conditions had a pore size of 10 μm. The stability of immobilized trypsin under these optimized conditions was evaluated using residual enzyme activity assays. There was a 30% decrease in enzymatic activity after 8 rounds of reuse, indicating that this immobilization process could produce a stable biocatalyst. The optimum pH and temperature profile were at pH 8.5 and 50 ºC, in addition, the thermal and storage stability of the trypsin was increased upon immobilization. The kinetic parameters K_m and V_{max} were 5.46 mM/min and 6100 μmol/mL/min, respectively.

Trypsin, Immobilization, Repeated Batch, Biocatalyst, Kinetic Parameters

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Introduction

Alginate is a naturally occurring structural component of marine brown algae and the capsular polysaccharide of some soil bacteria. It is a linear binary copolymer consisting of β-(1,4)-linked D-mannuronic acid (M) and α-L-guluronic acid (G) residues. The physical properties of alginate depend on the sequence of M and G residues as well as the average molecular weight and molecule-weight distribution of the polymer.

Alginate has some unique properties with respect to non-toxicity, biocompatibility, biodegradability, chelating ability, and hydrophilicity and is currently being used as an important biopolymer in the production of microcapsules designed for drug delivery, tissue engineering (Chico, Camacho, Pérez, Longo, Sanromán, Pingarrón & Villalonga, 2009; Sun, Wang, Zhong & Jiao, 2008; Wang, Wenk, Hu, Castro, Meinel, Wang, Li, Merkle & Kaplan, 2007) and the immobilization of enzymes, such as β-galactosidase, *Saccharomyces cerevisiae* invertase or fungal phenol oxidase (Quiroga, Llanes, Ochoa & Barberis, 2011; Won, Kim, Kim, Park & Moon, 2005; Taqieddin & Amiji, 2004).

Ca-alginate beads are one of the most widely used carriers for enzyme immobilization because they offer several advantages over enzymes in free solution including facilitation of the recovery and reuse of enzymes. Enzyme immobilization can be an alternative for the use of enzymes in food applications, including the use of trypsin, which can hydrolyse proteins into bioactive peptides (Muro-Urista, Álvarez-Fernández, Riera-Rodriguez, Arana-Cuenca, Téllez-Jurado, 2011), or in analytical applications, including biosensors, which are an essential quality control method used in medicine and food production.

In general, the trypsin immobilized can be consumed directly, improving blood circulation thereby increasing the supply of immune substances in the affected tissue (Srivastava & Singh, 2013).

Trypsin is the most widely used enzyme in protein fragmentation and selectively cleaves at lysine and arginine residues, typically resulting in peptides that have a mass range suitable for high-resolution/high-sensitivity mass mapping using mass spectrometry. The concept of using immobilized enzymes in protein analysis has received much attention in recent years because it offers several advantages over previous protocols (Kakuta, Takashima, Nakahata, Otsubo, Yamaguchi & Harada 2013; Ma, Zhang, Liang, Zhang & Zhang, 2007) including larger enzyme to substrate ratios, higher digestion efficiencies, and the possibility of repeated use.

Moreover, the rate of enzyme denaturation or inactivation is lower with immobilized enzymes than with free enzymes, which frequently lose their catalytic activity fairly quickly, leading to a lack of reproducibility. Immobilized trypsin can be used in batch-wise experiments or packed into columns and used in flow systems, such as immobilized-enzyme reactors (IMERs) (Ma *et al.* 2007).

Currently, immobilized proteins/enzymes are used routinely in the medical field to diagnose and treat various diseases. For example, immobilized antibodies, receptors, or enzymes are used in biosensors and ELISAs to detect various bioactive substances diagnostic of different disease states. Encapsulated enzymes are also used in bioreactors to remove waste metabolites and to correct inborn metabolic deficiencies (DiCosimo, McAuliffe, Poulose & Bohlmann, 2013).

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Although several supports have been tested for immobilizing this enzyme including polyelectrolyte microcapsules (PEMC), silica gel-supported macroporous chitosan beads, magnetic glycidyl methacrylate-based nanoparticles and ether sulfone and vinylidene fluoride membranes (Shen, Guo, Qi, Qiao, Wang & Mao, 2013; Starke, Went, Prager & Schulze, 2013; Garbers, Mitlöhner, Georgieva & Bäumler, 2007; Xi, Wu, Jia & Lin, 2005), Ca-alginate beads are advantageous because they increase enzyme stability compared to other supports (Quiroga *et al.* 2011) and have not been studied previously.

The concentrations of $CaCl_2$ and $C_6H_7O_6Na$ should be taken into account to avoid the diffusion of nutrients and product through the porous matrix. Ca-alginate beads have a wide pore-size distribution due to the open lattice structure of the matrix, which results in the release of biomacromolecules from these matrixes during storage. Alginate beads are also used to control enzyme diffusion into the surrounding solution (Ding, Shan, Xue, Zhu & Cosnier, 2009).

The aim of this present study was to optimize the concentrations of $CaCl_2$ and $C_6H_7O_6Na$ for trypsin immobilization and to compare the structure of the Ca-alginate beads based on their stability, porosity and enzymatic activity using bovine serum albumin (BSA).

Methodology

Materials

Sodium alginate ($C_6H_7O_6Na$), trypsin from bovine pancreas, bovine serum albumin (BSA) and $CaCl_2$ were obtained from Sigma-Aldrich. The viscosity of the 1 % (w/w) aqueous $C_6H_7O_6Na$ solution at 20 ºC was measured with a Rapid Visco-Analyzer (RVA, 3C, Newport Scientific PTY Ltd., Sydney, Australia) following the instruction manual, as 720 cps.

Experimental Design

A response surface method central composite routable experimental design was applied to determine the relationship between the independent variables ($C_6H_7O_6Na$ and $CaCl_2$ concentrations) and their responses as well as their interactions in an effective model. According to the model, it contains four full factorial design points, four axial points, and five central points. Two variables and two responses were involved in the experimental design.

The dependent variables measured were enzymatic activity and immobilization rate. The independent variables are the sodium alginate concentration and the $CaCl_2$ concentration. Preliminary trials were conducted before applying the design to determine the conditions under which the process yielded beads. The process variables and their respective levels are listed in Table 1. The data were analysed using the program Statistic 7.0 for Windows.

Preparation of Beads

The immobilization procedure was performed using the drip method reported by Ding *et al.* (2009). Different concentrations (Table 1) of $C_6H_7O_6Na$ and $CaCl_2$ were evaluated. The beads were prepared by dripping $C_6H_7O_6Na$ into a solution (10 mL) containing trypsin (1.37 U/mg protein). The resulting beads were stored in a $CaCl_2$ solution at 4 °C for 2 h to complete gel formation. The solution was then decanted, and the beads were collected and washed with deionized water for subsequent use.

Determination of enzyme activity

The enzyme activity was determined in triplicate using the method described by Kunitz (1965) modificated, as substrate was used as a BSA.

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The reaction mixture was made up of 100 μL of the free enzyme or two beads in a solution of 100 μL Tris-HCl buffer and 200 μL of the BSA dissolved in Tris-HCl 0.05 M, pH 8. These were incubated at 37 ºC for 1 h, and then the reaction was stopped by adding 500 μL of 10% trichloroacetic acid (TCA) (J. T. Baker). This was centrifuged at 13000 rpm for 5 min.

One unit of activity was defined as the amount of enzyme required to release 1 μmol of tyrosine per minute, under the assay conditions described.

Determining the immobilization rate

The enzyme:substrate complexes immobilized were filtered using quantitative cellulose filter paper (Whatman) under a vacuum, and washed three times with 50 mL of distilled water. The filtrates and flow-through were collected to determine the unabsorbed enzyme content using the Lowry method as modified by Peterson (1977) with BSA as a standard. The same method was used to quantify the protein in the enzymatic solution. The quantity of immobilized enzyme was calculated based on the difference between the proteins content in the enzymatic solution and the content in both the filtrates and flow-through combined. The immobilization rate was calculated using equation (1):

$$Immobilized\ enzyme\ (\%) = \frac{\text{Amount of immobilized protein}}{\text{Amount of initial protein}} * 100 \quad (1)$$

Determination of the optimal pH and temperature for the immobilized enzyme

The optimal pH of free and immobilized trypsin was carried at pH range 4.0-9.0 at 37 ºC using the proteolytic activity assays according to the method of Kunitz (1965) modificated, as substrate was used as a BSA. The optimal temperatures of the free and immobilized enzyme were determinate in the range 20-60 ºC at their optimal pH value.

Stability studies

The thermal stability of free and immobilized trypsin was determined by measuring the residual enzymatic activity of two different temperatures (55 y 65 ºC) in phosphate buffer (0.1 M, pH 7.5) for 150 min. After every 30 min time interval, a sample was removed and assayed for enzymatic activity as describe Bayramoğlu, Yilmaz & Arica, (2004). The results were given as percentage activity. Activity of the free and immobilized trypsin was expressed as a percentage of this residual activity compared to that of the initial activity.

Repeated use studies

The reusability of trypsin immobilized was evaluated by measuring its enzymatic activity before and after up to 20 repeated uses using the Lowry method as modified by Peterson (1977).

The immobilized enzyme activity (U/mg protein) was defined as amount of immobilized enzyme required to catalyse the hydrolysis of 1 μmole of BSA per minute. The reusability study was performed in triplicate.

Determination of the kinetic parameters

The kinetic studies of the free and enzyme and immobilized enzyme were conducted by measuring the activity with BSA as the substrate in accordance with the activity assay described above; the substrate concentration varied from 1.25 to 2.50 mg/mL. The apparent Michaelis-Menten constant (K_m) and maximum velocity (V_{max}) of immobilized trypsin, were calculated using the Lineweaver and Burk method and compared with the free enzyme.

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Scanning Electron Microscopy

The beads were mounted on brass stubs using double-sided adhesive tape, and Micrographs were taken with a scanning electron microscope (JSM- 6610LV, Jeol Ltd, Tokyo, Japan) at the required magnification (x1000) at room temperature. An acceleration voltage of 20 kV was used, and the image was detected with the secondary electron.

Results

Optimization

A central composite design was used to identify the optimal conditions for trypsin immobilization including the $C_6H_7O_6Na$ and $CaCl_2$ concentrations. After defining the experimental matrix, the experiments were conducted, and the initial activity (Y) of the immobilized enzyme was determined as shown in Table 2.

The variables that displayed a significance level lower than 0.05 (Prob>F) in these experiments were most relevant to the model. The results indicate that the $CaCl_2$ concentration (B), the square of the $C_6H_7O_6Na$ concentration (A^2), the $C_6H_7O_6Na$ concentration (A) and the square of the $CaCl_2$ concentration (B^2) were significant, as shown in equation (2):

$$EA = 39.33 + 3.94 * A + 63.73 * B + 69.25 * A^2 + 3.86 * B^2 \quad (2)$$

Where A and B are the coded values of the concentrations of $C_6H_7O_6Na$ and $CaCl_2$ concentrations, respectively, as shown in Table 2 and EA is enzyme activity.

The coefficient of determination (R^2) of the equation was 97 %, indicating that the experimental data were adequately adjusted and that these variables affect enzyme activity.

Therefore, 97 % of the variability in the experimental data could be explained by the empirical equation. Three additional experiments were performed under optimal conditions to validate the model, and the average enzymatic activity of these tests was 121.4 ± 0.42 U/mg protein. Based on the model [Equation (1)], the maximum enzyme activity was 128.9 ± 1.7 U/mg protein when 3 % $C_6H_7O_6Na$ and 1.36 M $CaCl_2$ were used (Treatment 4). To illustrate the effects of these variables on enzymatic activity, the response surface of these variables was constructed and is presented in Graphic 1.

The region of maximum enzymatic activity had higher alginate and $CaCl_2$ concentrations, therefore, the different concentrations of $C_6H_7O_6Na$ showed different levels of substrate diffusion into the beads. This is due to cross-linking, which is the main problem associated with the use of immobilized enzymes, especially when enzymes are acting on macromolecular substrates (Bencina, Bencina, Podgornik & Strancar, 2008). Because access of the substrate to the enzyme active site is limited, the accessible surface groups of the substrate also limit enzymatic activity.

This steric hindrance may consequently change the characteristic enzymatic cleavage patterns of the products derived from macromolecular substrates (Brena & Batista-Viera, 2006) and the immobilization rate was determined in order to examine these changes in our system. The results of the tests were compared with those reported by Ertan, Yagarb & Balkan (2006) who described that the optimum $C_6H_7O_6Na$ and $CaCl_2$ concentrations needed to achieve maximum enzymatic activity for *Aspergillus sclerotiorum* α-amylase immobilization were 3 % (w/v) y 0.3 M, respectively.

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Potumarthi, Subhakar, Pavani & Jetty (2008) described a statistically optimized calcium-alginate immobilization method for the production of *Bacillus licheniformis* NCIM-2042 alkaline protease in which they found that the optimum $C_6H_7O_6Na$ and $CaCl_2$ concentrations were 2.78 % (w/v) and 0.2 M, respectively. Similarly, a thermostable *Bacillus subtilis* α-amylase was immobilized by entrapment in Ca-alginate gel capsules and repeatedly used in batch processes of starch hydrolysis. The authors of this study found that the optimum $C_6H_7O_6Na$ and $CaCl_2$ concentrations for immobilization were 2 % (w/v) and 0.45 M, respectively (Konsoula & Liakopoulou-Kyriakides, 2006).

Thus, the maximum enzymatic activities for the different enzymes were achieved at $C_6H_7O_6Na$ concentrations of approximately 3 % (w/v). However, the $CaCl_2$ concentrations in the previous reports cited were lower (between 0.2 and 0.45 M) than those used in the present study (1.36 M), which may be due to the size of the trypsin substrate. Wang and Caruso (2005) reported that it is important to control the pore size of the beads and to treat the beads with an agent that encapsulates the enzyme (for example: polyvinyl diallylammonium, poly (allylamine hydrochloride) or $CaCl_2$ to form cross-links that prevent enzyme leakage. The inherent nature of the interactions in this method of entrapment provides high levels of activity and resistance to pH and temperature changes (Volodkin, Larianova & Sukhorukov, 2004).

Graphic 2 shows the influence of the $C_6H_7O_6Na$ and $CaCl_2$ concentrations on the immobilization rate (%) of trypsin, illustrating that at higher $C_6H_7O_6Na$ and $CaCl_2$ concentrations, the immobilization rate of trypsin increases.

These data are likely attributable to more complete gelation of the matrix due to the increased concentration of guluronic acid residues, which react with the calcium ions to form a matrix structure that is firmer. Sankalia, Mashru, Sankalia & Sutariya, (2006) reported that the degree of crosslinking is higher at higher $C_6H_7O_6Na$ concentrations given that increased contact time favours the efficiency of encapsulation. Likewise, when the $C_6H_7O_6Na$ concentration is low, the immobilization efficiency and substrate diffusion into the beads decreases. These data are confirmed by the steric restrictions mentioned above (Mahajan, Gupta & Sharma, 2010).

With the use of response surface methodology and overlapping surfaces, the optimal level of enzyme immobilization was calculated to find the optimal enzyme activity and immobilization rate values that would maximise enzymatic activity.

The conditions predicted by this model (Equation 2) for trypsin immobilization were then used to experimentally validate the model. We initially selected the central point of the optimal concentrations to ensure data reproducibility. The concentrations of the variables at the maximization point were: X_1= 2.92 % $C_6H_7O_6Na$ (w/v) and X_2= 1.28 M $CaCl_2$ (Graphic 3). Under these conditions, we achieved an enzymatic activity of 122 U/mg protein and an immobilization rate of 95 %, defined as the ratio of the enzymatic activity of the immobilized enzyme and the total activity of the soluble enzyme.

Effect of pH and temperature on activity

The effect of pH on the activity of free and immobilized trypsin was carried out in the pH range 4.0-9.0 and the results are presented in Graphic 4.

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Optimal conversion was obtained at pH 8 for the free enzyme, and optimum pH value for the immobilized enzyme was shifted to more alkaline region about pH 8.5. The immobilized trypsin has also a different pH activity profile and was broadened both in alkaline and acidic region. This displacement toward to more alkaline region could be possibly due to the secondary interaction between the enzyme and polymeric support (ionic interaction). Other researchers have reported similar observations upon immobilization of trypsin and other enzymes (Bayramoğlu, 2004).

The temperature dependence of the activities of the free and immobilized trypsin was studies in the temperature range 20-60 ºC (Graphic 5). The date revealed bell-shaped curves with optimum activity at 37 ºC for the free and at 50 ºC for the immobilized enzymes. The increase in optimum temperature was cause by the changing physical and chemical properties of the enzyme.

Thermal stability of free and immobilized trypsin

Thermal stability experiments were carried out with the free and the immobilized trypsin, which were incubated in the absence of substrate at two different temperatures. The Graphic 6 shows the heat inactivation curves at 55 and 65 ºC for the free and immobilized enzyme, respectively. The pattern of heat stability indicated that a smaller rate of thermal inactivation was observed for the immobilized enzyme.

At 55 ºC, the free enzyme retained 38% of its initial activity after 120 min of heat treatment, while the adsorbed enzyme showed significant resistance to thermal inactivation, retaining about 81 % of its initial activity after the same period.

At 65 ºC, the free enzyme retained 10 % of its initial activity after a 120 min of heat treatment, on the other hand, the immobilized trypsin was inactivated at much slower rate than the native form and the immobilized form showed significant resistance to thermal inactivation because retain about 63 % of its initial activity after the same time period.

These results suggest that the thermo stability of adsorbed trypsin becomes significantly at higher temperature. It should be noted that the higher stability of immobilized trypsin could partly be caused by the limitation of autolysis and partly by the protein conformational sytabilization. The extent of autolysis in the inactivation of trypsin is difficult to estimate in the native enzyme.

Moreover, in these experiment calcium ions were added to buffer solutions to limit auto destruction of enzyme. If the heat stability of enzyme increased upon adsorption, the potential application of this enzyme would be extended. Increased thermal stability has been reported for a number of adsorbed enzymes, and the polymer network and multi-point attachment in ionic adsorption method are supposed the tertiary structure of trypsin (Bayramoğlu, 2004).

Repeated use studies

The reusability studies were performed using the optimal conditions for trypsin immobilization, and the results are shown in Graphic 7. The relative activity for the rounds of reuse was calculated relative to the first batch, which was designated to have 100 % relative activity. There were no significant differences in the enzymatic activity of immobilized trypsin after 4 rounds of reuse, and the activity decreased by 30 % after 8 rounds of reuse.

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These results can be explained by the mechanical damage caused by rupturing the beads and thus releasing the enzyme from the beads. Similarly, Won *et al.* (2005) reported a 72 % loss of activity after 3 rounds of reuse for a lipase immobilized in alginate beads. These data are also consistent with the reports by Konsoula & Liakopoulou-Kyriakides (2006), who found that α-amylase immobilized on Ca-alginate beads retained 90 % of its initial efficiency after 20 batches of starch hydrolysis when immobilized with 2 % (w/v) and 0.45 M of $C_6H_7O_6Na$ and $CaCl_2$, respectively.

The results in the present study indicate that $C_6H_7O_6Na$ is a better immobilization substrate for trypsin than silica gel-supported macroporous chitosan beads when only retains 70 % of its activity after 4 rounds of reuse (Xi *et al.* 2005), whereas it retained 70 % of its activity after 8 rounds of reuse in our study.

Free and immobilized enzyme kinetic parameters

Kinetic analyses of the free enzymes and immobilized enzyme were conducted at optimal pH (8.5) and temperature (50 ºC) using the proteolytic activity assay with BSA as substrate. The Michaelis-Menten constant was determined using double-reciprocal plots showing $1/V$ *vs* $1/[S]$.

The kinetic parameters (K_m y V_{max}) for the free enzyme the K_m value was found to be 12.75 mM/min, and the V_{max} was calculated to be 5621 μmol/mL/min. K_m value was found to be 5.46 mM/min for the immobilized enzyme and the V_{max} was calculated to be 6100 μmol/mL/min. The K_m for the immobilized trypsin was significantly greater compared with the free enzyme, which might be due to the diffusional resistance of the gel. Similar changes in the K_m were also reported for Wang, Chen, Wang & Xing (2014).

Scanning electron microscopy studies

The scanning electron microscopy study was performed to topographically characterize the internal structure of the Ca-alginate beads containing trypsin and evaluate the relationship between the bead structure and trypsin activity. As shown in Figure 1, the Ca-alginate beads have irregular forms while maintaining a constant lattice-form array on the surface of the beads. In addition, when the concentrations of $C_6H_7O_6Na$ and $CaCl_2$ are increased, the surface has fewer folds.

The reduction in the number of folds is likely due to an increase in the viscosity and a delay in the penetration of calcium into the beads, resulting in a decline of cross-liking, roughness and porosity on the surface of the beads (Sankalia *et al.* 2006). Kakuta *et al.* (2013) reported that the structure, porosity and Ca-alginate size of the beads are influenced by the pore size used to extrude the alginate solution and its viscosity. These characteristics are very important because the pore size of the beads should be suitable enough for the substrate to enter and hydrolysis to occur (Potumarthi *et al.* 2008; Wu, Wang, Li, Lin & Wei, 2010).

Wu *et al.* (2010) also reported that the structure, porosity and Ca-alginate bead size are influenced by the opening used to extrude the $C_6H_7O_6Na$ solution and its viscosity, and in a decrease in the enzymatic activity of immobilized enzyme relative to soluble enzyme. Figure 2 shows a photomicrograph of the cross-section of the Ca-alginate beads containing trypsin under optimum conditions (trypsin immobilized activity of 120.87 U/mg protein using $C_6H_7O_6Na$ and $CaCl_2$ concentrations of 2.92 % (w/v) and 1.28 M, respectively) an x1000 magnification.

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The beads had an irregular lattice structure with an approximate pore size of 10 μm, which would allow the substrate to enter the beads and hydrolysis to occur, which is shown by the fact that there was measurable enzymatic activity.

Conclusions

The results of this study indicate that the optimal conditions for trypsin immobilization were 2.92 % $C_6H_7O_6Na$ and 1.28 M $CaCl_2$. These conditions resulted in pore sizes of 10 μm, which provides an optimal immobilization rate and maximum enzymatic activity. The enzyme immobilized under these conditions retained 70 % of its initial activity after 8 rounds of reuse. The optimum pH and temperature profile were at pH 8.5 and 50 ºC, in addition, the thermal and storage stability of the trypsin were increased upon immobilization. The kinetic parameters K_m and V_{max} were 5.46 mM/min and 6100 μmol/mL/min, respectively.

References

Bayramoğlu, G. Yilmaz, M. Arica, M.Y. (2004). Immobilization of a thermostable a-amilase onto reactive membranes: Kinetics characterization and application to continuous starch hydrolysis, Food Chem, 84, 591-599.

Bencina, M., Bencina, K., Podgornik, A., Strancar, A. (2008). Monolithic bioreactors for macromolecules, Method Mol. Biol, 421, 257-274.

Brena, B.M., Batista-Viera, F. (2006). Immobilization of enzymes and cells. In: Methods in Biotechnology. Guisan JM (ed). 2nd Humana Press Inc., Totowa, NJ, pp. 15-30.

Chico, B., Camacho, C., Pérez, M., Longo, M., Sanromán, M.A., Pingarrón, J.M., Villalonga, R. (2009). Polyelectrostatic immobilization of gold nanoparticles-modified peroxidase on alginate-coated gold electrode for mediator less biosensor construction, J. Electroanal Chem, 629, 126-132.

DiCosimo, R., McAuliffe, J., Poulose, A.J., Bohlmann, G. (2013). Industrial use of immobilized enzymes, Chem. Soc. Rev, 42, 6437-6474.

Ding, S.N., Shan, D., Xue, H.G., Zhu, D.B., Cosnier, S. (2009). Glucose oxidase immobilized in alginate/layered double hydroxides hybrid membrane and its biosensing application, Anal. Sci, 25, 421-1425.

Ertan, F., Yagarb, H., Balkan, B. (2006). Optimization of α-amylase immobilization in calcium alginate beads, Prep. Biochem. Biotech, 37, 195-204.

Garbers, E., Mitlöhner, R., Georgieva, R., Bäumler, H. (2007). Activity of immobilized trypsin in the layer structure of polyelectrolyte microcapsules (PEMC), Macromol. Biosci, 7, 1243-1249.

Kakuta, T., Takashima, Y., Nakahata, M., Otsubo, M., Yamaguchi, H., Harada, A. (2013). Preorganized hydrogel: self-healing properties of supramolecular hydrogels formed by polymerization of host-guest-monomers that contain cyclodextrins and hydrophobic guest groups, Adv. Mater, 25, 2849-2853.

Konsoula, Z., Liakopoulou-Kyriakides, M. (2006). Starch hydrolysis by the action of an entrapped in alginate capsules α-amylase from *Bacillus subtilis*, Process Biochem, 41, 343–349.
Kunit, N. (1965). Methods of enzymatic analysis. 2nd ed. Velag, Chenie. Acad. Press N.Y. London, pp. 807- 814.

ISSN-On line: 2414-8849

TOVAR-JIMÉNEZ, Xochitl, MURO-URISTA, Claudia Rosario and ARANA-CUENCA, Ainhoa. Optimization and characterization of the immobilization process of trypsin in calcium alginate beads. ECORFAN Journal-Republic of Guatemala. 2017.

Ma, J., Zhang, L., Liang, Z., Zhang, W., Zhang, Y. (2007). Monolith-based immobilized enzyme reactors: Recent developments and applications for proteome analysis, J. Sep. Sci, 30, 3050-3059.

Mahajan, R., Gupta, V.K., Sharma, J. (2010). Comparison and suitability of gel matrix for entrapping higher content of enzymes for commercial applications, Indian J. Pharm. Sci, 72, 223-228.

Muro-Urista, C.R. Álvarez-Fernández, F. Riera-Rodríguez, Arana-Cuenca, A. Téllez-Jurado, A. (2011). Review: Production and functionality of active peptides from milk, Food Sci. Technol. Int, 17, 293-317.

Peterson, G.L. (1977). A Simplification of the protein assay method of Lowry *et al*. which is more generally applicable, Anal. Biochem. 83, 346-356.

Potumarthi, R., Subhakar, C.H., Pavani, A., Jetty, A. (2008). Evaluation of various parameters of calcium-alginate immobilization method for enhanced alkaline protease production by *Bacillus licheniformis* NCIM-2042 using statistical methods, Bioresource Technol, 99, 1776–1786.

Quiroga, E., Llanes, C.O., Ochoa, N.A., Barberis, S. (2011). Performance improvement of araujiain, a cystein phytoprotease, by immobilization within calcium alginate beads, Process Biochem, 46, 1029-1034.

Sankalia, M.G., Mashru, R.C., Sankalia, J.M., Sutariya, V.B. (2006). Papain entrapment in alginate beads for stability improvement and site-specific delivery: Physicochemical characterization and factorial optimization using neural network modeling, *AAPS* Pharmscritech, 6, 209-222.

Shen, Y., Guo, W., Qi, L., Qiao, J., Wang, F., Mao, L. (2013). Immobilization of trypsin via reactive polymer grafting from magnetic nanoparticles for microwave-assisted digestion, J. Mater. Chem. *B*, 1, 2260-2267.

Srivastava, P.K., Singh, S.A. (2013). Review: Immobilization and applications of glucose-6-phosphate dehydrogenase, Prep Biochem Biotech, 43, 376-384.

Starke, S., Went, M., Prager, A., Schulze A. (2013). A novel electron beam-based method for the immobilization of trypsin on poly (ethersulfone) and poly (vinylidene fluoride) membranes, React. Funct. Polym, 73, 698-702.

Sun, W., Wang D.D., Zhong, J.H., Jiao, K.J. (2008). Electrocatalytic activity of hemoglobin in sodium alginate/SiO_2 nanoparticle/ionic liquid BMIMPF6 composite film, J. Solid State Electr, 12, 655-661.

Taqieddin, E., Amiji. M., (2004). Enzyme immobilization in novel alginate–chitosan core-shell microcapsules, Biomaterials, 25, 1937-1945.

Volodkin, D.V., Larianova, N.I., Sukhorukov, G.B. (2004). Protein encapsulation via porous $CaCO_3$ microparticles templating, Biomacromolecules, 5, 1962-1972.

Wang, W.Q., Wenk, E., Hu, X., Castro, G.R., Meinel, L., Wang, X., Li, C.M., Merkle, H., Kaplan, D.L. (2007). Silk coatings on PLGA and alginate microspheres for protein delivery, Biomaterials, 28, 4161-4169.

Wang, Y. Caruso, F. (2005). Mesoporous silica spheres as supports for an enzyme immobilization an encapsulation, Chem. Mater, 17, 953-961.

ISSN-On line: 2414-8849

Wang, Y., Chen, H., Wang, J., Xing, L. (2014). Preparation of active corn peptides from zein through double enzymes immobilized with calcium alginate–chitosan beads, Process Biochem, 49, 1682-1690.

Won, K., Kim, S., Kim, K.J., Park, H.W., Moon, S.J. (2005). Optimization of lipase entrapment in Ca-alginate gel beads, Process Biochem, 40, 2149-2154.

Wu, J., Wang, J.L., Li, M.H., Lin, J.P, Wei, D.Z. (2010). Optimization of immobilization for selective oxidation of benzyl alcohol by *Gluconobacter oxydans* using response surface methodology, Bioresource Technol, 101, 8936-8941.

Xi, F., Wu, J., Jia, Z., Lin, X. (2005). Preparation and characterization of trypsin immobilized on silica gel supported macroporous chitosan bead, Process Biochem, 40, 2833-2840.

ISSN-On line: 2414-8849