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INFLUENCE OF BLOOM NUMBER AND PLASTIFIERS ON GELATIN MATRICES PRODUCED FOR ENZYME IMMOBILIZATION

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Abstract - In this study the immobilization of lipase in matrices produced with gelatin with different Bloom values and with the addition of plastifiers was investigated to evaluate the influence of the Bloom value, as well as the capacity of the plastifiers to maintain the enzyme immobilized and the immobilization yield. The results indicated the need for crosslinking of the matrices with glutaraldehyde due to the high solubility in water, explained by the amino acid profile, which confirms the solubility of gelatin. Mannitol showed greater efficiency in the lipase immobilization, since it led to more porous structures and more uniform pores. These structures were also influenced by the gelatin concentration; greater concentrations associated with intermediate concentrations of plastifier led to matrices with a greater immobilization yield (87.92%). The X-ray diffraction analysis revealed that the structure of the immobilization matrices was partially crystalline.
Keywords: Gelatin; Glycerol; Mannitol, Immobilization; Enzyme.

INTRODUCTION

The use of some enzymes as catalysts in industrial processes is limited by the high costs of production and storage. During use, their stability reduces due to changes which occur, such as alterations in the pH, temperature or other factors, leading to conformational modifications and other cumulative effects. Since the enzymes are soluble, their recovery based on the mixture of the substrate and the product is not economically viable (Kotwal and Shankar, 2009). In this context, the immobilization of enzymes offers a means to minimize or eliminate the above-mentioned problems.

Gelatin, as an immobilizing agent, is of low cost, biocompatible and its structure facilitates multiple combinations of molecular interactions. Gelatin is used alone or mixed with other immobilizing agents (Sheelu *et al*., 2008; Gomez-Guillén *et al*., 2011), with most studies being focused on the latter. In most cases where gelatin is used as an immobilizer, the Bloom strength is not specified.

The Bloom strength or resistance of a gel is a measure of its hardness, consistency, firmness and compressibility at a certain temperature, evaluated through the load (in grams) required to produce a depression in the gel under normal conditions. The gel resistance is also dependent on the concentration

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and molecular weight of the gelatin. The Bloom index for commercial gelatins varies from 50 to 300 g (Ockerman and Hansen, 1994; Schrieber and Gareis, 2007). According to Segtnan and Isaksson (2004), the Bloom strength is the sum of the α and β fractions of the molecular structure of the gelatin.

The gelatin extraction temperature affects the Bloom index. An increase in the bloom index leads to a notable improvement in the mechanical properties and a significant reduction in the water absorption capacity (Lai, 2009). In general, gelatin extracted at lower temperatures is harder and has a higher Bloom index value (Bigi *et al*., 2004). Gelatins with high Bloom values have higher melting or gel points, the color is lighter and the odor more neutral (Schrieber and Gareis, 2007).

The hygroscopic nature of gelatin is its main disadvantage in terms of its use to produce films that act as a protection barrier. These films tend to swell or dissolve when placed in contact with water. Consequently, the current tendency in the conception of materials based on gelatin is focused on the development of better mechanical and water resistance properties through combining gelatin with biomolecules with different characteristics, such as lipids, protein isolated from soy and polysaccharides, hydrophobic and hydrophilic plastifiers, synthetic polymers and chemical modification agents (Gomez-Guillén *et al.,* 2011).

The addition of plastifiers to gelatin films provides characteristics such as an increase in the processability and resistance of the films, and the capacity to increase the free volume and molecular mobility, since these reduce the cohesive strength of the films through limiting the intermolecular forces responsible for the inter-chain interactions (Sothornvit and Krochta, 2000; Vanin *et al*., 2005; Vieira *et al*., 2011; Jiménez *et al*., 2012). These substances reduce the strain deformation, hardness and electrostatic charges. They also affect the degree of crystallinity, optic clarity, and electrical conductivity, influence the biological degradation, avoid fissures and, at high concentrations, can inhibit the formation of pores (Vieira *et al*., 2011).

Gelatin films are generally plastified using hydroxyl compounds, with polyols being frequently cited as good plastifying materials due to their capacity to reduce hydrogen bonding while increasing the intermolecular spacing through the reduction of forces, increasing the mobility of the polymeric chains, improving the film flexibility and decreasing the glass transition temperature (Cao *et al*., 2009; Rivero *et al*., 2010).

The selection of the plastifier used in gels is generally based on the compatibility between the plastifier and the protein, on the permanence in the film and on the plastification quality required. Despite the different possibilities for plastifiers that can be used in films, sorbitol and glycerol are the most commonly used. Besides influencing the film properties, some plastifiers, such as sorbitol, can also crystallize within the film, depending on the storage conditions, and the material can completely lose its characteristics (Vanin *et al.*, 2005).

The potential use of gelatin as an immobilizing matrix was studied in 1980 and demonstrated by the research group of Gianfreda, Parascandola and Scardi. The main advantage of the use of gelatin presented by the group of researchers was the facility to form a gel without the inactivation of the immobilized biocatalyst (Vujčić *et al*., 2011). Gelatin offers the advantage of a large variety of amino groups, which can be used to carry out the immobilization (Maalej-Achouri *et al*., 2009).

In general, gelatin gels can be modified through their amino, carboxyl and hydroxyl groups, with most of these changes occurring in lysine, hydroxylysine and N-terminal amino groups. The modifications using poly-functionals such as dialdehydes are based on the reaction of several side-groups of the gelatin, leading to crosslinking, that is, the gel acquires greater mechanical resistance, better stability and water resistance (Martucci *et al*., 2006, Schrieber and Gareis, 2007; Guo *et al*., 2012). On the other hand, the mass transfer is inhibited by the use of high concentrations of crosslinking agents (Drury and Mooney, 2003).

Of the crosslinking agents used for the modification of gelatin, glutaraldehyde has been widely cited since it has the advantage of rapid hardening action (less than 1 min with concentrations of 10-20%) and it also is readily available at low cost (Martucci *et al*., 2006).

In most cases the immobilization of the enzyme in a gelatin matrix is carried out through the mixture of enzymatic solutions with the gelatin gel and crosslinking agent. The resulting gel contains occluded enzymes, partially bonded to the gelatin through the formation of a *Schiff* base (Sheelu *et al*., 2008). Glutaraldehyde has two functional groups able to bind to free amino groups of lysine and hydroxylysine, amino acid residues of the polypeptide chains in the gelatin molecules. All of the free amino groups react with the aldehyde groups of glutaraldehyde to form *Schiff* bases (Mozafari and Moztarzadeh, 2010).

In this context, the aim of this study was to develop matrices for the immobilization of enzymes using gelatins with different bloom values and the added plastifiers glycerol and mannitol through the use of the experimental design technique. An analysis of the amino acid profile of the gelatins and the solubility of the matrices in water was carried out. After the immobilization, the migration was evaluated for the different combinations of gelatin and plastifier, with the plastifiers being assessed individually, and the immobilization yields and the morphological and structural characteristics of the matrices were determined.

MATERIALS AND METHODS

Materials

The gelatins used as immobilizing agents were bovine skin gelatins, with a 30 mesh grain size and Bloom values of 200, 220, 240 and 280 g (Gelnex), obtained on different days of production, however all characterized as gelatin type B. Glycerol and mannitol (Vetec) were used as plastifiers. The enzyme used in the immobilization was *Aspergillus niger* lipase (Granotec). Acetone, ethanol and gum arabic (Vetec), all of analytical grade, were used to determine the lipase activity. Olive oil (La Violetera), with an acidity of less than 1%, was used as the enzyme substrate.

Amino Acid Profile of Gelatins

In order to obtain the amino acid profile, acid hydrolysis was first carried out. The amino acid composition of the gelatins was then determined through pre-column PITC derivatization, followed by high performance liquid chromatography (HPLC) according to the methodology of White *et al*. (1986).

Preparation of Matrices and Lipase Immobilization

Four gelatins of different Bloom values (200, 220, 240 and 280 g) were tested for the immobilization, in order to verify the influence of this parameter. Table 1 shows the central composite rotatable design (CCRD) used to obtain the immobilization matrices, which gives the values for the variables and the levels (in parentheses). All of the experiments were carried out for each gelatin Bloom value studied. Also, for all experiments, the need for crosslinking, with the use of glutaraldehyde as the modifying agent, was verified. In parallel with the design experiments, experiments characterized as the control (C) were carried out with only gelatin (55%, central point of the experimental design, without plastifier) and gelatin crosslinked with glutaraldehyde. The results for these experiments are shown together with those for the experimental design.

Table 1: Central composite rotatable design used to obtain the gels – variables and levels (in parentheses).

Experiment	Plastifier % (w/v)	Gelatin % (w/v)
E1	2.5 (-1.0)	30 (-1.0)
E2	2.5 (-1.0)	80 (1.0)
E3	7.5 (1.0)	30 (-1.0)
E4	7.5 (1.0)	80 (1.0)
E5	1.5 (-1.41)	55 (0.0)
E6	8.5 (1.41)	55 (0.0)
E7	5.0 (0.0)	19.8 (-1.41)
E8	5.0 (0.0)	90.3 (1.41)
E9	5.0 (0.0)	55 (0.0)
E10	5.0 (0.0)	55 (0.0)

Each of the experiments in Table 1 was carried out for each gelatin Bloom number studied. Water was added to the gelatin samples in order to hydrate them and obtain a rigid matrix. The weight of each variable (grams) added was calculated in relation to the volume of water (5 mL) added for gel formation. The plasticizer was then added to this mixture, which was heated to 50 °C, melting the mixture by the use of heat with subsequent formation of a plasticized matrix. Among the chemicals used to crosslink gelatin, glutaraldehyde has been used extensively because it has the advantage of being a fast-acting hardener for colageneous materials (Martucci *et al.,* 2006). For the matrix in which glutaraldehyde was used, after cooling, 10 mL of a 2.5% (v/v) glutaraldehyde solution (Paula *et al.,* 2008) were added on one surface and the samples were stored for 30 min. They were then washed with distilled water. The matrices (with and without crosslinking) were cut into cubes of around 2 mm on each side. Concentrations of gelatin were chosen to evaluate a broad range, because there are gaps in the literature for the effects of gelatin at various concentrations, both on the gel and on the immobilization of enzymes. The definition of the concentrations of plasticizers were based on studies by Vanin *et al.,* (2005), Thomazine *et al.,* (2005), Bergo and Sobral (2007) and Rivero *et al.* (2010).

For the lipase immobilization the same procedure was used to obtain the matrices; however, during cooling to approximately 30 °C lipase (1.0%, w/v) was added and the mixture was homogenized. In the experiments the addition of lipase was performed in each individual beaker, and the mass corresponding

to the gelatin and plasticizer weighed individually for each assay, using a fixed amount of gelatin, plasticizer and lipase. The cubes obtained after cooling and cutting were repeatedly washed with distilled water for subsequent use.

Solubility in Water

Portions of the matrix (twenty 2 mm cubes) were placed in dry aluminum crucibles with 15 mL of distilled water and shaken gently at 20 ºC for 15 h. The solution was then filtered through Whatman nº 1 filter paper to recover the remaining undissolved film, which was dessicated at 105 ºC for 24 The solubility of the matrix in water was calculated according to Eq. (1) (Gómez-Estaca *et al*., 2011):

$$WS(\%) = \frac{A_0 - A_f}{A_0}.100 \quad (1)$$

where WS is the solubility of the matrix in water expressed as a percentage, A_0 is the initial mass in grams of the matrix, expressed as dry matter and A_f is the mass in grams of non-dissolved residue of the matrix after drying. All of the determinations were carried out in duplicate. Solubility tests were performed for matrices with added glycerol or mannitol, with and without crosslinking, for all the experiments of the experimental design.

Verification of the Matrix Capacity to Maintain the Enzyme Immobilized

After obtaining the solubility, tests were made to verify the capacity of the matrices to maintain the immobilized lipase. Only matrices crosslinked with glutaraldehyde were used, taking as a basis the previous results of solubility. This efficiency of the matrix to maintain the enzyme confined represents the capacity of the matrix to avoid the migration of the enzyme to the reaction medium. Only the samples which did not show migration were considered for the next step of the experiment. To this aim, 20 cubes of the matrix containing the lipase were incubated in 50 mL of 100 mmol.L^{-1} sodium phosphate buffer, pH 7.0, at 37 ºC and agitation of 160 rpm. Tests were carried out on 2 mL aliquots of the sodium phosphate buffer removed after 60 min of incubation for the enzymatic activity determination. Lipase activity was determined by a titration method. An emulsion of olive oil (10% w/v) and arabic gum (5% w/v) in 0.1 mol.L^{-1} sodium phosphate buffer, pH 7.0, was incubated with a sample of the enzymatic extract at 37 ºC and 160 rpm for 15 min. The reaction was stopped and the fatty acids extracted with a solution of acetone and ethanol (1:1). The fatty acids produced were titrated with 0.05 mol.L^{-1} NaOH (Freire *et al.,* 1997). One unit of lipase activity was defined as the amount of enzyme that produces 1 μmol of fatty acids/min, under the assay conditions. Experiments where enzymatic activity (U/mL) was detected after the incubation due to the presence of enzyme in the sodium phosphate buffer were considered to show migration. All of the determinations were carried out in duplicate.

Immobilization Yield

The immobilization yield was calculated through the relation between the enzymatic activity of the immobilized enzyme and that of the free enzyme, shown in Eq. (2) (Won *et al*., 2005).

$$IY\ (\%) = \frac{a_{immobilized}}{a_{free}}.100 \quad (2)$$

where IY is the immobilization yield (%), $a_{immobilized}$ is the activity of the immobilized enzyme (U/mL) and a_{free} is the activity of the free enzyme (U/mL). All of the determinations were carried out in duplicate.

Scanning Electron Microscopy (SEM)

In order to verify the morphology of the immobilization matrices, photomicrographs were obtained using scanning electron microscopy (SEM) (microscope Philips, model XL30). The gold coating was carried out in a BAL-TEC Sputter Coater, model SCD 005, for 120 s on the dried matrices (the samples of the matrices were previously dried at 30 °C for 24 hours). The photomicrographs of the surface were obtained of a crosslinked face and a cross-section of each immobilization matrix, which did not show lipase migration.

X-Ray Diffraction

In order to obtain the X-ray diffractograms a Philips X´Pert diffractometer was used, with CuKα radiation operating at 40 kV and 30 mA. The average values were obtained at a sweeping rate of 0.05°/s.

Statistical Analysis

The software Statistica 10.0® (Statsoft Inc.) was used to perform the Tukey test, to carry out the analysis of the effects, to calculate the regression coefficients and to obtain the response surfaces, all

of the responses being obtained at the 95% confidence level.

RESULTS AND DISCUSSION

Amino Acid Profile of the Gelatins

Gelatins are amino acid polymers with each amino acid residue united with its neighbor through a covalent peptide bond. Determining the amino acid sequence of a protein can provide information on its solubility characteristics and three dimensional structure. Table 2 shows the amino acid profile of the gelatins investigated in this study.

Table 2: Amino acid profile for gelatins used to develop the immobilization matrices.

Amino acid	Composition of amino acids (g/100 g of sample)			
	200 Bloom	220 Bloom	240 Bloom	280 Bloom
Aspartic acid	4.42	4.81	5.04	4.92
Glutamic acid	8.91	9.20	9.29	9.37
Hydroxyproline	9.23	9.43	9.41	9.84
Serine	3.17	3.48	3.30	3.60
Glycine	22.42	23.36	23.20	24.2
Histidine	0.70	0.92	0.71	1.11
Arginine	8.55	9.21	9.01	8.82
Threonine	1.62	1.67	1.73	1.74
Alanine	8.90	9.05	9.19	9.88
Proline	12.91	13.02	13.43	14.22
Tyrosine	0.58	0.60	0.61	0.49
Valine	1.99	2.26	2.26	2.32
Methionine	0.70	0.74	0.76	0.70
Cystine	0.48	0.62	0.46	0.49
Isoleucine	1.39	1.43	1.52	1.62
Leucine	2.68	2.94	3.05	3.01
Phenylalanine	1.81	2.23	2.29	1.91
Lysine	3.29	3.35	3.35	3.49
Total	93.75	98.32	98.61	101.72

It can be observed that the amino acid compositions of the gelatins have the same profile. Almost all of the amino acids found in the gelatin samples are classified as common amino acids (except for hydroxyproline, a product of the hydroxylation of proline). They differ from each other in terms of their side chains, which vary in structure, size and electrical charge, influencing the solubility of the amino acids in water (Nelson and Cox, 2011).

The amino acids which are in greatest quantity in the four gelatins tested are glycine, followed by proline, and, in similar quantities, hydroxyproline, or glutamic acid, arginine and alanine. Amide side chains of asparagines and glutamine are hydrolyzed to aspartate, glutamate and free ammonia; which, according to Devlin (2011), are included in the content of glutamic and aspartic acids. Tryptophan is not present due to its possible degradation in the hydrolysis stage employed in the method adopted to obtain the amino acid profile.

Glutaraldehyde (GTA) has two functional groups capable of binding to free amine groups of lysine and hydroxylysine residues of amino acids of the polypeptide chains in gelatin molecules. All free amino groups react with the aldehyde groups of glutaraldehyde to form *Schiff* bases (Mozafari and Moztarzadeh, 2010). It may be possible to activate all the amino groups in a protein with only one glutaraldehyde molecule per amino group or with two glutaraldehyde molecules (Betancor *et al.*, 2006). The treatment with 0.05% of glutaraldehyde is sufficient to crosslink about 60% of the ε-amino groups (Bigi *et al.*, 2001).

Solubility in Water

Solubility in water is a gel property which demonstrates tolerance to water and which can affect the immobilization matrix. With the solubilization of the matrix in water, the confined enzymes can be released to the reaction medium. Figure 1 shows the behavior in relation to the solubility of the matrices obtained with gelatins with Bloom values of 200 (a), 220 (b), 240 (c) and 280 (d) with glycerol or mannitol added, with and without crosslinking and also the control experiments (C).

With an increase in the Bloom value of the gelatin a decrease in the solubility of the immobilization matrices in water occurs, regardless of the experiment. This behavior was to be expected since high Bloom values lead to stronger gels. The matrices crosslinked with glutaraldehyde showed lower solubility in water than the matrices without crosslinking. All crosslinked films were stiffer than gelatin films without chemical treatment and turned yellowish, characteristic of the *Schiff* bases. For the control experiments (C) (not shown in Figure 1), for which the water solubility values were 99.94%, 78.88%, 59.94 % and 36.88% for gelatins with Bloom values of 200, 220, 240 and 280 g, respectively, there was no addition of plastifier and no crosslinking, which verifies that the Bloom strength alone is sufficient to differentiate between the solubility of one gelatin and another. The solubility values observed for the gels obtained using gelatin without crosslinking and plastifier are considered to be high. For the gelatin with a Bloom value of 200, there was almost total solubilization of the gel in water. None of the gels obtained in the control experiment (C) without crosslinking were appropriate for use in lipase immobilization, based on this property.

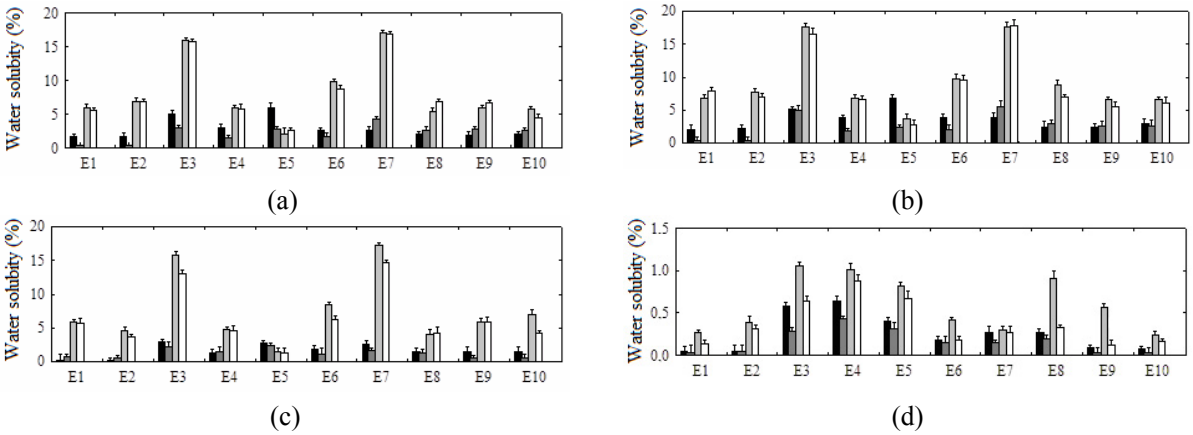


Figure 1: Solubility of the matrices in water: glycerol with crosslinking (■), mannitol with crosslinking (■), glycerol without crosslinking (■), mannitol without crosslinking (□).

Gelatin is a water soluble protein that results from partial hydrolysis of collagen. It is a nontoxic and biodegradable matrix with hydroxyl, amino and carboxyl functional groups effective in the formation of cross-links by inorganic as well as organic compounds (Vujčić *et al*., 2011).

All of the experiments without crosslinking but with the addition of plastifier showed lower solubility in relation to the control (with only the addition of gelatin without crosslinking). Since water is also a plastifier, the presence of the plastifiers used in the immobilization matrices in this study may have interfered with the interaction of water with the protein, reducing the solubilization in water.

When crosslinked with glutaraldehyde the control samples showed lower solubility in water, with values of 82.53%, 55.88%, 19.16% and 6.02% being obtained for the gelatins with Bloom values of 200, 220, 240 and 280, respectively. The use of glutaraldehyde reduced the solubility of the gel by 17.42% for the 200 Bloom, 29.16% for the 220 Bloom, 68.04% for the 240 Bloom and 83.68% for the 280 Bloom gelatins. According to Vanin *et al*. (2005), in order to reduce the water solubility of films based on gelatin, some authors have modified their physical and chemical properties using crosslinking agents such as glutaraldehyde and formaldehyde. Thus, they are no longer considered edible, but are of great interest for other applications, especially since they are biodegradable.

For the matrices produced with 200 Bloom gelatin (Figure 1a) the highest values for the water solubility were obtained for the samples with the greatest proportion of plastifier in relation to the gelatin mass used. Similar behavior was verified for matrices produced with 220 Bloom (Figure 1b) and 240 Bloom (Figure 1c) gelatins. Structural changes occur in the gel network through the action of the plastifiers, which can retard or facilitate the transport of water according to their concentration (Piermaria *et al.*, 2011). An increase in the plastifier concentration leads to an increase in the interaction with the water due to the reorganization of the protein network and consequent increase in the free volume (Thomazine *et al.*, 2005).

For the experiments carried out with 280 Bloom gelatin (Figure 1d), the values for the water solubility of the matrices with and without crosslinking were much lower than those for the other matrices produced with gelatins with a lower Bloom number. This demonstrates the good stability of the matrix in terms of lipase immobilization. The experiments in which the matrices were crosslinked and which showed lower solubility in water were E1, E2, E9 and E10 for glycerol and mannitol.

There is a clear trend in relation to the water solubility of the gels produced with gelatins with Bloom values of 200, 220, 240 and 280 g: the gels with the highest solubility in water were those without crosslinking. According to Bigi *et al.* (2001), appreciable amounts of gelatin are released in buffer solution from films crosslinked with GTA at low concentration, up to 18% after 4 weeks determined for the films crosslinked with 0.05% GTA solution. The cumulative gelatin release from the films crosslinked with 0.1% GTA solution is about 2 wt% after 1 week, and increases up to 9 wt% after 4 weeks. There is no appreciable release from the films crosslinked with GTA solutions at concentrations of 0.25% and greater. In this study, the concentration of the glutaraldehyde was 2.5%.

Immobilization and Determination of the Capacity of the Matrices to Maintain the Enzyme Immobilized

Immobilization Using Glycerol as the Plastifier

Figure 2 shows the results for the lipase activity corresponding to migration for the matrices produced with gelatins with Bloom values of 200 (a), 220 (b), 240 (c) and 280 (d), with the addition of glycerol.

The experiments using glycerol in which there was the least migration were those in which the lowest water solubility in water was observed.

The matrices produced with 280 Bloom gelatin showed statistically different results for enzymatic activity in relation to the other matrices, according to the Tukey test ($p<0.05$). The only experiment which gave statistically equal results was E1, with zero enzymatic activity for the 240 and 280 Bloom gelatins. The higher the gelatin Bloom number the lower the migration of the enzyme to the sodium phosphate buffer.

After the experimental design had been carried out, the effects of the variables studied were obtained in relation to the lipase migration (through the enzyme activity response). Only the results for the gelatins with Bloom numbers of 240 and 280 were analyzed, since the experiments indicated that, in these cases, there was no migration. Figure 3 shows the Pareto chart obtained with the migration results using glycerol and gelatin 240 Bloom and 280 Bloom.

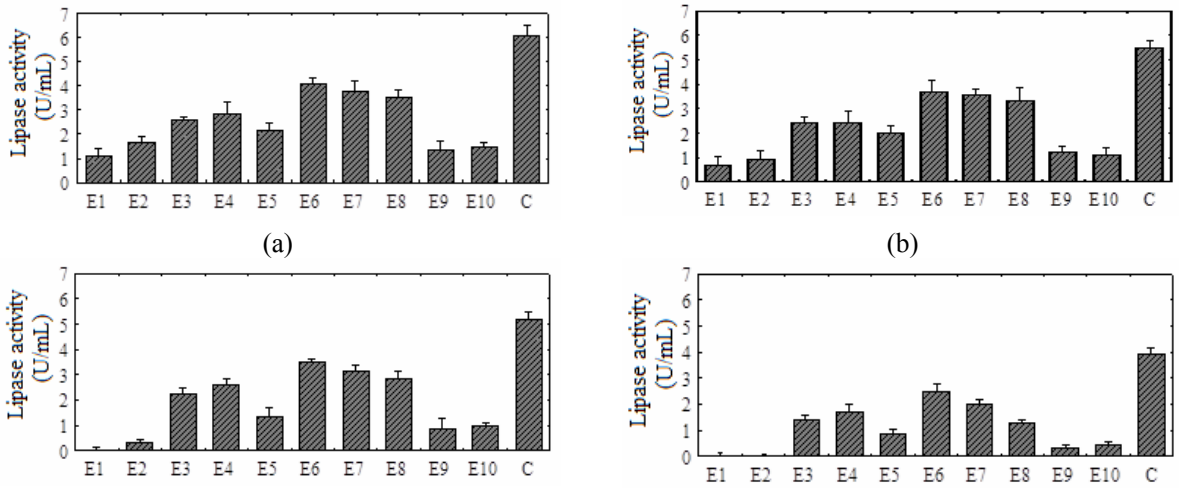


Figure 2: Lipase activity corresponding to migration for the matrices with glycerol added.

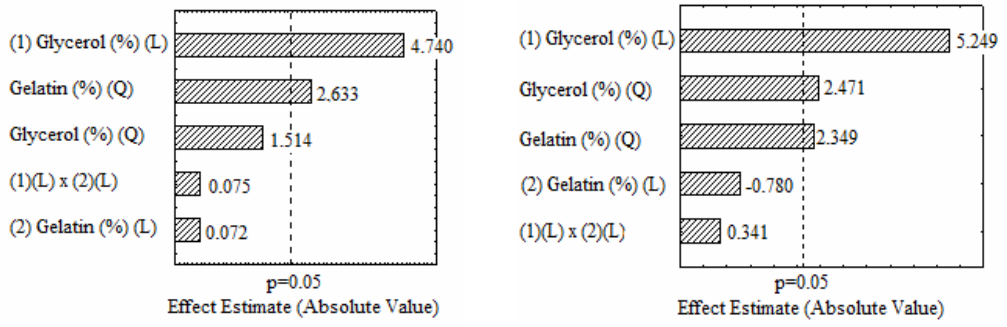


Figure 3: Pareto chart obtained for the migration results using glycerol and gelatin 240 Bloom (a) and 280 Bloom (b).

For the matrix produced with the 240 Bloom gelatin the glycerol (linear parameter) and gelatin (linear parameter) concentrations had a significant effect ($p<0.05$), which is positive for the process. Since the interaction variable did not show an effect, an analysis of the individual variables could be carried out. The positive effect of gelatin and of glycerol indicates that, when passing from level -1 to level +1 of the experimental design, an increase in the lipase activity occurs, that is, the migration is greater, which is not desirable for the immobilization. Similar effects occurred for the lipase migration in the matrix produced with 280 Bloom gelatin, the variables glycerol (linear parameter and quadratic parameter) and gelatin (linear parameter) having significant positive effects in relation to the enzymatic activity.

In order to obtain the mathematical model corresponding to the migration, the regression coefficients were calculated. Equations (3) and (4) provide the empirical mathematical models obtained through the regression coefficients related to the lipase immobilization for the 240 and 280 Bloom gelatins, respectively, in terms of the enzymatic activity.

$$A = 0.925 + 0.937GLY + 0.690G^2 \quad (3)$$

$$A = 0.681GLY + 0.425GLY^2 + 0.404G^2 \quad (4)$$

where A is the lipase activity in U/mL, GLY is the concentration of glycerol (%) and G is the concentration of gelatin (%).

The statistical analysis using the empirical mathematical model allowed the construction of the response surfaces shown in Figure 4 for the matrices produced with the 240 ($R^2 = 0.72$) and 280 ($R^2 = 0.70$) Bloom gelatins. It can be observed that lower gelatin concentrations and intermediate glycerol concentrations led to lower enzyme migration to the sodium phosphate buffer.

Immobilization Using Mannitol as the Plastifier

Figure 5 shows the results for the lipase activity corresponding to the migration for the 200 (a), 220 (b), 240 (c) and 280 (d) Bloom gelatins, respectively, with mannitol added.

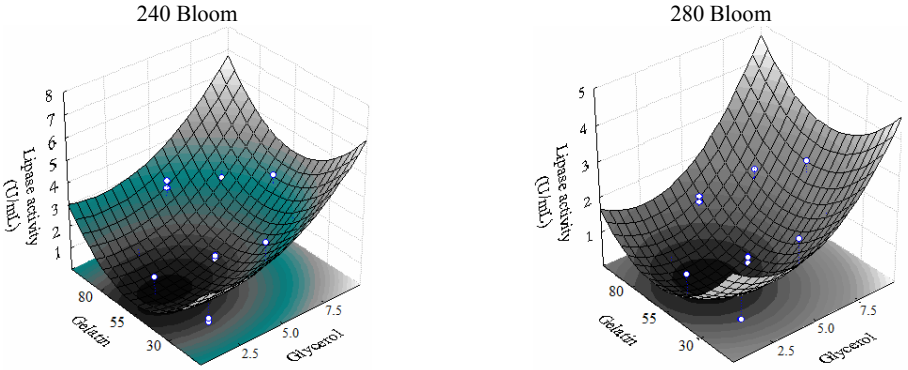


Figure 4: Response surfaces for the matrices produced with 240 and 280 Bloom gelatins and glycerol.

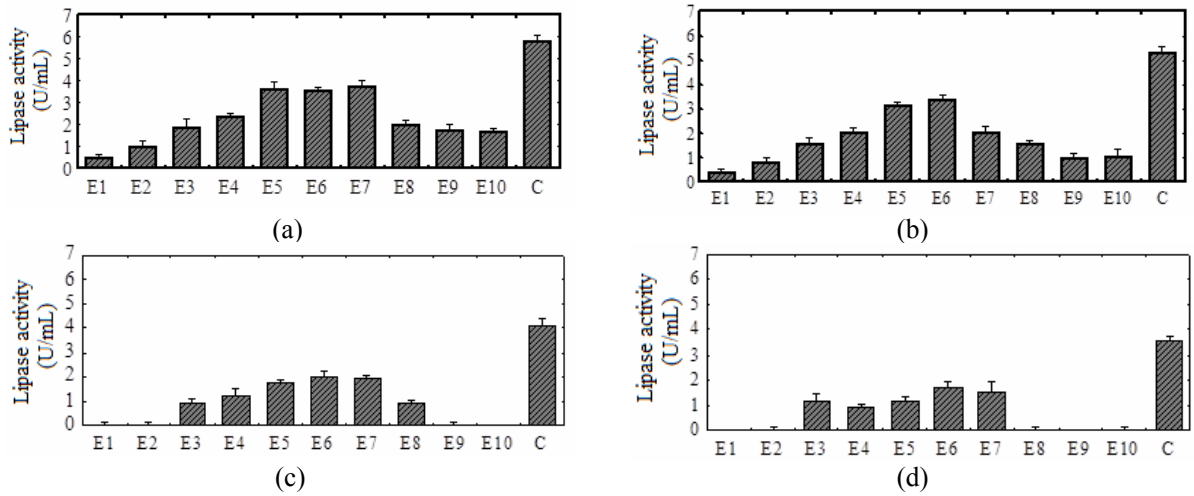


Figure 5: Results for lipase migration for matrices produced with 200 Bloom gelatin and mannitol

For the 200 and 220 Bloom gelatins lipase migration was observed in all experiments, as occurred in the case of the matrices with glycerol added.

It was verified from the Tukey test results that, for the same experiment in relation to the different gelatins, the results for most of the experiments with the 240 and 280 Bloom gelatin are statistically equal ($p>0.05$) and differ from those for the 200 and 220 Bloom gelatin experiments ($p<0.05$). The results for all of the experiments with 200 Bloom gelatin showed a significant difference compared with those for the experiments using 280 Bloom gelatin.

For the experiments with the addition of mannitol, a greater number of the immobilization matrices were efficient at maintaining the enzyme confined in comparison with glycerol.

The effects of the variables in relation to the matrices with mannitol added were investigated. Figure 6 shows the Pareto chart obtained for the migration results using mannitol and gelatin 240 Bloom and 280 Bloom.

For the experiments conducted with 240 Bloom gelatin only the concentration of mannitol was found to have a significant effect ($p<0.05$), this being positive. The gelatin concentration and the interaction between variables did not have an effect. The significant effects for the 280 Bloom gelatin were the mannitol and gelatin concentrations, the former being positive.

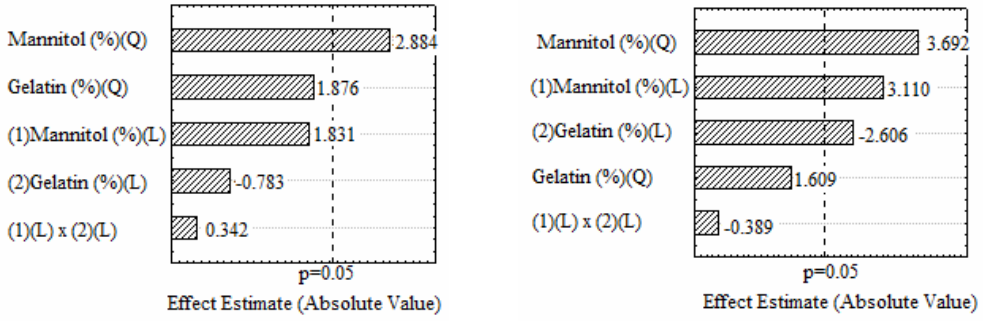
The regression coefficients were determined in order to obtain the mathematical model related to the migration. Equations (5) and (6) represent the empirical mathematical models obtained through the regression coefficients related to the lipase immobilization for the 240 and 280 Bloom gelatins, respectively, in terms of the enzymatic activity.

$$A = 0.659MAN^{2} \quad (5)$$

$$A = 0.354MAN - 0.296G + 0.557MAN^{2} \quad (6)$$

where A is the enzymatic activity in U/mL, MAN is the mannitol concentration (%) and G is the gelatin concentration (%).

The statistical analysis, applying the empirical mathematical model, allowed the construction of the response surfaces shown in Figure 7 for the matrices produced with 240 (R^2=0.89) and 280 (R^2=0.82) Bloom gelatins.



(a) (b)

Figure 6: Pareto chart obtained for the migration results using mannitol and gelatin 240 Bloom (a) and 280 Bloom (b).

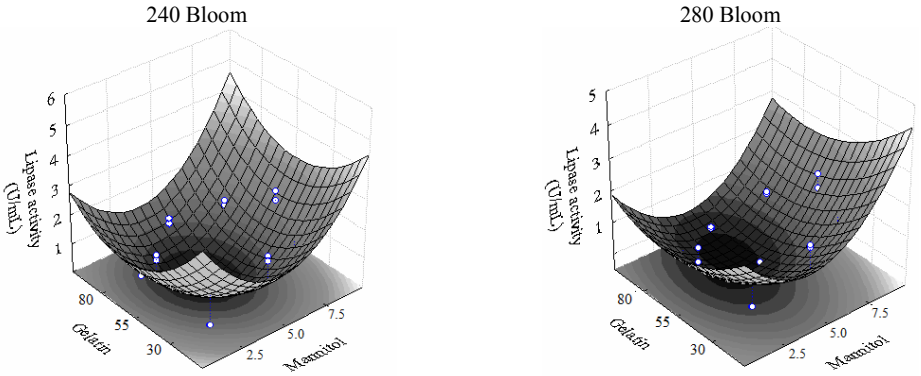


Figure 7: Response surfaces for the matrices produced with the 240 and 280 Bloom gelatins with mannitol added.

Immobilization Yield (IY)

After identifying the experiments in which migration did not occur, the immobilization yield was determined, which is the relation between the activity of the lipase in the immobilized and free forms and the higher the immobilization yield the closer the enzymatic activity of the lipase in the two forms. Figure 6 shows the results for the experiments with glycerol and mannitol, where E1-240-G is the experiment in which the matrix was produced with gelatin and plastifier concentrations corresponding to experiment E1, with 240 Bloom gelatin and glycerol as the plastifier. The same code was used for the matrices in Figure 8, where M stands for mannitol.

The lowest immobilization yields correspond to the experiment with the lowest concentration of gelatin (E1), obtaining values of 3.03% to 7.20%. The absence of enzymatic activity in the migration tests and low immobilization yield for these matrices can be explained by the lower concentration of gelatin associated with the plastifiers giving rise to more compact films, with a minimum quantity of pores. Thus, the absence of migration may be due to the homogeneity of the matrix, the enzyme being entrapped and the diffusion of the substrate through the matrix hindered.

On the other hand, the higher immobilization yields, corresponding to the experiments carried out with gelatin concentrations greater than 55%, have been explained by Vlierberghe *et al.* (2007), who stated that a greater gelatin concentration can result in a higher nucleation rate and thus a greater number of pores. This is due to the fact that, on heating the gelatin solutions, the molecules assume a random coil-type conformation. On cooling, reorganization begins and the nuclei appear, due to helical interactions (Tosh *et al.*, 2003). In this experiment the immobilization yield values were 77.31% to 87.92%.

Studies presented in the literature where different enzymes were immobilized in gelatin or gelatin mixtures or cross-linked gelatin exhibit very similar results. Assis *et al.* (2004a, 2004b), in a study of various media cross-linked with 2.5% glutaraldehyde for immobilization of pectin extract, obtained the highest yields of immobilization on Sepharose 81.7%, followed by gelatin gel, 78.0% after 48 hours of the process. Because gelatin is considerably more economically viable than Sepharose, it is better suited for immobilization, even with a small difference in the yield of immobilization. Naganagouda *et al.* (2007) immobilized α-galactosidase in a mixture of gelatin, sodium alginate and glycerol cross-linked with glutaraldehyde 5%, obtaining 64.3% immobilization. Tanriseven and Olcer (2008) immobilized glucoamylase in gelatin cross-linked with polyglutaraldeyde and obtained an immobilization yield of 85%.

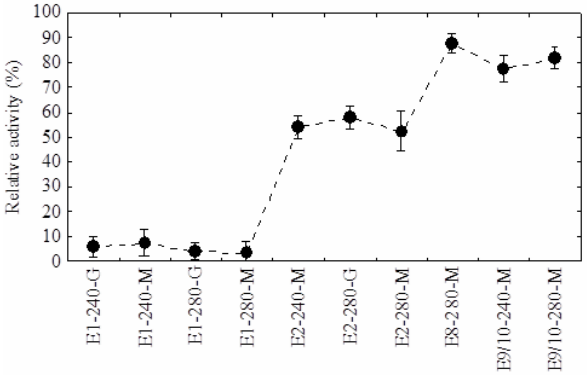


Figure 8: Immobilization yield for the experiments in which migration did not occur.

Scanning Electron Microscopy (SEM)

In order to better interpret the results for the immobilization yield, scanning electron microscopy (SEM) was carried out to analyze cross-sections of the immobilization matrices for which migration was not observed and which had the minimum and maximum immobilization yields.

Figure 9 shows the photomicrographs of cross-sections of the matrices corresponding to the experiments which gave the lowest immobilization yields. A low porosity was verified, which explains the low immobilization yields and is consistent with the affirmation that lower gelatin concentrations associated with plastifiers lead to more compact films. The lipase migration did not occur due to the formation of a uniform film without pores, which entrapped the enzyme. According to Kowalczyk and Baraniak (2011), a compact structure probably forms due to the cohesion forces, which are accentuated mainly during the drying of materials based on water.

Figure 10 shows the photomicrographs for the experiments that provided the highest immobilization yields. It can be observed that the morphology of the gels with the addition of plastifiers vary according to the concentrations of biopolymer and/or plastifier used. Al-Hassan and Norziah (2011) tested a concentration of 25% plastifier (glycerol or sorbitol) in films obtained from a mixture of manioc starch and fish gelatin in different proportions and obtained similar results, even though the concentration of plastifier was higher than those used in this study. They verified that the films with lower protein content had smoother surfaces and less pores.

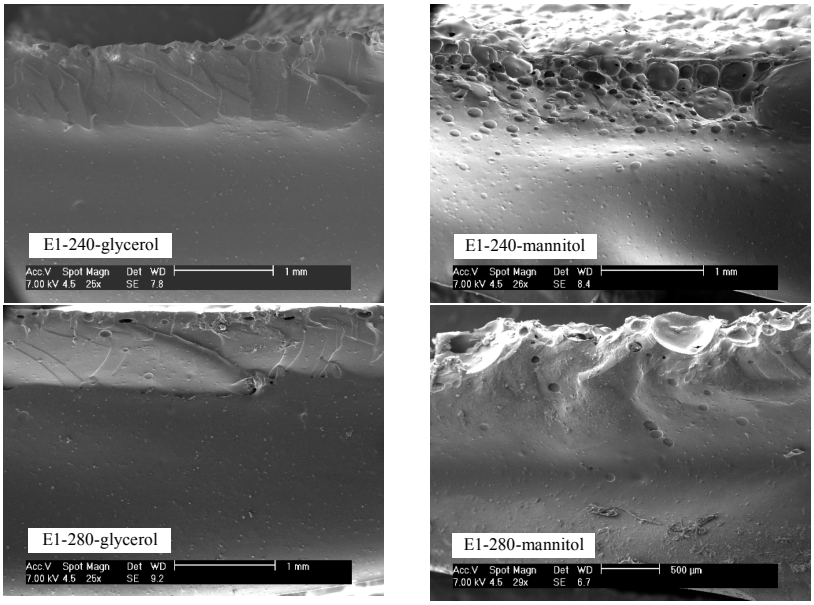


Figure 9: SEM micrographs of matrices with low immobilization yields.

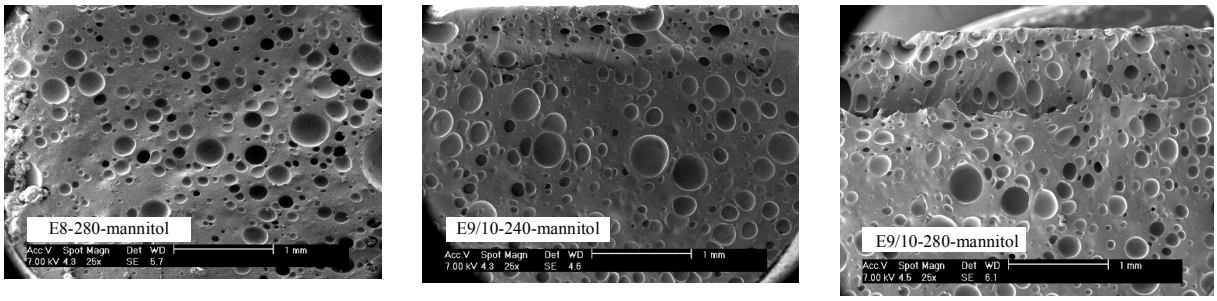


Figure 10: SEM micrographs of matrices with higher immobilization yields.

X-Ray Diffraction of Immobilization Matrices

Rivero *et al*. (2010) carried out X-ray diffraction (XRD) analysis of a type B gelatin sample, the same classification as that of the gelatin used in this study, and verified that the diffraction was characteristic of a partially crystalline material, with a broad peak located at 2θ=20º. It can be observed that, in all of the diffractograms shown in Figures 11 and 12, there is a peak at or close to an angle of 20º, which can thus be identified as the peak corresponding to gelatin. The values for the angles obtained for these peaks were in the range of 2θ=19.63º to 2θ=21.78º. The peaks in Figure 12 are of greater intensity than those in Figure 11. The peak intensity is related to the crystallinity of the sample, and thus these results demonstrate that the samples of experiment E1, with different gelatins and plastifiers, had lower crystallinity than those of the other experiments.

The presence of another peak was observed, for which the angle varied between 2θ=6.77º and 2θ=7.82º. According to Tanioka *et al.* (1996), the presence of these peaks indicate the reconstitution of the structure of the triple helix of collagen and, according to Rivero *et al.* (2010), peaks close to 8º are related to the diameter of the triple helix and their intensity is associated with the content of triple helices in the films. It was verified that the greatest intensities determined in relation to the peak close to 8º can be observed in Figure 12. This may characterize these matrices as being more stable since, on cooling, curling and cross linking of the bonds of the gelatin gel occurred, returning to a typical collagen structure which strengthens the stiffness of the gel. The result is essentially an open network formed by the association of the chains in the junction zones, rich in amino groups, strengthened by regions in which the helical structure of collagen has been reconstituted (Wong, 1995). According to Boanini *et al.* (2010), an increase in triple helix structures may be related to an increase in the thermal stability of gelatin gels.

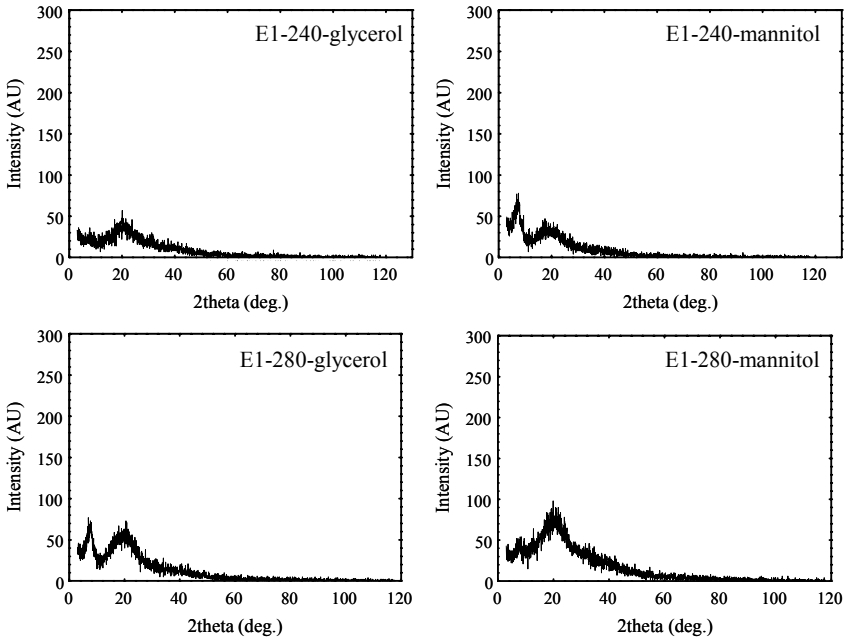


Figure 11: Diffratograms of matrices with lower immobilization yields.

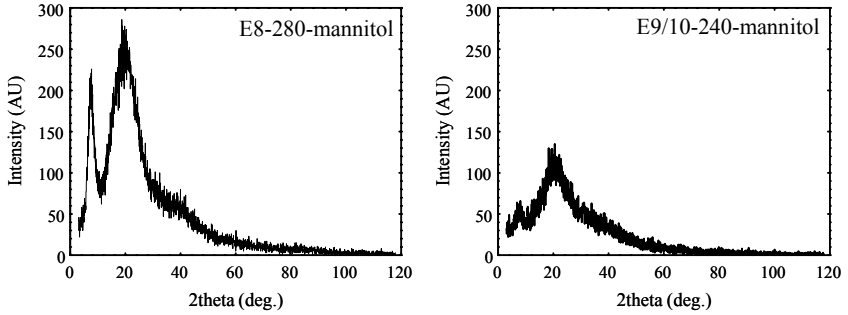


Figure 12: Diffratograms of matrices with higher immobilization yields.

CONCLUSIONS

Based on the concentrations of gelatin and plastifier used and the diverse tests carried out in this study, it can be concluded that the immobilization of lipase can be achieved with gelatin and the use of a plastifier. It was verified that the gelatin Bloom value influences the migration of the immobilized lipase, gelatins with higher Bloom values being more effective for the immobilization, maintaining the enzyme entrapped. The matrices crosslinked with glutaraldehyde had a solubility in water lower than matrices without crosslinking. In relation to the plastifiers, matrices with higher plasticizer concentrations have higher solubilities in water. Comparing the plasticizers, glycerol showed higher solubility in water. Mannitol provided satisfactory results, providing higher immobilization yields, reaching values above 80%. The morphology, as well as the degree of crystallinity of the matrices obtained, differed in relation to the gelatin concentration and plastifier used. By microscopy, it was found that the experiments with lower gelatin concentration resulted in matrices without the presence of pores throughout the cross section, with a small concentration of pores in the upper layer; these arrays corresponded to the experiments with low immobilization yield. The matrices that had higher yields of immobilization showed the highest amount of pores, which are more uniform. In X-ray diffraction, the matrices containing 280 Bloom gelatin and mannitol (E8 and E9/10 experiments) showed higher crystallinity.

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NOMENCLATURE

pH	hydrogen ion potential
T	temperature
U	units of enzyme activity
v/v	volume per volume
w/V	mass per volume

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