# COVALENT ATTACHMENT OF *ASPERGILLUS ORYZAE* LIPASE ON CHEMICALLY MODIFIED HYBRID MATRICES.

*A. A. Mendes[1]; D. S. Rodrigues [1]; W. S. Adriano[1]; E.J. Mammarella[2]; H. F. Castro[3]; R. L. C. Giordano[1*]*

1-Department of Chemical Engineering, Federal University of São Carlos, Rod. Washington Luiz, Km 235, 13565-905, São Carlos-SP, Brazil, e-mail: raquel@power.ufscar.br

2-Intec (UNL - CONICET) – Güemes 3450 – 3000, Santa Fé – Argentina

3-Engineering School of Lorena, University of São Paulo, Rod. Itajubá-Lorena, Km 74,5, 12600-970, CP 116, Lorena-SP, Brazil, e-mail: heizir@dequi.faenquil.br

## Abstract

This work aims to obtain thermo-stable immobilized derivatives on hybrid matrixes consisted of chitosan and different copolymers, as k-carrageenan, gelatin or alginate. Lipase from *Aspergillus oryzae* was immobilized by multipoint covalent attachment on chitosan-copolymer and pure chitosan (as control). The hybrid matrixes were chemically modified with 2,4,6 trinitrobenzene sulphonic acid (TNBS) to increase the support hydrophobicity followed by activation with Glycidol (GL), Epichlorohydrin (EP) or Glutaraldehyde (GA). Hybrid matrixes that provided immobilized derivatives with high hydrolytic activity were chitosan-alginate-TNBS activated with glycidol (281.5 U/g of gel), glutaraldehyde (231.5 U/g of gel), chitosan-alginate without TNBS activated with epichlorohydrin (308.7 U/g of gel) and chitosan-glycidol cross-linked with GA 1% (249.7 U/g of gel). For all immobilized derivatives, a 45-fold increase in the thermal stability was observed in relation to the free lipase.

## Introduction

Lipases (EC 3.11.3) are versatile catalysts with potential applications in a number of industrial processes. Important uses include their addition to detergents, the manufacture of food ingredients, pitch control in the pulp industry, and biocatalyst of transformations [1-2]. For many of these applications, it is recommended to use the enzyme in its immobilized form. With immobilized lipases, improved stability, reuse, continuous operation, the possibility of better control of reactions and hence more favourable economical factors can be expected.

Lipase has been immobilized by several methods, namely adsorption, cross-linking, adsorption followed by cross-linking, covalent attachment and physical entrapment [3]. However, the selection of an immobilization strategy should be based on process specifications for the catalyst, including such parameters as overall enzymatic activity, effectiveness of lipase utilization, deactivation and regeneration characteristics, cost of the immobilized procedure, toxicity of immobilization reactants and the desired final properties of the immobilised derivative [4].

Among immobilization methods available, multipoint covalent attachment is the most effective in terms of thermal stabilization and operational stabilities of the enzyme [5-7]. This behavior is due to the formation of some covalent linkages between the reactive groups of the enzyme (primary amino groups) and aldehyde groups of the support, reducing the aggregation in the isoelectric points and the performance of solvent on the active structure of the enzyme [5]. In the multipoint

immobilization attachment, high activated supports are required for the formation of two covalent linkages. Several enzymes, including lipases, have being immobilized by this technique for the production of biocatalysts with enhanced thermal stability [6-8].Several supports are suitable for this immobilizing procedure and in this work, chitosan a natural product derived from chitin was selected due to its several advantages in relation to other supports, including its lack of toxicity, chemical reactivity allowing easy fixation of enzymes and multiple physical forms [9].

This work aims to obtain high activity and thermal stable immobilized derivatives of *Aspergillus oryzae* lipase using hybrid matrixes of chitosan and different copolymers as k-carrageenan, gelatin and sodium alginate activated by glycidol, epichlorohydrin or glutaraldehyde. Chemical modification in the chitosan structure using hydrophobic agent (TNBS) and glutaraldehyde for cross-linking were also evaluated to produce a more favorable microenvironment for lipase immobilization. Other tested strategy was the addition of *Saccharomyces cerivisiae* yeast into chitosan beads to increase the porous formation in the hydrogel.

## 2. Material and Methods

*Material*

Lipase from *Aspergillus oryzae* (AOL) was kindly donated by Novozymes (Araucária, Brazil). Native chitosan (85% deacetylation degree) was purchased from Polymar S.A. (Ceará, Brazil). Glycidol (GL), epichlorohydrin (EP), k-carrageenan and 2,4,6-trinitrobenzene sulphonic acid (TNBS) were purchased from Sigma Aldrich Co. (St. Louis, MO, USA). Glutaraldehyde (GA), 25% v/v solution, and sodium alginate were obtained from Vetec (São Paulo, Brazil). Olive oil (low acidity), gelatin and yeast (*Saccharomyces cerevisiae*) were purchased at a local market. All other reagents used were of analytical grade.

*Methods*

*Preparation of chitosan beads*

Chitosan control (4% wt.) and chitosan-copolymers: chitosan (4% wt.)-gelatin (3% wt.), chitosan (2.5% wt.)-sodium alginate (2.5% wt.), chitosan (2.5% wt.)-k-carrageenan (2.5% wt.), chitosan (4% wt.)-yeast (10% wt.) and chitosan (2.5% wt.)-alginate (2.5% wt.)-yeast (10% wt.) were completely dissolved in 5% (v/v) acetic acid solution in a beaker and the contents were mixed for 15 h at room temperature. The chitosan solution was then injected into a 0.1 M NaOH solution (1:10 v/v) to form hydrogel beads at slow stirring for 24 h. The beads were finally filtered and rinsed thoroughly with distilled water. The beads were then stored in freezer for further use.

*Synthesis of hydrophobic beads with TNBS*

A total of 10 g of gel, were added 37.5 mL buffer bicarbonate pH 10.05 (100 mM) and 0.25 mL of TNBS. The mixture was stirred on a shaker operated at 150 rpm and 25 °C for 24 h. The beads were filtered and rinsed thoroughly with distilled water.

*Activation beads procedure*

Glyoxyl supports were prepared by etherification of chitosan with glycidol [10] and epichlorohydrin [11] and further oxidation of the resulting support-glyceril. Chitosan activated with glutaraldehyde was carried out according to the methodology described by Fernández-Lafuente et al. [12]. At end, activated chitosan beads were filtered and washed thoroughly with distilled water.

*Immobilization procedure*

Lipase was immobilized by multipoint attachment into chitosan control (4% wt.) and chitosan-copolymers: chitosan (4% wt.)-gelatin (3% wt.), chitosan (2.5% wt.)-alginate (2.5% wt.), chitosan (2.5% wt.)-k-carrageenan (2.5% wt.) unmodified and chemically modified with TNBS at pH 10.05 (100 mM buffer bicarbonate) during 12 h at room temperature, according to the methodology described by Guisán [10]. For all samples, enzyme loading was 5.0 mg of protein/ g of support. The immobilized derivatives were recovered by filtration, following by washings with 200 mM buffer phosphate pH 7.0. Finally the immobilized derivatives were thoroughly rinsed with distilled water.

*Cross-linking of glyceril-chitosan*
Cross-linking effect into glyceril-chitosan, using chitosan control and chitosan-alginate-TNBS, was evaluated by reaction with glutaraldehyde at different concentrations (0.5-1.0% v/w) and temperature of reaction (0-25 °C) in 200 mM buffer phosphate pH 7.0. Time of immobilization (12-24 h) was also studied for the evaluation of this parameter on the immobilization process.

*Cellular rupture with soap powder procedure*
Selected hydrogels, chitosan-alginate-TNBS and chitosan-cross-linked with GA, were supplemented with *Saccharomyces cerevisiae* yeast and incubated with a solution containing commercial soap powder (10% wt.) followed by the adjustment of the pH to 9.0 with NaOH 0.5 M solution. The reaction was carried out for 24 h at 40 °C for complete cellular rupture. Commercial soap powder contains hydrolytic enzymes such as proteases and lipases that are responsible by cellular rupture. The beads were filtered and thoroughly rinsed with distilled water. After the beads formation, AOL lipase was immobilized into selected supports activated with the three activating agents for 24 h.

*Thermal stability*
The soluble (0.2 mL) and immobilized lipase (0.1 g) were incubated in the presence of 1 mL buffer phosphate pH 8.0 (100 mM) at 70 °C for different time intervals. The inactivation constant ($k_d$) and half-life time ($t_{1/2}$) were determined by applying the exponential non-linear decay model [13].

*Determination of hydrolytic activity*
Hydrolytic activities of soluble and immobilized lipase were assayed by the olive oil emulsion method according to the modified methodology described by Soares et al. [14]. One international unit of activity was defined as the amount of enzyme that is necessary to liberate 1 μmol of free fatty acid per minute (1 IU) at pH 8.0 and 37 ºC.

*Determination of protein*
Protein was determined according to the methodology described by Bradford [15] using bovine serum albumin (BSA) as standard.

*Immobilization parameters*
Coupling yield percentage (CY) was determined from the ratio of immobilized protein to the initial protein concentration offered. Recovery activity percentage (RA) was determined from the ratio of recovered activity (immobilized) to the initial activity units offered. Stability factor (SF) was calculated from the ratio of half-life of the immobilized derivative to the soluble enzyme.

## 3. Results and Discussion

*Effect of the addition of copolymers on the chitosan beads*
According to Table 1, the use of glutaraldehyde as activating agent gave higher coupling yield than glycidol and epichlorohydrin reactants. High values of coupling yield were obtained for chitosan-gelatin beads in the absence or presence of TNBS (60-61%), chitosan control in the absence of TNBS (59.8%) and chitosan-alginate beads in the absence or presence of TNBS (59.4-53.3%). The lowest values were found for chitosan-k-carrageenan (37.3-39.6%) and chitosan control in the presence of TNBS (39.5%). Using glycidol as activating agent rendered protein fixation percentage in the range from 14.6% (chitosan-gelatin in the absence of TNBS) to 32.3% (chitosan-alginate in the absence of TNBS) and employing epichlorohydrin protein fixation percentage values were 20.2% (chitosan-gelatin in the absence of TNBS) and 29.4% (chitosan-alginate in the absence of TNBS). The protein fixation was not influenced by hydrophobization process employing TNBS.
High coupling yield was only verified for the support activated with glutaraldehyde, due to the high reactivity of this activating agent at basic pH [8]. The bifunctional glutaraldehyde reacts with the amine groups of chitosan to form Schiff bases, followed by covalent coupling of lipase. Glycidol and epichlorohydrin link through the carbon atoms, resulting in the rupturing of the epoxyde ring and the removal of a hydroxyl and chlorine atoms, respectively. Chitosan-gelatin cross-linked activated with glycidol produced an increase on the coupling yield, almost twice than chitosan-

gelatin without hydrophobization step. This may be justified due to the high number of amine groups present in the structure of the hybrid matrix that promoted several links with aldehyde groups of the glutaraldehyde. Gelatin is the partial derivative of collagen, which is the major component of skin, bone, cartilage, and connective tissue. Activated chitosan-alginate blend slight decreased the protein fixation (10-20%), independent of the activating agent used.

**Table 1:** Multipoint attachment of microbial lipase into chitosan control and chitosan-copolymers in the absence and presence of TNBS (immobilizing time 12 h).

| Support | TNBS | Activating agent | $A_{der.}$ (U/g) | CY (%) | RA (%) | $t_{1/2}$ (h) | SF |
|---|---|---|---|---|---|---|---|
| **Chitosan (4% wt.)** | Absence | GL | 109.1 | 20.1 | 40.0 | 1.34 | 15.2 |
| | | EP | 97.6 | 21.1 | 42.5 | 1.10 | 12.5 |
| | | GA | 95.3 | 59.8 | 26.2 | 0.59 | 7.10 |
| | Presence | GL | 108.9 | 24.3 | 43.5 | 1.22 | 13.9 |
| | | EP | 97.6 | 22.4 | 47.1 | 1.56 | 17.7 |
| | | GA | 90.8 | 39.5 | 31.9 | 0.77 | 8.75 |
| **Chitosan-gelatin (4% wt.-3% wt.)** | Absence | GL | 118.1 | 14.6 | 48.5 | 1.0 | 12.5 |
| | | EP | 99.9 | 20.2 | 40.0 | 0.90 | 11.2 |
| | | GA | 95.3 | 61.0 | 32.5 | 0.72 | 8.62 |
| | Presence | GL | 99.9 | 26.3 | 52.4 | 2.07 | 23.5 |
| | | EP | 121.6 | 25.0 | 74.2 | 2.19 | 27.3 |
| | | GA | 113.6 | 60.0 | 32.8 | 0.92 | 11.0 |
| **Chitosan-k-carrageenan (2.5% wt.-2.5% wt.)** | Absence | GL | 74.0 | 19.0 | 25.1 | 0.66 | 7.45 |
| | | EP | 50.0 | 21.6 | 16.1 | 0.46 | 5.24 |
| | | GA | 36.3 | 39.6 | 11.5 | 0.20 | 2.31 |
| | Presence | GL | 104.4 | 21.2 | 28.7 | 0.83 | 9.44 |
| | | EP | 140.7 | 24.3 | 50.2 | 1.45 | 16.5 |
| | | GA | 95.7 | 37.3 | 28.6 | 0.93 | 10.6 |
| **Chitosan-alginate (2.5% wt.-2.5% wt.)** | Absence | GL | 163.5 | 32.3 | 71.4 | 0.70 | 7.89 |
| | | EP | 308.7 | 29.4 | 94.1 | 0.90 | 10.2 |
| | | GA | 208.9 | 59.4 | 79.6 | 0.82 | 9.29 |
| | Presence | GL | 281.5 | 25.1 | 89.8 | 3.93 | 44.6 |
| | | EP | 254.2 | 23.6 | 79.3 | 3.99 | 45.2 |
| | | GA | 231.5 | 53.3 | 80.9 | 3.89 | 44.2 |

TNBS: 2,4,6 trinitrobenzene-sulphonic acid; GL: Glycidol; EP: Epichlorohydrin; GA: Glutaraldehyde; $A_{der}$: Hydrolytic activity of the immobilized derivate (U/g of gel); CY: Coupling yield (%); RA: Recovery activity (%); $t_{1/2}$: half-life time (h) and SF: Stability factor.

Cross-linking with TNBS was found to be effective in terms of hydrolytic activity of the immobilized derivatives and recovered activity percentage due to the produce a more favorable microenvironment for lipase immobilization. Reaction of TNBS with chitosan beads blocked $-NH_2$ groups present in the chitosan reduce the swelling degree and, consequently, increase substrate diffusion onto porous support and mechanical and thermal strength. In terms of hydrolytic activity, the immobilized derivate chitosan-alginate showed high catalytic activity using the three activant agent. Chitosan-alginate none chemically modified obtained the higher hydrolytic activity with support activated with epichlorohydrin (308.7 U/g of gel), followed of glutaraldehyde (208.9 U/g of gel) and glycidol (163.5 U/g of gel), resulting in recovery activity percentage of 94.1, 71.4 and 79.6% respectively. Employing chitosan-alginate chemically modified with TNBS, the hydrolytic activity of the gel activated with epichlorohydrin was reduced in 18% (254.2 U/g of gel) and glycidol and glutaraldehyde, respectively, was increased to 72% (281.5 U/g of gel) and 11% (231.5 U/g of gel). Hybrid matrix composed by chitosan-k-carrageenan reduced the recovery activity and, consequently, hydrolytic activity due to the high degree of swelling that reduced the substrate

diffusion onto porous support. Tapia et al. [16] evaluated the swelling behavior of polyelectrolytes complexes chitosan-carrageenan and chitosan-alginate on the prolonged drug release. According to the these authors, chitosan–alginate system is better than the chitosan–k-carrageenan system as prolonged drug release matrix because the complex chitosan-carrageenan have high swelling degree and the drug release is fast due to the lower number of ionized groups in your structure, reducing ionic interactions with chitosan. Chitosan-gelatin activated with glycidol and epichlorohydrin recovered 74.2 and 52.4% of the hydrolytic activity offered.

The Figure 1 shows the influence of the hybrid matrix composition on the thermal stability. Chitosan control cross-linked with TNBS showed lower thermal stability than hybrid matrix chitosan-alginate. After 3.5 h of incubation at 70 °C, was not observed hydrolytic activity for chitosan control cross-linked and for the hybrid matrix chitosan-alginate, total inactivation of the biocatalyst was detected after 9 h.
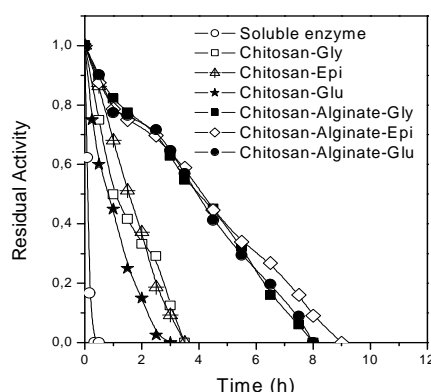


**Figure 1:** Thermal stability of lipase AOL soluble and immobilized into chitosan control and hybrid matrix chitosan-alginate cross-linked with TNBS. Inactivations were carried out at 70°C in 100 mM sodium phosphate buffer, pH 8.

Reaction of the amine group present in the chitosan structure with sulphonic group of the TNBS increased thermal stability of the biocatalyst. Chitosan-copolymers beads are formed by ionic interactions and controlled by pH of the system. If the pH increases, the protonation of chitosan decreases and induces a decrease of the ionic interaction. If the pH becomes too high, amino groups of chitosan are neutralized and ionic interaction is inhibited [9]. Cross-linking of chitosan-copolymers with TNBS reduce the influence of the pH because covalent interaction is more stable, providing higher rigidity to the composite structure. Activated chitosan-alginate cross-linked with TNBS was the hybrid matrix that provided the highest thermal stability, independent on the activating agent used. The half-time ($t_{1/2}$) values were varied from 3.89 to 3.99 h and the stability factor (SF), ratio of half-life of the immobilized derivate to the soluble enzyme, was 44.2 and 45.2. The second hybrid matrix with high thermal stability was chitosan-gelatin activated with glycidol and epichlorohydrin, which stabilizing 23.5 and 27.3 fold in relation to the soluble enzyme, respectively. This behavior was not obtained for the composite activated with glutaraldehyde.

*Effect of cross-linking of glyceril-support with glutaraldehyde*

Strategies have been carried out to improve the chemical stabilities of the hydrogel beads through chemical cross-linking involving bonds with chitosan amine groups such as glutaraldehyde, formaldehyde and glyoxal [17]. Chemical cross-linking reaction was found to reduce the solubility of the chitosan hydrogel beads in aqueous solutions of low pH values and produce a rigid polymer for use as matrix for immobilization of enzymes [17]. The cross-linking reaction reduced the disintegration of the chitosan beads (swelling degree) and increased the mechanical and thermal strength.

In this step, chitosan was activated with glycidol through glyceril-support formation and after was cross-linked with glutaraldehyde through different conditions, varying glutaraldehyde concentration, temperature of reaction and time of immobilization. Finally, oxidation with sodium periodate was carried out and the support was used to lipase immobilization. The Table 2 show the

influence of the cross-linking this reaction on the hydrolytic activity, coupling yield and thermal stability of the biocatalyst.

**Table 2.** Strategies fro to increase the thermal stability of the immobilized derivate in chitosan beads (4% w/w) activated by glycidol varying glutaraldehyde (GA) concentration, reaction temperature (T) of cross-linkage e time of immobilization (t).

| Run | Levels | | | Independent Variables | | | Response Variables | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | [GA] (%) | T (°C) | t (h) | CY (%) | $A_{der.}$ (U/g) | SF |
| 1 | -1 | -1 | -1 | 0.5 | 0 | 12 | 27.6 | 99.9 | 35.7 |
| 2 | +1 | -1 | -1 | 1.0 | 0 | 12 | 23.1 | 104.4 | 28.5 |
| 3 | -1 | +1 | -1 | 0.5 | 25 | 12 | 23.3 | 97.6 | 28.3 |
| 4 | +1 | +1 | -1 | 1.0 | 25 | 12 | 20.2 | 127.1 | 24.2 |
| 5 | -1 | -1 | +1 | 0.5 | 0 | 24 | 30.4 | 195.2 | 31.0 |
| 6 | +1 | -1 | +1 | 1.0 | 0 | 24 | 35.1 | 122.6 | 42.0 |
| 7 | -1 | +1 | +1 | 0.5 | 25 | 24 | 42.4 | 144.0 | 40.7 |
| 8 | +1 | +1 | +1 | 1.0 | 25 | 24 | 37.8 | 249.7 | 45.3 |
| Control | | | | - | - | - | 20.1 | 109.1 | 15.2 |

CY: Coupling yield (%); $A_{der}$: Hydrolytic activity of the immobilized derivate (U/g of gel) and SF: Stability factor

According to Table 2, the addition of glutaraldehyde at low concentration resulted in a slight increase on the coupling yield at 12 h. The increase of the time of immobilization allowed to increase protein fixation, higher twice (assays 7 and 8) than chitosan control. Hydrolytic activity also was influenced by the time of immobilization. The higher values obtained were for to assay 5 (195.2 U/g of support) and assay 8 (249.7 U/g of support). This increase on the activity recovered is due to the production of a more favorable microenvironment for lipase immobilization through reduction of the hydrophilic character of the support. Thermal stability after cross-liking was found to increase for all assays due to the N-acetylation of the amino group that prevents cleavage and, consequently, the depolymerization of the support [18]. The periodate ion attacks vicinal diols to cleave the carbon–carbon bond by an oxidation reaction, leading to the formation of a dialdehyde. Assays 6,7 and 8 were 3-fold more stable than lipase immobilized into chitosan control activated by glycidol, as showed in the Figure 2. The highest stability factor was 45.3 fold in relation to the soluble enzyme, obtained for the sample immobilized on the presence of gluraraldehyde (1% wt.) at 25°C during 24 h (assay 8). According to these results, hybrid matrix chitosan-alginate cross-linked with TNBS and chitosan activated with glycidol and cross-linked with glutaraldehyde (assay 8) showed similar behavior on the thermal stability, in order of 45 fold more stable than soluble lipase.
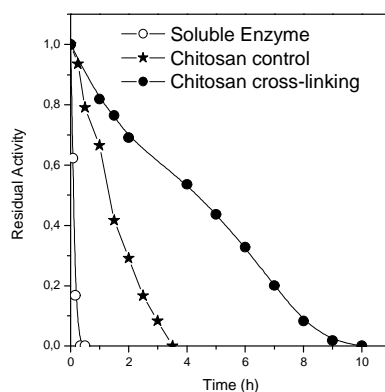


**Figure 2:** Thermal stability of lipase AOL soluble and immobilized into pure chitosan (control) and chitosan activated with glycidol and cross-linked with glutaraldehyde. Inactivation constant values were carried out at 70°C in 100 mM sodium phosphate buffer, pH 8.

After this study, the influence of the cross-linkage with glutaraldehyde at low concentration into chitosan-alginate-TNBS activated with either glycidol and epichlorohydrin was investigated on the immobilization parameters, such as coupling yield, activity recovered and thermal stability as shown in Table 3. All immobilization parameters were reduced with the cross-linkage with glutaraldehyde. This effect is due, mainly, to the diffusional limitations on the immobilized derivative. High aldehyde density on the support surface promotes reduction of the porous diameter influencing also in the increase of the thermal inactivation of the biocatalyst.

**Table 3:** Influence of the cross-linkage through glutaraldehyde (1% wt.) on the immobilization of lipase AOL into chitosan-alginate-TNBS immobilized at 24 h.

| Activant agent | $A_{der.}$ (U/g) | CY (%) | RA (%) | $t_{1/2}$ (h) | SF |
|---|---|---|---|---|---|
| **Glycidol** | 65.8 | 27.4 | 20.3 | 1.58 | 17.9 |
| **Epichlorohydrin** | 104.1 | 21.1 | 32.4 | 2.27 | 25.8 |

TNBS: 2,4,6 trinitrobenzene-sulphonic acid; GL: Glycidol; EP: Epichlorohydrin; GA: Glutaraldehyde; $A_{der}$: Hydrolytic activity of the immobilized derivate (U/g of gel); CY: Coupling yield (%); RA: Recovery activity (%); $t_{1/2}$: half-life time (h) and SF: Stability factor.

### *Influence of the yeast on the lipase immobilization into chitosan control and chitosan-alginate cross-linked with GA*

Activated Chitosan-yeast with glycidol and epichlorohydrin cross-linked with GA, activated chitosan-yeast with GA and activated chitosan-alginate-yeast-TNBS with the three activation agents were used as matrix for immobilizing lipase AOL. The influence of yeast supplementation was also evaluated as shown in Table 4.

**Table 4:** Influence of the yeast supplementation of yeast into chitosan hydrogels on the immobilization parameters.

| Support | Cross-linking agent | Activating agent | $A_{der.}$ (U/g) | CY (%) | RA (%) | $t_{1/2}$ (h) | SF |
|---|---|---|---|---|---|---|---|
| **Chitosan-yeast (4% wt.-10% wt.)** | GA | GL | 167.1 | 21.5 | 21.5 | 2.42 | 28.9 |
| | | EP | 180.9 | 22.4 | 29.9 | 1.55 | 18.6 |
| | - | GA | 191.8 | 58.4 | 35.2 | 0.99 | 11.8 |
| **Chitosan-alginate-yeast (2.5% wt.-2.5% wt.-10%wt.)** | TNBS | GL | 65.8 | 24.1 | 14.4 | 2.91 | 33.1 |
| | | EP | 76.7 | 24.8 | 15.6 | 2.64 | 30.0 |
| | | GA | 109.6 | 82.1 | 18.8 | 2.34 | 26.6 |

TNBS: 2,4,6 trinitrobenzene-sulphonic acid; GL: Glycidol; EP: Epichlorohydrin; GA: Glutaraldehyde; $A_{der}$: Hydrolytic activity of the immobilized derivate (U/g of gel); CY: Coupling yield (%); RA: Recovery activity (%); $t_{1/2}$: half-life time (h) and SF: Stability factor.

The highest coupling yield (82.1% of protein fixation) was obtained when the lipase was immobilized on chitosan-alginate-TNBS-yeast. High values of hydrolytic activity were detected for the chitosan-yeast, almost twice more active than chitosan control. Chitosan-alginate-TNBS-yeast slightly reduced the hydrolytic activity. Diffusional limitation can be responsible on the hydrolytic activity reduction for the chitosan-alginate-yeast, confirmed the reduction on the activity recovered. Perhaps, chitosan-alginate-yeast resulted into lower porous size formation than chitosan-yeast. In terms of stability factor, the immobilized derivates with higher thermal stability were chitosan-alginate and chitosan activated with glycidol.

Activated chitosan with epichlorohydrin and glutaraldehyde showed similar thermal stability to the chitosan control, without chemical modification. The supplementation of yeast, in comparison to the chitosan control, increased the thermal stability and showed to be an important tool for stabilizing enzymes. These supports studied, without yeast supplementation, gave positive effects on all the immobilization parameters. The influence of solvents, substrate and temperature on the supports with higher porous size is negative and also decreased the biocatalyst thermal stability.

According to the results obtained in this work, the best supports for lipase immobilization are chitosan-cross linked with GA and chitosan-alginate-TNBS as all immobilization parameters were found to appreciably increase.

## 4. Acknowledgments

## 5. References

**[1]**    H.F. Castro, A.A. Mendes, J.C. Santos, C.L. Aguiar, Quím. Nova, 27(2004) 146-156.

**[2]**    N.N. Ghandi, J. Am. Oil Chem. Soc, 74(1997) 621-634.

**[3]**    V.M. Balcão, A.L. Paiva, F.X. Malcata, Enzyme Microb. Technol, 18(1996) 392-414.

**[4]**    F.X. Malcata, H.R. Reyes, H.S. Garcia, C. G. Hill Jr, C.H. Amundson, J. Am. Oil Chem. Soc, 67(1990) 890-910.

**[5]**    R. Fernández-Lafuente, D.A. Cowan, A.N.P. Wood, Enzyme Microb. Technol, 17(1995) 366–372.

**[6]**    P.W. Tardioli, J. Pedroche, R.L.C. Giordano, R. Fernández-Lafuente, J.M. Guisán, Biotechnol. Progress, 19 (2003a) 352–360.

**[7]**    P.W. Tardioli, R. Fernández-Lafuente, J.M. Guisán, R.L.C. Giordano, Biotechnol. Progress, 19 (2003b) 565-574.

**[8]**    F. López-Gallego, T. Montes, M. Fuentes, N. Alonso, V. Grazu, L. Betancor, J.M. Guisán, R. Fernández-Lafuente, J. Biotechnol, 116(2005) 1-10.

**[9]**    J. Berger, M. Reist, J.M. Mayer, O. Felt, N.A. Peppas, R. Gurny, Eur. J. Pharm. Biopharm, 57(2004) 19-34.

**[10]**    J.M. Guisán, Enzyme Microb. Technol, 10(1988) 375-382.

**[11]**    M.M. Beppu, E.J. Arruda, R.S. Vieira, N.N. Santos, J. Memb. Sci, 240(2004) 227-235.

**[12]**    R. Fernandez-Lafuente, C.M. Rosell, V. Rodriguez, C. Santana, G. Soler, A. Bastida, J.M. Guisán, Enzyme Microb. Technol, 15(1993) 546–550.

**[13]**    A. Sadana, J.P. Henley, Biotechnol. Bioeng, 30(1987) 717-723.

**[14]**    C.M.F. Soares, H.F. Castro, G.M. Zanin, F.F. Moraes, Appl. Biochem. Biotechnol, 77/79(1999) 745-757.

**[15]**    M.M. Bradford, Anal. Biochem, 72(1976) 248-254.

**[16]**    C. Tapia, Z. Escobar, E. Costa, J. Sapag-Hagar, F. Valenzuela, C. Basualto, M. N. Gai, M. Yazdani-Pedram, Eur. J. Pharm. Biopharm, 57(2004) 65-75.

**[17]**    N. Li, R. Bai, Ind. Eng. Chem. Res, 44(2005) 6692-6700.

**[18]**    I.M.N. Vold, B.E. Christensen, Carbohydr. Res, 340(2005) 679-684.