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Immobilization of catalase from *Aspergillus niger* on inorganic and biopolymeric supports for H_2O_2 decomposition

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Abstract

This paper studies the H_2O_2 decomposition using supported catalase. The supports are γ-Al_2O_3, $FeCl_2$-treated γ-Al_2O_3, glutaraldehyde-treated chitosan and glutaraldehyde-treated cellulose. Catalase/γ-Al_2O_3 is inactive, but the treatment with $FeCl_2$ renders the oxide active. The extended Hückel type analysis (EHMO) indicates that the alumina–catalase interaction is strong, suggesting that a denaturation of the enzyme is accomplished when supporting on this oxide. Amongst all the samples, catalase/glutaraldehyde–cellulose is the most active, especially after 6 months of storage. After aging, catalase/glutaraldehyde–chitosan becomes inactive, probably due to the fact that NH_2 groups on chitosan react with the enzyme active sites.

Keywords: Oxidoreductases; Supported; Biocatalysts; Peroxide decomposition

1. Introduction

Several reports deal with the use of H_2O_2 to eliminate chemical bleaching before dyeing in cotton textile process [1–4]. Besides, H_2O_2 is used in large-scale municipal water treatments. Moreover, hydrogen peroxide has been applied to cold pasteurization of milk to prevent microbial pollution in the food chemistry [5,6]. For all these cases, it is desirable to eliminate residual H_2O_2.

Catalase (EC 1.11.1.6) is an abundant natural enzyme, which decomposes hydrogen peroxide to water and oxygen. The reaction rate is extremely fast: 1 mol of the enzyme is able to decompose 500 million-mol of H_2O_2 in 1 min. In this context, the application of H_2O_2/homogeneous catalase system in the textile industry has been studied [7], and it is worth to note that it reduces 48% of the energy consumption, 83% of the chemical costs, 50% of the water consumption and 33% of the processing time. Furthermore, the environmental impact is lowered since the traditional neutralization of the bleach with hydrosulphite or sodium thiosulphate is not performed [8].

Immobilization of catalase is an interesting way to broaden the application of this enzyme. Let's take one example: effective conventional peroxide bleaching is achieved at a high temperature and alkalinity. The use of traditional commercial catalase (non-supported) involves adjustment of pH and temperature to prevent catalase degradation; so, it is desirable to obtain an active supported system, stable at elevated temperature and resistant to pH variations. The immobilization of catalase on different supports has been previously studied [9,10]. The method of covalent binding for enzyme immobilization appears to be the most appropriate, when drastic environments and high stability are required. Besides, inorganic solids, for example alumina, could also be suitable supports due to its high mechanical resistance at high temperatures and pH. However, it has been reported that the activity of catalase strongly decreases when it is directly adsorbed on the alumina surface [11].

Taking into consideration the above comments in the present paper, we have focused our attention on the immobilization of catalase on three supports: alumina, chitosan and cellulose. For the three cases we have performed a pretreatment over the supports: with $FeCl_2$ in the case of the former support, with glutaraldehyde for the cases of the biopolymeric supports. It is known that catalase supported on bare alumina presents an extremely low efficiency. The treatment

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of alumina with $FeCl_2$, developed by Emery et al. [12], generates active enzymatic sites when an enzyme is supported on $FeCl_2$-treated support. The glutaraldehyde treatment of the biopolymeric supports improves the chemical and mechanical resistance. It has been reported that the amount of immobilized enzyme using glutaraldehyde-treated supports is higher than on other carriers [13].

We have tried to analyze the support effect on the catalytic performance of the catalase at room temperature and for low hydrogen peroxide concentrations, as those present in real industrial conditions. In this sense, the enzyme is not the main point to analyze, but the support–enzyme interaction and its catalytic effects on the reaction of H_2O_2 decomposition at 25 °C. Thermal inactivation of the supported systems and influence of pH media on the decomposition of H_2O_2 will be the subject of a forthcoming work.

2. Experimental

2.1. Materials and methods

Catalase from *Aspergillus niger* was provided by Novo Nordisk (Argentina) as a free sample of catazyme 25L (25,000 U/ml). U is catalase unit in μmol decomposed H_2O_2 per min at pH 7 and 25 °C. H_2O_2 (30 vol.%, 9.1%, w/v) was provided by Parafarm. $KMnO_4$ (Ernesto van Rossum y Cía S.R.L.) Pure F.A. was used. Buffer pH 7 (phosphate-based) was provided by Merck.

The three base supports were:

(a) γ-Al_2O_3 from Rhône Poulenc (120 m^2/g)
(b) Chitosan from shrimp shells, lot number TM 369. It was supplied by Primex Ingredients ASA, Norwegian, with 98.1% of dry material, deacetylation degree of 85.2%, 60 mesh powder, molecular weight of 70–80,000 Da. It contains not more than 15 ppm of heavy metals and 50 ppm of Fe. Fig. 1 shows the structure of Chitosan.
(c) Cellulose: The supplier of this biopolymer was "Dr. Theodor Schuchardt", München, Germany. Fig. 2 shows the structure of cellulose.

The reaction temperatures, the concentration of the catazyme solutions and the reaction times were different for each of the four cases. They were selected based on literature data [11,15,14]. For comparison, unsupported catalase was tested for the H_2O_2 decomposition. A UV-Vis spectrophotometer was used, with data acquisition for kinetic analysis of H_2O_2 decomposition.

2.2. Immobilization procedures

2.2.1. Alumina

One gram of support was preconditioned 1 h at 30 °C, pH 7 and 150 rpm. 0.2 ml of catazyme 25L were added to 20 ml of pH 7 buffer during 18 h. This procedure was repeated. The solid was dried at 40 °C. Three hundred milligrams of preconditioned Al_2O_3 were added to 1.5 ml of catazyme 25L and 1 ml of pH 7 buffer with magnetic stirring. After 18 h, the solution was filtered. Four washing steps with 15 ml of pH 7 buffer were carried out until no evidence of catalase in washings (checked by UV) was found. The solid was pale brown.

2.2.2. $FeCl_2$–alumina

After the preconditioning step, 55 mg of $FeCl_2$ were added (Riedel de Häen) to 20 ml of pH 7 buffer and 1 g alumina. The color of the solid changed from light brown to blue. This fact implies the reaction of $FeCl_2$ with the surface and oxidation of Fe^{2+}. After 18 h, 0.2 ml of catazyme was added. Finally, after 18 h the solid was repeatedly washed with 15 ml of pH 7 buffer. The color of this slurry is green-brownish and the resulting solid is brown.

2.2.3. Chitosan

Several films of chitosan were obtained by dissolving 1 g of the biopolymer in 100 ml of an acetic acid solution (0.8%, w/v). One milliliter of glutaraldehyde 25% (w/v) in 15 ml pH 7 buffer (1.56%, w/v) were added to a film of chitosan (272.5 mg). Reaction with glutaraldehyde generates destruction of film and formation of particles. After 1 h at 30 °C, the solution was filtered and the chitosan was washed three times with 15 ml of distilled water. Five milliliter of a

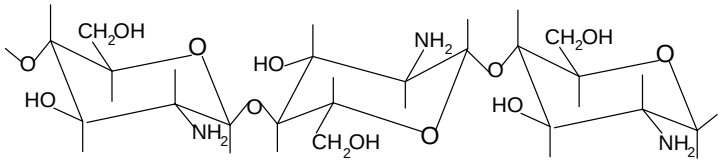

Fig. 1. Chitosan structure.

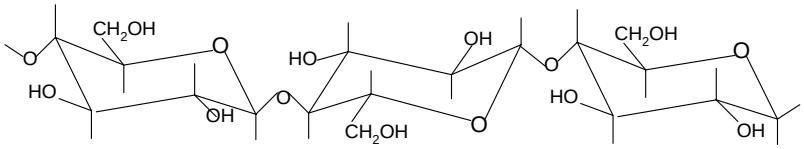

Fig. 2. Cellulose structure.

1/10 solution of catazyme 25L were added to 272.5 mg of glutaraldehyde–chitosan and 3 ml of pH 7 buffer. After 62 h at 5 °C, the reaction was stopped by increasing the temperature to 30 °C for 1 h.

2.2.4. Cellulose

Following a reported procedure [14], 2 ml of glutaraldehyde 25% (2.27%, w/v) were added to 1 g cellulose in 24 ml of pH 7 buffer. After 2 h at 30 °C the solution was filtered and the solid was washed three times with 15 ml of distillated water. Two milliliter of catazyme 25L were added to 1 g of cellulose, 3 ml of distillated water and 3 ml of pH 7 buffer. After 62 h at 5 °C, the reaction was stopped by increasing the temperature to 30 °C for 1 h.

The biopolymeric systems were treated with a 0.4N solution of NaCl to eliminate weakly adsorbed enzyme. All supported systems were stored at 2 °C.

2.3. Characterization of the supports and of the catalase/support

2.3.1. Fourier transformed infrared

FTIR spectra were obtained with a Nicolet Nexus 470 instrument. KBr windows were used to obtain spectra of powder of catalase/γ-Al_2O_3, catalase/$FeCl_2$–γ-Al_2O_3, glutaraldehyde–chitosan, catalase/glutaradehyde–chitosan and catalase/glutaraldehyde–cellulose.

2.3.2. Thermal gravimetric analysis

The thermal stability of cellulose and chitosan was evaluated by means of a thermogravimetric study conducted in a Perkin-Elmer thermal analyzer in the range 50–400 °C at a heating rate of 10/min under N_2 atmosphere.

SEM–EDX analysis performed on all the supports did not reveal differences between the biopolymers. Surfaces of *catalase/glutaraldehyde/biopolymers* and *catalase/$FeCl_2$/Al_2O_3* are different, as expected (results not shown).

2.4. Semiempirical study of aminoacid adsorption on γ-alumina

The interaction of the most important aminoacids with γ-Al_2O_3 was evaluated by means of a study of the extended Hückel type (EHMO). We have calculated the energy associated with the reaction between the lateral groups of enzymes and the most abundant planes of alumina. The considered planes were (1 0 0), (1 1 0)C and (1 1 0)D, while the aminoacids were Cysteine, Lysine, Threonine, Serine, Arginine, Tyrosine, Histidine, Glutamic acid, Tryptophan and Asparagine. Basically, we have carried out EHMO calculations to determine the formation and stabilization energies of an ideal system formed by a selected aminoacid placed at distances lower than 4 A to the alumina surface. This energy was obtained as the energy of adsorbed aminoacid on alumina minus the sum of the energies of the free aminoacid and alumina. The reported energies were found by moving the lateral chain of aminoacid 0.1 A perpendicular to the surface. All the energetic minima were found approaching the lateral chain of the aminoacid to the H of one OH group of the alumina surface.

2.5. Catalytic activity measurements: the decomposition of H_2O_2

2.5.1. Unsupported catalase

The kinetic data for peroxide decomposition were obtained using the calzyme method reported on the internet by Calzyme laboratories (http://www.calzyme.com/catalog/catalase.html). A wide range of peroxide concentrations from 8.76×10^{-4} to 0.0876 M was selected. The catalase concentration was 14.4 U/ml (or 2 μl of catazyme solution 25,000 U/ml), equivalent to 1×10^{-3} mg/ml, the temperature was set to 25 °C and the solution buffered at pH 7. Reaction was initiated when hydrogen peroxide solution was added to the cuvette containing the catalase and the buffer. The decrease of absorbance at $\lambda = 240$ nm was related to the decrease of hydrogen peroxide concentration. Additional tests were performed with a higher amount of catalase, similar to that corresponding to the catalase/support catalysts. However, the reaction was extremely fast and a large amount of O_2 bubbles were produced which limits an accurate measurement of H_2O_2 concentration.

2.5.2. Supported catalase

Reaction temperature was 25 °C. The amount of catalyst was in the 50–120 mg range. In a typical experiment, the supported enzyme was placed in 10 ml of the pH 7 buffer (20 ml for the case of cellulose), with magnetic stirring (150 rpm). After preconditioning for 15 min, 0.1–0.2 ml of 9% H_2O_2 was added. Once the reaction was started, at different selected reaction times, 1 ml of the reaction media was placed in 2 ml of a H_2SO_4 solution (3:50, v/v). No UV determination of hydrogen peroxide concentration was performed due to the presence of large amount of fine solids in the slurry. The concentration of the reactant was measured by means of a titration with $KMnO_4$. The activity is reported as μmol of H_2O_2 converted per gram of catalyst (or per gram of enzyme). The integral activity is considered as the total amount of peroxide reacted after 40 min of reaction.

2.5.3. Measurement of the amount of supported catalase

A calibration curve was obtained using $\lambda = 273$ nm for different catalase concentrations (correlation coefficient = 0.9988). This technique is widely used for the measurements of enzyme concentration, not only for catalase. Even lipase concentration in aqueous solution can be addressed with similar methods. The molar absortivity coefficient (ε) was 3.0×10^7 l/mol cm (considering a molecular weight of 250,000 g/mol for catalase). The amount of catalase retained by the support was calculated as the difference between the concentration of the initial solution and the concentration of the remaining solution.

Glutaraldehyde-treated cellulose, glutaraldehyde-treated chitosan (as film), alumina, and $FeCl_2$-treated alumina were tested for H_2O_2 decomposition. The test was done using 50–57 mg of catalysts in 10 ml pH 7 buffer with a H_2O_2 concentration = 0.0252 M. The hydrogen peroxide concentration was determined by means of a titration with $KMnO_4$. The variation of H_2O_2 with time was measured for all the cases. The initial and final hydrogen peroxide concentrations (after 40 min of reaction) were measured to compare different catalysts.

3. Results and discussion

3.1. Characterization of catalase/support

For all the samples, the amount of catalase immobilized on each support is reported in Table 1.

3.1.1. Catalase/γ-Al_2O_3 and catalase/$FeCl_2$–γ-Al_2O_3

It is well known that the electrostatic charge of alumina surface depends on the pH of the solution. Taking into account that the isoelectric point (the pH value corresponding to null charge) of alumina is 8 [16] and that corresponding to catalase is 5.4 [17], at the working conditions (pH 7), the enzyme is anionic and alumina is polarized positively. Thus, electrostatic forces between catalase and alumina are created. Taking into account the large surface area of the support, the amount of immobilized enzyme is rather low: 3.4 mg of enzyme per gram of alumina.

After the treatment with $FeCl_2$, new anchoring sites are formed on alumina, probably associated with Fe–Cl species. The concentration of these sites is relatively high, since more catalase is supported upon the modification with $FeCl_2$: 17.3 mg versus 3.4 mg in the case of non-treated alumina. As we will see in Section 3.2, catalase supported on the $FeCl_2$ modified alumina is active, while catalase/γ-Al_2O_3 is inactive, as already reported [12,18]. This fact would indicate that a denaturation of the enzyme arises on bare alumina.

Let us analyze the FTIR spectra corresponding to both samples: catalase/γ-Al_2O_3 and catalase/$FeCl_2$–γ-Al_2O_3, which are presented in Fig. 3. First of all, it is important to consider that we have not tried to perform a quantitative analysis of the data. When a comparison is carried out, no appreciable differences are seen between the two cases. This result does not explain the denaturation of catalase on alumina, since no evidence of new bands is observed. For both the cases, peaks at 2800–2900 cm^{-1} are assigned to CH_2–CH_3 and beyond 3000 cm^{-1} to retained water and N–H bonds. The supported protein spectrum presents bands at 1657 (amide I), 1541 (amide II), 1455 (δ-CH_2, δ-CH_3), 1390 and 1400 (ν-COO–), 1306 (γ-CH_2, γ-CH_2), 1245–1170 (amide III) and 1098 cm^{-1} (γ-NH_2, γ-NH_3, ν-C–N).

On the other hand, the EHMO theoretical study, which analyzes the enzyme–support interaction in an indirect way, has indicated a strong catalase–alumina interaction, suggesting that a degradation of the enzyme takes place upon supporting. In Table 2, the calculated relative stabilization energies for different adsorbed aminoacids on alumina are reported. We have considered three exposed planes of alumina, which are the most abundant: (1 1 0)C, (1 1 0)D and (1 0 0). We have calculated the energies associated with the adsorption of aminoacids on these surfaces. However, calculations indicated that for all the cases, the (1 1 0)D plane was the least reactive towards the aminoacids. Thus, we assumed that the enzyme is mainly adsorbed on both (1 1 0)C and (1 0 0) planes. The calculated stabilization energies corresponding to the aminoacids were remarkably high (in the −25 to −80 eV range), as it can be observed in Table 2, indicating that the organic moieties are strongly attracted to the alumina surface. This result suggests a possible change in the protein structure when it is supported on the inorganic solid, and concomitantly a drastic decrease in the activity of the adsorbed organic moiety. From the EHMO results it

Table 1
Catalase loading of the samples and activity of supported catalase for the decomposition of H_2O_2 after 40 min of reaction (A) at 25 °C

Support	mg enzyme/gram catalyst	At $[H_2O_2]_0$ = 0.0132 M or 2640 μmol H_2O_2/g catalyst μmol H_2O_2 converted[a]		At $[H_2O_2]_0$ = 0.02648 M or 5280 μmol H_2O_2/g catalyst μmol H_2O_2 converted	
		A (gram catalyst)	A (gram enzyme) × 10^{-3}	A (gram catalyst)	A (gram enzyme) × 10^{-3}
γ-Al_2O_3	3.4	–	–	63	18.5
$FeCl_2$–γ-Al_2O_3[b]	17.3	713	41	1372	79
Glutaraldehyde–cellulose	2.0	890	445	1044	522
Glutaraldehyde–cellulose[c]	2.0	2558	1279	4452	2226
Glutaraldehyde–chitosan	1.8	937	520	1375	764

Catalyst mass = 103–110 mg. Activity at 40 min: (μmol H_2O_2 present initially − μmol H_2O_2 found at 40 min reaction) per catalyst mass (g) or enzyme mass (g) in 1 ml basis. Activity per gram catalyst: A (g catalyst) = [(μmol H_2O_2/ml)$_{time=0}$ − (μmol H_2O_2/ml)$_{time=40\,min}$]/g catalyst per ml units = μmol H_2O_2/g catalyst. Activity per gram enzyme: A (g enzyme) = [(μmol H_2O_2/ml)$_{time=0}$ − (μmol H_2O_2/ml)$_{time=40\,min}$]/g enzyme per ml units = μmol H_2O_2/g enzyme.

[a] μmol: micromol.

[b] Pure $FeCl_2$ presents A (g) of 5.2 μmol/mg $FeCl_2$.

[c] After 6 months storage at 5 °C.

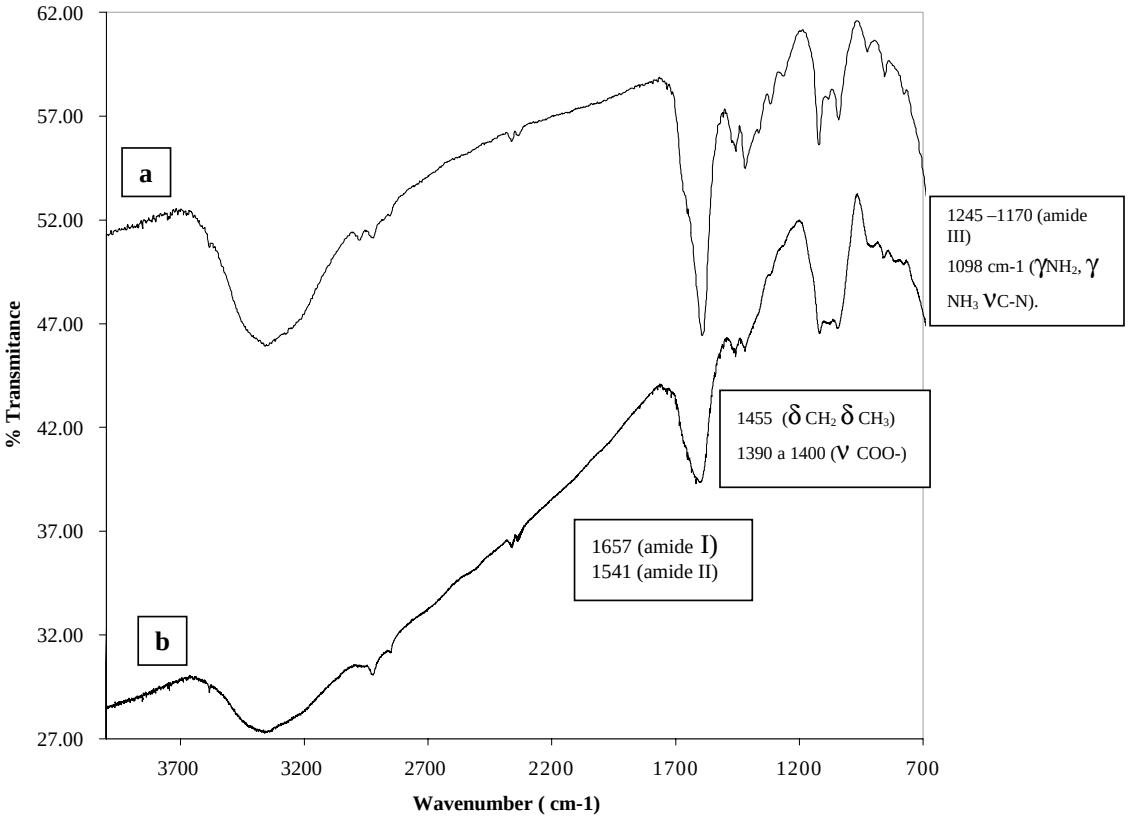

Fig. 3. (a) FTIR of catalase/γ-Al_2O_3 and (b) FTIR of catalase/$FeCl_2$–γ-Al_2O_3.

Table 2
Selected EHMO results-stabilization energy corresponding to the adsorption of lateral groups of the aminoacid onto the most favored planes ((1 0 0) and (1 1 0)C) of γ-alumina surface

Aminoacid	Stabilization energy range (eV)
Glutamic acid	(1 0 0) −31.45 (1 1 0)C −34.35
Cystein	(1 0 0) −87.72 (1 1 0)C −87.74
Arginin	(1 0 0) −59.6 (1 1 0)C −58.48
Histidine	(1 0 0) −34.75 (1 1 0)C −36.78
Aspargine	(1 0 0) −22.34 (1 1 0)C −25.10
Lysine	(1 0 0) −67.41 (1 1 0)C −71.23
Serine	(1 0 0) −68.92 (1 1 0)C −64.45
Triptophan	(1 0 0) −23.41 (1 1 0)C −22.18
Tyrosine	(1 0 0) −45.67 (1 1 0)C −43.28
Threonine	(1 0 0) −67.90 (1 1 0)C −68.54

was possible to conclude the following order in strength of lateral groups interaction with alumina:

Cysteine > Lysine > Threonine > Serine > Arginine > Tyrosine > Histidine > GlutamicAcid > Tryptophan > Asparagine

Histidine, tyrosine and arginine have shown strong adsorption of lateral groups (through imidazol, OH and terminal NH_2).

Since alumina is positively polarized, the protein must displace the counterions that neutralize the positive charge. Electrostatic effects could produce undesirable reactions after the protein adsorption. We shall revert to this matter in the discussion of catalytic results.

3.1.2. Catalase/glutaraldehyde–chitosan and catalase/glutaraldehyde–cellulose

We have not tested the organic supports without pretreatment since it has been reported that glutaraldehyde treatment improves the chemical and mechanical resistance of chitosan. Furthermore, the amount of immobilized enzyme is higher than on other supports [13].

It is important to establish that cellulose and chitosan are stable at the working temperatures. This is clearly concluded when the TGA results of biopolymers are considered. With increasing temperature, the weight of the chitosan powder

rapidly decreases until ca. 110 °C, slowly in the range 110–160 °C, and then very gradually. The thermogram showed a strong endothermic peak until 110 °C, practically no change in the 110–160 °C range, and another endothermic peak at higher temperature; the latter could be due to a partial decomposition of chitosan. These results indicate that all water molecules included in the chitosan powder are removed at 110 °C, as Ogawa et al. [19] have established.

In the case of cellulose, a sharp peak appears in the endotherm at 130 °C, probably due to adsorbed water. Both results indicate that the biopolymeric supports are stable under 100 °C.

The organic solids have retained approximately 2 mg of enzyme per g of support. This amount is relatively small, probably due to the low surface area of biopolymeric supports in comparison with alumina, approximately 5 and 100 m^2/g, respectively.

The FTIR spectra corresponding to catalase and catalase/glutaraldehyde–chitosan are shown in Figs. 4 and 5, respectively. First, we shall consider the FTIR corresponding to glutaraldehyde–chitosan. This spectrum shows bands at 3426–3180, 2921–2883, 1654, 1596, 1398 and 1078 cm^{-1}. The peak at 1654 cm^{-1} has been assigned to imine group [20,21]. This result throws some light on the reaction between glutaraldehyde and chitosan (i.e. a crosslinking of chitosan by glutaraldehyde). Different mechanisms have been proposed in the literature for this reaction. According to the FTIR results of the present work, the presence of the peak assigned to imine groups would indicate that the crosslinking is produced by a Schiff base formation [21,22], leading to an imine type crosslink. On the other hand, no carbonyl groups due to crosslinking have been found (at 1720–1730 cm^{-1}). This fact would indicate that the crosslinking is not accomplished by a reaction between Michael-type adducts and amine groups, as proposed by Muzzarelli [23].

It is interesting to note that the chemical crosslinking decreases the sensitivity of chitosan towards water. Unfortunately, the formation of chemical junctions induces a brittleness of the membrane. Certainly, we have noticed this problem during the preparation of glutaraldehyde–chitosan films.

Considering the FTIR spectra corresponding to catalase/glutaraldehyde–chitosan (presented in Fig. 5), the results have shown evidence of catalase presence on the supports. The spectrum in Fig. 5 shows the same features as in Fig. 3, although the quality is poorer probably due to the low surface area of the organic solid. The most important peak appears at 1587 cm^{-1}, as in case of catalase immobilized on the inorganic supports. The other minor bands are also coincident with those in Fig. 3. In case of

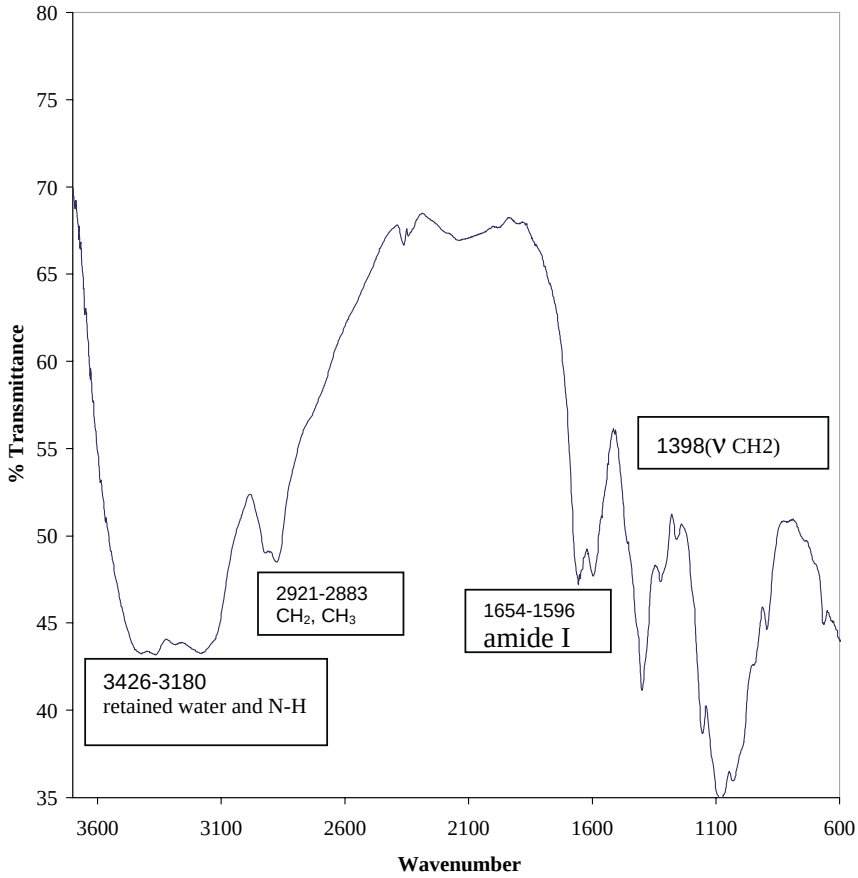

Fig. 4. FTIR of chitosan.

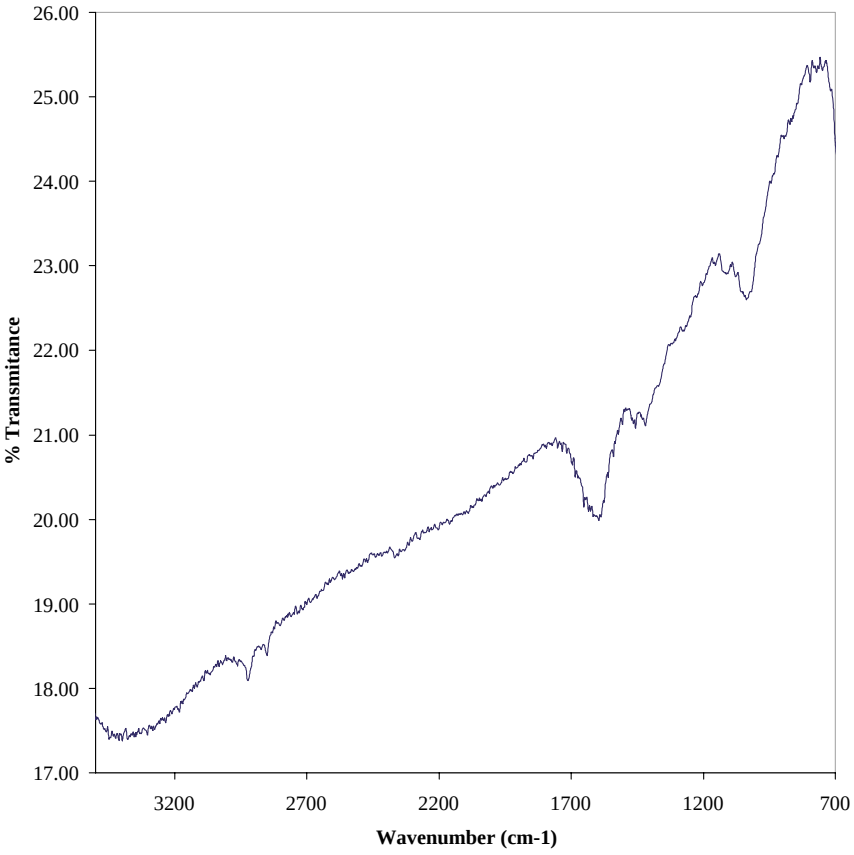

Fig. 5. FTIR of catalase/glutaraldehyde–chitosan.

catalase/glutaraldehyde–cellulose the shape of the spectrum (not shown) is similar, but not the relative intensities.

One could ask how the treated biopolymeric supports fix catalase. Surely, covalent bonds between the enzyme and glutaraldehyde are formed. We have observed that more enzyme was retained when sequential steps of catalase adsorption on glutaradehyde-treated supports were performed than when a catalase + glutaraldehyde co-impregnation method was carried out. The same is true for the system glucose oxidase–catalase [24]. These facts are probably related to the hydrophilic character of untreated chitosan and cellulose.

3.2. Activity of supported catalase for H_2O_2 decomposition

In Table 1, the activities of the supported catalase samples are shown, for two different hydrogen peroxide initial concentrations. The activity has been calculated as μmol of H_2O_2 decomposed per gram of catalyst or per gram of enzyme.

The activity order in peroxide decomposition (for the lower initial H_2O_2 concentration) was found to be: aged glutaraldehyde–cellulose $\gg$ glutaraldehyde–chitosan $>$ glutaraldehyde–cellulose $>$ $FeCl_2$–γ-Al_2O_3 $\gg$ γ-Al_2O_3. For the higher hydrogen peroxide concentration, the order is slightly different: catalase supported on the $FeCl_2$-treated inorganic support is more active than the enzyme supported on glutaraldehyde-treated cellulose on a per gram basis.

Next, we compare the kinetics for representative data obtained at a $[H_2O_2] = 0.026$ M. Fig. 6 shows that catalase supported on cellulose or chitosan presents a complex kinetic pattern, whereas when it is supported on the inorganic support, it shows an S shape of the kinetic course. Besides, it seems that the reaction approaches steady state conditions after 40 min for catalase/cellulose and catalase/alumina, whereas for the chitosan-supported enzyme this is not the case. Two steps are clearly observed in this case.

The most interesting result is that the activity of glutaraldehyde-treated cellulose notably increases when the supported system is stored for 6 months at low temperature. In Fig. 7, the decomposition of hydrogen peroxide along with time for fresh glutaraldehyde–cellulose supported catalase is compared with the reaction performed over the stored solid. Better performance of the latter is clearly observed. This is not the case for the chitosan system, for which the activity was totally lost after 6 months. The last result is in agreement with the work of Cetinus and Aztop [25], who indicated that the activity of catalase bovine on chitosan decreases 20% after 30 days of storage. We suppose that catalase, irreversibly reacted on the glutaraldehyde–chitosan surface inactive, slowly react with amino groups of chitosan and deactivates. It has been reported that in the interaction

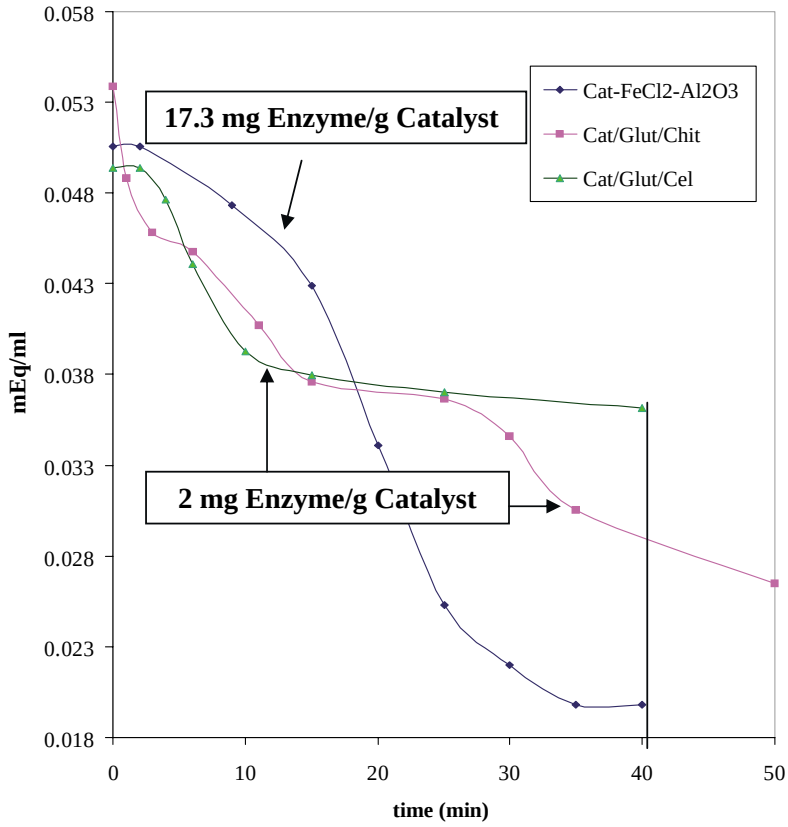

Fig. 6. Decomposition of H_2O_2 for catalase supported on chitosan, cellulose and $FeCl_2$-treated alumina, initial H_2O_2 concentration = 0.026 M, T = 25 °C, catalyst mass = 100–110 mg.

between the aldehyde and chitosan, only 33% of the chitosan NH_2 groups are reactive [26]. Thus, the surface of the treated chitosan presents a large concentration of amine groups. On the other hand, cellulose does not present NH_2 groups and consequently the catalase structure is stable on it.

Table 3 shows the Michaelis–Menten parameters of unsupported and supported catalase. The parameters were obtained by application of the program Leonora [27] to the kinetic patterns. Parameters are the maximum initial rate at the selected conditions of enzyme concentration and pH (V_m) and the H_2O_2 concentration at which the rate is $V_{max}/2$. These parameters are $V_m = 35,120 \pm 6762$ μmol/min mg enzyme and $K_m = 0.02664 \pm 0.00674$ M for non-supported catalase using 0.00116 mg enzyme per ml solution. For the case of 6 month stored glutaraldehyde-treated, cellulose supported catalase and a concentration of 0.02 mg enzyme/ml, K_m is 0.115 ± 0.0116 M, whereas V_{max} achieves 3135 ± 203.8 μmol/min mg enzyme. V_{max} decreased 10 times, whereas K_m increased four times comparing the non-supported catalase and the best (aged) supported catalase. Table 4 shows the kinetic results (initial rates) using catalase/glutaraldehyde/cellulose after 6 months storage. The kinetic data of supported catalase on biopolymeric supports show variations due to inherent problems at the initial stage of contact: instability and deactivation. Surprisingly, it is clear that after 6 months, catalase on cellulose has been stabilized in an effective way. The data of the non-supported catalase are included to point out the inevitable deactivation of the enzyme due to the immobilization. It is worth to note that the initial rates reported for homogeneous catalyst (at least 50 times higher than aged cellulose-supported catalase) were obtained with a mass of catalase of nearly 0.05 mg, whereas the supported

Table 3
Catalase–Michaelis–Menten kinetic parameters for non-supported catalase at pH 7 (a) and the best supported catalase (b) using Leonora program or linear adjustement of data following different procedures

Parameter	From Leonora program (b)	From Leonora program (a)	Lineweaver-Burk (a)	Eadie-Hofstee (a)	Hanes (a)
K_m (M)	0.115 ± 0.0116 M	0.02664 ± 0.00674	0.0306	0.0237	0.0259
V_{max} (μmol/min mg)	3135 ± 203.8	35120 ± 6762	39292	32849	34543

Catalase/glutaraldehyde/cellulose after 6 months storage at 5 °C, pH 7.

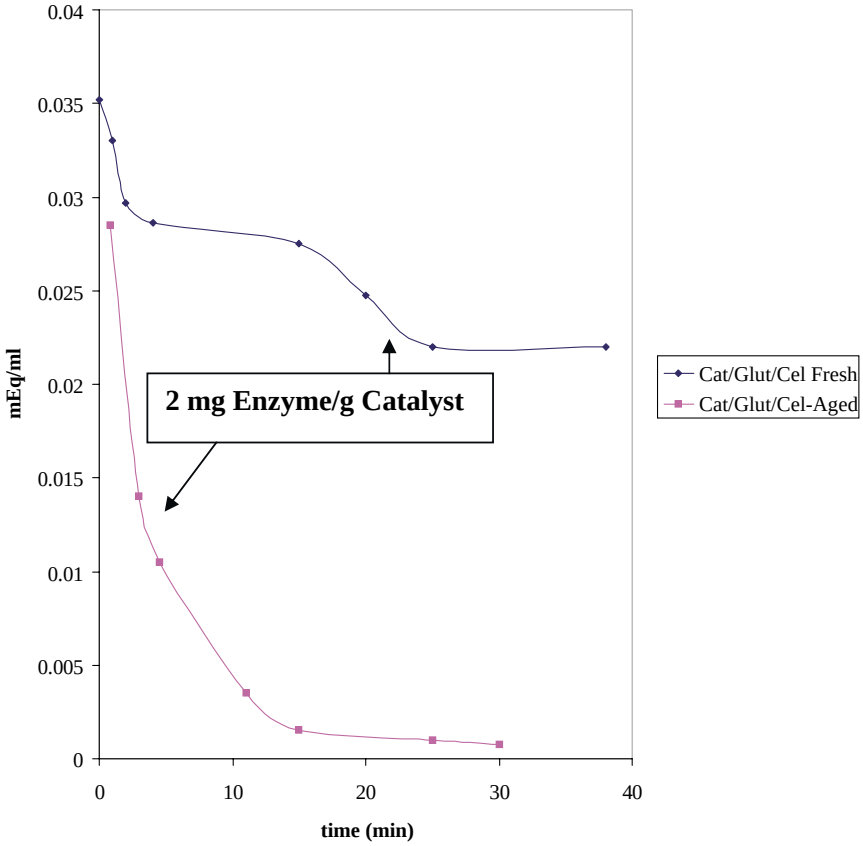

Fig. 7. Comparative kinetic pattern of fresh catalase/glutaraldehyde–cellulose and stored catalase/glutaraldehyde–cellulose, initial H_2O_2 concentration = 0.026 M, $T = 25\,^{\circ}C$, catalyst mass = 50 mg.

enzyme mass is nearly 0.1 mg for the data presented in Table 4.

In an additional experiment, with about 5 mg/ml of catalase/glutaraldehyde–cellulose after storage and an initial concentration of hydrogen peroxide of 0.0133 M, it was found that all H_2O_2 was decomposed after 40 min. Subsequently 0.1 ml of H_2O_2 were added and, although the initial activity was lower, in 40 min 97% of H_2O_2 was decomposed, indicating that no deactivation of the stored catalyst had occurred. This experiment was reproducible. From this result, the possibility of re-usage of the supported catalase in different batches, without catalyst deactivation is envisaged.

Table 4
Initial rate (H_2O_2 μmol/min mg enzyme) at pH 7 for catalase/glutaraldehyde–cellulose after 6 months of storage at 5 °C

Initial $[H_2O_2]$ (mol/l)	V_0 (μmol H_2O_2 converted/min mg enzyme)
0.0133	324
0.0237	394
0.0523	990
0.0701	1126
0.1029	1557
0.2433	2099

Values of V_0 for non-supported catalase are 14,671 μmol H_2O_2 converted/min mg enzyme at 0.0159 M H_2O_2 and 18,939 μmol H_2O_2 converted/min mg enzima at 0.0315 M H_2O_2 using a total amount of 0.0436 mg enzyme. Temperature: 25 °C; mass of catalyst: 45–55 mg; mass of enzyme: 0.09–0.11 mg; 10 ml buffer pH 7.

The nature of the changes due to aging are difficult to elucidate. The preparation procedure and the storage conditions were the same for both supported catalase samples. The microbial contamination could be disregarded since chitosan and cellulose are both commercial grade and both were stored at the same conditions (0–5 °C) and isolated from contact in closed vials.

One more possibility must be taken into account in order to explain the activation of the catalase/glutaraldehyde–cellulose with time of storage: a multipoint covalent attachment of catalase with the support surface could be developed with time. It is possible that after some months of aging the intricate structure of the enzyme is disentangled and these conformational changes account for the improved activity with aging. At the same time, this could explain the lack of activity in chitosan-based supported systems, since the conformational changes would allow NH_2 groups of the support to react with the enzyme in a detrimental way.

While cellulose-supported catalase is more active than catalase supported on chitosan, we ought to consider a practical point of view: cellulose is a fine powder, thus a filtration

step is required after reaction. In the case of chitosan as support, the catalyst separation is faster.

We now consider the two inorganic supports: bare Al_2O_3 and $FeCl_2$-treated alumina. One can observe that activity is null for the former, while the latter system is remarkably active. The highest activity is not only due to an increase in the amount of the fixed catalase, as it can be deduced when specific activity per gram of enzyme is compared (see Table 1, compare rows 1 and 2). Probably a degradation reaction between alumina and the catalase occurs, as suggested by EHMO calculations. The denaturation of the enzyme probably takes place at the adsorption step due to a reaction of lateral nucleophilic groups of catalase (terminal OH, imidazol, NH_2) with acidic sites of the alumina. Another possibility is that the affinity of alumina with iron produces a decomposition of heme groups. Anyway, the inactivity of catalase upon supporting on alumina suggests that the tetrameric structure of the enzyme is destroyed. It is well-known that this structure is responsible for the catalytic activity [27]. Regarding the effect of the pretreatment with the iron salt, supported $FeCl_x$ species could act as a separator between the protein and the acidic sites of alumina. Besides, iron species would anchor the OH, NH_2, COOH and imidazol groups of the protein.

Performing a comparison between $FeCl_2$–γ-Al_2O_3 and the biopolymeric supports, we can observe in Table 1 that the activity, expressed per mass of catalyst, is not very different for the three fresh catalysts. However, when the specific activity (per mass of enzyme) is taken into account, catalase on $FeCl_2$-treated alumina shows lower activity. Besides, an additional experiment carried out at 25 °C and at the same conditions with a low mass of catalase/support (40 mg instead of 100 mg) indicated the existence of diffusion limitations when catalase/$FeCl_2$–alumina is the catalyst. Activity was increased to 3633 A (μmol H_2O_2/g catalyst) or 210 A (μmol H_2O_2/g enzyme) using $[H_2O_2] = 0.026$ M. This fact is the subject of a forthcoming paper.

In summary, the nature of the support is extremely important to the development of the active structure of catalase. Bare alumina presents sites that are too reactive towards catalase. The tetrameric structure of the enzyme probably is destroyed when supported on alumina. The presence of $FeCl_x$ species on the surface of the inorganic solid passivates, in some way, the surface and creates new sites, which are more effective in supporting active forms of catalase. In the case of the organic supports, glutaraldehyde-treated cellulose and chitosan, the main difference is the presence of NH_2 groups on the latter polymer. The interaction NH_2–catalase is harmful for the activity of the enzyme. Since this interaction increases with time, only stored samples are inactive. On the contrary, on glutaraldehyde-treated cellulose, beneficial enzyme–support interaction exist, mainly after aging. Thus, catalase/glutaradehyde–cellulose is the best catalyst for hydrogen peroxide decomposition. Although it is beyond the scope of the present study, we suggest that catalase/glutaraldehyde–cellulose catalysts would present a wide range of applications in the enzymatic catalysis field, since several active sites of catalase are developed when supporting it on cellulose-treated with glutaraldehyde.

4. Conclusion

When fresh samples of catalase supported on bare γ-Al_2O_3, $FeCl_2$–γ-Al_2O_3, glutaraldehyde–chitosan and glutaradehyde–cellulose are tested for hydrogen peroxide decomposition, the most active system is catalase/glutaraldehyde–cellulose. Catalase supported on bare alumina is inactive, while the treatment with $FeCl_2$ notably improves the efficiency of the alumina as a support for catalase.

The effect of aging of supported catalase on biopolymers is extremely important. The activity of catalase/glutaraldehyde–cellulose is notably increased after some months of storage, probably due to the formation of multipoint covalent enzyme–support bonds developed with time. On the contrary, in case of catalase/glutaraldehyde–chitosan, a long-term interaction between catalase and NH_2 groups of chitosan destroys the fundamental active structure of the enzyme.

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