Available online at www.sciencedirect.com
SCIENCE DIRECT
Enzyme and Microbial Technology 38 (2006) 1005–1012
ENZYME AND MICROBIAL TECHNOLOGY
www.elsevier.com/locate/emt

Activity of magnetite-immobilized catalase in hydrogen peroxide decomposition

F. Horst, E.H. Rueda [a], M.L. Ferreira [a,b,*]

[a] *Planta Piloto de Ingeniería Química (PLAPIQUI-UNS-CONICET), Camino La Carrindanga km 7, 8000 Bahía Blanca, Argentina*
[b] *Dpto de Química, Universidad Nacional del Sur, Avda Alem 1253, 8000 Bahía Blanca, Argentina*

Received 30 April 2005; received in revised form 10 August 2005; accepted 31 August 2005

Abstract

The present work analyzes the activity in decomposition of H_2O_2 using magnetite-immobilized catalase. The support of catalase is a glutaraldehyde-treated magnetite (Fe_3O_4). The data obtained in the H_2O_2 decomposition are analyzed. The fitting of the initial rate of the H_2O_2 decomposition versus hydrogen peroxide concentration data is discussed using a specific program for enzyme kinetics modeling (Leonora). The free catalase from *Aspergillus niger* (3.5 or 10 U/mL) does not show substrate inactivation up to 0.4 M H_2O_2. The immobilized catalase at low catalyst concentration shows substrate inhibition. Using 1 mg/mL of supported catalase the predicted maximum activity is higher than in the case of the free catalase at similar catalase concentration, although the optimum temperature is lower (40 °C versus 60 °C).

Keywords: Magnetite; Catalase; Hydrogen peroxide; Enzymatic; Supported catalase

1. Introduction

Several methods are been used to degrade the bleaching agent hydrogen peroxide [1,2] and to reduce the huge water consumption in the textile industry (100 L/kg textiles) [3]. Bleaching with peroxide is done at temperatures above 60 °C and pH values above 9. Optimum activity of catalase is achieved at moderate temperatures (20–50 °C) and neutral pH, therefore, the commercial use of catalase requires adjustments of pH and temperature for the bleaching effluent treatment [4,5]. The addition of chemicals such as sodium bisulphite or hydrosulphite for the reduction of H_2O_2 would lead to unfavorable high salt concentration in the process. Catalase is one of the most effective biocatalyst but the cost of the enzyme can be reduced by the repeated use of the immobilized enzyme. Inorganic supports (alumina) [6] and biopolymers are been used (gelatine, chitosan, cellulose) [7–10]. Different coupling methods were tested and the most successful was glutaraldehyde [6,11]. Recently, some of us have published a report about the activity of catalase supported on chitosan, cellulose and $FeCl_2$-treated alumina [12].

Biocompatible ferromagnetic particles have been effectively used as potential drug carriers since 1970. Magnetic supports can be prepared by copolymerisation of magnetic particles within polymers, onto which the biomolecules can be immobilized. Magnetic particles can also be coated with layers of common support materials – agarose or dextran – and the biomolecules covalently linked to their surfaces [13]. Small magnetic particles exist as stable colloidal suspensions or ferrofluids that will not aggregate, thus allowing a uniform distribution in a reaction mixture. Recent studies demonstrated that it is possible to immobilize 97% bovine serum albumin (BSA) by covalent binding to magnetic particles by coupling it to CDI, without loss of its properties. Several clinically important enzymes, like alkaline and acidic phosphatase and lactate dehydrogenase were also immobilized, and 50–80% activity was retained by the immobilised preparation. The linking of BSA, glucose oxidase (GOD) chymotripsin, streptokinase and dispase, directly to magnetic particles using 1-[3-(dimethylamino) propyl]-3-ethylcarboiimide hydrochloride or CDI as the coupling agent was proposed. The binding is due to the presence of hydroxyl groups on the surface of fine magnetic particles from Fe_3O_4. The optimal conditions depend on the kind of immobilized proteins and enzymes, the pH of the reaction mixture and the ratio of each reagent in the reaction mixture. The direct binding of enzymes and proteins to magnetic particles has

* Corresponding author. Tel.: +54 291 4861700; fax: +54 291 4861600.
E-mail address: mlferreira@plapiqui.edu.ar (M.L. Ferreira).

0141-0229/$ – see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.enzmictec.2005.08.035

Nomenclature

$K_{m\alpha}$	MM constant for the α phase
$V_{m\alpha}$	Maximum rate of the α phase
k_3^*	Related to the reaction of Cpd I with *S* through the fast loop
k_5	related to the reaction of Cpd III
$f_{Cpd\ I}$	$[Cpd\ I]/[E_{fast}] = V_{max\alpha}/k_2(K_{m\alpha} + S)$
$[E_{fast}]$	$[Ferric] + [Ferric - H_2O_2] + [Cpd\ I] + [Cpd\ I - H_2O_2]$

several other potential applications in the field of biotechnology [13,14].

Literature is abundant on the kinetic behavior of the catalase/H_2O_2 system [14,17]. A gradual decrease of the reaction rate due to the enzyme destruction by H_2O_2 was observed during the β phase; several kinetic equations have been developed to account for this irreversible inactivation [18]. Inhibition associated with the transition from α to β phase is reversible. These two processes, reversible inhibition and irreversible inactivation are confounding variables in catalase assays. Recent reports demonstrated differences between the catalases from *Bovine Liver* (BLC) and from *Aspergillus niger* (ANC) regarding catalytic behavior at high H_2O_2 concentrations: ANC obeyed Michaelis Menten type kinetics and the inactivation rate was always first order; in contrast BLC showed a kinetic behavior typical of substrate inhibition and the decay of enzyme activity with time showed two distinct phases [14]. ANC is known to be a catalase, not a catalase-peroxidase oxidoreductase [14]. The fungal catalase is more stable than the bovine liver enzyme when they are subjected to high concentrations of peroxide.

This work presents the results about free and immobilized catalase from *Aspergillus niger* for hydrogen peroxide decomposition, at low catalase concentration. Magnetite and glutaraldehyde have been selected as support and coupling agent resepctively. The kinetics has been analyzed following Michaelis Menten equation for free and immobilized catalase, using a program for enzyme kinetics modeling (Leonora).

2. Materials and methods

2.1. Materials

Catazyme 25L (25,000 U/mL catalase from *Aspergillus niger*-240,000 Da) was gently donated (Novo, Argentina). Glutaraldehyde (25%, w/v, 2.5 M, Merck) and commercial H_2O_2 (30 vol.% O_2 or 9.1%, w/v, H_2O_2 2.67 M, Profarma) were used. Buffer pH 7 was prepared using 500 mL KH_2PO_4, 0.1 M and 291 mL NaOH, 0.1 M. A H_2O_2 2.647 M solution was used as mother liquor. $KMnO_4$ 0.65 M was used for the titration method. The $[H_2O_2]$ range was 0.025–0.44 M at the reaction media.

2.2. Free catalase activity

2.2.1. Titration method

Decomposition of hydrogen peroxide was selected as the reaction test, at 18 °C. Different amounts of Catazyme 25L were used (3, 5 and 10 U/mL) with a final volume of 25 mL of buffered solution. Aliquots of 1 mL were withdrawn from the test solution at selected times and diluted with sulfuric acid solution, where catalase was inactived. Titration of the remaining hydrogen peroxide at each time was done with $KMnO_4$. From the mother liquor ($[H_2O_2] = 2.68$ M) different volumes were used, being the $[H_2O_2]$ at the reaction media from 0.026 to 0.44 M. Different $KMnO_4$ solutions were prepared to avoid undesirable errors in the quantification of H_2O_2 and to consume between 10 and 20 mL of the titration solutions.

2.2.2. Determination of evolved O_2

To check the importance of the O_2 evolution detection, several measurements of the oxygen evolution were performed with free catalase, using a dissolved Oxygen meter of Extech Instruments. In a total amount of 80 mL, using an H_2O_2 concentration of 0.02962–0.416 M, we adjusted the needed amount of catalase to be at the same concentration that in the experiences using the titration method at the lowest concentration (3 U/mL). In order to compare the previous results with a peroxidase to establish a different behavior, several studies using horseradish peroxidase (HRP) as catalyst in the H_2O_2 decomposition were included. HRP is a known peroxidase.

2.3. Catalase immobilization

Two hundred milligrams magnetite (Fe_3O_4), prepared in our lab [19] was added to 5 mL buffer pH 7 and 1 mL of glutaraldehyde were added (25%, w/v), at a constant temperature of 90 °C stirring for 1 h. The solution was filtered and the solid washed with 20 mL bidistilled water three times, for approximately 1 h. After this, 2 mL of Catazyme 25L solution were put in contact with 200 mg magnetite. The amount of immobilized catalase was obtained using 273 nm as the wavelength and a calibration curve constructed with the Catazyme 25L ($\varepsilon = 3.0 \times 10^7$ L M^{-1} cm^{-1} for a molecular weight of 250,000 Da). This method is sensitive to protein concentration of catalase. The catalase concentration at the support is of 0.045 wt.% catalase/total weight. From here and thereafter, when catalyst is mentioned, it implies supported or immobilized catalase. The magnetite has an average particle size of about 300 nm. This kind of oxide has low area BET (lower than 45 m^2/g) and the pore size is near 9 A. There is no room for the catalase to be into these pores.

2.4. Activity of the immobilized catalase in decomposition of H_2O_2

First of all, a "mother solution" with 2 mg catalyst/mL was prepared for diluted catalyst preparations −10 mg of catalyst in 5 mL. Another concentrated solution with 20 mg catalyst/mL was prepared for the high concentration tests—100 mg of catalyst in 5 mL. Different volumes were selected to choose an adequate supported catalyst concentration in the reaction media, diluted to 25 mL (0.5–1, 1.5 and 2 mL or 0.0208, 0.04, 0.06, 0.08 mg/mL). In milligrams of catalase 1.2×10^{-4}, 2×10^{-4}, 4×10^{-4} mg/mL were used for free catalase and 0.1×10^{-4}–4×10^{-4} mg/mL of catalase in magnetite-supported catalyst or 0.021–0.08 mg/mL of whole *catalyst*. The $[H_2O_2]$ was 0.0503 M. The highest concentration was selected and the complete range of hydrogen peroxide was analyzed following the same procedure as in the case of free catalase. A total volume of 25 mL was achieved with the needed amount of hydrogen peroxide to obtain the adequate concentration. A 10 times higher concentration of supported catalase was also analyzed to compare between free and supported enzyme at similar catalase concentration. Blank experiments using magnetite (without catalase supported) were also performed. The stirring was enough to allow the particles to be dispersed in the solution.

2.5. The effect of temperature on the activity

The temperature effect was analyzed using 3 U/mL Catazyme for the free catalase and 1 mg/mL for immobilized catalase. The activity of these systems was evaluated using $[H_2O_2] = 0.1037$ M at temperatures of 30, 40, 49 and 77 °C, besides 18 °C.

2.6. Kinetic analysis of activity of free and immobilized catalase

The proposed mechanism for catalase-mediated H_2O_2 decomposition is the following [14] (being Cpd I, Compound I)

$$Enzyme + H_2O_2 \leftrightarrow Ferric - H_2O_2 \rightarrow Cpd\ I + H_2O$$

$$\text{Cpd I} + H_2O_2 \leftrightarrow \text{Cpd I} - H_2O_2 \rightarrow \text{Ferric} + H_2O + O_2$$

Until now, attempts to study the elementary steps for the formation and catalatic degradation of the Cpd I have failed. In the presence of high levels of hydrogen peroxide, catalases from a number of sources show two phases of catalatic activity, termed α and β [15]. Manuscript of Lardinois et al. [14] presented a complete cycle for the catalatic reaction, involving a fast loop and a slow loop. The fast loop involves only the Cpd I as the intermediate. The slow loop takes place in parallel with the fast loop in the presence of high levels of H_2O_2. In the fast loop, only the Cpd I is in high concentration. The Cpd III only is reduced to the native enzyme very slowly [16]. Cpd I is considered to be a reactive intermediate with high formal oxidation state of +5, instead +3 for the remainder enzyme. This compound is capable of oxidizing a range of reducing substrates by a mechanism involving two sequential single-electron steps.

Kinetic results at 18 °C were analyzed considering a Michaelis Menten (MM) behavior and Lineweaver–Burk (LB). Different adjustments considering or not substrate inhibition were taken into account, using the program Leonora [20]. This program is intended to allow kinetic data to be fitted by any reasonable method including linear/non-linear least squares, robust regression, least absolutes, median regression, minimax fitting and on screen graphical examination regression method. The classic MM equation was used and also the equation that considers substrate inhibition.

2.7. Evolution of O_2

The O_2 evolution with time at each set of concentrations can be determined analytically by integrating the Lardinois equation, from Ref. [14], with initial condition $[O_2] = 0$. In this equation the not common parameters are $K_{m\alpha}$, k_5^*, k_5, $f_{\text{Cpd I}}$. These parameters are explained in the nomenclature section. However, further details can be found in Ref. [14].

The main point here is to consider that the O_2 evolution must be compared with the H_2O_2 determination to gain knowledge about the mechanism, especially using the lower concentration. The equipment reports the data in mg O_2/L. In this case, only initial rates of H_2O_2 decomposition were used in the analysis, as usual. Our data of O_2 evolution were fitted with the equations presented in Refs. [14,16] for the evolution of O_2 concentration *in time*. The rate of O_2 evolution was the half of the H_2O_2 decomposition. Considering that some was lost in the compound III formation, obviously the initial rate can be considered free of this effect. The complete picture involves several reactions that can be "trapping" H_2O_2.

2.8. The activity of free and immobilized catalase—pH effects

For free and immobilized catalase, the pH was varied between 5 and 11. Reaction conditions were 5 μL catalase Catazyme 25 L in 25 mL total volume with buffer or adequate pH, 18 °C and 0.107 M H_2O_2. In the case of immobilized catalase the catalyst concentration was 1 mg/mL.

3. Results

3.1. Free catalase

Results for the three concentrations of Catazyme tested, at initial $[H_2O_2] = 0.831$ N, show that the higher free catalase concentrations are more effective in the hydrogen peroxide elimination in absolute terms (results not shown). Initial rate of H_2O_2 decomposition versus hydrogen peroxide concentration follows MM behavior apparently but no saturation was found in the range used. Data followed MM in all the range of hydrogen peroxide concentrations for 10 U/mL. At a lower concentration of catalase deviations from MM were observed. Table 1 shows the results of the adjustments using different linearizations of MM.

3.2. Immobilized catalase

When supported catalase is analyzed, linearity is found between the initial rate and the catalyst concentration, similarly to the free catalase. However, it is clear that using 10 U/mL other reactions affect the efficient use of the enzyme. The relation between the initial rate of hydrogen peroxide decomposition, using 0.1037 M hydrogen peroxide as the initial concentration, and the catalyst concentration is proportional. The relation between catalyst mass and activity is linear (see Fig. 1). When an analysis MM is performed on the immobilized catalase using 0.0833 mg catalyst/mL, increasing the hydrogen peroxide concentration, the initial rate increases and, after getting an optimum value, the activity decreases (see Fig. 2). No catalytic activity for hydrogen peroxide decomposition of bare magnetite was detected in blank experiments.

3.3. Effect of experimental variables

Fig. 3 shows the effect of temperature in the initial rate of hydrogen peroxide decomposition for free and immobilized catalase. Free catalase shows the maximum activity at 60 °C, whereas, the immobilized catalase reaches at 40 °C. It seems that immobilization decreases the thermostability of the catalase.

Table 1
Parameters for soluble catalase obtained by linearization or using Leonora program

Parameters	10 μL		5 μL		3 μL	
	LB	Leonora	LB	Leonora	LB	Leonora
K_m (mol)	0.15	0.136 ± 0.023	0.1	0.090 ± 0.041	0.098	0.119 ± 0.014
V_{max}[a]	87413	78150 ± 8140	90910	90000 ± 18200	77592	96850 ± 4500

Parameters	10 μL (Leonora)	5 μL (Leonora)
K_S (mol)	0.04991	0.01624
K_{Si}	1.067	0.39035
V_{max}	44710	35950

[a] (μmol/min mg enzyme).

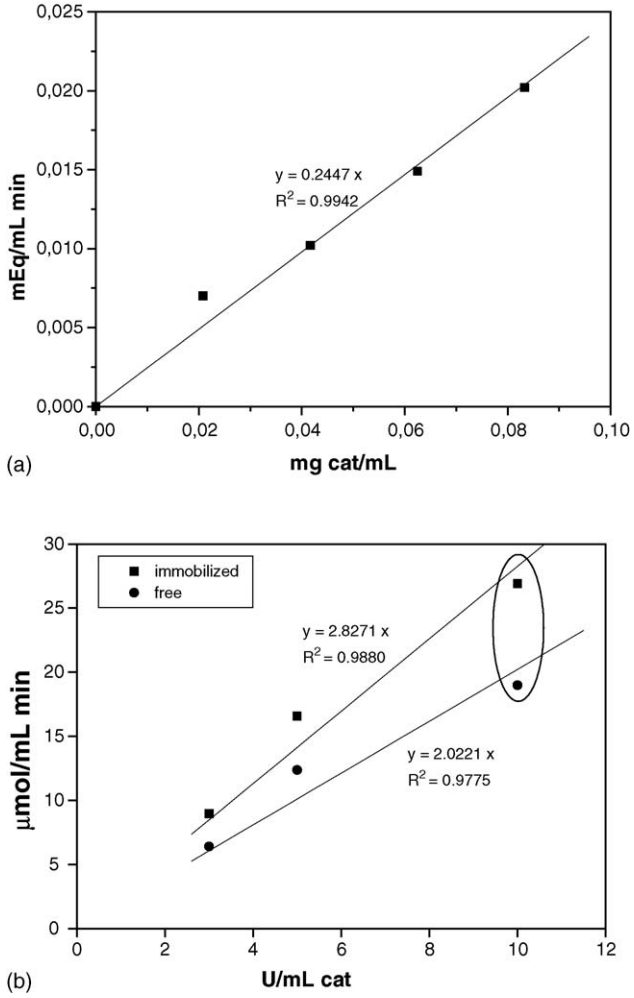

Fig. 1. Effect of catalyst mass-on initial rate of hydrogen peroxide decomposition: (a) immobilized catalase; (b) free catalase-[H_2O_2] = 0.0503 M.

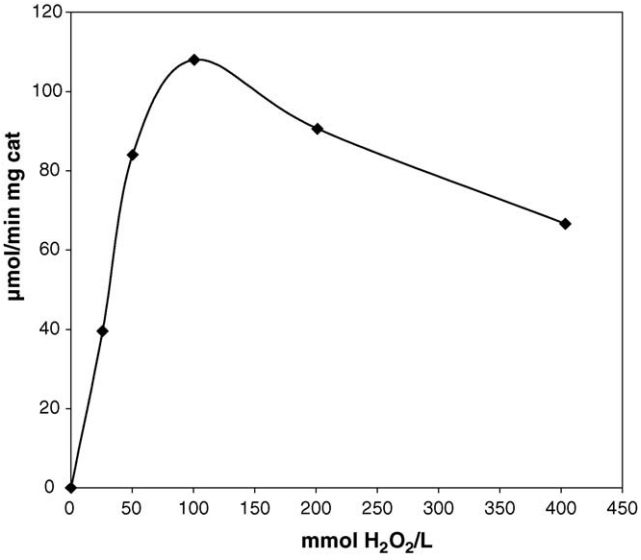

Fig. 2. Initial rate in mEq/μg enzyme min: free catalase = 3 U/mL (0.21 μg/mL); immobilized catalase = 1 mg catalyst/mL (0.4598 μg/mL) vs. 1/*T*.

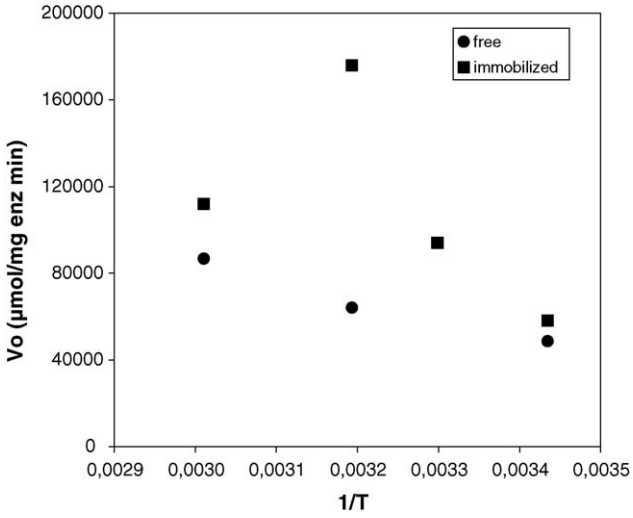

Fig. 3. Effect of temperature in free and immobilized catalase.

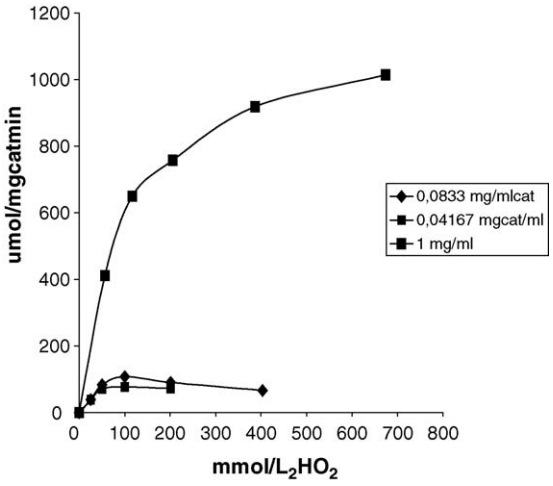

Fig. 4. MM plot for immobilized catalase, 0.083 mg catalyst/mL.

The supported catalyst was tested using different concentrations to evaluate the dependence with the hydrogen peroxide concentration. Using 0.083 mg catalyst/mL as catalyst concentration strong substrate inhibition is found and the initial rate does not follow pure MM (Fig. 4). However, using 1 mg catalyst/mL, the initial rate of hydrogen peroxide decomposition follows MM and a complete curve up to saturation can be obtained in the studied range. Table 2 shows the concentration of supported and soluble catalase used in the experiments.

Table 2
Concentrations in μg/mL for soluble and supported catalyst

Parameter	Supported catalyst		10 U/mL (V_t = 25 mL)	5 U/mL (V_t = 25 mL)	3 U/mL (V_t = 25 mL)
Catalyst (μg/mL)	83.3	1000	0.833	0.4165	0.21
Enzyme (μg/mL)	0.0383	0.4598	0.833	0.4165	0.21

Table 3
Parameters of MM analysis with and without substrate inhibition

Parameters	0.0833 mg/mL[a]	1 mg/mL
K_{inh} (mol)	0.117	–
K_m (mol)	0.111	0.104
V_{max}[b]	309179	233800
V_{max}[c]	–	508262

[a] V_{max} = 234782 at 0.2 N H_2O_2. μmol/min mg enzyme 105,700 μmol/min g catalyst (for 0.0833 mg/mL) Value obtained at the experimental optimum (see Fig. 4).
[b] μmol/min g catalyst.
[c] μmol/min mg enzyme.

Table 3 shows the parameters obtained from the kinetic analysis for different concentrations of supported catalyst. The maximum experimental initial rate found using 0.0833 mg catalyst/mL was 234,782 μmol/min mg enzyme. Because of substrate inhibition there is no possibility to reach the maximum initial rate predicted by MM (672,130 μmol/min mg enzyme) in this case. The analyzed equations were the pure MM and MM with inhibition in presence of high substrate concentrations. Fig. 5 shows the effect of pH in the performance of free and immobilized catalase. pH 7 is the best for the free catalase, but activity increases steadily with the immobilized catalase, being higher at pH 11 than at pH 7. This is a very important result. The magnetite-immobilized catalase could be applied to alkaline pH *increasing its activity* in comparison to neutral pH. In Fig. 6 the O_2 evolution as a function of H_2O_2 concentration is observed. Table 4 shows the results for the use of Lardinois equation for the O_2 evolution.

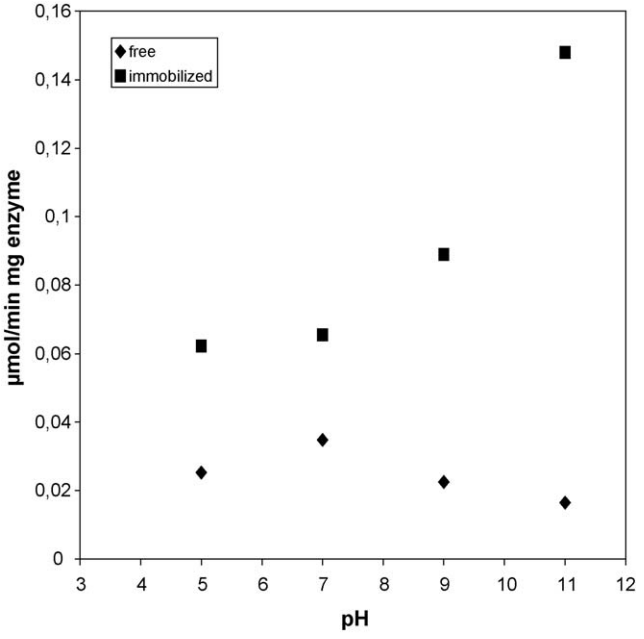

Fig. 5. Effect of pH in enzymatic activity of free and magnetite immobilized catalase: free catalase = 5 U/mL; magnetite immobilized catalase = 1 mg/mL.

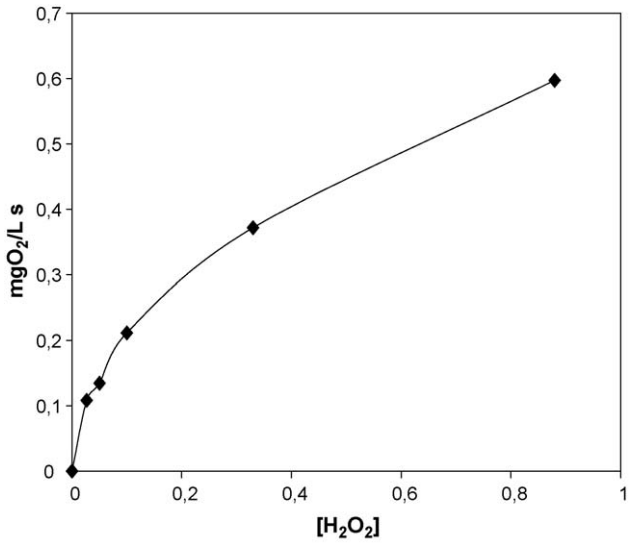

Fig. 6. MM parameters $V = 0.699$ mg O_2/Ls or 0.01085 mol H_2O_2/min mg enzyme; $K_m = 0.2$ M; substrate inhibition $V = 0.4054$ mg O_2/Ls, $K_s = 0.0871$ M.

Table 4
Parameters of our own experiments following Ref. [14] obtained for the O_2 evolution using the low concentration of catalase

M [H_2O_2]	$f_{Cpd\ I}$	$va - bt = 0$	$k_5 + k_3 f_{Cpd\ 1} S$	k_5	k_3
0.0257	0.01332504	4.10944E−06	0.350273963	0.35	0.75
0.0503	0.00869338	5.24733E−06	0.380437277	0.38	1
0.1	0.00555556	6.66667E−06	0.300416667	0.3	0.75
0.2	0.00641026	1.53846E−05	0.350961538	0.35	0.75
0.4	0.00305556	1.46667E−05	0.350916667	0.35	0.75
0.88	0.00231481	2.44444E−05	0.351527778	0.35	0.75

3.4. The deactivation with time and the deactivation at high hydrogen peroxide concentration

The free and immobilized catalysts were contacted successively with high hydrogen peroxide concentrations. Fresh hydrogen peroxide concentrated solution was added after the activity achieved a plateau. Table 5 shows the initial rate for free and immobilized catalase and the percentage of conversion after 15 min. It is clear that, although the initial rate can be higher for the magnetite supported catalase, the deactivation

Table 5
Reuse experiments

Use	Initial rate			% Conversion	
	Free catalase (μmol/min mg enzyme)	Immobilized catalase (μmol/min)		Free	Immobilized
		mg catalyst	mg enzyme		
1°	30000	50	111100	98.27	45.23
2°	22125	19.5	43329	91.07	22.22
3°	18250	16.5	36663	68.42	15.66
4°	4750	0	0	23.38	0

Conversions (% of decomposed hydrogen peroxide) after 15 min experiences done with reaction conditions; immobilized catalase = 1 mg/mL; free catalase = 0.2 μL/mL [H_2O_2] = 0.033 M.

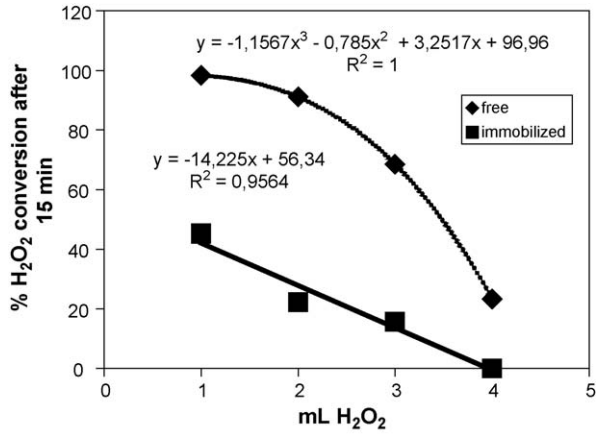

Fig. 7. Percentage conversion after 15 min with different H_2O_2 adding immobilized catalase concentration = 1 mg/mL; free catalase = 0.2 μL/mL $[H_2O_2]$ = 0.033 M.

Table 6
Parameters reported by Ref. [14] and by our work

	Parameters	
	Lardinois	This work
V_{max}	3.62E05 mol O_2/mol enzyme	3.49E02 mol O_2/mol enzyme
K_m	0.322 M	0.2
[E]	1.5–6 μM	1 nM
$[H_2O_2]$	0.01–2 M	0.02–0.88
pH	7.8	7
k_{cat}	241333–60333	349000

K_{cat} in V_{max} (mol O_2/mol enzyme)/[E] (μM enzyme).

is more important in this case than in the case of the free catalase. The deactivation is higher for the supported catalase than for the free catalase. However, the contact with high hydrogen peroxide concentrations deactivates irreversibly the catalase and this is shown by the loss of enzymatic activity that Fig. 7 presents. The higher deactivation is clear after the third adding of fresh hydrogen peroxide. The deactivation of horseradish peroxidase is much higher than in the case of ANC, in fact, the evolution of O_2 happens in steps. Totally different profiles are obtained with H_2O_2 in this case (results not shown).

3.5. Comparison with Lardinois group

Differences were found between the rate of O_2 evolution and the rate of H_2O_2 decomposition. The reported data from Ref. [14] are summarized in Table 6. It is very important that in our case we used three orders of magnitude lower enzyme concentration and the V_{max} is then three orders of magnitude lower. K_{cat} data for O_2 evolution are extremely coherent, considering that the catalase concentration is different, the pH is slightly lower in our case and we are comparing different labs. O_2 evolution with free peroxidase from Horseradish was much lower than with free catalase, at the same concentrations.

4. Discussion

Magnetite has the inverse spinel ($MgAl_2O_4$) structure, with a cubic close packed oxygen array, and iron in both four- and six-fold coordination; magnetite is frequently observed in nature as octahedral crystals exhibiting {1 1 1} faces [23]. Glutaraldehyde is supposed to react with magnetite in the same way as Al_2O_3. Recent reports about aldehyde adsorption demonstrated that there are several reaction posibilities of aldehydes with bare oxides surfaces: Strong coordination to Lewis acidic sites, adsorbed terminal etoxy species and aldolization between two molecules on surface, this last possibility for glutaraldehyde [25]. Strong Lewis coordination is the previous step to the oxidation on surface oxygen sites and the formation of a glutaracetate adsorbed. Anyway, the proposal of a strong coordination/reaction of glutaraldehyde to the bare magnetite surface is supported sufficiently. Although we used glutaraldehyde, the amount of supported catalase we used was enough at the high concentration of 1 mg catalyst/mL to have near 4.5×10^{-4} mg enzyme/mL. This concentration in mass is similar to the concentration obtained for free catalase (see Table 2). In this sense we can compare the results for the supported and free catalase. From Fig. 2 it is evident that using the lowest free catalase concentrations there are no problems, but with the highest some of the enzyme is not properly used because the activity per mg enzyme decreases.

In our case, we found that, with 0.045 mass% catalase considering the overall catalyst, the maximum *experimental* activity per mg of catalyst (support + glutaraldehyde + catalase) is about 1000 μmol/min mg catalase or 2.2×10^6 μmol/min mg enzyme. Maximum experimental activity using free catalase was 74,857 μmol/min mg enzyme or 0.075×10^6 μmol/min mg enzyme. Obviously, per mg basis, the maximum activity found for free catalase is 75 times higher than the activity of supported catalase. Per mg enzyme basis it seems that the immobilized catalase is 30 times more active than free catalase. To obtain this value, we checked the absorbance before and after the immobilization in the remaining solution. The main assumption of this is that the enzyme at the support has the same activity as the enzyme at solution, a consideration that can be untrue some times.The best fitting results are obtained when substrate inhibition is included in our hydrogen peroxide concentration range. Our catalase concentration is almost three orders of magnitude lower than in the case of Ref. [14]. The irreversible inactivation takes place and we saw this in the successive adding of further hydrogen peroxide. This irreversible inactivation could be assigned to the low rate of conversion of Cpd III into native form and to a low rate of conversion of Cpd I to Cpd II for ANC. From Table 4 it is clear that depending on the hydrogen peroxide concentration the fraction of Cpd I decreases. The rate of oxygen release during the α–β transition increases with hydrogen peroxide concentration. This transition is controlled by the respective values of $k_5 + k_3 f_{Cpd\,1} S$. k_5 is found in the 0.3–0.35 range and k_3 from 0.75 to 1 range (see Table 4). Values of Lardinois et al. [14] with the same units are 0.35 s^{-1} for k_5 and 0.8 $M^{-1}\,s^{-1}$ for k_3^*.

Free and magnetite-immobilized catalase are active in hydrogen peroxide decomposition. Catalase from *Aspergillus niger* is reported by Novo to decrease only laterally activity at pH range 8–10 and we confirmed this point. The elimination achieves 90% using 10 U/mL for the highest concentration tested after only 16 min. No strong substrate inhibition was found in the range of hydrogen peroxide concentration tested for free catalase, although other authors reported it [18].

We discarded any photometric method using Ti compounds such as the peroxotitan cation $([TiO_2]aq)^{+2}$ at 415 nm or the absorption of hydrogen peroxide at 240 nm. Any photometric method would be affected by the size of the particles of the magnetite. We cannot measure the change in the UV/vis method with magnetite in the reaction media, not even with a recirculation system, because of the effect of the support (dispersion, spurious absorptions) in the UV/vis absorption. The titration method looks simple, easy to perform and without the kind of errors we can have with the UV/vis method in the presence of finely divided solids.

When supported catalase from *Aspergillus* was analyzed the values obtained for MM with substrate inhibition were $K_m = 0.087$ M, $K_i = 0.56$ M and $V_{max} = 295$ μmol/min g catalyst [18]. When chitosan as film is the support V_{max} of immobilized the catalase is nearly 1022 μmol/min mg protein and no data to calculate per g catalyst were provided. When chitosan beads were used, K_m is 0.0775 M and V_{max} achieved 122 μmol/min mg protein [21,22]. In this case, our catalyst presents a V_{max} of 288 μmol/min mg catalyst or 288,000 μmol/min g catalyst, whereas for alumina-supported catalysts at the same conditions authors of Ref. [21] reported 160 μmol/min g catalyst. V_{max} for free catalase is nearly 90,000 μmol/min mg enzyme at the low concentrations used in our experiments (see Table 2). A factor of three is involved when we compare free and immobilized V_{max} at the same catalase concentration. The K_m is almost unaltered but the V_{max} increases three to six times comparing free and supported catalase at the concentrations evaluated (0.0833 and 1 mg/mL). Reports from some of us presented similar K_m to the obtained in this case (0.115 M) and a maximum reaction rate of 3135 ± 203.8 μmol/min mg enzyme (or 6270 per g catalyst) for aged cellulose-supported catalase. In the same work, V_{max} near 35,120 μmol/min mg enzyme and K_m near 0.025–0.030 M were obtained using UV/vis method for activity measurements and a catalase concentration of 16.7 U/mL [12].

When the supported catalyst is used, 100 mg of chitosan or cellulose-supported catalase in 10 or 20 mL are usually spent [12]. In this case, we had a catalyst concentration of 5–10 mg/mL. However, employing magnetite as support, we are using 0.0833 mg/mL or even 1 mg supported catalase/mL (a catalase concentration 10 times lower), in a similar concentration to free catalase in the case of using 5 U/mL. The maximum activity is high for supported catalyst when similar enzyme concentrations are compared between free and supported catalase in the present manuscript. Using 1 mg catalyst/mL (4.5×10^{-4} mg enzyme/mL) there are no problems with inactivation due to H_2O_2. Novo reports 60 °C as the optimum temperature for free catalase and our results confirmed this (see Fig. 3). When catalase is supported, maximum activity is found at 40 °C, although at 60 °C it is still active. Thermostability is lower for the supported catalase, compared with the free catalase.

The supported catalase increases its activity at higher pH (11). From Fig. 5, it is evident that the H_2O_2 decomposition is higher at pH 11 than at pH 7 using immobilized catalase, opposite to the free catalase. Several authors reported that immobilized catalase on alumina support increases its stability to alkaline pHs [2]. In this case, with magnetite as support, the relative activity of the catalyst *increases* at alkaline pH versus neutral or acidic ones. This kind of behavior is uncommon. Generally the profiles of the immobilized and free catalase are similar, but the immobilized catalase is generally more stable at higher values of pHs [15,24].

5. Conclusion

Catalase supported on magnetite has excellent perspectives as a catalyst for hydrogen peroxide decomposition. Glutaraldehyde-magnetite as catalase support is highly effective. K_m is almost unchanged but V_{max} increases comparing the free and supported catalase at the same concentrations of enzyme in the reaction media. The maximum rate obtained with the supported catalase is up to six times higher than for the free catalase, although the substrate inhibition is a problem and amounts of 1 mg supported catalyst/mL (or higher) are needed. Magnetite shows a stabilizing effect on catalase in terms of activity, although it seems that the supported catalase at the concentrations tested is not as thermally stable as the free catalase. However, the practical use of magnetite as support includes the easy recovering of the supported catalase and its potential re-use. Further works are being carried out to optimize the conditions for use (and reuse) of supported catalase.

Acknowledgements

M.L. Ferreira acknowledges the financial support from *Foundation Antorchas* (Argentina). Authors acknowledge financial support from CONICET and UNS and C. Zenobi by the obtention of the experimental data using supported catalase at different concentrations.

References

[1] Costa SA, Tzanov T, Carneiro AF, Paar A, Gübitz GM, Cavaco Paulo A. Studies of stabilization of native catalases using additives. Enzyme Microb Technol 2002;30:387–91.
[2] Costa S, Tzanov T, Gudelj M, Robra K-H, Cavaco-Paulo A, Gübitz GM. Thermo-alkali-stable catalases from newly isolated *Bacillus* sp. for the treatment and recycling of textile bleaching effluents. J Biotechnol 2001;89:147–53.
[3] Gudelj M, Fruhwirth GO, Paar A, Lottspeich F, Robra K-H, Cavaco-Paulo A, et al. A catalase-peroxidase from a newly isolated thermoalkaliphilic *Bacillus* sp. with potential for treatment of bleaching effluents. Extremophiles 2001;5:423–9.
[4] Owen R., Química de los alimentos; Fennema, Ed. Acribia, 2001.
[5] Robert K Murray, Daryl K Granner, Peter A Mayes, Victor W Rodwell, editors. Harper's Biochemistry. 25th ed. McGraw-Hill Companies; 2002 (Lange Series).

[6] Costa SA, Tzanov T, Paar A, Gudelj M, Gübitz GM, Cavaco-Paulo A. Immobilization of catalases from *Bacillus* SF on alumina for the treatment of textile bleaching effluents. Enzyme Microb Technol 2001;28:815–9.
[7] Kohjiya AS, Maeda K, Yamashita S. Immobilization of catalase by polyion complex. J Appl Polym Sci 1991;43:2219–22.
[8] Selli E, Ambrosio A, Bellobono IR. Enzymatic activity under tangential flow conditions of photochemically grafted membranes containing inmobilized catalase. Biotechnol Bioeng 1993;41:474–8.
[9] Solas MT, Vicent C, Xavier L, Legaz ME. Ionic adsorption of catalase on bioskin: kinetic and ultrastructural studies of pressure and inhibition on kinetics and deactivation. J Biotechnol 1994;33:63–70.
[10] Emine A, Leaman T. Characterization of immobilized catalases and their application in pasteurization of milk with H_2O_2. Appl Biochem Biotechnol 1995;50:291–303.
[11] Lilly MD. Immobilized enzymes. In: Mosbach Klaus, editor. From Series Methods in Enzymology, vol. XLIV. Londres: Academic Press; 1976. p. 46–53.
[12] Eberhardt A, Pedroni V, Volpe MA, Ferreira ML. Immobilization of catalase from *Aspergillus Niger* on inorganic and biopolymeric supports for H_2O_2 decomposition. Appl Catal B: Environ 2004;47:153–63.
[13] Koneracká M, Kopcanský P, Timko M, Ramchand CN, Sequeira Ade, Trevan M. Direct binding procedure of proteins and enzymes to fine magnetic particles. J Mol Catal B: Enzym 2002;18:13–8.
[14] Lardinois MO, Mestdagh MM, Rouxhet PG. Reversible inhibition and irreversible inactivation of catalase in presence of hydrogen peroxide. Biochim Biophys Acta 1996;1295:222–38.
[15] Acosta M, García Cánovas F, Hiner AN, Arnao MB, Hernández Ruiz J. Catalase-like activity of horseradish peroxidase, relationship to enzyme inactivation by H_2O_2. Biochem J 2001;354:107–14.
[16] Ghademarzi M, Moosavi-Mohadevi, Ghademarzi M. Influence of different types of effectors on the kinetic parameters of suicide inactivation of catalase by hydrogen peroxide 1999;1431:30–36.
[17] Dunford BH. Heme Peroxidases. New York: Wiley-VCH; 1999.
[18] Vasudevan PT, Weiland RH. Deactivation of catalase by hydrogen peroxide. Biotechnol Bioeng 1990;36:783–9.
[19] Ferreira ML, Rueda EH, Ambrosio K. Magnetite-supported hematin as a biomimetic of horseradish peroxidase phenol removal by polymerization. Biocatal Biotrans 2004;22:35.
[20] Cornish-Bowden A. Leonora program. In: Analysis of Enzyme Kinetic Data. Oxford University Press; 1995.
[21] Cetinus AS, Oztop HN. Immobilization of catalase into chemically crosslinked chitosan beads. Enzym Microb Technol 2003;32:889–94.
[22] Cetinus AS, Oztop HN. Immobilization of catalase on chitosan film. Enzyme Microb Technol 2000;26:497–501.
[23] Barbieri AW, Weiss W, Van Hove MA, Somorjai GA. Magnetite Fe_3O_4(1 1 1): surface structure by LEED crystallography and energetics. Surf Sci 1994;302:259–79.
[24] Costa S, Tzanov T, Carneiro F, Gübitz GM, Cavaco-Paulo A. Recycling of textile bleaching effluents for dyeing using immobilized catalase. Biotechnol Lett 2002;24:173–6.
[25] Rasko J, Kiss J. Adsorption and surface reactions of acetaldehyde on TiO_2, CeO_2 and Al_2O_3. Appl Catal A: Gen 2005;287:252–60.