ELSEVIER

Catalysis Today 79–80 (2003) 333–339

CATALYSIS TODAY

www.elsevier.com/locate/cattod

Lactose hydrolysis by immobilized β-galactosidase: the effect of the supports and the kinetics

M. Di Serio [a], C. Maturo [a], E. De Alteriis [b], P. Parascandola [b], R. Tesser [a], E. Santacesaria [a,*]

[a] *Dipartimento di Chimica, Università di Napoli "Federico II", via Cinthia, 80126 Naples, Italy*

[b] *Dipartimento di Fisiologia Generale ed Ambientale, Università di Napoli "Federico II", via Mezzocannone 16, 80134 Naples, Italy*

Abstract

The kinetic behaviour of β-galactosidase from *Kluyveromices marxianus* (*Saccharomyces*) *lactis*, immobilized on different oxides supports, such as alumina, silica, and silicated alumina has been studied. We observed a strong dependence of the immobilized enzyme activities on the chemical nature and physical structure of the supports. In particular, when the particle sizes of the supports are increased, the enzymatic activity strongly decreases.

The hydrolysis of lactose, promoted by the mentioned enzyme immobilized on small commercial silica spheres, has been studied in different operative conditions, by changing: feed rate, reagent and products concentration and temperature, while pH has been kept constant (7.0). A depressing effect originated by both the reaction products, has been observed. Therefore, a kinetic model based on the Michaelis–Menten mechanism, in which the depressing effects of both the hydrolysis products (galactose and glucose) are also considered, have been developed and the related parameters determined.

Keywords: β-Galactosidase; Immobilized enzymes; Hydrolysis

1. Introduction

A large part of the human population show lactose intolerance and has difficulty in consuming milk and dairy products [1]. The problem can be conveniently solved using the enzyme lactase, β-galactosidase, that hydrolyses lactose into the not dangerous compounds glucose and galactose. The enzyme can be used directly or immobilized. The use of free enzyme is quite expensive, but when it is immobilized by coupling it to an adequate matrix, the resulting bio-catalyst may be reused several times, thus lowering the costs [2]. Enzymes have been immobilized in the past by using different methods such as, for example, physical adsorption, covalent bonding, gel entrapping, etc. [3]. However, despite the large interest in the β-galactosidase immobilization, it is difficult to obtain a catalyst showing simultaneously: (i) high activity, (ii) high stability, (iii) optimal mechanical properties (shape, size, density and attrition resistance). As a matter of fact, although special immobilized β-galactosidase reactors for continuous hydrolysis of lactose on dairy products were developed, for example, by SNAM Progetti in Italy and by Sumitomo Chemicals in Japan, in the 1970s, free enzyme is still widely used in dairy industry around the world [4]. This suggests that the β-galactosidase immobilization procedure has not been yet optimized and justifies, therefore, the papers recently published on the subject [4–12].

* Corresponding author. Tel.: +39-081-6740-27; fax: +39-081-6740-026.
E-mail address: santacesaria@chemistry.unina.it (E. Santacesaria).

0920-5861/03/$ – see front matter © 2003 Elsevier Science B.V. All rights reserved.
doi:10.1016/S0920-5861(03)00059-2

In these papers the enzyme is, normally, linked to a support by chemical bonds, because, this renders very stable the bio-catalyst preventing the enzyme leakage [5]. Some polymeric supports have been used in the development of the immobilized enzyme but inorganic carriers, due to their physical properties, are more suitable for industrial use, offering some advantages such as high mechanical strength, high density, thermal stability, ease of handling, high flow rates in continuous reactors and the ease with which they can be regenerated by simple pyrolysis [6].

For these reasons, we have studied, in the present work, the behaviour of β-galactosidase, immobilized with covalent bond on different oxides such as alumina, silica, and silicated alumina. We observed, in particular, a strong dependence of the immobilized enzyme activities on both the chemical nature and physical structure of the supports. Some suggestions have been given to explain the observed behaviours. In particular, different runs have been made in this work on, respectively, pellets and powders of the same material. As, the activity strongly decreases with the particle size, a compromise must be adopted in the choice of the particle dimension to be used in practice. For this reason we performed a kinetic study of the reaction carried out on the enzyme immobilized on spherical pellets (250–500 μm) of silica, a commercial product normally used as chromatographic support. The resulting kinetic data, collected in a continuous fixed bed reactor, have been interpreted by using a kinetic model based on the Michaelis–Menten mechanism opportunely modified for considering the rate inhibition, experimentally observed, induced by both the reaction products. The model and the obtained kinetic parameters have been used to assess a preliminary evaluation of the industrial feasibility and constitute a base of reference to improve and optimize the covalent bonding immobilization procedure on inorganic supports.

2. Experimental

2.1. Materials, reagents and methods

The enzyme (Maxilact 5000), a highly purified liquid preparation of β-galactosidase derived from *Kluyveromices marxianus* (*Saccharomyces*) *lactis*, was provided by Gist-Brocades France S.A. The specific activity of the enzyme solution and of the immobilized enzyme was determined by using the *o*-nitrophenyl-β-D-galactopyranoside (ONPG) substrate [13]. The activity of the mother enzyme solution corresponded to about 5100 neutral lactose unit/g (NLU/g). One NLU is the quantity of enzyme which will form 1.30 μmol of *ortho*-nitro-phenol (ONP) from ONPG substrate under the conditions of the assay. The assay is based on a 10 min hydrolysis of the ONPG substrate at 30 °C and pH = 7. The mother solution contained about 35 mg of proteins per gram of solution.

The properties of the commercial silica and alumina supports, used for the enzyme immobilization, are reported in Table 1. The specific surface area and pore size distribution of these supports have been obtained by N_2 absorption, at 77 K (see Table 1). Fluka furnished all other reagents.

2.1.1. Enzyme immobilization on powdered supports

In order to avoid the influence of particle size, all the supports have been powdered and sieved, by selecting for the immobilization particles with diameter of less than 40 μm. The adopted immobilization technique occurs in three different steps [14]. In the first one (*aminoalkylation*), the oxide surface is silanized by contacting 5 g of solid, previously calcined, at 300 °C for 6 h, with 90 ml of γ-aminopropyltrietoxysilane (γ-APTS) solution (0.025 mol/dm^3), in refluxed toluene, for 4 h. Then,

Table 1
Characteristic of the used supports

No.	Support	Mean particles diameter (mm)	Specific area (m^2/g)	Pore volume (cm^3/g)	Specific area of pores diameter >150 Å (m^2/g)
1	Millipore Silica 150A	0.25–0.50	209	0.55	37
2	Millipore Silica 60A	0.20–0.50	381	0.83	21
3	Alumina Akzo	2.4	194	0.50	45
4	Alumina Enghelard	2.5–4	184	0.43	23

the obtained solid is washed with toluene and acetone and dried at room temperature for 24 h. In the second step (*coupling*) 0.4 g of the obtained solid is treated, at 4 °C under agitation for 24 h, with 150 ml of buffered phosphate aqueous solution (pH = 7.0) containing 1 wt.% of glutaraldehyde. One of the two aldehyde groups of the glutaraldehyde reacts with an amino group of the γ-APTS leaving the other one free to react with an amino group of the enzyme in the last step of preparation. The obtained solid is firstly washed with the buffered phosphate solution (pH = 7) and then (*lactase grafting*) is put in contact, at 4 °C for 24 h, under agitation, with 100 ml 20 NLU/g enzyme solution obtained by diluting Maxilact 5000 solution with the buffered phosphate solution (pH = 7). The obtained solid is then washed several times with the buffered phosphate solution (pH = 7) until no enzymatic activity was found in the washing solution. The residual activity of the filtered solution has been determined and the difference with respect to the initial solution and the comparison with the activity of the grafted enzyme give the yield of the immobilization procedure. The activity data obtained for these powdered supports are reported in Table 2 (runs 1–4).

2.1.2. Enzyme immobilization on silicated alumina

The surface chemical characteristics of the alumina support, reported in Table 1, have been modified by grafting silicon alkoxides. The grafting reaction was performed by contacting, at room temperature, for 5 h, the powdered alumina support with a solution of silicon tetraethoxide (TEOS) dissolved in anhydrous ethanol. After the reaction, the solid is recovered by filtration, washed with solvent, dried over night at 50 °C, and then calcined in air, at 300 °C, for 2 h. Silicated alumina supports have been prepared by repeating both grafting reaction and calcination steps for three times. The most important difference between the original alumina support and silicated alumina is the surface acidity and a potentiometric titration of the surface shows that the zero point charge (ZPC) of the solid decreases, from 7.4 to 5.6 [15], as a consequence of the coating. The immobilization of the enzyme on silicated alumina has been performed as previously described. Also in this case enzymatic activities have been determined on, respectively, the initial and the residual solution and of the solid, in order to evaluate the grafting yield. The activity data obtained for these supports are reported in Table 2 (runs 5 and 6).

2.1.3. Enzyme immobilization on pelletized support

In order to evaluate the influence of the particles size of the supports, enzyme immobilization has been carried out on spherical pellets of silica, having a mean diameter 0.25–0.50 mm (Millipore Silica 150A). In this case the aminoalkylation of silica has been performed as previously described, while the second and the third immobilization steps have been carried out by packing 4 g of the support in an isothermal jacketed glass column (1.5 cm × 25 cm) reactor and by recycling with a pump firstly a buffered aqueous solution containing 1 wt.% of glutaraldehyde, at 4 °C for 24 h, and secondly a 20 NLU/g of the enzyme solution, again at 4 °C and for 24 h (pump fed = 1 cm^3/min, enzyme total solution volume = 1000 cm^3).

The column and the fed solutions were both cooled by using an ultrathermostat. The immobilized enzyme has then been washed by feeding the buffered phosphate solution (pH = 7.0) on the packed bed until no enzymatic activity was found in the washing solution. The activity obtained for this support is always reported in Table 2 (run 7).

Table 2
Activity of immobilized enzymes in ONPG hydrolysis

No.	Support	Mean particles diameter (mm)	Adsorbed enzyme (NLU/g)	Specific activity (NLU/g)
1	Powdered Millipore Silica 150A	<0.040	3600	155
2	Powdered Millipore Silica 60A	<0.040	4400	98
3	Powdered Alumina Akzo	<0.040	4150	73
4	Powdered Alumina Enghelard	<0.040	4350	47
5	Silicated Alumina Akzo	<0.040	4250	171
6	Silicated Alumina Enghelard	<0.040	4425	143
7	Millipore Silica 150A	0.25–0.50	4850	14

Table 3
Kinetic runs on packed reactor containing 4 g of immobilized lactase on Millipore Silica 150A (14 NLU/g)

Run	T (°C)	Feed rate (cm^3/min)	Lactose concentration ($\times 10^2$ mol/dm^3)	Galactose concentration ($\times 10^2$ mol/dm^3)	Glucose concentration ($\times 10^2$ mol/dm^3)	Lactose conversion (%)	
						Experimental	Calculated
1	4	4	2.8	0	0	19.3	22.9
2	20	4	2.8	0	0	52.2	51.4
3	37	4	2.8	0	0	80.2	79.7
4	4	4	6.9	0	0	13.1	11.9
5	20	4	6.9	0	0	35.4	33.1
6	37	4	6.9	0	0	63.0	63.2
7	4	4	13.9	0	0	7.0	6.7
8	20	4	13.9	0	0	22.1	21.6
9	37	4	13.9	0	0	50.0	48.5
10	4	4	27.8	0	0	4.0	3.6
11	4	10	6.9	0	0	6.9	5.4
12	20	10	6.9	0	0	16.9	17.3
13	37	10	6.9	0	0	37.0	38.2
14	20	16	6.9	0	0	10.3	11.8
15	4	4	13.9	5.6	0	1.2	2.6
16	20	4	13.9	5.6	0	11.3	10.6
17	37	4	13.9	5.6	0	31.5	32.3
18	4	4	13.9	0	5.6	4.2	3.1
19	20	4	13.9	0	5.6	13.5	12.3
20	37	4	13.9	0	5.6	34.0	35.5

2.1.4. Kinetic runs in a packed bed reactor

Kinetic runs of lactose hydrolysis have been performed by using the previously described continuous packed bed tubular reactor containing the enzyme immobilized on silica sphere of 0.25–0.50 mm. The runs have been performed by changing, respectively, feed rates, reagent and products concentration and temperature. Solutions fed were taken at constant pH (7.0) buffering with phosphates. All the runs performed and related conditions are reported in Table 3. The outlet solution was withdrawn every 15 min and analysed by using an HPLC method [7] to control the achievement of steady-state condition and to collect kinetic data. After 40 h of use, the deactivation of the immobilized enzyme was tested carrying out a run using the same condition of run 1 and the immobilized enzyme has not shown deactivation. This means that covalent bond enzyme immobilization is very stable and also that enzyme leaching is, in this case, negligible.

3. Results and discussion

As it can be seen from activities data reported in Table 2, we have a low yield (not exceeding 5%) in retaining enzymatic activities after the immobilization. This arises by comparing the activity of the solutions before and after the immobilization with the one shown by the solid. This behaviour can be explained by assuming that bulky enzyme molecules fill the macropores before reacting with glutaraldehyde groups. Many enzyme molecules remain so trapped in plugged pores. Ladero et al. [8] observed that enzyme immobilized on silica-alumina has, in their conditions, an activity of 50% lower than the one shown by free enzyme, but immobilized enzyme was less than 1/20 with respect to the present work. It seems reasonable, therefore, a lower percentage of activity with respect to the free enzyme, in our case, by considering the greater amount of adsorbed enzyme. This is a consequence of different effects such as: the enzyme molecules aggregation [9], a greater number of plugged macropores, a not uniform enzyme distribution and, eventually, a low effectiveness factor. However, from data reported in Table 2 it is possible to observe a strong dependence of the activity on the pore size distribution of the supports (see Table 1) having the same chemical composition. Moreover, the higher active immobilized enzyme is the one anchored on supports with higher pore fraction having diameter size larger than

150 Å, even if, this solid has a lower specific surface area (see Table 1). This result is the consequence of the size of the enzyme molecules (150 Å × 50 Å or 120 Å × 70 Å) [16] that cannot be anchored inside the micropores and partially also inside the mesopores. An experimental evidence seems to be that activity is affected by the fraction of specific surface area corresponding to pores with diameter greater than 150 Å. We have estimated these values for the different used supports that are reported in Table 1. By comparing supports that are similar for chemical properties but having different pores distributions, it is interesting to note a rough correlation between activity and specific surface area of the pores with diameter greater than 150 Å. Data reported in Table 2 show also that chemical characteristics of silica surface are better than those of alumina, because, two solids having quite similar physical structures (supports 1 and 3, of Table 1, having similar surface area and pore volume) show after enzyme immobilization, different activities. As a consequence, the alumina performances can be improved by modifying the chemical characteristics of the surface, by coating it, for example, with silica, as it can be seen, always in Table 2, runs 5 and 6. Since the physical properties of the supports employed in the runs 1, 3 and 5 of Table 2 were similar, the differences in their behaviours depend, very probably, on their different chemical properties, in particular the different acid and hydrophobic character of the surface.

However, the most important characteristic of the supports, for obtaining higher activities seems to be the particle size. As a matter of fact, the immobilized enzyme activity collapses with the increase in the mean particles diameter (see Table 2). An activity loss of about 90% can be observed from activity data obtained for Silica Millipore 150A in spherical pellets and powdered, respectively (runs 1 and 7 of Table 2).

This activity loss cannot easily be interpreted considering that bulky enzyme molecules are anchored only in the large pores while reagent and products molecules can hardly diffuse only through the narrower pores and are, therefore, prevented to reach the active sites. Besides, active sites could be distributed inside the macropores in a non-uniform way [9]. This situation cannot be treated, according to us, with the classical internal diffusion approach, also considering that large pores are plugged by enzyme molecules and their aggregates. Therefore, only a small fraction of the enzyme molecules resulted active, because accessible, when concentrated enzyme solutions are used in the grafting. According to Ladero et al. [8,9], in fact, in order to increase the enzyme immobilization yields very dilute solutions of enzyme must be used. We used, on the contrary, concentrated solutions with the aim to obtain saturation in grafting.

Table 3 reports the lactose conversions data found in the kinetic runs, performed in a continuous packed bed reactor, after that steady-state conditions were achieved. The values reported in Table 3 correspond to the mean value measured during 1 h of runs (mean experimental error 5%). From initial lactose concentration and conversions data it is easy to calculate the product concentrations considering the very simple stoichiometry of the reaction. Data reported in Table 3 clearly show a depressing effect of the products on the hydrolysis reaction rates (see runs 7–9 and 15–20 in Table 3). Both the products, galactose and glucose, decrease the rate of hydrolysis.

A mechanism for the enzymatic reaction, justifying the observed depressing effects of both the products can be the following:

$$\mathrm{E} + \mathrm{L} \overset{K_\mathrm{M}}{\rightleftharpoons} \mathrm{EL} \overset{k}{\rightarrow} \mathrm{E} + \mathrm{Ga} + \mathrm{Gl},$$

$$\mathrm{E} + \mathrm{Ga} \overset{K_{\mathrm{I}1}}{\rightleftharpoons} \mathrm{EGa}, \qquad \mathrm{E} + \mathrm{Gl} \overset{K_{\mathrm{I}2}}{\rightleftharpoons} \mathrm{EGl}$$

where L is the substrate (lactose), Ga and Gl are the products (galactose and glucose), E the immobilized enzyme, EL, EGa and EGl are the complexes of enzyme with substrate and products. From this scheme the following equation for the lactose hydrolysis rate (r) can be derived:

$$r = \frac{(\eta k)(W/V_\mathrm{R})C_\mathrm{L}}{K_\mathrm{M}(1 + (C_\mathrm{Ga}/K_{\mathrm{I}1}) + (C_\mathrm{Gl}/K_{\mathrm{I}2})) + C_\mathrm{L}} \qquad (1)$$

where K_M, $K_{\mathrm{I}1}$ and $K_{\mathrm{I}2}$ are equilibrium formation complexes constants (mol/dm^3), ηk the product of effectiveness factor and the kinetic constant (mol/g min), C_i the concentration of ith component (mol/dm^3), W the catalyst weight (g), V_R the reactor volume (dm^3).

Eq. (1) can be introduced in the lactose mass balance equation for a plug-flow reactor:

$$FC_\mathrm{L}^0 \, \mathrm{d}\lambda = r \, \mathrm{d}V \qquad (2)$$

where F is the feed rate (dm^3/min), C_L^0 the initial lactose concentration (mol/dm^3), λ the conversion. After

Table 4
Kinetic parameters

$(\overline{\eta k})$ mol/(g min)	$(1.2 \pm 1.2) \times 10^7 \exp[-(7687 \pm 270)/T]$
K_M (mol/dm^3)	$(4.5 \pm 21) \times 10^8 \exp[-(7045 \pm 1470)/T]$
K_{I1} (mol/dm^3)	0.42 ± 0.06
K_{I2} (mol/dm^3)	0.56 ± 0.09

the introduction of Eq. (1), Eq. (2) can be integrated and we obtain:

$$\left[K_M + \frac{C^0_{Ga}}{K_{I1}} + \frac{C^0_{Gl}}{K_{I2}} + \left(\frac{1}{K_{I1}} + \frac{1}{K_{I2}}\right) C^0_L\right] \ln(1-\lambda) - \left[1 - \left(\frac{1}{K_{I1}} + \frac{1}{K_{I2}}\right)\right] C^0_L \lambda = -\frac{(\overline{\eta k})W}{F} \quad (3)$$

where C^0_{Ga} and C^0_{Gl} are the galactose and glucose concentration (mol/dm^3), and $(\overline{\eta k})$ is the mean value of the product of effectiveness factor and the kinetic constant. Eq. (3) is a non-linear algebraic equation in λ that can easily be solved with a numerical methods such as the bisection method. The kinetic parameters of the model, determined by mathematical regression analysis [17] extended to all the data of Table 3, are reported in Table 4. The proposed model describes quite well all the performed kinetic runs (mean error 13%, correlation index 0.997), as it can be seen from Table 3. As it can be seen we have reported as apparent kinetic parameter a mean value of the product of the effectiveness factor and the kinetic constant, considering η approximately constant, because conversions are normally less than 0.40. This approximation explains the large 95% confidence limits observed for this parameter. The obtained results are not completely in agreement with those reported by other authors [7,8]. These authors, on the basis of their kinetic data, from batch runs related to an immobilized lactase from *Kluyveromices fragilis* on silica alumina, reported a depressing effect on the reaction rate only for galactose considering the effect of glucose negligible but we have demonstrated this effect by feeding glucose together with the reagent (see runs 18–20 of Table 3). The unique difference in our kinetic runs with respect to the mentioned authors is that we operated in a continuous packed bed reactor instead of a batch one, i.e. using, therefore, a more sensible device.

The activity of silica spherical pellets is relatively high, even if, the obtained activity yield (0.3%) is very low with respect to the total enzyme immobilized (about 30 mg of proteins/g). From the kinetic parameters obtained by us for the immobilized enzyme on silica pellets (Table 4) and the ones reported by Ladero et al. [8] for the immobilized enzyme on silica–alumina particles, the initial activities of the two different examined bio-catalysts have been calculated and compared. Former bio-catalyst containing about 30 mg/g of total proteins anchored, has shown an activity that is double of the one shown by the second, containing lower enzyme loading (about 2 mg of proteins/g). In this last case the low amount of loaded enzyme gives place to higher activity yield (50%) [8,9]. This suggests the opportunity of further investigations to obtain the optimisation of the catalyst preparation procedure giving place to the highest possible specific activity and yield.

Using the described model and the obtained kinetic parameters it is possible to foresee the productivity of a 1 m^3 industrial reactor, in which a 10% lactose solution with a feed rate of 13 m^3/h gives place to a conversion of 95%. As it can be seen, the activity shown by the used spherical SiO_2 pellets is already enough for industrial purposes also considering the high density of this material compared with that of polymeric supports.

4. Conclusions

In the present work we observed that β-galactosidase immobilization with covalent bonds on different oxides such as, alumina, silica and silicated alumina is possible giving place to active bio-catalysts.

The activity is strongly affected by both pore structure and chemical properties (acid–base and hydrophobicity). Silica has given better performances than alumina for a similar porous structure. Silicated alumina has shown results that are comparable with the ones obtained with silica confirming the importance of the chemical environment. The activity of the immobilized enzyme strongly decreases with the particle size. On the other hand, it is opportune to avoid the use of powdered catalysts in industrial reactors.

Spherical silica pellets of diameter 0.25–0.50 mm have shown acceptable activity in the lactose hydrolysis. For this reason we have made a kinetic study of the reaction on this catalyst in a continuous packed

bed reactor. The obtained results allowed us, first of all, to demonstrate the industrial feasibility, even if, the yield of the enzyme immobilization must be consistently improved. The reaction rates have been interpreted with a Michaelis–Menten kinetic model modified for the inhibiting effect of both the reaction products. The main depressing effect is that galactose, in agreement with the findings of other authors [8,9], but the effect of glucose is not negligible and has been evidenced for the first time in this work.

Acknowledgements

Thanks are due to Consiglio Nazionale delle Ricerche (Target Project on Biotechnology) and to Murst (Murst-Cofin 40% to Palma Parascandola No. MM05C63814) for the financial support.

References

[1] V. Gekas, M. Lòpez-Leiva, Process Biochemistry 20 (1985) 2.
[2] P.S.J. Cheetham, in: A. Wiseman (Ed.), Handbook of Enzyme Biotechnology, Horwood Limited, New York, 1985.
[3] G.F. Bickerstaff, Immobilization of Enzymes and Cells, Humana Press, Totawa, 1997.
[4] Q.Z.K. Zhou, X.D. Cheng, J. Food Eng. 48 (2001) 69.
[5] C. Carpio, P. Gonzàlez, J. Ruales, F. Batista-Viera, Food Chem. 68 (2000) 403.
[6] C. Giacomini, A. Villarino, L. Franco-Frauguas, F. Batista-Viera, J. Mol. Catal. B 4 (1998) 313.
[7] A. Santos, M. Ladero, F. García-Ochoa, Enz. Microb. Technol. 22 (1998) 257.
[8] M. Ladero, A. Santos, F. García-Ochoa, Enz. Microb. Technol. 27 (2000) 583.
[9] M. Ladero, A. Santos, F. García-Ochoa, Biotechnol. Bioeng. 72 (2001) 458.
[10] S.T. Yang, M.R. Okos, Biotechnol. Bioeng. 33 (1989) 837.
[11] A. Bodolo, E. Gomez, J.L. Gomez, J. Batista, M.F. Maximo, F. Diaz, Proc. Biochem. 26 (1991) 349.
[12] C.R. Carrara, A.C. Rubiolo, Proc. Biochem. 31 (1996) 243.
[13] FCCIV General Tests and Assays 801.
[14] P.J. Robinson, P. Dunnil, M.D. Lilly, Biochem. Biophys. Acta 242 (1971) 659.
[15] P. Iengo, M. Di Serio, V. Solinas, D. Gazzoli, G. Salvio, E. Santacesaria, Appl. Catal. A 170 (1998) 255.
[16] R.A. Messing, in: Immobilized Enzymes for Industrial Reactors, vol. 63, Academic Press, New York, 1975.
[17] G. Buzzi Ferraris, Ing. Chim. Ital. 4 (1968) 171.