MILENA G. ŽUŽA
SLAVICA S. ŠILER-MARINKOVIĆ
ZORICA D. KNEŽEVIĆ

Faculty of Technology and Metallurgy, University of Belgrade, Belgrade, Serbia

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PREPARATION AND CHARACTERIZATION OF PENICILLIN ACYLASE IMMOBILIZED ON SEPABEADS EC-EP CARRIER

This paper reports the covalent immobilization of penicillin G acylase from E. coli on Sepabeads EC–EP, an epoxy–activated polymethacrylic carrier, and describes the properties of the immobilized enzyme. Due to its versatility to mediate hydrolysis of penicillins and semi–synthetic ß–lactam antibiotics synthesis reactions, the selected enzyme belongs to a class of biocatalysts of great industrial interest. The immobilized enzyme was characterized in its pH and thermal stability and reaction kinetics. The immobilization of penicillin acylase resulted in a slightly different pH activity profile and temperature optima, indicating that the immobilization by this method imparted the structural and conformational stability to this enzyme. The immobilized enzyme also retained a high catalytic activity and showed the increased thermal stability compared with a free enzyme. By comparison of decimal reduction time values obtained at 50° C, it can be concluded that the immobilized enzyme was approximately 5–fold more stable than a free enzyme. The immobilization procedure developed is quite simple and easily reproduced, and provides a promising solution for the application of penicillin acylase for the purpose of 6–aminopenicillanic acid production.

Key words: Penicillin G acylase; Covalent immobilization; Epoxy–activated polymethacrylic carrier, Sepabeads EC–EP; Thermal stability.

Penicillin acylases (penicillin amidohydrolase; EC 3.5.1.11) catalyzes the hydrolysis of penicillins into 6–aminopenicillanic acid (6–APA) and an organic acid which depends on the type of the penicillin hydrolysed [1]. According to their preferential substrate they are classified in three sub–groups: penicillin V acylase, penicillin G acylase and ampicillin amidase [2]. Penicillin G acylase (PGA) which catalyzes the hydrolysis of penicillin G, is the most important acylase because penicillin G is the cheapest raw material for the production of 6–APA, the nucleus from which a range of semi–synthetic penicillins are made. The standard industrial practice to produce semi–synthetic ß–lactam antibiotics employs a chemical route, with protection/de–protection of reactive groups, low temperatures (–30°C or less) and organochloride solvents. Hence, their enzymatic synthesis has received great attention as a possible "green chemistry" alternative [3].

To fully exploit the technical and economical advantages of highly cost penicillin acylases, it is recommended to use them in an immobilized form [4]. The reason for this is that a free enzyme, as a biocatalyst, is lacking of long–term stability under the process conditions and is difficult to recover and recycle from the reaction mixture, making the reuse of the enzyme impossible. Hence, the idea of immobilizing the enzyme on a rigid solid support enabling an easy separation and the possibility of operation in a packed–bed or fluidized–bed reactor has been of great industrial interest for many years [5]. Of all the immobilization methods, the covalent attachment is more advantageous than others, since diffusional restrictions to the substrate or products are considerably decreased. Moreover, the formation of the rigid enzyme–carrier linkage provides both kinetic and thermodynamic stabilization of the three–dimensional structure of the active catalytic site and often improves the enzyme thermal stability.

The PGA immobilization technology has been improved in the last decades. PGA has been covalently immobilized to various carriers such as microparticulate and monolithic silica carriers [6], poly(vinyl acetate–co–diviyl benzene) beads [7], activated agarose [8], grafted nylon membranes [9] and others. However, various problems associated with these carriers were also reported. One common problem is the lack of active sites on the polymer. Consequently, most previous studies used glutaraldehyde as a non–specific cross–linking agent to fix the enzyme on the polymeric matrix, but with a low immobilization yield and a low final enzyme activity the results were often unsatisfactory. Therefore, the development of new PGA immobilization techniques using inexpensive and industrially applicable carriers is of economical importance.

Epoxy–activated carriers are almost–ideal ones to perform a very easy immobilization of enzymes at both laboratory and industrial scale. Epoxide groups are convenient for the covalent binding of enzymes, since they are able to directly react with amino, hydroxyl or sulfhydryl groups of enzymes depending on pH of the

Author address: M.G. Žuža, Faculty of Technology and Metallurgy, Karnegijeva 4, University of Belgrade, 11000 Belgrade, Serbia
E–mail: milenazuza@yahoo.com
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buffer used. Thus, it is not necessary to activate the carrier or the enzyme to achieve the covalent immobilization. Moreover, the N–C, O–C or S–C bonds formed by the epoxide groups are extremely stable, so that the epoxide–containing commercial polymers such as Eupergit® or Sepabeads can be successfully used for the immobilization of enzymes and proteins [10,11].

Sepabeads EC–EP is a commercial polymethacrylic carrier presenting suitable characteristics as a support for enzyme immobilization. It is very stable and has good chemical, mechanical and other properties such as a hydrophilic nature, a wide pore distribution and almost ideal spherical beads, a simple immobilization procedure, a high protein binding capacity, a low swelling tendency in a high molar solution and, in common solvents, a high flow rate in column procedures, an excellent performance in stirred batch reactors, etc. Although a number of studies have shown that epoxide–containing polymers are good carriers for the enzyme immobilization, their potential for binding penicillin acylases has not been fully explored. In a previous paper we investigated the optimal condition for covalent immobilization of penicillin G acylase from *Escherichia coli* on Sepabeads EC–EP [12]. In this paper, the immobilized enzyme was characterized by evaluating the potential effects of immobilization on its pH and thermal stability and reaction kinetics, especially in comparison with a free enzyme. Finally, mathematical models for the enzyme thermal inactivation and penicillin G hydrolysis are reported.

EXPERIMENTAL

Materials

Penicillin G acylase (E.C. 3.5.1.11) from *Escherichia coli* (PGA) was a gift from DSM (The Netherlands). The enzyme was a crude preparation with the specific activity of 167 U mg^{-1} protein and 90% (w/v) protein based on Lowry's method for protein assay [13]. Sepabeads EC–EP (particle sizes 200–240 μm, average pore diameter 30–40 nm, specific volume 2.8–3.3 cm^3/dry g) was kindly donated by Resindion S.R.L. (Mitsubishi Chemical Corporation, Milan, Italy). 6–Aminopenicillanic acid (6–APA), penicillin G, *p*–dimethylaminobenzaldehyde (PDAB), Folin Ciocalteu's Phenol Reagent were purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals were reagent–grade.

Immobilization of penicillin G acylase

As previously described [12], the enzyme immobilization on Sepabeads EC–EP carrier involves a direct enzyme binding on polymers via epoxide groups (Figure 1). Unmodified Sepabeads EC–EP (500 mg of wet carrier) was incubated with 10 cm^3 of the native enzyme attachment solution in 1.25 M potassium phosphate buffer (7.5 U/g wet support, pH 8.0). The immobilization was carried out at 25°C in a shaking water bath (130 strokes per min). After the 48h incubation, the beads were collected by the vacuum filtration using a glass filter (Whatman), washed with 1 M NaCl (3x20 cm^3) and the potassium phosphate buffer (pH 8.0; 3x20 cm^3), and stored in it at 4°C until use.

The efficiency of immobilization was evaluated in terms of the enzyme and activity coupling yields. The enzyme coupling yield, Y_E(%) and the activity coupling yield, Y_A(%) were calculated as follows:

$$Y_E(\%) = (P_1/P_0)x100 \quad (1)$$

$$Y_A(\%) = (SA_2/SA_1)x100 \quad (2)$$

where P_1 is the amount of the immobilized enzyme; P_0 the initial amount of the enzyme; SA_1 and SA_2 are specific activities of free and immobilized penicillin acylase, respectively.

The amount of the bound enzyme was determined indirectly from the difference between the amount of the enzyme introduced into the coupling reaction mixture and the amount of the enzyme in the filtrate and in the washing solutions. The amount of the bound enzyme per g of dry Sepabeads was calculated by considering that 3.93 g of a wet polymer correspond to 1 g of a dry support (experimentally determined water retention in the polymer was 74.56%).

Enzyme activity assay

The enzyme activity of penicillin acylase was determined by measuring the penicillin G enzymatic product 6–APA spectrophotometrically [14]. One unit of penicillin acylase was defined as the amount of the enzyme required to produce 1 μmol of 6–APA per minute under the assay conditions (4% w/v penicillin G as a substrate solvated in 0.1 M phosphate buffer, pH 7.92, at 37°C). The amount of 6–APA was determined with the PDAB method. The experimental procedures were as follows: firstly, the solutions of 20% v/v of acetic acid (A), 0.5% w/v of NaOH (B) and 0.5% (w/v) of PDAB in methanol (C) were prepared; secondly, A,B and C were added to 0.5 ml of the sample solution in

Figure 1. Schematic illustration of the covalent method for the enzyme immobilization on Sepabeads EC–EP carrier

sequence according to the volume ratio 2:1:0.5; finally, after the 20 minute reaction at room temperature, the absorbance value of the solution was determined at 415 nm.

Kinetics assay

Kinetic parameters of the free and immobilized enzyme were determined by measuring the enzyme activity with various substrate concentrations (0.25 to 4% of penicillin G in 0.1 M phosphate buffer, pH 7.92). The enzyme concentration was constant (0.1 g of the immobilized enzyme or a correspondent amount of the free enzyme). All data are the averages of duplicate samples and were reproducible within ±5% of accuracy. Mean and standard deviation of the results from at least two independent experiments were calculated by using Microsoft Excel (Redmond, WA, USA) software.

Assuming Michaelis–Menten type reaction kinetics, the apparent Michaelis–Menten constant, K_m, and the maximum apparent initial rate, V_{max} of the free and immobilized enzyme were calculated directly from the model by nonlinear regression analysis using the MATLAB software (version 6.5, Release 13, The MathWorks, Juc, Matick, MA, USA). The extent of mass–transfer control could be expressed by the effectiveness factor, EF (considered as the ratio between maximum rates of the reaction catalyzed by immobilized and free penicillin acylase under the same reaction conditions).

pH and temperature profile

The effect of pH on a free and immobilized enzyme was studied by assaying the preparations at different pH values in the range of 4.5 to 9.3 at 37°C. The assays were allowed to take place in 0.1 M potassium phosphate buffers. The buffers were prepared by mixing different volumes of aqueous solutions of K_2HPO_4, KH_2PO_4 and H_3PO_4. The titration solutions were KOH (0.625 M) for pH 5.5; 6.3; 7.3; 7.9; 8.7 and 9.3 and H_3PO_4 (0.1 M) for pH 4.5. The effect of the temperature on the activities of free and immobilized penicillin acylase was also determined at temperatures of 8 to 75°C under the assay conditions (4% (w/v) penicillin G as substrate solvated in 0.1 M phosphate buffer, pH 7.92).

Thermal stability assays

The thermal stability assays were performed at 50°C in an aqueous medium (100 mM phosphate buffer, pH 7.92) by using the equivalent number of the activity units of biocatalysts (free and immobilized penicillin acylase on a solid support). Biocatalysts were dissolved in the buffer and incubated in a constant temperature water bath. The samples were taken at different times and the residual activity was determined taking an unheated control to be 100% active.

Kinetic inactivation model

In this study, the model considering two first–order enzyme deactivation steps has been used to describe the experimental thermal deactivation data for both free and immobilized enzymes [15]. In the model, the transition of a fully active native enzyme, *E* to a final enzyme form, E_2 with or without residual activity, can be represented schematically as:

$$E \xrightarrow[\alpha_1]{k_1} E_1 \xrightarrow[\alpha_2]{k_2} E_2$$

where k_1 and k_2 are the first–order deactivation rate constants and α_1 and α_2 are the ratio of specific activities E_1/E and E_2/E in %, respectively. This mechanism leads to a model, where the residual enzyme activity at time *t* can be determined by the following equation:

$$A = A_1 \exp(-k_1 t) + A_2 \exp(-k_2 t) + A_3 \quad (3)$$

$$A = [100 + (\alpha_1 k_1)/(k_2 - k_1) - (\alpha_2 k_2)/(k_2 - k_1)]\exp(-k_1 t) + [(\alpha_2 k_1)/(k_2 - k_1) - (\alpha_1 k_1)/(k_2 - k_1)]\exp(-k_2 t) + \alpha_2 \quad (4)$$

The one–step deactivation model can be obtained from the two–step model, considering that the final enzyme form is E_1, and that $k_2 = 0$. In the proposed kinetic model, the adjustable parameters are rate constants k_1 and k_2, and the enzyme residual activity, α_1 and α_2 which were determined by the least–squares fits to the experimental data by using the MATLAB software.

RESULTS AND DISCUSSION

Characterization of the immobilized enzyme

The method for the penicillin acylase immobilization on Sepabeads EC–EP involves a direct enzyme binding on polymers via epoxy groups. In the previous paper, this procedure has been reported to yield an immobilized enzyme with a high specific activity under the optimal condition.[12] In other words, the activity coupling yield has been shown to depend on the enzyme concentration in the attachment solution, and the maximum yield was achieved by working at a low enzyme concentration. Thus, in the current work, the enzyme attachment solution containing 7.5 U/g wet support was used and about $35.6 \cdot 10^{-3}$ mg of the pure enzyme was immobilized per g of wet Sepabeads (0.14 mg per g of dry polymer) with an enzyme coupling yield of 79.1%. The specific activity of the immobilized enzyme was around 150 U/mg enzyme bound which corresponds with an activity coupling yield of 89.4%.

It is important to know if the immobilization on Sepabeads via epoxy groups would have any effects on the enzyme and its process performance. The basic characteristics of an immobilized enzyme include its specific activity, thermal and pH stability as well as kinetic properties. Thus, in this paper, a comparative study between free and immobilized penicillin acylase is provided in terms of the reaction kinetics, thermal

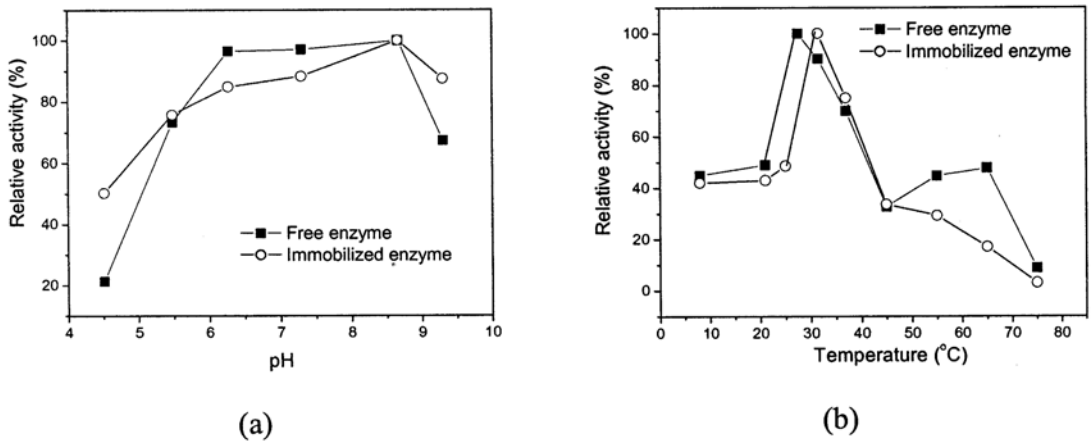

Figure 2. Effects of pH (a) and temperature (b) on the activities of free and immobilized penicillin acylase. The enzyme activity was determined under the assay conditions (4% (w/v) penicillin G as substrate solvated in 0.1 M buffer at indicated pH and temperature).

stability and the activity profile as a function of pH and temperature. Penicillin G hydrolysis for the production of 6–APA was chosen as the model reaction system.

The effect of immobilization on optimum pH and temperature

The activity of the immobilized penicillin acylase was investigated in the pH range 4.5–9.3 at 37°C and compared with a free one. The results are presented in Figure 2a.

The pH activity profile of the immobilized enzyme was slightly narrower than that of the free enzyme without having much effect on pH optima. The trends of the curves are similar in both cases. This similar behavior is related to the electrically neutral character of the carrier, which did not alter the pH of the immobilized enzyme microenvironment. However, the immobilized enzyme shows a higher activity at lower (pH<5.5) and higher pH values (pH>8.7) than the soluble one, indicating that the immobilization of the penicillin acylase imparted structural and conformational stability to this enzyme.

The effect of temperature on the activities of the free and immobilized enzyme was also studied and the results are shown in Figure 2b. It appears that the optimal reaction temperature shifted from 27.5°C for the free penicillin acylase to 31.5°C for the immobilized enzyme, suggesting better thermal stability of the immobilized enzyme.

Effect of immobilization on the enzyme thermal stability

When evaluating an immobilized enzyme system for industrial application, it is important to know about the enzyme inactivation. Moreover, a proper kinetic deactivation model and kinetic parameters are necessary for the design and the operation of an enzyme reactor. Therefore, the thermal stability of the immobilized enzyme was studied at 50°C in an aqueous

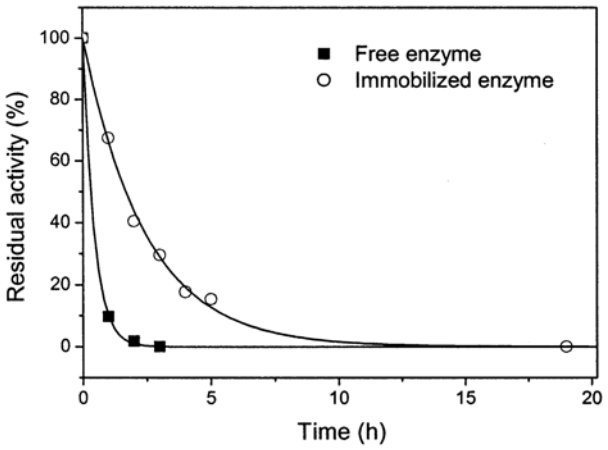

Figure 3. Thermal inactivation of free and immobilized enzymes at 50°C in an aqueous medium (100 mM phosphate buffer, pH 7.92). The lines represent the best fits of the two steps first–order kinetic deactivation model. Starting activities were taken as 100%.

medium (100 mM phosphate buffer, pH 7.92) and compared with that of the free one. The results are presented in Figure 3.

The results show that the immobilization of the enzyme on Sepabeads EC–EP offers a high thermo protection. For example, the immobilized enzyme treated at 50°C for 2 h still held a significant activity of around 40%, whereas the free enzyme completely lost its original activity at this condition. This fact is of real significance in commercial applications.

In order to interpret and analyze the obtained experimental results, the model based on the two–step first–order degradation kinetics was used to describe the deactivation curves, Eq. (4). The points on the graph are experimental data and the solid lines represent the best fits of theoretical model predictions. It seems that the first–order enzyme degradation kinetics fits experimental data well for a soluble enzyme, suggesting that the native enzyme loses its activity in only one step ($\alpha_1 = 0$,

Table 1 Best–fit parameters of the two–step deactivation model, equation (4), for free and immobilized penicillin acylase at 50°C (100 mM phosphate buffer, pH 7.92)

Biocatalyst	k_1, h^{-1}	k_2, h^{-1}	α_1	α_2 (A_3)	D^*, h	F^{**}
Free enzyme	2.32	0	100	0	1.14	1.0
Immobilized enzyme	0.41	0.05	99.8	0.06	5.88	5.16

*D is decimal reduction time (time required for one log10 reduction in the activity, i.e. 90% reduction in the activity)
**F is stabilization factor (considered as the ratio between decimal reduction time of immobilized and free enzymes)

k_2 = 0) at 50°C. However, the first–order model somewhat underestimated the residual activity of the immobilized enzyme at long times, implying a biphasic mechanism of inactivation. The best–fit values of deactivation rate constants (k_1 and k_2), enzyme residual activities, (α_1 and α_2) and decimal reduction time, *D* are listed in Table 1.

The comparison of kinetic parameters demonstrated that the immobilization process has enhanced the thermo stability of penicillin acylase. At 50°C, the immobilized enzyme degraded with a rate constant almost six times lower than the soluble enzyme. Thus, it appears that the immobilization results in not only an altered inactivation kinetics, but also in an improved.

Kinetic properties

The reaction kinetics of immobilized penicillin acylase was compared with that of the free enzyme using penicillin G hydrolysis as a model reaction. The initial reaction rates for free and immobilized penicillin acylase were determined at different concentrations of substrate ranging from 0.25 to 4% and the results are shown in Figure 4. Although the reaction may be too complex to be described by the simplified Michaelis–Menten kinetic model, considering that the most penicillin acylases are inhibited by their products, the model was used to compare the experimental results obtained for immobilized enzyme to those obtained for free enzyme. The values of kinetic parameters of free and immobilized enzyme are obtained directly by fitting the experimental data to the kinetic model.

The nonlinear regression analysis indicated that the quality of the fit was quite good, with r^2 value of 0.960 and 0.949 for free and immobilized enzyme, respectively. The K_m value for the free enzyme was determined to be 5.99±1.46 mmol dm^{-3}, whereas V_{max} value was 128.98 ±5.37 μmol min^{-1} mg^{-1}. The apparent value of K_m for the immobilized enzyme was 29.85 ±8.7 mmol dm^{-3}, approximately 5–fold higher than that of the free enzyme, suggesting significant mass transfer limitations in the immobilized enzyme system. Similarly, the value of V_{max} for the immobilized enzyme seems to be approximately 9–fold lower than that of the free enzyme (V_{max}=14.65±1.55 μmol min^{-1} mg^{-1}; EF=0.11). However, as the exact concentration of PGA in the immobilized enzyme system is unknown, the calculation of k_{cat} from the obtained values of V_{max} is not possible. Thus, the comparison of efficiencies of free and immobilized enzyme systems is futile. The increase in K_m values for immobilized enzymes has also been reported by various authors [7,16]. Wang et al.[16] reported that, owing to the influence of the support, the K_m value of immobilized penicillin acylase was 7–fold higher than that of the free enzyme. These changes in kinetic parameters may be a consequence of either a structural change in the enzyme occurring upon

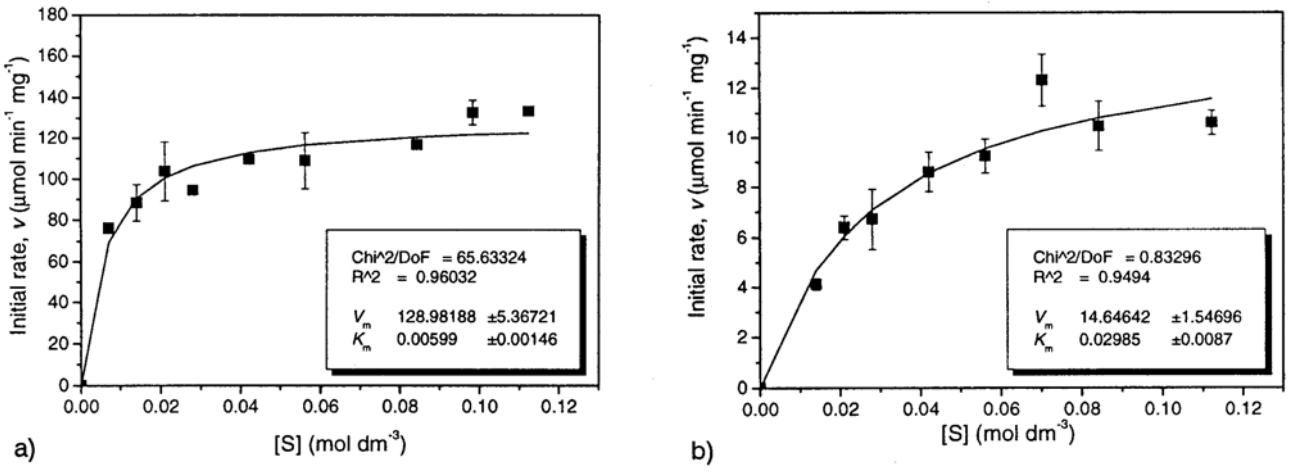

Figure 4. The effect of penicillin G concentration on initial rates of free (a) and immobilized penicillin acylase (b). The lines represent the best fits of the Michaelis–Menten kinetic model. Assay conditions: t=37°C; 0.1 M phosphate buffer, pH 7.92; 0.1 g immobilized enzyme or a correspondent amount of free penicillin acylase.

immobilization, or lower accessibility of the substrate to the active sites of the immobilized enzyme. Moreover, these results suggest that the mass transfer rate strongly participated in the overall reaction rate catalyzed by the immobilized enzyme.

CONCLUSION

An approach is presented for the stable covalent immobilization of penicillin G acylase from *E. coli* on Sepabeads EC–EP with a high retention of hydrolytic activity. Penicillin G hydrolysis for the production of 6–APA was chosen as the model reaction system. The immobilization procedure developed is quite simple and easily reproduced, and involves the direct enzyme binding on polymers via epoxide groups.

It appears that penicillin G acylase from *E. coli* has been successfully immobilized on Sepabeads EC–EP using the covalent binding method. Immobilization has been shown to affect the pH and temperature profile of the enzyme activity. The optimal pH for the immobilized enzyme activity was found to be 8.7. A slightly higher value for optimum temperature (31.5°C) was found for the immobilized enzyme in comparison with that displayed by the free one (27.5°C). The study of thermal stability at 50°C revealed that the immobilization produces an appreciable stabilization of the biocatalyst, changing its thermal deactivation profile. The immobilized enzyme appears to have favorable kinetic properties in the industrially feasible reaction system of penicillin G hydrolysis. The method for the enzyme immobilization is simple, inexpensive, and scaleable for industrial applications. Thus, Sepabeads EC–EP can be used as a low cost carrier for highly active and stable biocatalyst.

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NOMENCLATURE

A_1 – parameter of the fitted exponential decay in Eq [3] (%)

A_2 – parameter of the fitted exponential decay in Eq [3] (%)

D – decimal reduction time (h)

E, E_1 and E_2 – enzyme forms having different specific activities

EF – effectiveness factor as the ratio between maximum apparent initial rate of immobilized and free enzymes

F – stabilization factor as the ratio between decimal reduction time of immobilized and free enzymes

k_1 – first–order deactivation rate constant for the first step in Eqs [3] and [4](h^{-1})

k_2 – first–order deactivation constant for the second step in Eqs [3] and [4] (h^{-1})

K_m – apparent Michaelis–Menten constant (mmol dm^{-3})

P_1 – amount of the immobilized enzyme (mg)

P_0 – initial amount of enzyme (mg)

SA_1 – specific activity of free penicillin acylase (IU mg^{-1} enzyme)

SA_2 – specific activity of immobilized penicillin acylase (IU mg^{-1} enzyme)

t – time (h)

V_{max} – maximum apparent initial catalytic rate (μmol min^{-1} mg^{-1})

Y_A – activity coupling yield (%)

Y_E – enzyme coupling yield (%)

GREEK LETTERS

α_1 – ratio of enzyme specific activity E_1/E (%)

α_2 – ratio of enzyme specific activity E_2/E (%)

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