Enzyme and Microbial Technology 47 (2010) 268–276

Contents lists available at ScienceDirect

Enzyme and Microbial Technology

journal homepage: www.elsevier.com/locate/emt

Evaluation of the incidence of diffusional restrictions on the enzymatic reactions of hydrolysis of penicillin G and synthesis of cephalexin

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ARTICLE INFO

Article history:
Received 15 March 2010
Received in revised form 9 July 2010
Accepted 12 July 2010

Keywords:
Penicillin acylase
Diffusional restrictions
Penicilllin G
Cephalexin ;β-Lactam antibiotics

ABSTRACT

The impact of mass transfer limitations on penicillin G acylase immobilized in glyoxyl-agarose particles of different sizes and enzyme loads was evaluated for the reactions of hydrolysis of penicillin G and synthesis of cephalexin under the hypothesis that the impact of internal diffusional restrictions on the catalytic potential of the enzyme will be of a greater magnitude for a fast reaction of hydrolysis than a slower reaction of synthesis. Experimental evidences were obtained from batch reactor operation where increase in enzyme load and particle size has a much stronger impact on the former reaction. Additional evidences were obtained by the impact of the above biocatalyst properties on the apparent Michaelis constants for substrates. Michaelis constants of penicillin G acylase catalysts of different enzyme load and particle sizes varied between 0.73 and 4.55 mM and between 3.0 and 11.1 mM for the hydrolysis of penicillin G and the synthesis of cephalexin respectively. Michaelis constants for penicillin G acylase biocatalysts subjected to progressive size reduction were reduced from 5.0 to 0.46 mM and from 7.2 to 2.1 mM for hydrolysis of penicillin G and synthesis of cephalexin respectively. Higher fluctuation between these values in hydrolysis of penicillin G reflects higher impact of diffusional restrictions on this reaction. Thièle modulus for the substrate was much higher for penicillin G hydrolysis than for cephalexin synthesis in a wide range of substrates concentrations. Ratio between moduli for hydrolysis of penicillin G and synthesis of cephalexin was 15 at saturating concentration of phenylglycine methyl ester and increased at lower concentrations of such substrate. Results highlight the importance of designing the biocatalyst according to the reaction in which it will be used, being particularly important in the case of penicillin G acylase that is currently being used both in reactions of hydrolysis and synthesis.

1. Introduction

Penicillin G acylase (penicillin amido hydrolase, E.C. 3.5.1.11) (PGA) is a remarkably versatile enzyme [1,2] that has been used for a long time in the large scale production of 6-aminopenicillanic acid (6-APA) as an intermediate for the synthesis of derived penicillins [3,4] and, more recently, for the synthesis of derived penicillins and cephalosporins like amoxicillin and cephalexin [5,6]. This is an outstanding feature, since the same enzyme providing the β-lactam nucleus, be it 6-APA or 7-amino-3-deacetoxy cephalosporanic acid (ADCA) can, under appropriate conditions, catalyze the reverse reactions of synthesis of the corresponding β-lactam antibiotics from those nuclei and appropriate side-chain precursors [7]. Enzymatic synthesis of β-lactam antibiotics can be conducted under thermodynamic [8] or kinetic control [9]; the latter, despite being more complex [10], is a better strategy because substrate conversion is not restrained by reaction equilibrium and higher productivities are attained [11]. According to this mechanism, the nucleophile must be bound to the acyl–enzyme complex before the acyl group is transferred to the nucleophile [12]. In both strategies of synthesis the use of non-conventional (non-aqueous) media is favourable by displacing the equilibrium towards synthesis in the first strategy [13] or by selectively depressing the competing hydrolytic reactions in the second [14,15]. Many strategies have been proposed to reduce water activity to favour synthesis over hydrolysis; organic cosolvents has been the most thoroughly studied [16–18], but the use of greener solvents like ionic liquids [19,20] or aqueous medium at high substrate concentrations [21–23] are preferred options from the standpoint of green chemistry.

Immobilization of PGA is an absolute prerequisite for its use as a biocatalyst in the reactions of hydrolysis and synthesis and there is ample information on PGA immobilization [24]. Glyoxyl-agarose immobilized PGA catalysts are suitable for scientific and

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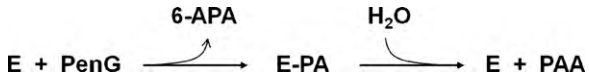
doi:10.1016/j.enzmictec.2010.07.010

technological purposes because of the multicovalent but not distorting attachment that provides significant enzyme stabilization and good mechanic properties [25]. Reactivity of the glyoxyl groups with non-ionized amino groups is high, so the enzyme is bound to the support very rapidly at pH 10 by the ε-amino group of one of its lysine residues. Nevertheless, more contact time is needed for the correct alignment of the enzyme and the establishment of multiple covalent enzyme-support bonds that are required for the proper stabilization of the enzyme structure. The bonds formed are very weak Schiff's bases whose energy of formation does not compensate the energy required for the production of a significant distortion of the protein structure, keeping the protein functionality [26]. Many enzymes have been immobilized on this support with high recovery of activity and high stabilization factors [26]. PGA has been immobilized in glyoxyl-agarose with 70% of recovered enzyme activity and stabilization factors (ratio of half-life of the biocatalyst to the half-life of the free enzyme) as high as 8000 have been obtained [26]. These properties make such catalysts adequate for the hydrolysis and synthesis of β-lactamic antibiotics [27], better results being obtained when compared to other good PGA catalysts such as CLEAs [28]. Regular spherical geometry of glyoxyl-agarose particles makes this support adequate for the systematic study of diffusional restrictions, since biocatalyst shape can be accurately described and particle size distribution determined by simple microscopic imaging and digital analysis. This is a major issue, since it allows a rigorous analysis of mass transfer limitations, which is desirable for the development of a reaction-diffusion model [29]. It is recognized that mass transfer limitations that affect immobilized PGAs is a major hurdle for process improvement [24] but, despite this, not much information is available in the open literature dealing with this crucial aspect of PGA biocatalysts performance [29,30]. Theory about mass transfer limitations in biocatalysts has been proposed by Shuler et al. [31] and Engasser and Horvath [32]. According to the theory, mass transfer reduces the efficiency of the catalyst and increases the values of the observed Michaelis–Menten constant (K_M). Enzyme catalyzed reactions in heterogeneous systems are complex because of the high number of steps and parameters involved in the reaction mechanism, even without considering thermal inactivation and pH dependence. Substrates and products profiles are generated inside the biocatalyst particles as a consequence of mass transfer limitations, reducing reaction rates because of substrate depletion and the accumulation of potentially inhibitory products. Effect of pH on enzyme activity is more significant if pH gradients are present inside the catalyst. PGA biocatalysts are exposed to pH gradients because of the generation of protons in the reactions of antibiotic hydrolysis and synthesis. pH gradients will affect enzyme activity and stability, so a buffered medium is needed to avoid these effects [33]. However, it has been reported that pH gradients do not develop at buffer concentrations over 50 mM [33]. Phosphate buffer species not only arrest H^+ ions, but act as H^+ transporters from the catalyst matrix to the liquid bulk minimizing the dynamic pH gradients [34]. Phosphate species have higher diffusion rates than substrates and products of reaction so that pH gradients inside the biocatalyst are avoided.

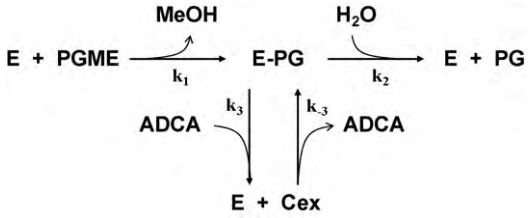
In this article, the impact of mass transfer limitations on PGA immobilized in glyoxyl-agarose particles of different sizes and enzyme loads is evaluated both for the reactions of hydrolysis of penicillin G and the kinetically controlled synthesis of cephalexin (Cex). Reaction mechanism for hydrolysis of penicillin G has been presented by Kheirolomoom et al. [35]. Substrate and product inhibition were observed and a rate equation (Eq. (1)) was validated for the following mechanism.



$$v = \frac{k \cdot E_0 \cdot S}{K\left[1+(S/K)+(S^2/KK_S)+(P_1/K_1)+(P_2/K_2)+(S \cdot P_2/K \cdot K_2)+((P_1/K_1)\cdot(P_2/K_2))\right]} \quad (1)$$

where E is penicillin acylase, PenG is penicillin G, 6-APA is 6-aminopenicillanic acid, E-PA is acyl–enzyme complex formed by penicillin acylase and phenylacetate and PAA is phenylacetic acid.

Schroën et al. [10,11] proposed a simplified reaction scheme for the kinetic controlled synthesis of Cex:



The reactions take place simultaneously during synthesis as seen in the reaction scheme above, where E is penicillin acylase, PGME is phenylglycine methyl ester, MeOH is methanol, E-PG is the acyl-enzyme complex, ADCA is 7-amino-3-deacetoxicephalosporanic acid, Cex is cephalexin and PG is phenylglycine.

Rate equations are summarized as follows:

Synthesis of Cex

$$v_S = \frac{k_1 k_3 \cdot E_0 \cdot \mathrm{PGME} \cdot \mathrm{ADCA}}{k_1 \cdot \mathrm{PGME} + k_2' + k_3 \cdot \mathrm{ADCA} + k_{-3} \cdot \mathrm{Cex}} \quad (2)$$

Hydrolysis of Cex

$$v_H = \frac{k_2' k_{-3} \cdot E_0 \cdot \mathrm{Cex}}{k_1 \cdot \mathrm{PGME} + k_2' + k_3 \cdot \mathrm{ADCA} + k_{-3} \cdot \mathrm{Cex}} \quad (3)$$

Hydrolysis of PGME

$$v_E = \frac{k_1 k_2' \cdot E_0 \cdot \mathrm{PGME}}{k_1 \cdot \mathrm{PGME} + k_2' + k_3 \cdot \mathrm{ADCA} + k_{-3} \cdot \mathrm{Cex}} \quad (4)$$

Mass balance equations for substrates inside PGA catalyst include mass transfer modelled by Fick's first law of diffusion. The constant k_2' corresponds to k_2 multiplied by the water molar concentration.

$$\frac{\partial S}{\partial t} = D_{eS} \cdot \left(\frac{\partial^2 S}{\partial r^2} + \frac{2}{r} \cdot \frac{\partial S}{\partial r}\right) - \frac{k \cdot E_0 \cdot S}{K\left[1+(S/K)+(S^2/KK_S)+(P_1/K_1)+(P_2/K_2)+(S \cdot P_2/K \cdot K_2)+((P_1/K_1)\cdot(P_2/K_2))\right]} \quad (5)$$

$$\frac{\partial \mathrm{PGME}}{\partial t} = D_{eA} \cdot \left(\frac{\partial^2 \mathrm{PGME}}{\partial r^2} + \frac{2}{r} \cdot \frac{\partial \mathrm{PGME}}{\partial r}\right) - \frac{k_1 k_3 \cdot E_0 \cdot \mathrm{PGME} \cdot \mathrm{ADCA}}{k_1 \cdot \mathrm{PGME} + k_2' + k_3 \cdot \mathrm{ADCA} + k_{-3} \cdot \mathrm{Cex}} \quad (6)$$

$$\frac{\partial \mathrm{ADCA}}{\partial t} = D_{eB} \cdot \left(\frac{\partial^2 \mathrm{ADCA}}{\partial r^2} + \frac{2}{r} \cdot \frac{\partial \mathrm{ADCA}}{\partial r}\right) - \frac{k_1 k_3 \cdot E_0 \cdot \mathrm{PGME} \cdot \mathrm{ADCA}}{k_1 \cdot \mathrm{PGME} + k_2' + k_3 \cdot \mathrm{ADCA} + k_{-3} \cdot \mathrm{Cex}} \quad (7)$$

The hypothesis underlying this work is that the impact of internal diffusional restrictions on the catalytic potential of the enzyme will be of a greater magnitude for the case of hydrolysis (fast reaction) than for the case of synthesis of β-lactam antibiotics (slower reaction), being in both cases strongly dependent on particle size and enzyme load. Conditions for both reactions were selected from previous works, where optimal temperature and pH for each reaction were experimentally determined [36,37]. Low substrate (sub-optimal) concentrations were used to appreciate the impact of diffusional restrictions more clearly.

2. Materials and methods

2.1. Materials

PGA from *Escherichia coli*, with 400 ± 20 IU_H/mL and 16.6 ± 1 mg/mL protein, was a gift from Antibióticos S.A. (León, Spain). Cross-linked 6% agarose spherical beads (Sepharose 6B-CL) was a product from GE Healthcare (Uppsala, Sweden), used as raw material for fractionation. Penicillin G potassium salt was kindly provided by Natsus S.A. (Lima, Perú); 6-APA, phenylacetic acid (PAA), (R)-(−)-2-phenylglycine methyl ester hydrochloride 97% pure (PGME) and Cex hydrate were from Sigma (St Louis, MO, USA); ADCA was kindly provided by Antibióticos S.A; (R)-(−)-2-phenylglycine (PG) was from Aldrich (Milwaukee, WI, USA). All other reagents were of analytical grade and purchased either from Sigma or Merck (Darmstadt, Germany).

2.2. Analyses

Initial reaction rates of penicillin G hydrolysis were determined, at different concentrations in 100 mM sodium phosphate buffer pH 7.8 and 30 °C, using a pH-stat (Mettler Toledo, DL50) to titrate the H^+ produced by the hydrolysis of penicillin G as it is converted into PAA; 50 mM NaOH was employed as titrant solution [38]. One international unit of penicillin G activity (IU) was defined as the amount of enzyme that hydrolyzes 1 μmole of penicillin G per minute from 10 mM penicillin G solution under the above conditions. Protein was determined according to Bradford's method [39], immobilized protein being determined by difference between offered protein and protein remaining in solution. Substrates and products of hydrolysis of penicillin G and synthesis of cephalexin were identified and analyzed by HPLC using a Jasco delivery system PU-2089plus with a Jasco UV 2075 UV–Vis detector and a LC-NetII/ADC Jasco HPLC/PC integrator. The column used was a Kromasil C_{18} (150 mm × 4.6 mm) from Análisis Vínicos (Madrid, España). Samples for penicillin G hydrolysis assays were eluted with a sonicated mixture of 30% acetonitrile and 70% 10-mM phosphate buffer pH 3.0 at a flow rate of 1 mL/min, and analyzed in the UV detector at 220 nm. Elution times were 1.3, 6.0 and 7.4 min for 6-APA, PAA and penicillin G respectively. Samples for cephalexin synthesis assays were eluted with a sonicated mixture of 10% acetonitrile and 90% 10-mM phosphate buffer pH 6.0 at a flow rate of 1 mL/min, and analyzed in the UV detector at 220 nm. Elution times were 1.6, 1.9, 3.3 and 7.4 min for ADCA, PG, Cex and PGME respectively. Concentration of substrates and products were calculated from calibration curves using stock solutions. HPLC samples were always assayed in duplicate, differences among them never exceeding 3%.

2.3. Gel fractionation

Sepharose 6B-CL beads were sieved obtaining a fine fraction of particles of diameter lower than 63 μm (R30), an intermediate fraction of particles of diameter between 125 and 63 μm (R50), and a coarse fraction of particles of diameter higher than 125 μm (R72). An extra-coarse fraction of particles of diameter higher than 250 μm (R140) was also obtained and used solely for the determination of the effective diffusion coefficients (Section 2.5). Particle size distributions were determined by image analysis using the software Image Tool v2.0.

2.4. Preparation of glyoxyl-agarose immobilized penicillin G acylase biocatalysts

Glyoxyl-agarose immobilized PGA biocatalysts were prepared by multi-point covalent attachment of the enzyme, through ε-amino groups of lysine, to glyoxyl-agarose gel beads of Sepharose 6B-CL, as previously described [37], using the fine (R30) and coarse (R72) fractions of glyoxyl-agarose, numbers referring to the mean radius of particle size distribution. Enzyme loads for both fractions were 150, 400 and 650 IU/g, which cover the range of actual industrial PGA biocatalysts [40]. In this way, six different biocatalysts were prepared as indicated in Table 2. To check the time-course of enzyme immobilization, hydrolytic activity over the artificial substrate NIPAB was measured to the supernatant. Direct quantification of the immobilized enzyme activity after the process was determined by titration of the phenylacetic acid produced by hydrolysis of PenG. Immobilized protein was determined by difference between loaded and residual protein in the supernatant.

2.5. Evaluation of mass transfer limitations with immobilized penicillin G acylase biocatalysts

In order to study the effect of internal (intraparticle) diffusional restrictions (IDR) on enzyme kinetics, conditions were established to make external (film) diffusional restrictions (EDR) negligible. As previously reported for similar catalysts, EDR became negligible at agitation speeds higher than 400 rpm, where initial reaction rates were not affected in a wide range of substrates concentrations [41]. Further experiments were conducted at 600 rpm to neatly evaluate the effect of IDR.

To test the hypothesis that IDR has a stronger impact on the hydrolysis of PenG (fast reaction) than on the synthesis of Cex (slower reaction), a set of experiments was designed consisting in batch reactions of hydrolysis (at 10 mM PenG) and synthesis (at 15 mM ADCA and 45 mM PGME) with all PGA biocatalysts. Reactor operation was designed to have the same enzyme activity in each case, meaning different masses of biocatalysts according to their different specific activities (IU/g_{cat}) shown in Table 2. Reactors operated at controlled conditions of pH, temperature and agitation speed. Operating conditions for hydrolysis of PenG and synthesis of Cex were pH 7.8 and 30 °C and pH 7.4 and 14 °C respectively, corresponding to their previously determined optimum values [23,36].

To further support the hypothesis, apparent Michaelis constant (K_M^{app}) of all PGA biocatalysts were determined from initial reaction rate data, at varying substrate(s) concentration both for the hydrolysis of PenG and the synthesis of Cex. Experiences were done at the corresponding pH and temperatures indicated above. For the reaction of PenG hydrolysis, initial reaction rates at varying PenG concentrations were determined. For the case of Cex, initial reaction rates of Cex synthesis were determined at varying ADCA concentrations (up to 30 mM) and constant PGME at 100 mM concentration to focus on ADCA mass transfer limitations. Other evidence in favour of the hypothesis was built by conducting both reactions with PGA biocatalysts previously subjected to mechanical rupture to reduce particle size. As the size of the biocatalyst particle is reduced, it is expected to become less affected by IDR. Values of K_M^{app} were evaluated as a function of rupture time, the experiments being done with PGA650R50 (intermediate fraction) subjected to mechanical attrition by high speed stirring (>1000 rpm) with magnetic bar. Finally, the hypothesis was tested by evaluating the Thièle moduli of all biocatalysts in the reactions of hydrolysis of Pen G and synthesis of Cex. Intrinsic kinetic parameters were conventionally determined from initial rate data and non-linear regression to the corresponding rate equations. PenG hydrolysis by PGA is subjected to competitive inhibition by PAA and non-competitive inhibition by 6-APA [36] and kinetic parameters were determined accordingly. Kinetically controlled synthesis of Cex was considered to proceed according to the mechanism proposed by Schroën et al. [42] and the kinetic parameters were determined based on that mechanism. To do so initial reaction rates of synthesis of Cex and hydrolysis of PGME (reaction in the absence of ADCA) were determined at varying substrate concentrations. Diffusion coefficients for substrates and products of the reactions of hydrolysis and synthesis were determined by measuring effusion rates according to the method proposed by Grünwald [43].

3. Results and discussion

3.1. Gel fractionation

A statistical analysis of particle size distribution for all agarose fractions used was made which is presented in Table 1. Fine and coarse fractions were used to assess the impact of particle size on IDR. Intermediate fraction was used for evaluating the impact of biocatalyst grinding on K_M^{app} (Section 3.3.2). Extra-coarse fraction was used for the determination of effective diffusion coefficients (Section 3.3.3).

3.2. Immobilization of penicillin G acylase at different enzyme loads in glyoxyl-agarose particles of different sizes

Biocatalyst were prepared with enzyme loads of 150, 400 and 650 IU/g using the fine ($d < 63$ μm) and coarse ($d > 125$ μm) fractions of glyoxyl-agarose, being designated as R30 and R72 respectively in reference to the mean radius of that distribution (see Table 1). Results of enzyme immobilization are summarized in Table 2. Protein immobilization yield was in all cases higher than enzyme immobilization yield in terms of activity. In fact, most of the offered protein was immobilized, but the amount of immobilized enzyme activity expressed in the biocatalyst varied according to the amount of enzyme offered and the biocatalyst particle size. Unbound enzyme increased with enzyme load and particle size. In all cases, immobilization of enzyme was completed in 3 h and that contact time was used, being long enough to ensure the homoge-

Table 1
Particle size distribution of glyoxyl-agarose fractions. Dimensions for diameter (d) and radius are in (μm).

	Particle size				
	Fine fraction $d < 63$ (R30)	Intermediate fraction $63 < d < 125$ (R50)	Coarse fraction $d > 125$ (R72)	Extra-coarse fraction $d > 250$ (R140)	Non-fractionated
Nr. of particles	195	362	114	115	–
Mean radius	30.0	48.1	71.6	141.1	48.5
Standard deviation	5.1	13.0	10.1	41.4	16.2
Minimum radius	12.6	14.2	47.6	54.0	12.6
Maximum radius	40.5	79.2	91.1	264.1	91.1
Median value	30.2	47.2	72.6	132.6	46.8
Equivalent radius R_e*	32.4	58.9	75.7	168	63.8

* Radius of the particle representing 50% of the cumulative volume of the particles sampled.

Table 2
Immobilization of PGA in glyoxyl-agarose of different particle size distributions (R30 and R72) at different enzyme loads (150, 400 and 650 IU/g_{cat}).

Catalyst	Expressed specific activity (IU/g_{cat})	Expressed activity (% of offered)	Immobilized protein (mg/g_{cat})	Immobilized protein (% of offered)
PGA150R30	131	87,3	7.8	96
PGA400R30	321	80.2	20.1	96
PGA650R30	499	76.8	31.3	97
PGA150R72	116	77.3	7.0	91
PGA400R72	262	65.5	19.8	95
PGA650 R72	344	52.9	34.0	97

neous distribution of the enzyme within the biocatalyst particle. Homogeneous distribution is a reasonable assumption since this time is higher than the one required for the enzyme protein to diffuse within the support.

3.3. Evaluation of the impact of internal diffusional restrictions on immobilized penicillin G acylase

3.3.1. Batch hydrolysis of penicillin G and synthesis of cephalexin

Reactor operation, both for the hydrolysis of penicillin G and synthesis of Cex at their optimum conditions, was conducted with all biocatalysts. Reactor operation for the hydrolysis of penicillin G is presented in Fig. 1 for R30 and R72 biocatalysts respectively at all three enzyme loads. Differences among operation curves are bigger for R72 than R30 biocatalysts meaning that the effect of enzyme load is stronger for larger size particles. Reactor operation for the synthesis of Cex is presented in Fig. 2 for R30 and R72 biocatalysts respectively at all three enzyme loads. Up to the point of maximum yield of conversion there are no significant differences with respect to enzyme loads both for R30 and R72 biocatalysts; particle size does not have a significant effect either. These results suggest that the impact of IDR is higher in the reaction of hydrolysis of

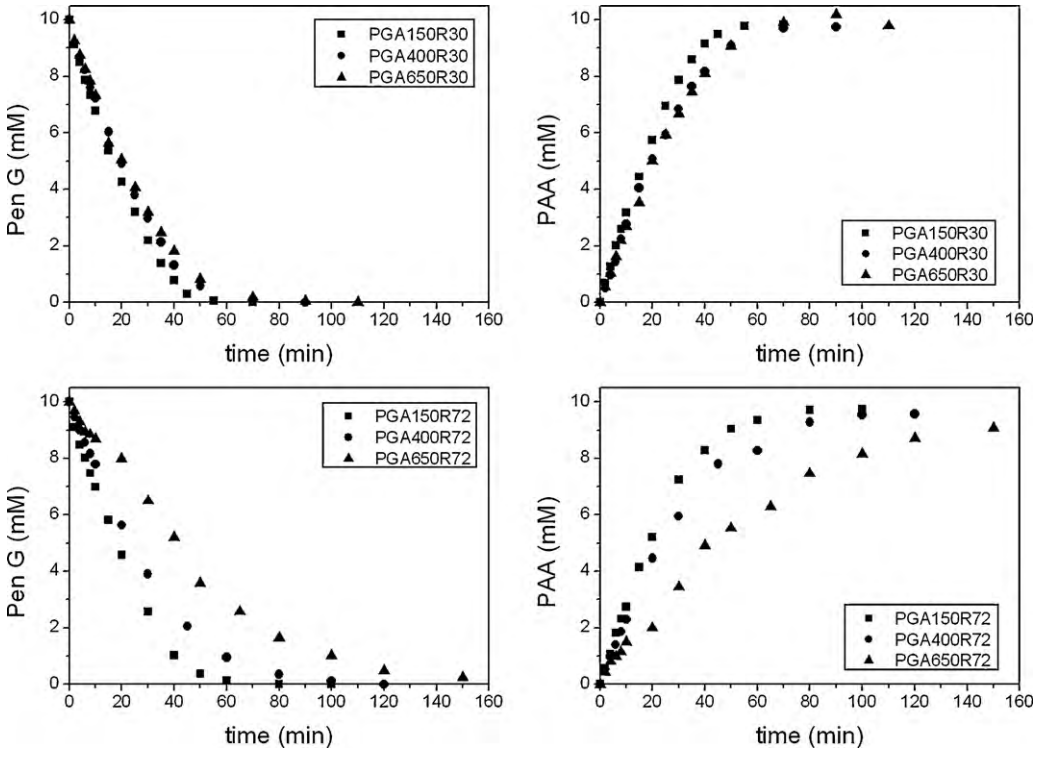


Fig. 1. Operation curves of batch reactor for the hydrolysis of penicillin G (PenG) with biocatalysts of different sizes (R30 and R72) and enzyme loads (150, 400 and 650 IU/g_{cat}), showing PenG and PAA profiles.

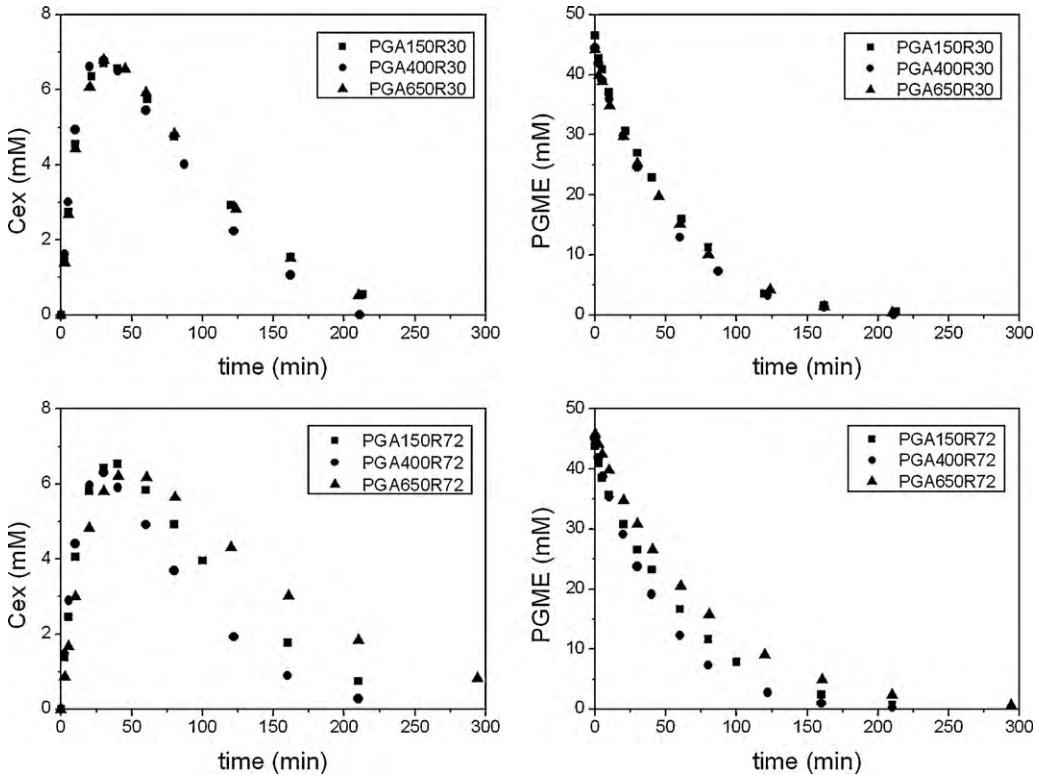


Fig. 2. Operation curves of batch reactor for the synthesis of Cex with biocatalysts of different sizes (R30 and R72) and enzyme loads (150, 400 and 650 IU/g_{cat}), showing Cex and PGME profiles.

penicillin G than in the reaction of synthesis of Cex, which supports the hypothesis. However, substrates concentrations are not comparable so that it is necessary to evaluate the incidence of IDR independently from substrates concentrations.

3.3.2. Determination of apparent Michaelis–Menten constants (K_M^{app}) for substrates in the reactions of hydrolysis of penicillin G and synthesis of cephalexin

Apparent K_M of an immobilized enzyme (K_M^{app}) is the value of K_M (Michaelis–Menten constant) determined under the influence of mass transfer limitation and should then increase according to the magnitude of IDR. So, to further support the hypothesis, K_M^{app} of all PGA biocatalysts were determined from initial reaction rate data at varying substrates concentration. Results for the reaction of hydrolysis of penicillin G and synthesis of Cex are presented in Fig. 3A and B respectively. Values of K_M^{app} were determined by non-linear regression to the Michaelis–Menten curve and are summarized in Table 3. Results indicate that, as expected, K_M^{app} values increase with particle size and enzyme load both for the reaction of hydrolysis of penicillin G and synthesis of Cex. However, the effect of IDR is stronger in the former case as can be appreciated also in Table 3, where the ratio of K_M^{app} with respect to the biocatalyst less affected by IDR (PGA150R30) is presented for both reactions, being that ratio always higher for the hydrolysis of PenG than for the synthesis of Cex. This further validates the hypothesis that the fast reaction of hydrolysis of penicillin G is more affected by IDR than the slower reaction of Cex synthesis.

Values of K_M^{app} were then evaluated on progressively ruptured biocatalyst particles to examine the different impacts for the reaction of hydrolysis of penicillin G and synthesis of Cex. Initial rates of reaction with the ruptured biocatalysts are presented in Fig. 4A and B for the reactions of hydrolysis of penicillin G and synthesis of Cex respectively. An increase in normalized reaction rate (v_0/V_{max}) and a decrease in K_M^{app} is observed with respect to rupture

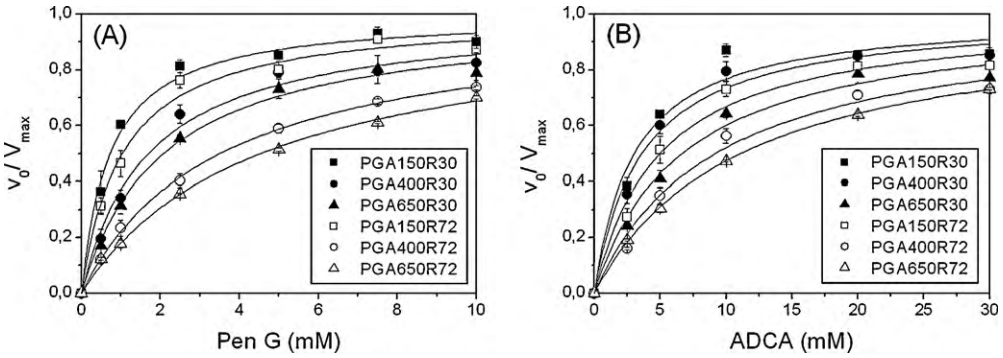


Fig. 3. (A) Normalized initial reaction rates of penicillin G (PenG) hydrolysis (v_0/V_{max}) at varying PenG concentrations with PGA biocatalysts of different sizes and enzyme loads; (B) normalized initial reaction rates of cephalexin synthesis (v_0/V_{max}) at varying ADCA concentrations and 100 mM PGME. Symbols correspond to data points; lines correspond to the respective fitted Michaelis–Menten equation.

Table 3
Values of K_M^{ap} of PGA catalysts of different particle size and enzyme loads in the reaction of hydrolysis of penicillin G at pH 7.8 and 30 °C and synthesis of cephalexin (Cex) at pH 7.4 and 14 °C.

Catalyst	K_M^{ap} (mM)		$K_M^{ap}/K_{M\ PGA150R30}^{ap}$	
	Hydrolysis of penicillin G	Synthesis of Cex	Hydrolysis of penicillin G	Synthesis of Cex
PGA150R30	0.73	3.03	1	1
PGA400R30	1.70	3.61	2.33	1.19
PGA650R30	2.09	6.66	2.74	2.19
PGA150R72	1.04	4.98	1.43	1.64
PGA400R72	3.52	9.2	4.82	3.04
PGA650R72	4.55	11.1	6.23	3.66

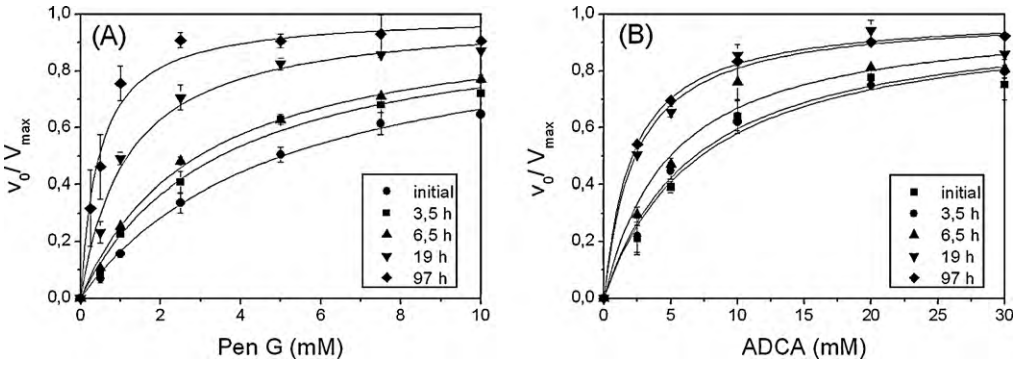


Fig. 4. (A) Normalized initial reaction rates of penicillin G (PenG) hydrolysis (v_0/V_{max}) at varying PenG concentrations for mechanically ruptured PGA650R50 biocatalyst; (B) normalized initial reaction rates of cephalexin synthesis (v_0/V_{max}) at varying ADCA concentrations and 100 mM PGME for mechanically ruptured PGA650R50 biocatalyst. Symbols correspond to data points; lines correspond to the respective fitted Michaelis–Menten equation.

Table 4
Values of K_M^{app} of PGA catalyst of intermediate size and high enzyme load (PGA650R50) as a function of mechanical rupture time in the reaction of hydrolysis of penicillin G at pH 7.8 and 30 °C and synthesis of Cex at pH 7.4 and 14 °C. $K_{M,0}^{app}$ is the value of the intact biocatalysts.

Rupture time (h)	K_M^{app} (mM)		$K_M^{app}/K_{M,0}^{app}$	
	Hydrolysis of penicillin G	Synthesis of Cex	Hydrolysis of penicillin G	Synthesis of Cex
0	5.04	7.23	1	1
3.5	3.48	6.79	0.690	0.939
6.5	2.95	4.95	0.585	0.685
19	1.18	2.35	0.234	0.325
97	0.465	2.12	0.092	0.293

time. Values of K_M^{app} were determined by non-linear regression to the Michaelis–Menten curve and are summarized in Table 4. The magnitude of variation is different for each reaction as can be appreciated from the values of $K_M^{app}/K_{M,0}^{app}$ whose relative variation with rupture time is more pronounced for the hydrolysis of penicillin G than for the synthesis of Cex. This implies that the incidence of IDR is higher in the former giving additional support to the hypothesis.

3.3.3. Determination of the Thièle modulus of all biocatalysts in the reactions of hydrolysis of penicillin G and synthesis of cephalexin

Thièle modulus is a parameter whose magnitude reflects the impact of IDR in any catalyzed reaction. Intrinsic kinetic parameters and effective diffusion coefficients of all reacting species are required to determine the corresponding Thièle moduli.

It was assumed that the intrinsic kinetic parameters of immobilized PGA correspond to those of the soluble enzyme; this assumption has been sustained [29,42] and it is acceptable as long as no significant configurational effects arise as a consequence of immobilization, which is the case for glyoxyl-agarose immobilized enzymes as a consequence of the open pore structure of the support and its geometric congruence with the enzyme [25,29,44]. Values of intrinsic kinetic parameters are summarized in Table 5.

Effective diffusion coefficients inside glyoxyl-agarose particles (D_e) for all substrates and products of the reactions of hydrolysis of penicillin G and synthesis of Cex were determined by measuring effusion rates of all species from saturated glyoxyl-agarose particles. Results are summarized in Table 6 and compared to the diffusion coefficient of the corresponding species in solution (D_0), estimated according to the equation proposed by Vorlop [43]. Results obtained for D_e correlate well with the molar mass of each substance and their values differ only slightly from the correspond-

Table 5
Intrinsic kinetic parameters for the reactions of hydrolysis of penicillin G and synthesis of cephalexin with penicillin G acylase, according to reaction mechanism in Refs. [9–11,29,34].

Species	Parameter	Value	Units
Enzyme	k	2460	min^{-1}
PenG	K	0.13	mM
	K_S	821	mM
PAA	K_1	1.82	mM
6-APA	K_2	48	mM
Acyl–enzyme complex formation from acyl donor	k_1	56.5	$(mM\,min)^{-1}$
Nucleophilic attack of acyl–enzyme complex by water (hydrolysis)	k'_2	3407	$(min)^{-1}$
Nucleophilic attack of acyl–enzyme complex by ADCA	k_3	101.1	$(mM\,min)^{-1}$
Acyl–enzyme complex formation from cephalexin	k_{-3}	14,3	$(mM\,min)^{-1}$

Table 6
Effective diffusion coefficients (D_e) for substrates and products of the reactions of penicillin G hydrolysis and cephalexin synthesis at 30 °C and pH 7.8. D_0 is the diffusion coefficient in water at the same conditions (30 °C and pH 7.8) estimated according to the equation of Vorlop [42]. Values in parenthesis are the values at 14 °C and pH 7.4 corresponding to the conditions of Cex synthesis.

Substance	$D_e \times 10^{10}$ (m^2/s)	$D_0 \times 10^{10}$ (m^2/s)	D_e/D_0
PAA	7.33	7.72	0.95
6-APA	5.89	6.38	0.92
PenG	5.30	5.34	0.99
PGME	5.97 (5.65)	7.13 (6.75)	0.84
ADCA	6.03 (5.71)	6.41 (6.07)	0.94
Cex	5.37 (5.09)	5.25 (4.98)	1.02
PG	6.00 (5.68)	7.39 (7.00)	0.81

ing D_0, which was expected because of the open pore structure and high hydrophilicity of the support.

Thiële modulus for the penicillin G in the reaction of hydrolysis of penicillin G with PGA (Φ) is deduced by adimensionalization of Eq. (5) and expressed as:

$$\frac{\partial s}{\partial \tau} = \left(\frac{\partial^2 s}{\partial \rho^2} + \frac{2}{\rho}\cdot\frac{\partial s}{\partial \rho}\right) - R^2 \cdot \frac{k \cdot E_0}{K \cdot D_{eS}} \cdot \frac{s}{[1 + s + ss' + p + q + sq + pq]} \quad (8)$$

$$\frac{\partial s}{\partial \tau} = \left(\frac{\partial^2 s}{\partial \rho^2} + \frac{2}{\rho}\cdot\frac{\partial s}{\partial \rho}\right) - 9\Phi^2 \cdot \sigma \quad (9)$$

$$\Phi = \frac{R}{3}\cdot\sqrt{\frac{k \cdot E_0}{K \cdot D_{eS}}} \quad (10)$$

where R is the particle radius and E_0 is the total enzyme concentration and σ is the dimensionless reaction rate. The equivalent radius (R_e, as defined in Table 1) is a sound representation of the size distribution in a given population of biocatalyst particles [29].

The synthesis of Cex is a two-substrate reaction so that in principle two Thiële moduli can be proposed, one for each substrate. In this case, the resulting rate equations are conveniently transformed to Michaelis–Menten type equations before defining the moduli. Thiële modulus obtained for each substrate is in this case apparent in the sense that it is dependent on the concentration of the other reacting species. Thiële modulus for PGME (Φ_A) and ADCA (Φ_B) may be deduced from Eqs. (6) and (7), respectively, and expressed as:

$$\frac{\partial \mathrm{PGME}}{\partial t} = D_{eA} \cdot \left(\frac{\partial^2 \mathrm{PGME}}{\partial r^2} + \frac{2}{r}\cdot\frac{\partial \mathrm{PGME}}{\partial r}\right) - \frac{V'_A \cdot \mathrm{PGME}}{K'_A + \mathrm{PGME}} \quad (11)$$

Table 7
Thiële moduli of PGA catalysts for penicillin G in the reaction of hydrolysis of penicillin G (Φ) and for PGME (Φ_A) and ADCA (Φ_B) in the reaction of synthesis of cephalexin at PGME 45 mM; ADCA 15 mM. E_0, enzyme load; R_e, equivalent radius (see Table 1).

Catalyst	E_0 (mM)	R_e (μm)	Φ	Φ_A	Φ_B
PGA150R30	0.0532	32.4	1.92	0.06	0.09
PGA400R30	0.1303	32.4	3.01	0.09	0.14
PGA650R30	0.2030	32.4	3.75	0.11	0.17
PGA150R72	0.0470	75.7	4.22	0.12	0.19
PGA400R72	0.1064	75.7	6.35	0.19	0.29
PGA650R72	0.1399	75.7	7.28	0.21	0.34

$$\frac{\partial a}{\partial \tau} = \left(\frac{\partial^2 a}{\partial \rho^2} + \frac{2}{\rho}\cdot\frac{\partial a}{\partial \rho}\right) - R^2 \cdot \frac{V'_A}{K'_A \cdot D_{eA}} \cdot \frac{a}{1+a} \quad (12)$$

$$\frac{\partial a}{\partial \tau} = \left(\frac{\partial^2 a}{\partial \rho^2} + \frac{2}{\rho}\cdot\frac{\partial a}{\partial \rho}\right) - 9\Phi_A^2 \cdot \sigma_A \quad (13)$$

$$\Phi_A = \frac{R}{3}\cdot\sqrt{\frac{k_3 \cdot E_0 \cdot \mathrm{ADCA}}{D_{eA} \cdot K_A[1 + (\mathrm{Cex}/K_P) + (\mathrm{ADCA}/K_B)]}} \quad (14)$$

$$\frac{\partial \mathrm{ADCA}}{\partial t} = D_{eB} \cdot \left(\frac{\partial^2 \mathrm{ADCA}}{\partial r^2} + \frac{2}{r}\cdot\frac{\partial \mathrm{ADCA}}{\partial r}\right) - \frac{V'_B \cdot \mathrm{ADCA}}{K'_B + \mathrm{ADCA}} \quad (15)$$

$$\frac{\partial b}{\partial \tau} = \left(\frac{\partial^2 b}{\partial \rho^2} + \frac{2}{\rho}\cdot\frac{\partial b}{\partial \rho}\right) - R^2 \cdot \frac{V'_B}{K'_B \cdot D_{eB}} \cdot \frac{b}{1+b} \quad (16)$$

$$\frac{\partial b}{\partial \tau} = \left(\frac{\partial^2 b}{\partial \rho^2} + \frac{2}{\rho}\cdot\frac{\partial b}{\partial \rho}\right) - 9\Phi_B^2 \cdot \sigma_B \quad (17)$$

$$\Phi_B = \frac{R}{3}\cdot\sqrt{\frac{k_1 \cdot E_0 \cdot \mathrm{PGME}}{D_{eB} \cdot K_B[1 + (\mathrm{Cex}/K_P) + (\mathrm{PGME}/K_A)]}} \quad (18)$$

where V'_A and V'_B are maximum apparent reaction rates of synthesis of Cex at saturating concentrations of PGME and ADCA respectively, K_A, K_B and K_P are Michaelis-type constants of PGME, ADCA and Cex respectively, K'_A and K'_B are apparent Michaelis-type constants of PGME and ADCA respectively, D_{eA} and D_{eB} are effective diffusion coefficients of PGME and ADCA respectively and a and b are dimensionless concentrations of PGME and ADCA respectively.

Values of Thiële modulus for the reaction of hydrolysis of penicillin G and synthesis of Cex are presented in Table 7 that shows that the former are significantly higher than the latter at the substrates concentrations considered. As seen in Fig. 5, if the concentration of substrates (PGME and ADCA) is increased, Φ/Φ_A and Φ/Φ_B (Eqs. (10), (14) and (18)) keep always significantly higher than 1 in the whole range of possible ADCA and PGME concentrations up to the limit of tractability of the system (around 800 mM PGME). This

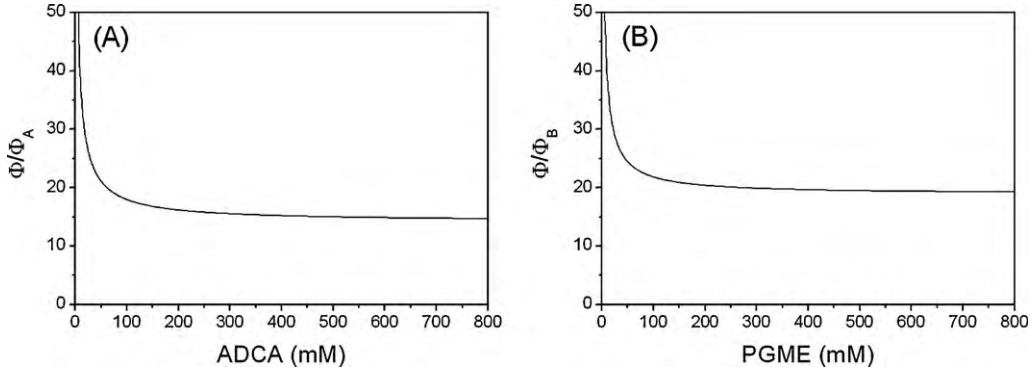


Fig. 5. (A) Ratio of Thiële modulus of penicillin G in the reaction of hydrolysis of penicillin G (Φ) to Thiële modulus for the substrate PGME (Φ_A) at varying ADCA concentrations in the reaction of synthesis of cephalexin; (B) ratio of Thiële modulus of penicillin G in the reaction of hydrolysis of penicillin G (Φ) to Thiële modulus for the substrate ADCA (Φ_B) at varying PGME concentrations in the reaction of synthesis of Cex.

observation further supports the hypothesis. Results suggest that an immobilized enzyme not well suited for performing hydrolysis reactions because of severe mass transfer limitations may be adequate for performing the slower reaction of synthesis. This is particularly important in the case of PGA which is a dual enzyme performing both hydrolytic and synthetic reactions.

4. Conclusions

The impact of mass transfer limitations in immobilized PGA in glyoxyl-agarose proved to be strongly dependent on enzyme load and particle size. However its impact is quite different for the reaction of hydrolysis of penicillin G than for the reaction of synthesis of Cex. This is supported by experimental evidence in the performance of batch reactors with PGA biocatalysts of different loads and particle sizes both in the hydrolysis of penicillin G and in the synthesis of Cex. In the former, particle size and enzyme load have a significant effect on reactor performance while in the latter the effect is mild.

Further evidences of the different impact of mass transfer limitations in both reactions were obtained by determining K_M^{app} for substrates. K_M^{app} varied significantly with particle size and enzyme load for penicillin G in the reaction of hydrolysis, but not so for ADCA in the reaction of synthesis. In addition, when the PGA biocatalysts were subjected to particle size reduction by mechanical rupture, K_M^{app} decrease was much more pronounced in the former case.

Thièle moduli for substrates in both reactions were determined from the values of the intrinsic kinetic parameters and effective diffusion coefficients. Thièle moduli were significantly higher for the substrate in the reaction of penicillin G hydrolysis than for each of the substrates in the reaction of Cex synthesis in a wide range of substrates concentrations. Even though the impact of mass transfer limitations can be reduced by working at high substrate concentrations, as is usual in industrial processes, as reaction proceeds concentrations will drop making the impact of mass transfer limitations significant even under such conditions, because industrial production also requires high substrate conversions.

Results obtained reflect the higher sensitivity to mass transfer limitations in a fast reaction of hydrolysis as compared to a slower reaction of synthesis with immobilized hydrolases. This suggests that enzyme biocatalysts should be designed according to the characteristics of the reaction in which they will be used. This is quite important in the case of PGA, traditionally used as a hydrolase for producing intermediate antibiotic precursors, but now important as well as a catalyst for performing reactions of synthesis of β-lactam moieties and other relevant organic compounds.

Acknowledgements

This work was funded by Grant 1080122 from Fondecyt, Chile. The authors wish to thank Ms. Rosa Arrieta for her valuable analytical support.

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