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RESEARCH ARTICLE

Diffusional restrictions in glyoxyl-agarose immobilized penicillin G acylase of different particle size and protein loading

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Abbreviations: 6-APA: 6-aminopenicillanic acid
EDR: external diffusional restrictions
IDR: internal diffusional restrictions
LP: large particles size
PAA: phenylacetic acid
PGA: penicillin G acylase
SP: small particles size

Particle size and enzyme protein loading are design parameters of enzyme immobilization affecting biocatalyst performance that can be varied within broad margins. Their effect on mass transfer limitations at different bulk penicillin G concentrations has been studied with glyoxyl agarose immobilized penicillin G acylase biocatalysts of average particle size of $5 \cdot 10^{-5}$m and $10 \cdot 10^{-4}$m at protein loadings from 15 to 130 mg/g_{gel}. Internal diffusional restrictions were evaluated for such biocatalysts: Thiele modulus varied from 1.17 for the small particles at the lower protein load to 5.84 for the large particles at the higher protein load. Effectiveness factors at different bulk substrate concentrations were determined for all biocatalysts, values ranging from 0.78 for small particle size at 25 mM penicillin G to 0.15 for large particle size at 2 mM penicillin G. Enzyme protein loading had a strong impact on the effectiveness factors of immobilized penicillin G acylase, being it more pronounced in the case of large particle size biocatalysts. At conditions in which 6-aminopenicillanic acid is industrially produced, all biocatalysts tested were mass-transfer limited, being this information valuable for reactor design and performance evaluation.

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Penicillin G acylase (penicillin amidohydrolase; E.C. 3.5.1.11) is used as a bulk enzyme in the production of 6-aminopenicillanic acid (6-APA) by hydrolysis of penicillin G or V (Shewale and Sudhakaran, 1997; Parmar et al. 2000) and more recently in the synthesis of 6-APA and 7-amino-desacetoxycephalosporanic acid (7-ADCA) derived semi-synthetic penicillins and cephalosporins (Illanes and Wilson, 2006; Du et al. 2009; Pchelintsev et al. 2009). Biocatalyst engineering has been a central issue in penicillin acylase biocatalysis (Kallenberg et al. 2005; Chandel et al. 2008); substantial improvements in penicillin acylase stabilization have been obtained by directed immobilization to solid supports (Basso et al. 2003; Montes et al 2007; Sun et al. 2009), aggregation (Rajendhran and Gunasekaran, 2007), site-directed mutagenesis and directed evolution (Rajendhran and Gunasekaran, 2004; Serra et al. 2009), and functional screening (Gabor et al. 2005). Immobilization is the most powerful approach for increasing operational stability and multi-point covalent attachment to activated agarose gels is one of the most effective systems, being extensively used for immobilizing penicillin G acylase (Mateo et al. 2005). We have optimized the protocol for penicillin G acylase (PGA) immobilization in glyoxyl-agarose by multi-point covalent attachment, selecting the best protecting agent and optimizing the time of immobilization for increased stability (Illanes et al. 2003). However, despite all the reports on immobilized penicillin acylase, few have been specifically devoted to evaluate mass transfer limitations that certainly play a major role in biocatalyst performance (van Roon et al. 2006; Gonçalves et al. 2008). Most kinetic parameters reported are actually apparent and likely to vary significantly according to reactor operation conditions (Schroën et al. 2002). In this work we have evaluated mass-transfer limitations on glyoxyl agarose (gel spherical beads) immobilized *E. coli* PGA as a catalyst for the hydrolysis of penicillin G, which is a fast reaction, being a good system to test their influence on biocatalyst performance. The effect of protein loading (amount of immobilized enzyme protein per unit mass of support), particle size (average diameter) and bulk substrate (penicillin G) concentration was studied on biocatalyst performance under mass transfer limitations. This information is useful to determine how immobilization parameters affect biocatalyst performance and highlights the importance of considering them with respect to the end-use of the enzyme.

MATERIALS AND METHODS

Materials

PGA from *Escherichia coli*, with 40 ± 2 IU/mg protein was a gift from Antibióticos S.A. (León, Spain). The enzyme was centrifuged and dialysed prior to use and remained fully stable for more than a year stored at 5ºC. Cross-linked 6% agarose spherical beads (Sepharose 6B-CL) was a product from GE Healthcare (Uppsala, Sweden). Penicillin G potassium salt was kindly provided by Natsus S.A. (Lima, Perú); 6-APA and phenylacetic acid (PAA) were from Sigma (St Louis, MO, USA). All other reagents were

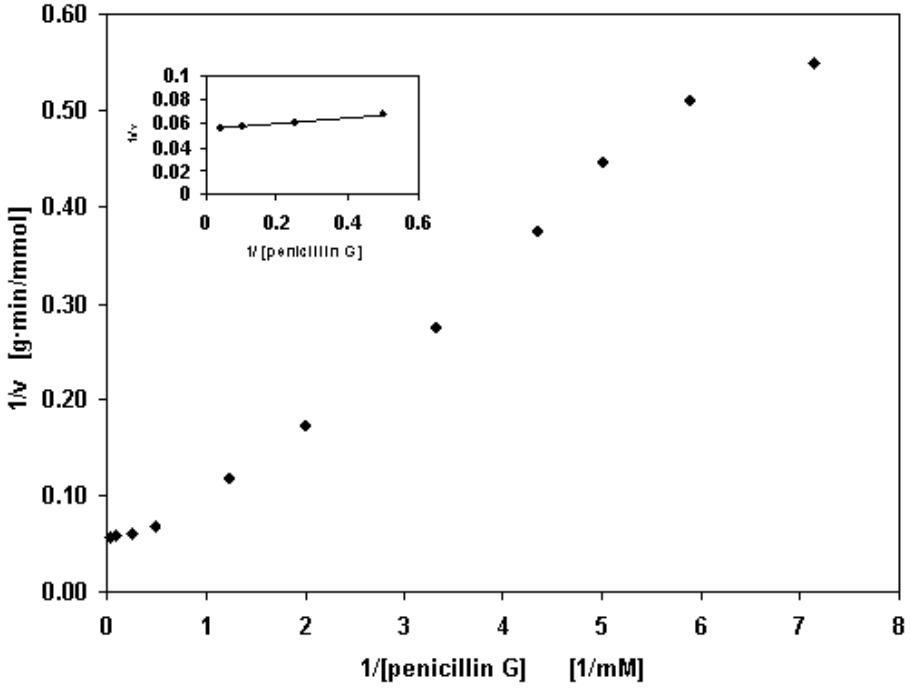

Figure 1. Lineweaver-Burk plot for immobilized penicillin acylase (SP1). v: initial reaction rate of penicillin G hydrolysis (mmoles/min·$g_{protein}$); Insert is an amplification of the zone at high substrate concentrations where a linear relationship is observed that allows determining the intrinsic parameters of the enzyme.

Table 1. Protein immobilization yield of large and small particle (LP and SP) size Sepharose 6B-CL immobilized penicillin G acylase at different protein loadings.

Biocatalyst	Loaded protein ($g\ g_{gel}^{-1}$)	Immobilized protein ($g\ g_{gel}^{-1}$)	Protein immobilization yield (%)
LP1	0.016	0.0154	97
LP2	0.035	0.0341	98
LP3	0.054	0.053	98
LP4	0.073	0.0691	95
LP5	0.092	0.0824	90
LP6	0.13	0.0912	70
SP1	0.016	0.0154	97
SP2	0.035	0.0341	98
SP3	0.054	0.0529	98
SP4	0.073	0.0698	96
SP5	0.092	0.0843	92
SP6	0.13	0.0893	69

of analytical grade and purchased either from Sigma or Merck (Darmstadt, Germany).

Analyses

Initial reaction rates of penicillin G hydrolysis were determined, at different penicillin G concentrations in 100 mM sodium phosphate buffer pH 7.8 and 30ºC, 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 (Wilson et al. 2009). The amount of H^+ produced can be easily converted into the amount of hydrolyzed penicillin G according to the stoichiometry of the reaction. One international unit of PGA activity (IU) was defined as the amount of enzyme that hydrolyzes 1 µmole of penicillin G per min from 10 mM penicillin G solution under the above conditions. Specific reaction rates were determined per mg of protein, protein being assayed according to Bradford (1976).

Gel fractionation

Sepharose 6B-CL spherical beads, as obtained, have a Gaussian-type size distribution ranging from 45 to 165 µm in diameter. Beads were screened obtaining three fractions: large particles of diameter higher than 100 µm (LP); medium particles of diameter between 100 and 65 µm, and small particles of diameter smaller than 65 µm (SP). The intermediate size fraction was discarded and work was done with the large and small size fractions. Particle size distributions were determined by image analysis using the software Image Tool v2.0.

Enzyme immobilization

Glyoxyl agarose was prepared as previously described (Illanes et al. 2005). PGA was immobilized by multi-point covalent attachment in glyoxyl-agarose gel beads of Sepharose 6B-CL, based on a reported procedure, but using PAA instead of penicillin G sulfoxide as protecting agent during immobilization (Alvaro et al. 1990). Different protein loadings were contacted both for LP and SP as shown in Table 1. The immobilized PGA biocatalysts were stored as wet gels at 5ºC. No enzyme inactivation or leakage was detected during prolonged storage. Density of biocatalysts was 960 g/L.

Determination of intrinsic kinetics and mass transfer parameters

Kinetic parameters for the free PGA and intrinsic kinetic parameters for the immobilized biocatalysts were determined by linearization of initial reaction rate data at

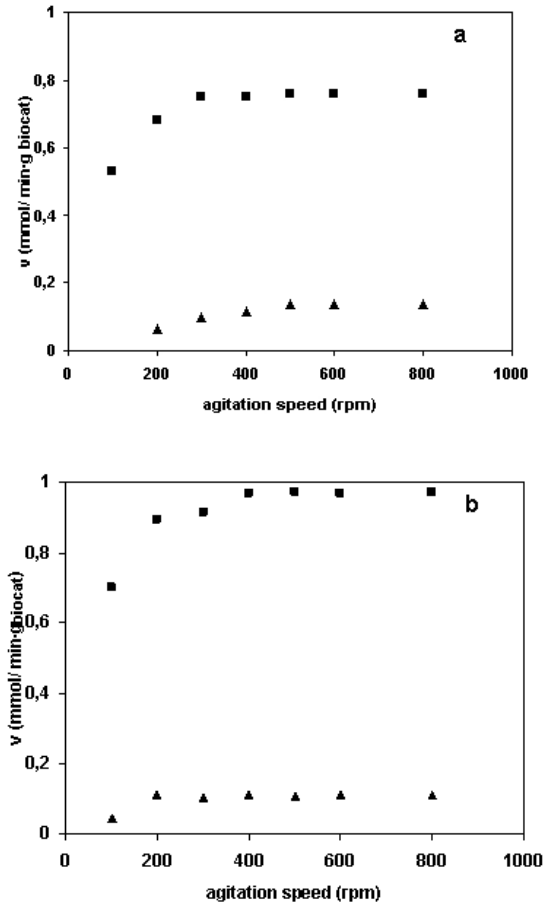

Figure 2. Effect of agitation speed on external diffusional restrictions of immobilized penicillin G acylase biocatalysts.
a) Large particle size biocatalyst (LP6).
b) Small particle size biocatalyst (SP6).
■: 10 mM penicillin G; ▲: 0.2 mM penicillin G.

different penicillin G concentrations in 100 mM sodium phosphate buffer pH 7.8 and 30ºC. Values obtained by linearization were checked by non-linear regression to the kinetic rate equation. Intrinsic kinetic parameters for the immobilized PGA were determined from the linear portion of the Lineweaver-Burk plot of the biocatalyst less affected by internal diffusional restrictions (the one with the smaller size and lower protein loading).

Effective diffusion coefficients (D_{eff}) for penicillin G, PAA and 6-APA were determined through effusion experiments from glyoxyl-agarose particles, by non-linear regression to Equation 1 (Grünwald, 1989):

$$c_t = c_\infty - c_\infty \cdot e^{-\frac{\pi^2 \cdot D_{eff}}{R^2} \cdot t} \quad [1]$$

Where c_t and c_∞ are the concentration of the species at a time t and infinite time and R is the radius of the particle.

Determination of conditions for negligible external diffusional restrictions of PGA biocatalysts

In order to study the specific effect of internal (intraparticle) diffusional restrictions (IDR) on enzyme kinetics, conditions were determined to make external (film) diffusional restrictions (EDR) negligible. To do so, the effect of agitation speed on initial reaction rate was determined at the condition in which IDR are higher: high protein load and low substrate concentration (Kheirolomoom et al. 2002). Experiments were then conducted at high protein loads (LP-6 and SP-6 biocatalysts), both at high (10 mM) and low (0.2 mM) substrate concentrations (Table 1). Initial reaction rates of penicillin G hydrolysis were determined at different agitation speeds up to a point in which that rate was no longer affected. The agitation speed at which the initial reaction rate for the worst case (large size particles at low substrate concentration) was not affected was selected to perform all subsequent experiments.

Determination of internal diffusional restrictions of PGA biocatalysts

Initial rate data were determined at different substrate concentrations for PGA biocatalysts of different particle sizes and enzyme protein loadings (Table 1). Effectiveness factor (η) for each type of biocatalyst was defined as:

$$\eta = \frac{V_{eff}}{V_{int}} \quad [2]$$

Where v_{eff} is the effective reaction and v_{int} is the intrinsic reaction rate (reaction rate in the absence of internal diffusional restrictions). Thiele modulus of each biocatalyst was:

$$\Phi = \frac{R}{3}\sqrt{\frac{V_{max}}{K_M \cdot D_{eff}}} \quad [3]$$

Where R/3 is the equivalent length of the biocatalyst spherical particles, V_{max} is the maximum intrinsic reaction rate, K_M is the intrinsic Michaelis constant for penicillin G and D_{eff} is the effective diffusion coefficient for penicillin G inside the biocatalyst particle. All experiments were conducted at conditions where EDR were negligible. Experiments were done in duplicate and samples were analyzed in duplicate or triplicate.

Table 2. Initial reaction rates (v) for free (FPGA) and immobilized (SP1) penicillin acylase at different penicillin G concentrations at pH 7.8 and 30ºC, and calculated kinetic parameters. In the case of SP1, kinetic parameters are intrinsic.

FPGA		SP1	
[penicillin G] (mM)	v (mmol min^{-1} g_{prot}^{-1})	[penicillin G] (mM)	v (mmol/min·g_{prot})
0.08	18.7		
0.1	21.2		
0.14	24.4	0.14	1.82
		0.17	1.96
		0.20	2.24
0.23	29.3	0.23	2.67
		0.3	3.65
0.5	35.0	0.5	5.75
		0.8	8.41
		2	14.78
		4	16.51
10	41.6	10	17.40
		25	17.73
K_M (mM)	V_{max} (mmol/min·g_{prot})	K_M (mM)	V_{max} (mmol/min·g_{prot})
0.10	42.0	0.45	18.18

RESULTS AND DISCUSSION

Gel fractionation

Sepharose 6B-CL beads were screened, selecting a fraction of large particles with a mean diameter value of 105.1 μm and a standard deviation of 30.1 μm, denoted as LP, and a fraction of small particles with a mean diameter value of 51.1 μm and a standard deviation of 8.8 μm, denoted as SP. LP represented 47% of the total gel mass and SP represented 7%; LP and SP fractions were used to assess the effect of particle size on diffusional restrictions.

Enzyme immobilization

Results of protein immobilization yields are presented in Table 1. As seen, almost the same amounts of immobilized protein per unit mass of gel were obtained for LP and SP in the range of protein loadings considered, allowing a sound evaluation of the differential effect of particle size and protein loading on diffusional restrictions.

Determination of intrinsic kinetics and mass transfer parameters

Kinetic parameters for the free PGA and intrinsic kinetic parameters for the immobilized biocatalysts were determined using penicillin G concentrations in the range from 0.08 to 10 mM and from 0.14 to 25 mM, respectively. From those data, kinetic parameters were determined for the free enzyme by linearization of initial reaction rate data at different penicillin G concentrations in 100 mM sodium phosphate buffer pH 7.8 at 30ºC; intrinsic kinetic parameters for immobilized PGAs were determined under the same conditions as above and estimated from the linear portion of the Lineweaver-Burk plot (corresponding to penicillin G concentrations higher than 2 mM), using the biocatalyst less affected by IDR (SP1) under conditions

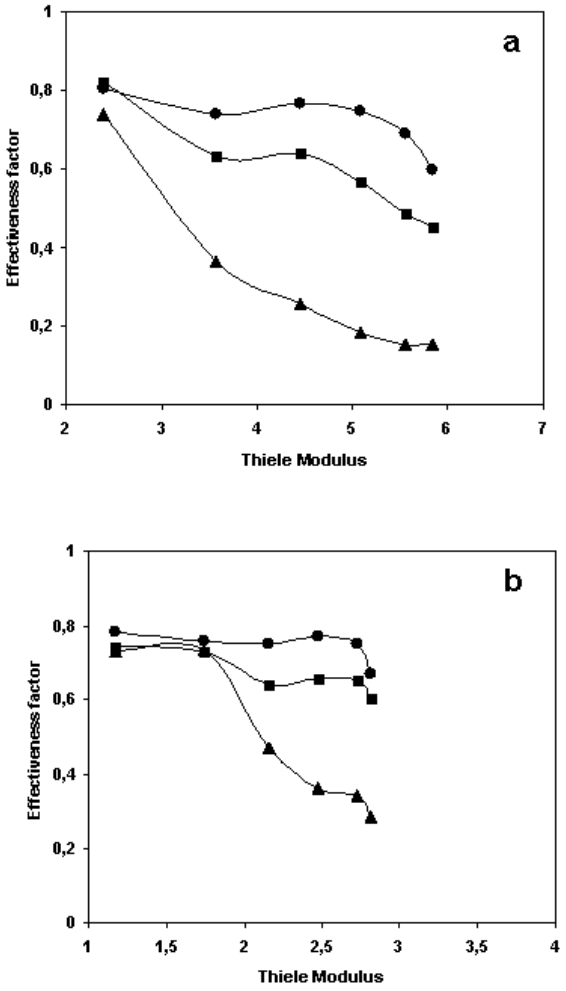

Figure 3. Effect of Thièle modulus and penicillin G concentration on effectiveness factor of immobilized penicillin acylase in large (a) and small (b) glyoxyl agarose gel particles. ●: 25 mMpenicillin G; ■: 10 mM penicillin G; ▲: 0.2 mMpenicillin G.

where EDR were negligible. A straight line was obtained at penicillin G concentration higher than 2 mM (see enlarged section in Figure 1), showing that under such conditions the effect of IDR was negligible, so that parameters determined from that data can be considered as intrinsic. Table 2 presents the results for reaction rates and kinetic parameters calculated for free and immobilized PGA. Intrinsic parameters of the immobilized enzyme differ from those of the free enzyme, suggesting that factors other than diffusional restrictions play a role in determining the catalytic properties of the immobilized enzyme. Conformational change, as a consequence of immobilization, is a plausible explanation but we have no direct evidence of this change. However, when doing the study of immobilization there was a fraction of initial activity not recovered in the biocatalyst or in the supernatant after immobilization; if enzyme inactivation during immobilization is ruled out, and we have information to prove that it is insignificant, then this result is an indirect proof of possible reduction of enzyme activity due to conformational changes. Even though there is ample information in the literature about the fact that immobilization on glyoxyl agarose is a very mild system because of the open pore structure and geometric congruence between the enzyme and the support (Mateo et al. 2005; Pedroche et al. 2007), the difference between the intrinsic K_m of SP1 and the K_m of the free enzyme (Table 2) suggests that conformational effects are not negligible. Lineweaver-Burk plot for SP1 shown in Figure 1 has the characteristic shape for a biocatalyst subjected to IDR with a clear departure from linearity as substrate concentration is decreased and a clear inflection point (Engasser and Horvath, 1973). Therefore, even SP1 is subjected to diffusional restrictions at moderate substrate concentrations. Data points in Figure 1 represent the average of two separate experiments in which three different measurements for each point were averaged. The differences between the separate experiments and among the samples were found to be less than 5% and 3%, respectively.

Determination of conditions for negligible external diffusional restrictions of penicillin acylase biocatalysts

The effect of agitation speed on initial reaction rate was determined at high protein loads (LP6 and SP6), both at high (10 mM) and low (0.2 mM) substrate concentrations. The data points in Figure 2, which represent the average of three separate experiments, showed that the differences were 5% or less.

Large particles were more sensitive to EDR, as expected, but at agitation speeds higher than 400 rpm initial reaction rates were not affected at either substrate concentration, meaning that EDR can be considered negligible above that speed. Further experiments were all conducted at 600 rpm. At such agitation speed no particle attrition or enzyme leakage was observed even for prolonged time.

Determination of internal diffusional restrictions of penicillin acylase biocatalysts

The effective diffusion coefficient (D_{eff}) for substrate (penicillin G) and products of hydrolysis (6-APA and PAA) were 5.30×10^{-10}, 5.89×10^{-10} and 7.33×10^{-10} m^2/s respectively, as determined from Equation 1 using effusion data for each compound. Reported values represent the average of two separate effusion experiments for each compound, differences being below 5% in all cases. The value for penicillin G was the smallest, so that diffusional restrictions for the substrate are likely to be the most significant. D_{eff} for penicillin G was somewhat higher than the estimated value of 4×10^{-10} m^2/s for penicillin G in free solution at 28ºC; it was 80% higher than the estimated value from reaction data with Eupergit 250 L-immobilized penicillin acylase at such temperature and 50% higher than

Table 3. Effect of internal diffusional restrictions on glyoxyl-agarose immobilized penicillin acylase of different particle size and protein loadings. LP: large particle size (average diameter 105.1 μm); SP small particle size (average diameter 51.1 μm); IP: immobilized protein in g/g_{biocat}; v: initial effective reaction rate in moles/min x $g_{protein}$; η: biocatalyst effectiveness factor; V_{max}: intrinsic maximum reaction rate in mM/min; Φ: substrate Thiele modulus. Subscripts in v and η refer to penicillin concentration (mM).

Biocatalyst	IP	v_2	v_{10}	v_{25}	η_2	η_{10}	η_{25}	V_{max}	φ
LP1	0.0154	10.91	14.26	14.26	0.738	0.820	0.805	268.8	2.40
LP2	0.0341	5.32	11.02	13.11	0.360	0.633	0.740	595.1	3.57
LP3	0.0530	3.79	11.15	13.57	0.256	0.641	0.765	925.0	4.45
LP4	0.0691	2.72	9.85	13.23	0.184	0.566	0.746	1206.0	5.08
LP5	0.0824	2.28	8.44	12.19	0.154	0.485	0.688	1438.1	5.55
LP6	0.0912	2.27	7.89	10.59	0.153	0.453	0.597	1591.7	5.84
SP1	0.0154	10.79	12.91	13.88	0.730	0.742	0.783	268.8	1.17
SP2	0.0341	10.82	12.72	13.48	0.732	0.731	0.760	595.1	1.74
SP3	0.0529	6.98	11.15	13.31	0.472	0.641	0.751	923.3	2.16
SP4	0.0698	5.38	11.42	13.64	0.364	0.656	0.769	1218.2	2.48
SP5	0.0843	5.07	11.38	13.30	0.343	0.654	0.750	1471.3	2.73
SP6	0.0893	4.21	10.53	11.90	0.285	0.605	0.671	1558.5	2.81

the value reported in agar gel and in rat cerebral cortex using pressure microinjection and ion-selective microelectrodes selective for penicillin (Lehmenkühler et al. 1988; Spieβ et al. 1999; Tischer and Kasche, 1999). Because of the open pore structure and the high content of water of glyoxyl agarose gel, diffusion rates inside it may not differ significantly from that in water.

Initial reaction rate data determined for PGA biocatalysts of different particle sizes and enzyme protein loadings are presented in Table 3. Values represent the average of duplicates with differences below 3% in most cases and close to 5% in only two cases. Intrinsic reaction rates are those presented for SP1 at the corresponding substrate concentration; η values were calculated according to Equation 2. Intrinsic maximum reaction rate was 18.18 (mmoles/min x g_{prot}) as indicated in Table 2. The corresponding values of maximum reaction rates (mM/min) for each biocatalyst are presented in Table 3. The values of Φ calculated for each biocatalyst according to Equation 3 ranged from 1.17 for SP1 to 5.84 for LP6 (Table 3). This is reasonable since SP1 is the less susceptible to IDR, while the opposite holds for LP6. Figure 3 is the graphical representation of data shown in Table 3 which exhibits a better appreciation of the impact of particle size, protein loading and substrate concentration on IDR.

As expected, η decays sharply as Φ increases both for LP and SP biocatalysts, but this impact moves to higher values of Φ as the bulk substrate concentration increases. None of the biocatalysts tested can be considered free of IDR, but η around 0.8 could be obtained for LP and SP at low protein loadings (0.015 to 0.03 g/g_{biocat}) and high bulk substrate concentration (25 mM). At low protein loadings, η for LP and SP did not differ significantly, which is reasonable since the system moves away from mass transfer limitations. As the protein loading increases much higher Φ and, consequently, much lower η were obtained for LP when compared to SP, which is the consequence of exacerbated mass transfer limitations when enzyme activity in the biocatalyst is high.

Initial penicillin G concentration for 6-APA production at industrial scale is between 150 and 300 mM and conversion is usually around 95% (Kumar et al. 1996; Shewale and Sudhakaran, 1997). Penicillin G concentrations considered in this study (10 and 25 mM) lie somewhere between initial and final values (in the case of sequential batch reactor operation) or inlet and outlet values (in the case of continuous packed bed reactor operation) so that IDR will be relevant in biocatalyst performance under actual process operating conditions. Variation of η with substrate concentration is valuable information for proper reactor

design with biocatalysts subjected to mass transfer limitations (Illanes and Altamirano, 2008).

CONCLUDING REMARKS

Mass transfer limitations are relevant in the case of immobilized enzymes within gel matrices, limiting their catalytic potential. Particle size and protein loading, both affecting Φ, have been studied as relevant variables for determining the impact of IDR on immobilized enzyme kinetics, which was evaluated in terms of the η of the biocatalysts. Protein loading had a strong impact, being more pronounced in the case of large particle size biocatalysts. Bulk substrate concentration was determinant on the values of Φ over which IDR (as reflected by the values of η) are relevant, being higher at higher substrate concentrations.

At conditions of 6-APA production, all biocatalysts tested were mass-transfer limited, as is usually the case for immobilized enzymes. However, if these catalysts are used for the slower reactions of synthesis of derived penicillins or cephalosporins from the corresponding β-lactam nuclei, this is not necessarily so and biocatalysts not good enough for hydrolysis might be adequate for synthesis. Therefore, this information is valuable for selecting the appropriate biocatalyst for a given reaction.

REFERENCES

ALVARO, Gregorio; FERNÁNDEZ-LAFUENTE, Roberto; BLANCO, Rosa M. and GUISÁN, José M. Immobilization - stabilization of penicillin acylase from *Escherichia coli*. *Applied Biochemistry and Biotechnology*, November 1990, vol. 26, no. 2, p. 181-195.

BASSO, Alessandro; DE MARTIN, Luigi; EBERT, Cynthia; GARDOSSI, Lucia; LINDA, Paolo and SIBILLA, Fabrizio. Organically modified xerogels as novel tailor-made supports for covalent immobilisation of enzymes (penicillin G acylase). *Tetrahedron Letters*, July 2003, vol. 44, no. 31, p. 5889-5891.

BRADFORD, Marion M. A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, May 1976, vol. 72, no. 1-2, p. 248-254.

CHANDEL, Anuj K.; RAO, L. Venkateswar; NARASU, M. Lakshmi and SINGH, Om M. The realm of penicillin G acylase in β-lactam antibiotics. *Enzyme and Microbial Technology*, February 2008, vol. 42, no. 3, p. 199-207.

DU, Li-Li; WU, Qi; CHEN, Chun-Xiu; LIU, Bo-Kai and LIN, Xian-Fu. A two-step, one pot enzymatic synthesis of ampicillin from penicillin G potassium salt. *Journal of Molecular Catalysis B: Enzymatic*, June 2009, vol. 58, no. 1-4, p. 208-211.

ENGASSER, Jean-Marc and HORVATH, Csaba. Effect of internal diffusion in heterogeneous enzyme systems: evaluation of true kinetic parameters and substrate diffusivity. *Journal of Theoretical Biology*, November 1973, vol. 42, no. 1, p. 137-155.

GABOR, Esther M.; DE VRIES, Erik J. and JANSSEN, Dick B. A novel penicillin acylase from the environmental gene pool with improved synthetic properties. *Enzyme and Microbial Technology*, February 2005, vol. 36, no. 2-3, p. 182-190.

GONÇALVES, L.R.B.; FERREIRA, A.L.O.; FERNÁNDEZ-LAFUENTE, R.; GUISÁN, J.M.; GIORDANO, R.C. and GIORDANO, R.L.C. Influence of mass transfer limitations on the enzymatic synthesis of β-lactam antibiotics catalyzed by penicillin G acylase immobilized on glyoxil-agarose. *Bioprocess Biosystem Engineering*, August 2008, vol. 31, no. 5, p. 411-418.

GRÜNWALD, Peter. Determination of effective diffusion coefficient - an important parameter for the efficiency of immobilized biocatalysts. *Biochemical Education*, April 1989, vol.17, no. 2, p. 99-102.

ILLANES, Andrés; CABRERA, Zaida; WILSON, Lorena and AGUIRRE, Carolina. Synthesis of cephalexin in ethylene glycol with glyoxyl-agarose immobilised penicillin acylase: temperature and pH optimisation. *Process Biochemistry*, September 2003, vol. 39, no. 1, p. 111-117.

ILLANES, Andrés; ALTAMIRANO, Claudia; FUENTES, Martín; ZAMORANO, Francisca and AGUIRRE, Carolina. Synthesis of cephalexin in organic medium at high substrate concentrations and low enzyme to substrate ratio. *Journal of Molecular Catalysis B: Enzymatic*, August 2005, vol. 35, no. 1-3, p. 45-51.

ILLANES, Andrés and WILSON, Lorena. Kinetically-controlled synthesis of β-lactam antibiotics. *Chimica Oggi/ Chemistry Today*, 2006, vol. 24, no. 5, p. 27-30.

ILLANES, Andrés and ALTAMIRANO, Claudia. Enzyme reactors. In: ILLANES, Andrés. ed. *Enzyme Biocatalysis: Principles and Applications*. United Kingdom; Springer, 2008, p. 205-251.

KALLENBERG, Agnes I.; VAN RANTWIJK, Fred and SHELDON, Roger A. Immobilization of penicillin G acylase: the key to optimum performance. *Advanced Synthesis and Catalysis*, June 2005, vol. 347, no. 7-8, p. 905-926.

KHEIROLOMOOM, Azadeh; KORASHEH, Farhad and FAZELINIA, Hossein. Influence of external mass transfer limitation on apparent kinetic parameters of penicillin G acylase immobilized on nonporous ultrafine silica particles. *Journal of Bioscience and Bioengineering*, February 2002, vol. 93, no. 2, p. 125-129.

KUMAR, Ramachandran; SURESH, A. Krishnamurthy and SHANKAR, H. Subbaraman. Kinetics and reaction engineering of penicillin G hydrolysis. *Journal of Chemical Technology and Biotechnology*, July 1996, vol. 66, no. 3, p. 243-250.

LEHMENKÜHLER, A.; KERSTING, U. and NICHOLSON, C. Diffusion of penicillin in agar and cerebral cortex of the rat. *Brain Research*, March 1988, vol. 444, no. 1, p. 181-183.

MATEO, Cesar; ABIÁN, Olga; BERNEDO, Marieta; CUENCA, Emma; FUENTES, Manuel; FERNÁNDEZ-LORENTE, Gloria; PALOMO, Jose M.; GRAZU, Valeria; PESSELA, Benevides C.C.; GIACOMINI, Cecilia; IRAZOQUI, Gabriela; VILLARINO, Andrea; OVSEJEVI, Karen; BATISTA-VIERA, Francisco; FERNÁNDEZ-LAFUENTE, Roberto and GUISÁN, Jose M. Some special features of glyoxyl supports to immobilize proteins. *Enzyme and Microbial Technology*, September 2005, vol. 37, no. 4, p. 456-462.

MONTES, Tamara; GRAZÚ, Valeria; MANSO, Isabel; GALÁN, Beatriz; LÓPEZ-GALLEGO, Fernando; GONZÁLEZ, Ramón; HERMOSO, Juan A.; GARCÍA, José L.; GUISÁN, José M. and FERNÁNDEZ-LAFUENTE, Roberto. Improved stabilization of genetically modified penicillin G acylase in the presence of organic cosolvents by co-immobilization of the enzyme with polyethyleneimine. *Advanced Synthesis and Catalysis*, February 2007, vol. 349, no. 3, p. 459-464.

PARMAR, A.; KUMAR, H.; MARWAHA, S.S. and KENNEDY, J.F. Advances in enzymatic biotransformation of penicillins to 6-aminopenicillanic acid (6-APA). *Biotechnology Advances*, July 2000, vol. 18, no. 4, p. 289-301.

PCHELINTSEV, N.A.; YOUSHKO, M.I. and ŠVEDAS, V.K. Quantitative characteristic of the catalytic properties and microstructure of cross-linked enzyme aggregates of penicillin acylase. *Journal of Molecular Catalysis B: Enzymatic*, April 2009, vol. 56, no. 4, p. 202-207.

PEDROCHE, Justo; YUST, Maria M.; MATEO, Cesar; FERNÁNDEZ-LAFUENTE, Roberto; GIRÓN-CALLE, Julio; ALAIZ, Manuel; VIOQUE, Javier; GUISÁN, José M. and MILLÁN, Francisco. Effect of the support and experimental conditions in the intensity of the multipoint covalent attachment of proteins on glyoxyl-agarose supports: correlation between enzyme-support linkages and thermal stability. *Enzyme and Microbial Technology*, April 2007, vol. 40, no. 5, p. 1160-1166.

RAJENDHRAN, Jeyaprakash and GUNASEKARAN, Paramasamy. Recent biotechnological interventions for developing improved penicillin G acylases. *Journal of Bioscience and Bioengineering*, 2004, vol. 97, no. 1, p. 1-13.

RAJENDHRAN, Jeyaprakash and GUNASEKARAN, Paramasamy. Application of cross-linked enzyme aggregates of *Bacillus badius* penicillin G acylase for the production of 6-aminopenicillanic acid. *Letters in Applied Microbiology*, January 2007, vol. 44, no. 1, p. 43-49.

SCHROËN, C.G.P.H.; FRETZ, C.B.; DE BRUIN, V.H.; BERENDSEN, W.; MOODY, H.M.; ROOS, E.C.; VAN ROON, J.L.; KROON, J.P.; STRUBEL, M.; JANSSEN, A.E. and TRAMPER, J. Modelling of the enzymatic kinetically controlled synthesis of cephalexin. Influence of diffusional limitation. *Biotechnology and Bioengineering*, November 2002, vol. 80, no. 3, p. 331-340.

SERRA, Immacolata; CECCHINI, Davide A.; UBIALI, Daniela; MANAZZA, Elena M.; ALBERTINI, Alessandra M. and TERRENI, Marco. Coupling of site-directed mutagenesis and immobilization for the rational design of more efficient biocatalysts: the case of immobilized 3G3K PGA from *E. coli*. *European Journal of Organic Chemistry*, March 2009, vol. 2009, no. 9, p. 1384-1389.

SHEWALE, Jaiprakash G. and SUDHAKARAN, Vayalombron K. Penicillin V acylase: its potential in the production of 6-aminopenicillanic acid. *Enzyme and Microbial Technology*, May 1997, vol. 20, no. 6, p. 402-410.

SPIEβ, A.; SCHLOTHAUER, R.C.; HINRICHS, J.; SCHEIDAT, B. and KASCHE, V. pH gradients in immobilized amidases and their influence on rates and yields of β-lactam hydrolysis. *Biotechnology and Bioengineering*, February 1999, vol. 62, no. 3, p. 267-277.

SUN, H.; BAO, X.Y. and ZHAO, X.S. Immobilization of penicillin G acylase on oxirane-modified mesoporous silicas. *Langmuir*, February 2009, vol. 25, no. 3, p. 1807-1812.

TISCHER, Wilhelm and KASCHE, Volker. Immobilized enzymes: crystals or carriers? *Trends in Biotechnology*, August 1999, vol. 17, no. 8, p. 326-335.

VAN ROON, J.L.; ARNTZ, M.M.H.D.; KALLENBERG, A.I.; PAASMAN, M.A.; TRAMPER, J.; SCHROËN, C.G.P.H. and BEEFTINK, H.H. A multicomponent reaction-diffusion model of a heterogeneously distributed immobilized enzyme. *Applied Microbiology and Biotechnology*, September 2006, vol. 72, no. 2, p. 263-278.

WILSON, Lorena; ILLANES, Andrés; SOLER, Lorena and HENRÍQUEZ, María José. Effect of the degree of cross-linking on the properties of different CLEAs of penicillin acylase. *Process Biochemistry*, March 2009, vol. 44, no. 3, p. 322-326.