Research Article

A new biocatalyst: Penicillin G acylase immobilized in sol-gel micro-particles with magnetic properties

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The present work focuses on the development and basic characterization of a new magnetic biocatalyst, namely penicillin G acylase (PGA), immobilized in sol-gel matrices with magnetic properties, ultimately aimed for application in cephalexin (CEX) synthesis. A mechanically stable carrier, based on porous xerogels silica matrixes starting from tetramethoxysilane (TMOS), was prepared leading to micro-carriers with medium sized particles of 30 μm, as determined by scanning electron microscopy. An immobilization yield of 95–100% and a recovered activity of 50–65% at 37°C, as determined by penicillin G (PG) hydrolysis (pH STAT method), were observed. These results clearly exceed those reported in a previous work on PGA immobilization in sol-gel, where only 10% of activity was recovered. The values of activity were kept constant for 6 months. Immobilized PGA (682 $U/g_{dry\ weight}$) retained high specific activity throughout ten consecutive runs for PG hydrolysis, suggesting adequate biocatalyst stability. The CEX synthesis was performed at 14°C, using the free and immobilized PGA in aqueous medium. Phenylglycine methyl ester was used as acyl donor at 90 mM and 7-aminodeacetoxycephalosporanic acid was the limiting substrate at 30 mM. The CEX stoichiometric yield after 1-h reaction was close to 68% (23 mM CEX/h) and 65% (19 mM CEX/h), respectively.

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1 Introduction

One of the goals of applied biocatalysis is the development of new tools and approaches for improving a wide array of production processes, aiming at high productivity along with reduced energy and raw material consumption as well as generation of less waste and toxic side-products. Within this scope, the development of enzymatic-based processes for antibiotic production is a most active research area. This is hardly surprising, because semi-synthetic penicillins (*e.g.* amoxicillin, ampicillin, ticarcillin) and cephalosporins (*e.*g. cephalexin (CEX), cefaclor, cefadroxil) correspond to 65% of the ever rising worldwide production of antibiotics, exceeding 45 000 tons in 2000 [1]. On its own, CEX has an annual consumption of almost 3000 tons, the largest within the world market for cephalosporins, and generates 30–40 kg waste per kg of end product [2]. The enzymatic synthesis of cephalosporins catalyzed by penicillin G acylase (PGA, EC 3.5.1.11) carried out in aqueous medium and under milder conditions has been proposed as a potential alternative to the chemical methods [3]. PGA from *Escherichia coli* is furthermore the most studied and used enzyme for commercial purposes [4–6].

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Abbreviations: 7-ADCA, 7-aminodeacetoxycephalosporanic acid; **AOT,** sodium dioctyl sulfosuccinate; **CEX,** cephalexin; **D-PG,** D-phenylglycine; **D-PGM,** D-phenylglycine methyl ester; **PG,** penicillin G; **PGA,** penicillin G acylase; **SEM,** scanning electron microscopy; **TMOS,** tetramethoxysilane

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Availability of inexpensive enzyme catalysts with improved specificity, activity, purity and stability is a key issue in the development of successful methodologies for the enzyme-based production of β-lactam antibiotics. In industrial practice, the use of an immobilized enzyme is required for reasons of downstream processing and recyclability [7].

A relatively recent approach for enzyme immobilization is based on a sol-gel process, which allows the room temperature synthesis of silica glasses, suitably modified to exclude the typical harsh conditions that would cause enzyme denaturation [8]. The group of Avnir [9] broadened the use of sol-gel entrapment to include a wide range of enzymes such as phosphatase, trypsin, aspartase, glucose oxidase, carbonic anhydrase, chinitase and monoamine oxidase. Reetz [10, 11] showed it to be effective and efficient with lipases.

A well-established sol-gel processing technique consists of the hydrolysis of the adequate precursors in aqueous solutions to produce soluble hydroxylated monomers, followed by polymerization and phase separation to yield a hydrated metal or semi-metal oxide hydrogel. Removal of water from the wet gel, which usually brings along changes in the structure of the pores and in the gel network, results in a porous xerogel. The most widely used precursors are alkyl-alkoxysilanes because they react readily in water [12, 13]. The characteristics and properties of a particular sol-gel inorganic network are related to a number of factors that affect the rate of hydrolysis and condensation reactions, such as pH, temperature, and time of reaction, reagent concentrations, aging temperature and time, and drying [13].

Such materials offer the same beneficial properties as traditional silica-based matrices. Furthermore, the amount of enzyme that can be immobilized is not limited by the available surface area on the matrix, because the enzyme is also entrapped within the polymeric matrix as it forms, and becomes homogeneously distributed throughout the material [11]. This method has been employed for the entrapment of a wide range of enzymes, antibodies, and whole cells [8, 9]. When applied to PGA, the activity recovery was a poor 10%, which the authors ascribed to diffusion limitation in the silica matrix [7, 14]. Taking into consideration the advantages of sol-gel immobilization, this work aimed at the development and basic characterization of a suitable strategy for PGA immobilization in sol-gel matrices with magnetic properties and preliminary evaluation of its application in CEX synthesis.

2 Materials and methods

2.1 Materials

PGA solution (26.6 mg/mL protein, 35 U/mg) from *Escherichia coli*, tetramethoxysilane (TMOS) ≥99%, sodium dioctyl sulfosuccinate (AOT), 6-nitro-3-(phenylacetamido)benzoic acid (NIPAB), magnetite nanopowder, (R)-(-)-2-phenylglycine methylester hydrochlorid 97% (D-PGM), and acetaminophen ≥99% were all purchased from Sigma-Aldrich (USA). Isooctane and CEX vetranal® was supplied from Riedel de Haën (Germany), D-(-)-α-phenylglycine ≥99% (D-PG) was purchased from Fluka (USA) and 7-aminodeacetoxycephalosporanic acid (7-ADCA), commercial grade, was supplied from DSM (Amsterdam). Penicillin G (PG) was obtained from Fersinca (Mexico). All other reagents used were either laboratory or analytical grade.

2.2 PGA sol-gel biocatalyst

A solution containing 100 μL TMOS (2.32 M) and 40 μL HCl (1.37 mM) was sonicated in a Transsonic T 460 sonicating water bath for 10 min until the hydrolysis reaction was complete, according to method described in Clark *et al*. [15]. In a typical immobilization procedure, 75 μL of PGA was suspended in 85 μL of the magnetic suspension – 10% w/v in 100 mM phosphate buffer pH 7.5, and then mixed with the sol solution. To obtain micro-particles, 300 μL of the sol-gel solution with enzyme was immediately added to 6 mL of 150 mM AOT/isooctane solution, before gelation. The resulting mixture was vortexed (VF2 Janke&Kunkel, IKA Labortechnik) for 1 min, washed twice with 100 mM phosphate buffer, pH 7.5 and aged (incubation under given conditions, *viz*. pressure and temperature, which allows for strengthening the gel network through further polymerization, alongside with exclusion of the solvent from the network, due to shrinkage, and concomitant evaporation of the solvent) at 2–8°C, in the refrigerator or at room temperature under controlled water activity (a_w = 0.75), during 1 week. A water activity controlled environment was established by incubating Eppendorf tubes with sol-gel in a closed container with a saturated solution of sodium chloride [16, 17]. The micro-particles obtained were suspended in 1 mL of the same phosphate buffer. The particles were either used or stored at 2–8°C.

2.3 Enzyme activity assay and activity retention upon consecutive runs

One unit of PGA activity (U) for the soluble and immobilized enzymes is defined as:

2.3.1 pH STAT method

The amount of enzyme required to produce 1 mmol of 6-APA (6-amino penicillanic acid) per minute at 37°C and pH 8.0. Enzyme activity was determined in a small batch magnetic stirred conical reactor with automatic pH correction by the pH STAT method [18], using a 4% w/v penicillin solution in 20 mM phosphate buffer pH 8.0, at 37°C. In order to evaluate the mechanical stability of the biocatalyst, PG hydrolysis was performed during 10 min at 37°C and pH 8.0 (pH STAT method) in a magnetic stirred conical reactor and in a differential recycled reactor using the sol-gel immobilized PGA in aqueous medium.

2.3.2 NIPAB method

The amount of enzyme required to produce 1 μmol of 3-amino-6-nitrobenzoic acid (NABA) at 30°C. The activity assay was based on the conversion of 6-nitro-3-(phenylacetamido)benzoic acid (NIPAB) to 3-amino-6-nitrobenzoic acid (NABA). The release of NABA in the assay mixtures was followed by recording the increase in absorbance at 405 nm and 30°C. The activity assay mixture contained 900 μL of 50 mM phosphate buffer pH 7.0 and 50 μL of 6 mM NIPAB in 50 mM phosphate buffer pH 7.0. The reaction was started by the addition of 50 μL of the enzyme solution (soluble or immobilized).

2.3.3 Effect of methanol

In order to evaluate the influence of methanol in the deactivation of PGA, a solution of free PGA was incubated in phosphate buffer 100 mM with methanol 35% v/v, pH 7.6 at 37°C for 1 h and in the same buffer solution but without methanol. Activity was determined by NIPAB method at specific times. Triplicates of each experiment were performed. The SD did not exceed 5%.

2.3.4 Activity retention

Activity retention upon immobilization was defined as the ratio between the specific activity of the enzyme immobilized on the support and the specific activity of the free enzyme. Activity retention for PG hydrolysis in consecutive batch runs was defined as the ratio between the specific activity of the immobilized enzyme in a given batch and the specific activity of the immobilized enzyme in the first batch. Enzyme activity was determined according to the pH STAT method.

The activity retention upon incubation of PGA in the presence of methanol was defined as the ratio between the specific activity of the free enzyme at a specific time and the specific activity of PGA at the start of the run. Enzyme assays were done by NIPAB method.

2.4 Synthesis of CEX with free and immobilized PGA

Synthesis of CEX was performed in temperature controlled and magnetically stirred conical reactors with 10 mL of reaction medium. Substrate concentrations were 30 mM 7-ADCA, and 90 mM D-PGM. The biocatalyst (20 mg) or 10 μL of free PGA per mL of reaction, corresponding to 455 or 239 U/mmol 7-ADCA (pH STAT method), respectively, were used. The reactions were performed at 14°C, in 50 mM phosphate buffer pH 8.0, with a pH shift from the initial pH 8.00 to pH 7.65 and pH 7.50, respectively, at the end of reactions. During synthesis, pH and temperature were monitored and samples were taken to analyze product and substrates, in order to determine yield and volumetric productivity. Yield was defined as the maximum molar conversion of 7-ADCA into CEX (%) and productivity as the concentration of CEX produced per unit time and unit reaction volume at maximum yield (mM/h). Specific productivity was determined per gram of dry biocatalyst ($mM \times h^{-1} \times g^{-1}$). Triplicates of each experiment were performed. Standard deviation did not exceed 5%.

2.5 Scanning electron microscopy (SEM)

Dry particles of biocatalyst were put on a double carbon tape and analyzed in a Field Emission Scanning Electron Microscope (Jeol JSM-7001F).

2.6 HPLC analysis of reactants and products

The methodology for HPLC analysis was adapted from Patent US6060268 [19]. Substrates (7-ADCA and D-PGM) and products (CEX and PG) of synthesis were identified and analyzed by HPLC using a Merck Hitachi delivery system L-6000 with a Perkin Elmer LC 90 UV UV-vis detector and a Merck Hitachi D-2500 (Chromato-Integrator) integrator. The column used was a ChromSpher 5 C18 S250x4.6 from Varian. Samples were eluted in isocratic mode with a mixture of 40% v/v acetonitrile, 60% v/v 5 mM phosphate buffer containing 0.2% m/v SDS, pH 3.1, at a flow rate of 1 mL/min, and analyzed in the UV detector at 214 nm. Chromatography was performed at room temperature. Elution times were 3.22, 4.00, 4.85, 9.62 and 13.85 min for acetaminophen (internal

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standard), D-PG, 7-ADCA, CEX and D-PGM, respectively. Concentrations of substrates and products were calculated from calibration curves using stock solutions with internal standard.

2.6.1 Internal standard solution

Internal standard (acetaminophen, 0.25 g/L) was dissolved and diluted to 50.0 mL with 50 mM phosphate buffer pH 8.00 to form the internal standard solution.

2.6.2 Reactants and products standard solutions

To form standard solutions (1 g/L) all reagents were dissolved and diluted to 50.0 mL with 50 mM phosphate buffer pH 8.00.

2.6.3 Dilution buffer

The dilution buffer consisted of 25% acetonitrile in 2 mM phosphate buffer, pH 5.00 [19].

2.6.4 Solutions for assessment of linearity in HPLC analysis

Five concentrations of each reagent (0.5, 0.25, 0.125, 0.08, 0.04 g/L) were prepared by dilution of standard solutions with 50 mM phosphate buffer pH 8.00. These solutions were diluted 1:2 with internal standard solution (0.25 g/L) and 1:10 with dilution buffer. Linear response was observed for the range of concentrations tested.

2.6.5 Sample preparations

Of each sample, 50 µL was diluted 1:50 in 50 mM phosphate buffer pH 8.00, further diluted 1:2 with internal standard solution (0.25 g/L) and finally diluted 1:10 with dilution buffer.

2.7 Assay for protein concentration

The concentration of protein in the enzyme solution was determined before immobilization and both in the supernatant and in the effluents from the washing steps, after immobilization, through mass balance based on a calibration curve of PGA relating the absorbance at 280 nm with protein concentration in previously prepared solutions with known concentrations. The amount of protein entrapped in the support was calculated by mass balance. Yield of immobilization was calculated as the ratio of the amount of protein entrapped in the sol-gel matrix to the initial amount.

3 Results and discussion

This work was aimed primarily at PGA immobilization by entrapment in a silica matrix with magnetic properties. Stable xerogel carriers containing magnetite were produced from TMOS, to yield an immobilized biocatalyst in the form of polydispersed micro-carriers, with medium-sized particles of 30 mm, as determined by scanning electron microscopy (SEM). A typical SEM micrograph of the micro-carriers is given in Fig. 1.

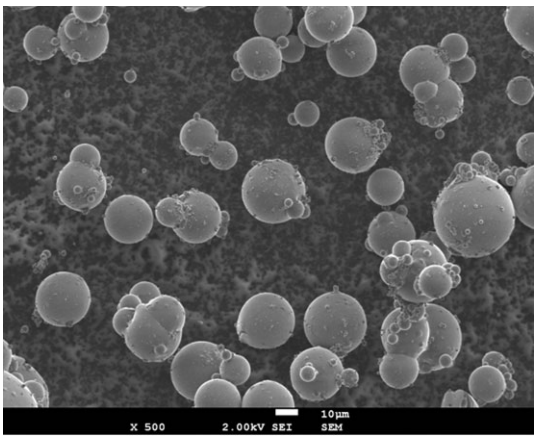

Figure 1. SEM micrograph of sol-gel micro-particles with encapsulated magnetite and PGA (bar match 10 µm).

The separation of the biocatalyst from the reaction mixture is commonly performed by simple filtration or centrifugation. The nature of the biocatalyst particles used in the present work allows for magnetic separation, an alternative method, which overcomes particle size limitations and reduces biocatalyst losses due to manipulation in standard separation processes, namely at the small scale used. The magnetic nature of the particles, due to the presence of magnetite, considerably eased the recovery of the biocatalyst from the reaction media, which was performed using a magnetic concentrator, thus avoiding the use of filters, which is an interesting feature for a large-scale application [7].

Immobilization of PGA (682 $U/g_{dry\ weight}$) in these carriers allowed for an immobilization yield of 95–100%, whereas the activity retention upon immobilization was of 50–65% at 37°C, as determined by the pH STAT method, depending on the aging. These results clearly exceed those reported in a previous work on PGA immobilization in sol-gel, where only 10% of activity was recovered [7]. PGA leakage during immobilization was assessed as described in Section 2 and under the experimental conditions tested, PGA leakage did not occur.

There are three main reasons responsible for the loss of activity during immobilization. One of these is due to methanol produced during gel formation. Theoretically, four molecules of methanol are produced during the hydrolysis and condensa-

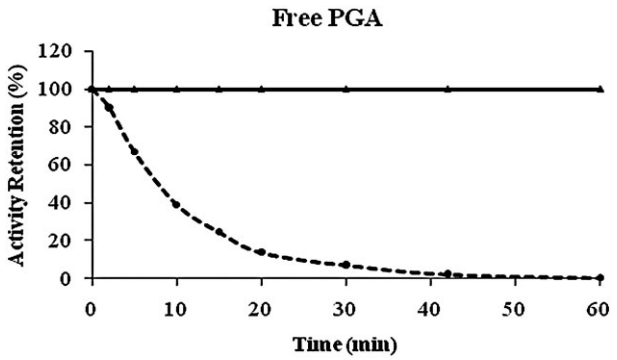

Figure 2. Thermal stability in phosphate buffer 100 mM pH 7.6 (—) and in the same buffer solution with 35% methanol v/v (----) at 37°C. Enzyme assays were performed by NIPAB method.

tion, from each molecule of TMOS. Assuming an ideal solution behavior, the amount of methanol released during the hydrolysis and condensation of TMOS, and taking into account the dilution of methanol into the reaction mixture when the enzyme solution is added, leads to high methanol concentrations (roughly 35% v/v), which can prove toxic to PGA. In Fig. 2, the deactivation of free PGA in phosphate buffer 100 mM pH 7.6 and with methanol 35% v/v at 37°C is presented, where the toxic effect of methanol in PGA is evidenced. One way to overcome this drawback is to remove the alcohol via evaporation under vacuum in order to get a fully hydrolyzed solution before adding PGA. Assays were performed accordingly, with the removal of 41, 30, 22 and 14% m/m of methanol. These values were calculated by mass balance at the trap used to retain methanol, assuming that the mass difference is just assigned to methanol release. In the first two assays, the condensation occurred instantly in the Eppendorf tube when the enzyme solution was added, preventing the procedure to follow. In the third assay, the condensation occurred in the tip after mixing the enzyme solution, before the addition to AOT/isooctane, and with the removal of 14% methanol the procedure was completed. However, the activity retention upon incubation in the presence of methanol was almost the same as when immobilization was performed without methanol extraction. The use of additives that showed promising results when challenged to prevent protein denaturation due to the presence of alcohol, such as PEG and glycerol [8], is currently being undertaken. Physical loss of biocatalyst during the immobilization procedure could also be accounted for the activity decrease. Through an overall mass balance, a mass loss of about 19.3% of the initial mass could be ascribed to mass retained in the tips, centrifuge tube and in the Eppendorf tube used for sol-gel drying. Physical stress can also lead to some partial denaturation of the enzymes. The silica matrix forms around the trapped biomolecule, but some shrinkage always occurs during the condensation process and the drying of the gel [8]. The effect of matrix aging in biocatalytic activity was assessed by testing supports with 3 and 7 days of aging at 2–8°C, the former presenting an activity retention for PG hydrolysis of 65%, whereas the activity retention for PG hydrolysis in the latter was 50%. When used in successive batch runs, particles with less aging displayed lower operational stability, which can be tentatively ascribed to lower mechanical resistance (Fig. 3). Since the moisture level in the refrigerator is not controlled, this aging strategy could prevent thorough reproducibility of data., Therefore, a different methodology was implemented in order to perform aging in a better-controlled moisture environment. This was achieved by equilibrating the particles with a satu-

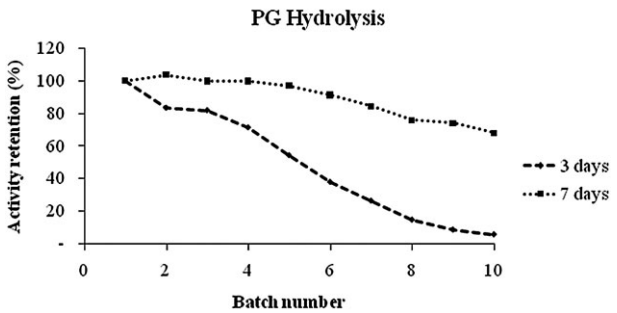

Figure 3. Activity retention for PG hydrolysis in micro-particles with 3 and 7 days of aging determined according to the PG hydrolysis standard method (pH STAT method). Operational runs were performed at pH 8.0 in a magnetic stirred reactor.

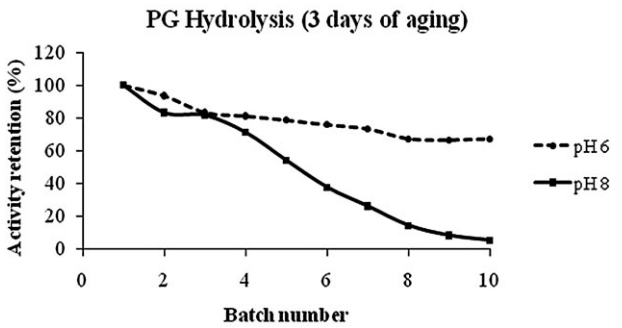

Figure 4. Activity retention for PG hydrolysis in micro-particles with 3 days of aging determined according to the PG hydrolysis standard method (pH STAT method). Operational runs were performed at pH 6.0 and pH 8.0 in a magnetic stirred reactor.

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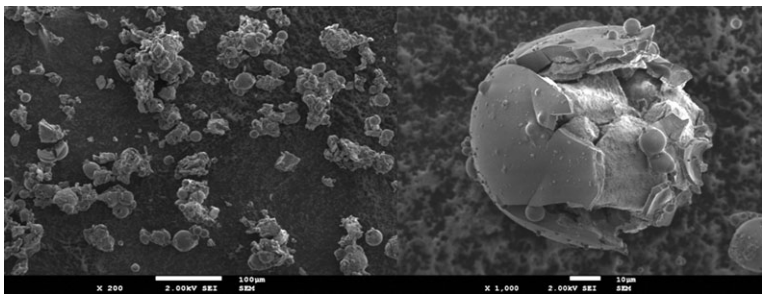

Figure 5. Example of micro-particles degradation (magnetic stirred reactor) after 10 runs of PG hydrolysis.

rated salt solution. In this particular case, the option was sodium chloride (a_w = 0.75, [16, 17]) in order to obtain dry particles (dry particles corresponds to a_w <0.8) at room temperature.

Incubation in an alkali environment has been shown to increase the solubility of the sol-gel [13] and concomitantly lead to particle loss, hence the significant decay of activity throughout consecutive runs (Fig. 4). Further work was nevertheless performed at pH 8.0 since this corresponds to the standard activity assay (pH STAT) for PGA.

The catalytic activity displayed by immobilized PGA was also affected by abrasion. The activity decrease observed in Fig. 6 for a bioconversion performed in a magnetic stirred reactor is likely to be mostly due to the particle attrition promoted by the magnetic bar and the conical shape of the reactor, which causes the degradation of some particles (Fig. 5) and the concomitant loss of biocatalyst throughout the consecutive runs. When a differential, non-stirred, small packed-bed reactor, with reaction media recirculation was used, particle attrition was avoided and activity retention for PG hydrolysis remained constant (Fig. 6).

The dependence of activity on temperature and pH was similar for free and for immobilized PGA (Figs. 7 and 8). The optimum temperature and pH values are 45°C and 7.5, respectively. The energy of activation also remained roughly unchanged following immobilization, since 43.9 and 45.1 kJ/mol, for free and for immobilized enzyme, respectively, were determined from Arrhenius plots. These figures are similar to those described in Fonseca *et al*. [18]. Both the free and the immobilized forms of the enzyme were able to carry out hydrolysis and synthesis reactions efficiently, in aqueous media (Figs. 9–11). Figure 9 shows that, apart from experimental errors, the performances of free and immobilized PGA are very similar. In the synthesis reactions, the free and immobilized enzyme performed at low substrate concentrations (30 mM 7-ADCA, 90 mM D-PGM) and high enzyme load (239 and 455 U/mmol 7-ADCA, respectively) in a magnetic stirred reactor. In these conditions, a maximum conversion yield of 68 and 65%, respectively, was obtained, after 1 h of reaction, with a productivity of 23 and 19 mM/h, respectively, a specific productivity of 95 $mM \times h^{-1} \times g^{-1}_{dry\ biocatalyst}$ and a maximum S/H ratio (the synthesis/hydrolysis ratio) defined as mol product per mol hydrolyzed side chain donor formed of 2.53 (at 60 min) for free PGA and 2.45 (at 10 min) for immobilized enzyme. Illanes *et al*. [20] obtained a similar result for specific productivity (93 $mM \times h^{-1} \times g^{-1}$) but using a large amount of co-solvent (40% v/v ethylene glycol) at high substrate concentrations (150 mM 7-ADCA;

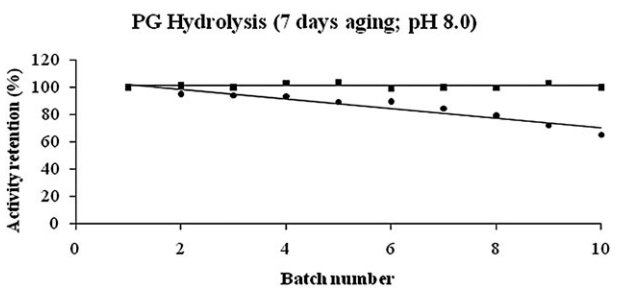

Figure 6. Activity retention for PG hydrolysis in micro-particles with 7 days of aging determined according to the PG hydrolysis standard method (pH STAT method). Operational stability runs were performed at pH 8.0 in a magnetic stirred reactor (●) and in a differential recycled reactor (■).

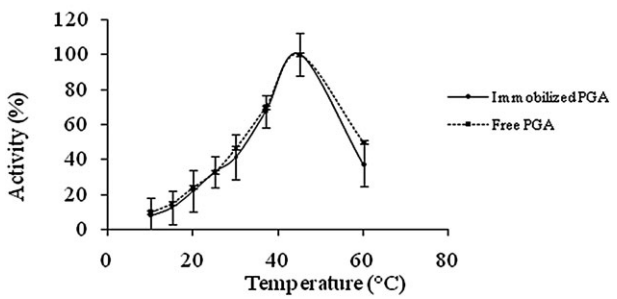

Figure 7. Relative activity versus temperature at pH 8.0 (pH STAT method) for free and immobilized enzyme.

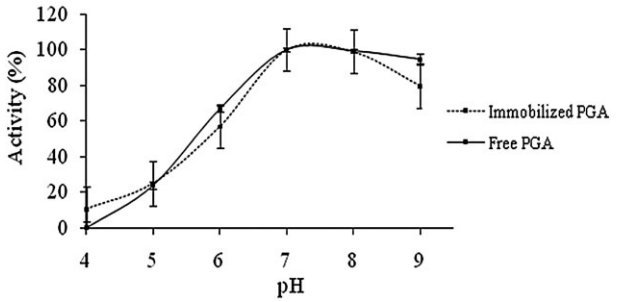

Figure 8. Relative activity versus pH at 37°C (pH STAT method) for free and immobilized enzyme.

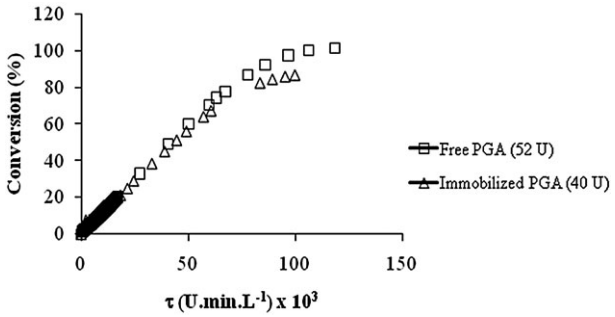

Figure 9. Penicillin G hydrolysis (pH STAT method) with micro-particles of biocatalyst. Runs were performed at 37°C and pH 8.0, 20 mM phosphate buffer, for an initial PG concentration of 4% m/v. The variable τ represents the normalized residence time ($U \times min \times L^{-1}$) [18, 21].

450 mM D-PGM) and low enzyme load (80 U/mmol 7-ADCA). These preliminary results are thus encouraging and are likely to allow for a "green process" and concomitant improvement in terms of costs and environment.

The objectives of immobilization are easy recovery and reuse, high operational and mechanical stability and activity per unit volume, which were mostly achieved by the use of magnetic sol-gel particles. This allowed for good activity recovery after immobilization; the magnetic properties make the biocatalyst recovery more straightforward as compared to the manipulation in filtration or centrifugation, and catalytic activity remained roughly unchanged upon consecutive batch runs.

4 Concluding remarks

The PAG from *Escherichia coli* was successfully immobilized in a silica xerogel matrix with magnetic properties. The biocatalyst was catalytically active and able to perform hydrolysis and synthesis reactions efficiently. PGA tolerated the presence of magnetite in the entrapment particles. Magnetic sol-gel particles thus allowed for high activity recovery after immobilization; the magnetic properties dramatically ease biocatalyst recovery stability, thereby complying with key goals of biocatalyst immobilization. It is still necessary to optimize the synthesis reaction conditions (pH, reagent concentrations and temperature) in order to depress hydrolytic activity of the product and the acyl donor (D-PGM). To minimize biocatalyst degradation caused by particle attrition of magnetic stirring, a differential recycled reactor compatible with the developed magnetic biocatalysts was tested, with the aim to develop a system in which the biocatalyst can be recycled many-fold, a requirement for making biocatalysis economically feasible.

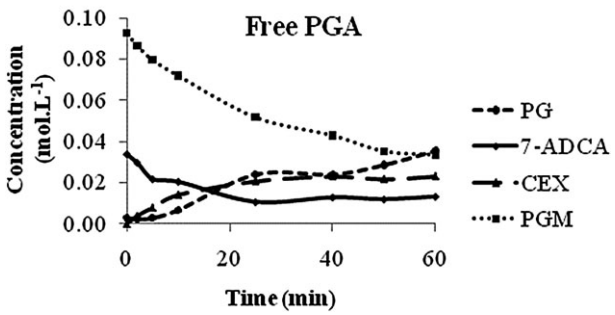

Figure 10. Cephalexin synthesis performed by free PGA. Runs were carried out at 14°C. The pH shifted from 8.0 to 7.5 at the end of the reaction. A conversion yield of 68% was observed, along with a productivity of 23 mM/h.

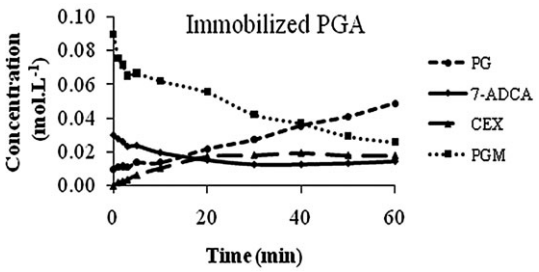

Figure 11. Cephalexin synthesis performed by micro-particles of biocatalyst. Runs were carried out at 14°C. The pH shifted from 8.0 to 7.65 at the end of the reaction. A conversion yield of 65% was observed, along with a productivity of 19 mM/h and a specific productivity of 95 $mM \times h^{-1} \times g^{-1}$.

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The authors have declared no conflict of interest.

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