J Sol-Gel Sci Technol (2011) 58:545–556
DOI 10.1007/s10971-011-2426-7

Optimization in the immobilization of penicillin G acylase by entrapment in xerogel particles with magnetic properties

Susana Bernardino · Nídia Estrela · Vanessa Ochoa-Mendes · Pedro Fernandes · Luís P. Fonseca

Received: 18 November 2010 / Accepted: 10 February 2011 / Published online: 24 February 2011

Abstract Biocatalysis presents a sound alternative to chemical synthesis in the field of drug production, given the highly selective nature of biological catalysts. Penicillin G Acylase (PGA) from *E. coli* is currently used to hydrolyze penicillin G (PG) and catalyzes the synthesis of β-lactam antibiotics. In this work, particular emphasis is given to recent developments in penicillin G acylase immobilization, by entrapment simultaneously with nanomagnetic particles in a silica matrix. The sol–gel biocatalytic particles were prepared either by a conventional method (crushed powder) or by a more recent approach, based in an emulsion system using 150 mM AOT/isooctane, which allowed for the formation of spherical micro- and nanobeads. The effects on PGA activity of different sol–gel precursors, additives, enzyme concentration, aging, drying conditions and mechanical stability were evaluated. After these optimization studies, a mechanically stable carrier based on porous xerogels silica matrixes, starting from tetramethoxysilane (TMOS) with 65–67% PGA activity yield in these carriers allowed an immobilization yield of 74 mg protein $g^{-1}_{dry\ sol-gel}$ and 930 $Ug^{-1}_{dry\ sol-gel}$ for specific activity were obtained.

Keywords Penicillin G acylase · Enzyme immobilization · Sol–gel · Entrapment · Micro-emulsion · Magnetic particles

S. Bernardino
ESTM—School of Tourism and Maritime Technology, Marine Resources Research Group, Polytechnic Institute of Leiria, 2520-641 Peniche, Portugal

S. Bernardino · N. Estrela · V. Ochoa-Mendes · P. Fernandes · L. P. Fonseca
IBB—Institute for Biotechnology and Bioengineering, Centre for Biological and Chemical Engineering, Instituto Superior Técnico, Av. Rovisco Pais, 1049-001 Lisbon, Portugal

S. Bernardino (✉) · N. Estrela · V. Ochoa-Mendes · P. Fernandes · L. P. Fonseca
Department of Bioengineering, Instituto Superior Técnico, Av. Rovisco Pais, 1049-001 Lisbon, Portugal
e-mail: susana.bernardino@ipleiria.pt

N. Estrela
IBB—Institute for Biotechnology and Bioengineering, Centre for Molecular and Structural Biomedicine, Universidade do Algarve, 8005-139 Faro, Portugal

1 Introduction

Enzymes are a significant catalyst of many industrial processes because of their ability to improve reaction rates and product specificity [1, 2]. The industrial production of β-lactam antibiotics and their intermediates is undergoing a remarkable transformation, where the traditional chemical conversions based on stoichiometry, are being replaced by enzyme-catalyzed processes with more environmental benefit approaches—the so-called "green chemistry". In contrast to the complex solvent-based chemical approach, the one-step enzymatic conversion is regio-and stereospecific and can be performed mainly in aqueous medium and under mild conditions [3]. Compared with the chemical process, the enzymatic process has economic, environmental and operational advantages [4, 5]. Penicillin G acylase (PGA) (EC 3.5.1.11), a N-terminal nucleophile hydrolase with molecular dimensions of $7.0 \times 5.0 \times 5.5$ nm, is an industrially important enzyme, which is mainly involved in the production of 6-aminopenicillanic acid (6-APA) and more recently in the industrial synthesis of semi-synthetic β-lactam antibiotics [6]. The application of PGA in the production of β-lactam antibiotics has been

extensively studied by many research groups. Within this scope, PGA from *Escherichia coli* is one of the most studied and used enzyme for commercial purposes [7]. Active PGA from *E. coli* is a heterodimer with a 23 kDa β-subunit and a 62 kDa β-subunit [8] closely intertwined, with no obviously discrete domains [9]. The kidney-shaped enzyme possesses a centrally located deep depression. In its bottom are located the active sites of the enzyme [9, 10]. PGA enzymes have proved effective in their role as biocatalysts for both synthesis and hydrolysis, but their industrial application is often limited by the lack of long term operational stability, and of adequate steps for the recovery and re-use of the enzyme. Improved biocatalysts and higher bioconversion yields than those currently obtained are thus required [11, 12]. Immobilization of enzymes provides a suitable tool to help tackling with all these requirements.

Enzyme immobilization overcomes major problems that are inherent to the use of soluble enzymes. These include product contamination, difficulty of separation from the reaction mixture, and limited reuse, while enabling the use of enzymes in continuous mode of operation [12, 13]. A further benefit of immobilization, which is often quoted, is enhanced stability, under both storage and operational conditions, since immobilization provides a protective (micro)environment towards denaturation by heat or organic solvents, or by autolysis [14]. However, immobilization brings along some drawbacks, such as: (a) loss of activity following immobilization and diffusion limitations, which reduce the productivity of the immobilized enzyme system as compared to the free form; and (b) increased process costs, inherent to the immobilization step and to the processing of the spent immobilized biocatalyst [15]. The major techniques for immobilization reported in the literature include covalent binding, ionic and hydrophobic adsorption, aggregation and entrapment [3, 12, 16].

In recent years, a number of methods have been reported that achieve high volumetric activities, the most efficient being physical aggregation of enzymes and subsequent chemical cross-linking, CLEAS (cross-linked enzyme aggregates) [16, 17]. Several enzymes have been successfully immobilized using this strategy, including PGA. The widespread use of this methodology is somehow hampered by the ability of enzymes to resist chemical cross-linking. Furthermore, diffusion limitations are also still prone to occur, due to aggregation [18, 19]. The difficulty in the handling and in the recovery of biocatalyst particles, due to their low mechanical stability, is also a main drawback of CLEAs immobilization [20].

One promising method for the immobilization of enzymes is a sol–gel technique, where enzymes are confined within a chemically inert silica support, which is mostly, but not exclusively, composed of SiO_2 materials [21–24]. A well-established sol–gel processing technique consists of hydrolyzing the adequate precursors in aqueous solutions to produce soluble hydroxylated monomers, followed by polymerization and phase separation, resulting in a hydrated metal or semi-metal oxide hydrogel. Removal of water from the wet gel, which is usually accompanied by changes in the structure of the pores and of the gel network, results in a porous xerogel [25, 26]. The sol–gel method allows to produce the support material and to entrap the enzyme in a single step, starting from a solution containing both the matrix precursors and the enzyme. When the gelation occurs, the enzyme remains entrapped and homogeneously dispersed in the gel. The characteristics and properties of a particular sol–gel inorganic network are related to a number of factors, which affect the rate of hydrolysis and condensation reactions, such as, pH, temperature and time of reaction, reagent concentrations, aging temperature and drying conditions [27].

There are several advantages of the sol–gel matrixes, in general: (a) they are easily obtained in a variety of forms: monoliths, optically grade polished monoliths, thin films, fibers, powders, etc., that can be miniaturized to micron size, and can be furthermore attached to most other materials (plastic, paper, metals, etc.); (b) the process occurs at near room temperature, which avoids protein denaturation; (c) the easy insertion of substituting groups into the matrix provides the entrapped enzymes with beneficial microenvironments; (d) the matrixes have controllable surface area, average pore size and pore size distribution; (e) they are thermally stable, much beyond the range of temperatures which is of relevance for biocatalysts; (f) are not photodegradable and, in the case of SiO_2 matrixes, are not involved in light-induced reactions with the matrix; (g) do not degrade electrochemically; (h) can be transparent well into the UV range ($\sim$250 nm for SiO_2), and are highly suitable for optical applications; (i) allow control of conductivity; (j) enhance the stability of the encapsulated molecules, as a result of the rigidity of the cage; (k) prevent leaching of proteins due to the effective caging; (l) have a potential advantage over the use of surfaces in the form of adsorption or covalent bonding, since adsorbed molecules easily leak out, and covalently attached molecules are prone to chemical degradation of the anchoring bond [21, 28, 29]. Nevertheless, it has been reported that enzyme leakage is one of the main drawbacks of the physical entrapment of a biocatalyst using the sol–gel technique [30, 31]. This problem has occasionally led to the design of protocols for the preparation of matrixes having a pore size large enough to allow the flow of substrates and products, but small enough to prevent the leakage of the entrapped biocomponent [18, 32].

A very large number of enzymes (e.g. alkaline phosphatases, glucose oxidases, lipases, among many others) have been trapped successfully within sol–gel, while retaining their catalytic activity [21, 26, 33], but for PGA only 10% activity recovery was reported [34]. Trapped enzymes usually exhibit better activity and longer lifetimes than free enzymes. During encapsulation, they remain trapped within a silica cage, so mobility within this confined space is restricted. Unfolding is prevented, thus avoiding denaturation, even when exposed to harsh conditions (viz. high temperature, extreme pH environments, or organic solvents) [24, 35–37].

Diffusion limitations within the porous network are prone to occur in the sol–gel immobilization method, especially in the case of large monoliths [21, 38]. To address this disadvantage, different strategies were developed for the production of small particles. The most common sol–gel encapsulated enzyme systems have been produced in crushed powder form, from the dried xerogel state. However, this method yields irregular shapes and sizes, and it is hardly scalable [39]. Although spray-drying and microwave assisted sol–gel methods for the production of spherical beads have been implemented, these procedures require high temperatures, which are not suitable for in situ enzyme immobilization [40]. A sol–gel emulsion technology has, however, been developed, that combines the emulsion and sol–gel technology, and has been used to prepare nano- or micro-particles [24, 39]. This process is suitable for the immobilization of biological molecules, because of the room temperature and relatively mild processing conditions required. Nevertheless, since the filtering characteristics that are desirable in industrial practice put a lower limit of 0.1–0.2 mm on the particle size of immobilized catalysts, diffusion limitations are prone to take place. Biocatalyst removal using a magnetic field would be a good alternative, even in the presence of solid products, since this approach prevents imposing any lower limit on the particle size [16, 26, 41–43].

Magnetic particles themselves cannot be used in practical applications, since they tend to form large aggregates, which may result in the alteration of magnetic properties, and they can undergo rapid biodegradation when they are directly exposed to the biological system. The fabrication of inert host particles containing the magnetic particle core (magnetite/silica composite particles) is a good technique for preventing such limitation [44]. Previous results demonstrated the efficient immobilization of PGA on sol–gel matrices [45]. The present study aims to highlight the effect of key variables on the yield of PGA immobilization, retention of activity, reaction conversion and mechanical resistance of the produced bicatalysts based on sol–gel technology with magnetic properties.

2 Materials and methods

2.1 Materials

PGA solution from *Escherichia coli* (26.6 mg mL^{-1} protein, as reported by the supplier (Sigma) from absorbance measurement at 280 nm and specific activity of 35 U mg^{-1}), tetramethoxysilane (TMOS) $\geq$ 99%, sodium dioctyl sulfosuccinate (AOT), polyvinyl alcohol (9,000–10,000 31,000–50,000; 70,000–100,000) (PVA), 6-nitro-3-(phenylacetamido)benzoic acid (NIPAB), magnetite nanopowder, were all purchased from Sigma–Aldrich (USA). Methyltrimethoxysilane (MtrMOS) and polyethylene glycol 570–630 (PEG) were supplied by Fluka (USA). Lyophilized PGA from *E. coli* (15 U mg^{-1}) was provided by CPC Biotech S.R.L. (Italy), isooctane was supplied by Riedel de Haën (Germany). Penicillin G (PG) was obtained from Fersinca Gb (Mexico). Gelatin was obtained from Merck (USA). BCA™ Protein Assay Kit was obtained from Thermo Scientific (USA). All other reagents used were either laboratory or analytical grade.

2.2 Electrophoretic analysis

SDS–PAGE was performed using 10% polyacrylamide gel as described by Schägger (2006), [46]. After electrophoresis the gels were stained with Coomassie Brilliant Blue R-250.

2.3 PGA biocatalyst preparation

2.3.1 Sol–gel entrapment (crushed method)

Homogeneous sol was typically prepared by sonicating a solution containing 100 μL of the alkoxysilane precursors in four different combinations (100 μL TMOS; 60 μL MTrMOS + 40 μL TMOS; 60 μL TMOS + 40 μL MTrMOS; 100 μL MTrMOS) and 40 μL of 10 mM HCl in a water sonication bath (Transsonic T 460) at 4°C for 10 min [46, 47]. To the freshly prepared sol (140 μL) were either added 75 μL of PGA solution (Sigma) suspended in 85 μL of the magnetic suspension—10% (w/v)—or 100 μL of 50 mg mL^{-1} PGA solution (CPC Biotech S. R. L.) suspended in 60 μL of magnetic nano-particles suspension 20% (w/v) in 100 mM phosphate buffer, pH 7.5. Biocatalysts without magnetic properties were produced replacing the magnetite suspension by phosphate buffer only. The resulting sol–gel was dried at atmospheric pressure in open 1.5 mL Eppendorf tubes at room temperature, at 2–8°C in the refrigerator, or at room temperature under controlled water activity ($a_w = 0.75$) for at least 24 h to allow polymerization and aging [45]. The resulting sol–gel was then crushed in a

mortar to a fine powder, and suspended in 1 mL of the same 100 mM phosphate buffer pH 7.5. The biocatalyst suspension was either immediately used or stored at 2–8°C.

2.3.2 Sol–gel entrapment (micro-emulsion method)

According to this methodology, enzyme immobilization was achieved by mixing the sol–gel solution containing the enzyme with the solvent containing already the surfactant [48]. Thus, a sol–gel solution was prepared with TMOS as described previously in Crushed method. To obtain micro-particles or nano-particles by emulsion sol–gel methodology, 300 μL of the sol–gel solution with enzyme were immediately added to 6 mL of 150 mM AOT/isooctane solution, before gelation. The resulting mixture was either placed under vortex (VF2 Janke & Kunkel, IKA Labortechnik) for 1 min to produce micro-particles, or sonicated with a sonifier (Branson Sonifier 250) during 1 min, in continuous operation mode at 40 W, allowing to obtain only nano-particles, and then washed twice with 100 mM phosphate buffer pH 7.5. The silica beads were collected either by magnetic separation, using a Dynal® MPC™-1 (Invitrogen Corporation, Carlsbad, California, USA), with *Neodymium-Iron-Boron permanent magnets,* B_r (Remanence): 12,200 Gauss 1,220 mTesia, ${}_bH_c$ (Coercive Force): 11,400 Oersteds 410 kA/m, ${}_jH_c$ (Intrinsic Coercive Force): 17,000 Oerstedt 1,350 kA/m, BH_{max} (Maximum Energy Product): 38 × 106 GOe 306 kJ/m^3; or by centrifugation during 2 min at 14,000 rpm (centrifuge 5417R from Eppendorf, rotor F45-30-11). Beads were then dried and aged as described in Crushed method. The micro and nano-particles obtained were suspended in 1 mL of the same 100 mM phosphate buffer pH 7.5. The particles were either immediately used or stored at 2–8°C.

2.3.3 Modifications of the immobilization procedure

Gelatin and other additives, prepared as 5% (w/v) solutions in water (gelatin and PVA) or in 100 mM phosphate buffer pH 7.5, were added to the enzyme solution, prior to mixing with the sol–gel solution (Micro-emulsion method, without magnetite), to obtain a final additive concentration of 1.4% (w/v).

2.4 Enzyme activity assay

One unit of PGA activity (U) for the soluble and immobilized enzymes is defined as described by Bernardino et al. (2009), [45], briefly:

2.4.1 pH STAT method

The amount of enzyme required to produce 1 μmol 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 [49], using a 4% (w/v) penicillin solution in 20 mM phosphate buffer pH 8.0, at 37°C in a Titrino 702 SM (Metrohm) with a LL Micro glass electrode (Metrohm).

2.4.2 NIPAB method

The amount of enzyme required to produce 1 μmol of 3-amino-6-nitrobenzoic acid (NABA) at 30°C and pH 7.0. The activity assay was based on the hydrolysis 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 in a Hitachi U—2000 spectrophotometer. 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), under magnetic agitation at 800 rpm (Electronic Stirrer Model 300—Rank Brothers, LTD).

2.5 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.

2.6 Mechanical stability

In order to evaluate the mechanical stability of the biocatalyst, ten consecutives runs of PG hydrolysis were performed by the pH STAT method (as described in activity assay) in: (a) a conical reactor, either under magnetic stirring or under 120 rpm mechanical stirring, promoted by a four pitched-bladed turbine; (b) a fixed bed reactor, operated as a differential reactor. The leakage of PGA from the sol–gel matrix was investigated by removing the immobilized beads from the bioconversion medium, and allowing incubation to proceed further. For the quantification of leakage the activity of the immobilized PGA was assessed for 10 min (pH STAT method); once immobilized PGA was removed, the reaction was followed for another 10 min, to assess if the reaction proceeded further. The bioconversion medium was then concentrated 10-fold by ultra-filtration in a stirred cell, UHP—43 mm (Cole-Parmer Instrument), fitted with a 10 kDa ultra-filtration membrane of regenerated cellulose (Sigma), and the retentate screened for the presence of enzyme by 12%

SDS–PAGE gel electrophoresis. The activity of the retentate was detemined according to the NIPAB method.

2.7 SEM

Dry particles of biocatalyst were placed on a double carbon tape and analyzed in a Field Emission Scanning Electron Microscope (Jeol JSM-7001F). Some particles were gold-coated with a Polaron E5100 sputter coater (±20 nm thick).

2.8 Assay for protein concentration

The concentration of protein in the enzyme solution was determined spectrophotometrically in a Hitachi U-2000 spectrophotometer by direct reading at 280 nm or by the Pierce BCA Assay. The quantification of protein was performed before immobilization, and both in the supernatant and in the effluents from the washing steps, after immobilization. The amount of protein entrapped in the support was calculated by mass balance. The yield of immobilization was calculated as the ratio of the amount of protein entrapped in the sol–gel matrix to the initial amount of protein. Yields were expressed as a percentage. All assays were performed in triplicate.

2.8.1 Abs 280 nm

The quantification of protein concentration by absorbance at 280 nm was performed using as reference a calibration curve previously established using standards of well-known PGA or BSA concentrations.

2.8.2 Pierce BCA assay (BCA™ protein assay kit)

The quantification of protein concentration was performed according to the methodology suggested by the supplier [50, 51], and using as reference a calibration curve. This was previously established, based in standards of well known PGA or BSA concentrations.

3 Results and discussion

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, catalyst nature (acid or basic) and concentration, $[H_2O]/[Si]$ molar ratio, aging temperature and time, and drying [27]. To optimize PGA immobilization in silica sol–gel matrixes, multiple immobilization conditions were compared by varying the precursors, water content, use of additives, aging, drying conditions and enzyme concentration. The mechanical stability of the optimized biocatalyst was investigated by incubating the sol–gel particles in consecutive batch runs, performed in both magnetic and mechanical stirred reactors, and in a differential fixed bed reactor.

3.1 Effect of precursor for sol–gel immobilization using crushed method

The different combinations of sol–gel precursors has been shown to affect enzyme activity, suggesting that each enzyme has a unique preference for a particular silica gel matrix, which depends on the characteristics of the precursors [39]. The action of the hydrophobic moieties as well as the polymeric additives is multifold because they can modify the interactions between the gel network and the enzymes [52]. In order to examine the effects of the precursors on PGA activity, two different precursors for the formation of the sol–gel matrix, TMOS and MTrMOS, were investigated. If only TMOS is used as precursor, the wet gels mostly carry Si–OH sides groups which gives a hydrophilic character to the gels. If the proportion of MTrMOS is sufficiently high, the proportion of hydrophobic groups ($-CH_3$) in the pore surface will be enough to give a hydrophobic character to the gels, so that polar liquids such as water do not wet the surfaces covered by such hydrophobic groups. Control experiments allowed to establish that the gels presented no activity in the absence of PGA (data not shown). The relative activity of PGA was measured according to the ratio of TMOS and MTrMOS, in order to determine the optimum ratio between the two precursors (Table 1).

The use of TMOS for sol–gel formation consistently allowed for higher enzyme activity, as compared to the use of MTrMOS or the use of mixtures of TMOS and MTrMOS. The condensation is faster using only TMOS, a feature that can be ascribed to the refractive nature of the organometalic $Si-CH_3$ bond towards hydrolysis. The hydrolysis and condensation of alkoxisilane MtrMOS, $(CH_3)Si(OCH_3)_3$, is therefore more difficult to promote than the hydrolysis and condensation of the alkoxide TMOS, $Si(OCH_3)_4$, the former requiring more basic catalysts to accelerate the process [52].

Table 1 Immobilized PGA (Sigma) activity (NIPAB method) according to the ratio of precursors

Ratio (%) (TMOS:MTrMOS)	100:0	60:40	40:60	0:100
Inmob yield (%)	97	77	52	41
Sol–gel activity (NIPAB) (%)	37	16	12	7
Supernatant activity (NIPAB) (%)	ND	20	41	57

ND non detectable

Source: Bernardino et al. (2010)

Accordingly, when mixtures of TMOS and MTrMOS were used, the gel network was initially mostly built by TMOS, while MTrMOS mostly covers the pore surface in a second stage reaction [52]. This could explain the results that suggest the preference of PGA for more hydrophilic supports. Thus, using only TMOS, PGA is encapsulated in a hydrophilic sol–gel matrix, whereas using a mixture of both or only the precursor MTrMOS, PGA can not be totally encapsulated. Taking into account that MTrMOS condensation is slower and confers a hydrophobic character to the gel, PGA is not retained in the sol–gel matrix, being most likely preferentially forced towards the surface of the gel and ultimately into the surrounding macro-environment as the gel is formed, due to decreased electrostatic interaction with the surface, which can explain the increase in the activity in the supernatant and the decrease in immobilization yield, as the proportion of MTrMOS increases. Another possibility is that MTrMOS lead to more open structure i.e. high pore size of the silica matrix network due to the present of $–CH_3$ group which increase the leakage of the enzyme during immobilization and washing steps, an hypothesis put forward previously [53]. However, data presented by Kawakami and Yoshida point differently, since these authors observed a decrease in the pore size in sol–gel particles with the increase of MTrMOS to TMOS ratio, up to a virtually non-porous solid when only MTrMOS was used [54], The microenvironment is a key issue in enzyme encapsulation [12, 26] and PGA encapsulation is clearly favored using more hydrophilic precursors. Therefore, TMOS was applied as a single precursor for all experiments in which the sol–gel emulsion system (Micro-emulsion method) was used.

3.2 Crushed xerogel versus micro-emulsion sol–gel method

The immobilized PGA prepared by the micro-emulsion method displayed higher activity when compared to the biocatalyst prepared according to the conventional crushed xerogel silica matrix method (Fig. 1). The crushing of the silica particles yields irregular shapes and sizes (Fig. 2) which leads to a heterogeneous mixture, hardly reproducible, and diffusion limitations are likely to be enhanced given the presence of the relatively large particles. The extent and the process of mixing play a crucial role in the shape and the size of the beads prepared by the micro-emulsion method. Thus, under very mild mixing conditions irregular beads were obtained with a relatively large average particle size (around 100 μm) [24, 39]. Under the vortex agitation (maximum speed) used in the present work, the beads obtained were spherical (Fig. 3) and polydispersed (<30 μm): Furthermore, for particles within such size range, mass transfer limitations of substrates and products are not significant [39]. Smaller, nano-particles

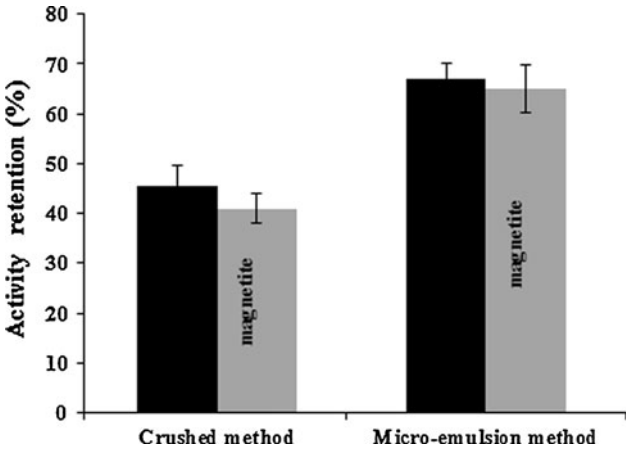

Fig. 1 Crushed xerogel versus reverse microemulsion sol–gel method, using only TMOS, with and without magnetite as additive. Activity retention (pH STAT method) of the immobilized PGA (Sigma) sol–gel particles relative to the initial activity of soluble PGA

were obtained under ultrasound agitation (<100 nm). However, when a magnetic field was applied to perform the recovery of the superparamagnetic silica composite nanospheres, the process was quite ineffective. The poor magnetic response of the nanospheres can tentatively be ascribed to the low loading density of the superparamagnetic iron oxide, in this case, magnetite (Fe_3O_4), in the sol–gel nanoparticles, which makes the recovery of magnetic beads very difficult as it was also observed by Li et al. (2008) and Kobayashi et al. (2008) [29, 44]. The recovery of the sol–gel nano-beads without magnetite was performed by centrifugation, but again, the process was not fully effective, so further work was performed with micro-particles. In this study, activity retention was determined by pH STAT method, after one week aging at room temperature under controlled water activity ($a_w = 0.75$). The highest activity retention yield was 67%, for micro-emulsion method, and 45% for crushed particles without magnetite. There are three main reasons responsible for the loss of activity during PGA immobilization [48]: (a) methanol produced during gel formation; (b) physical loss of biocatalyst during the immobilization procedure; and (c) physical stress within the silica sol–gel matrix. The small differences observed in particles with magnetite (65 and 41%, respectively, micro-emulsion and crushed sol–gel) were probably due to physical loss, which occurred in the tip of the micropipette during the experimental manipulation.

3.3 Effect of the ratio of TMOS to PGA solution

The effects of the ratio of TMOS to the enzyme solution on enzyme activity were also examined in order to determine the optimum volume ratio, for a fixed amount of PGA (2 mg). As described above, the $[H_2O]/[Si]$ molar ratio is

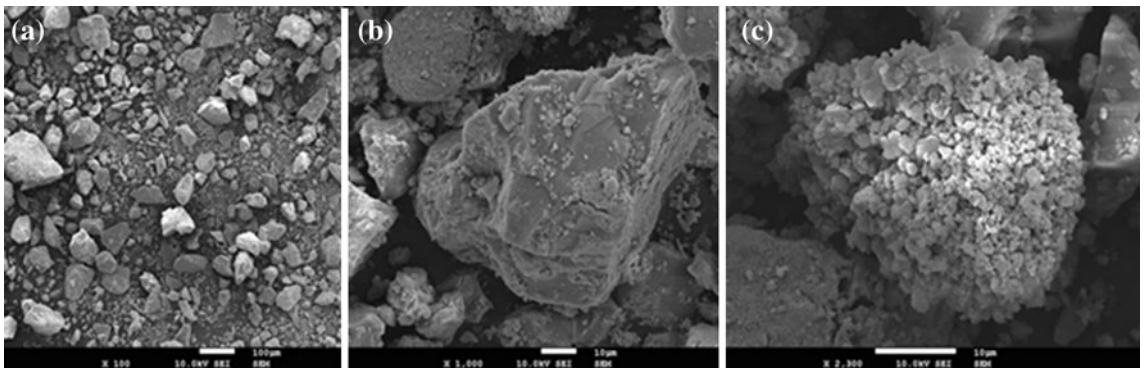

Fig. 2 SEM micrograph of silica sol–gel particles crushed in a mortar (Crushed method) with encapsulated magnetite and PGA. The particles were gold coated with a Polaron E5100 sputter coater (±20 nm thickness). In Fig. 2 **a** bar match 100 μm and in Figs. 2 **b** and **c** bar match 10 μm

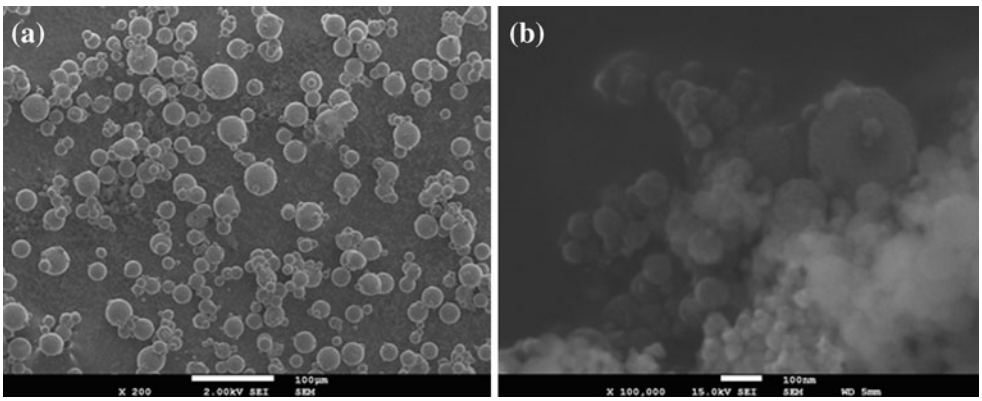

Fig. 3 SEM micrograph of PGA and magnetite entrapped on silica xerogel produced by sol–gel microemulsion (Micro-emulsion method). **a** micro-particles (bar match 100 μm); **b** nano-particles (bar match 100 nm). SDS–PAGE 10%. *Lane 1*: molecular-mass markers; *lane 2*: PGA 26.6 mg mL^{-1} (Sigma) 1:50; *lane 3*: reaction media PG hydrolysis 10 times concentrated; *lane 4*: PGA 50 mg/mL (CPC Biotech) 1:100

an important parameter that influences the structure and properties of the sol–gel inorganic network [27, 52]. The activity results according to the ratio of TMOS to the PGA solutions, which are presented in Table 2, show that enzyme activity increased as the amount of PGA solution used increased. When the volumetric ratio of the enzyme to the precursor solution was 6:10 the enzyme activity was negligible, whereas the highest activity was obtained when the ratio of the enzyme to precursor solution was 16:10. This ratio was therefore used for all subsequent experiments. For a ratio of 20:10, the condensation occurred too slowly. Bergerone et al. (2008), [55], suggested that when the amount of water added to hydrolysed silica precursors was low, the enzyme conformation was frozen during gelation. This proposal was consistent with the observed catalytic activity of β-galactosidase, which was higher in wet gels than in dry ones [51].

Table 2 Immobilized PGA (CPC Biotech) activity (pH STAT method) according to the volume ratio of TMOS to enzyme solution

Volume ratio (PGA:TMOS)	6:10	8:10	10:10	12:10	14:10	16:10	20:10
PGA (μl)	60	80	100	120	140	160	200
TMOS (μl)	100	100	100	100	100	100	100
HCL (μl)	40	40	40	40	40	40	40
Activity retention (%)	6	16	29	41	50	67	ND
Activity ($Ug^{-1}_{dry\ sol-gel}$)	98	239	396	517	586	733	ND

Amount of PGA (CPC Biotech) per immobilization; 2 mg. Sol–gel particles produced as described in Micro-emulsion method

ND non detectable

Source: Bernardino et al. (2010)

3.4 Effect of additives

The addition of additives such as polyethylene glycol (PEG), polyvinyl alcohol (PVA), and proteins like albumin, gelatin and others, can have a stabilizing effect on

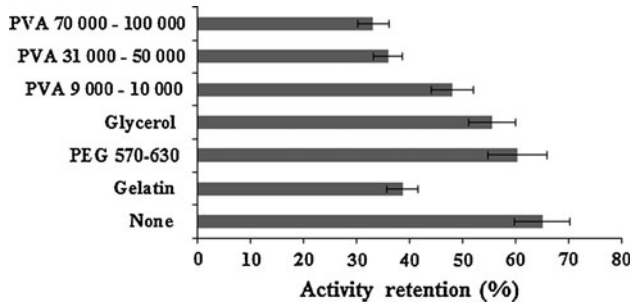

Fig. 4 Effect of various stabilization reagents on the recovery of immobilized PGA. The sol–gel particles were prepared as described in Micro-emulsion method. The beads contained 1.4% (w/v) of each additive

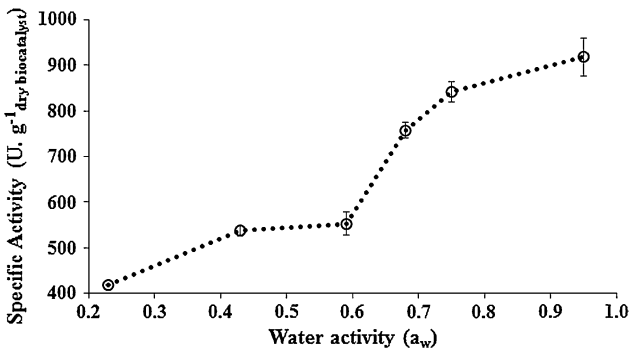

Fig. 5 Effect of water activity. Specific activity by pH STAT method of the immobilized PGA (CPC Biotech) sol–gel particles (micro-emulsion method) after 7 days incubation at room temperature (RT) under controlled water activity

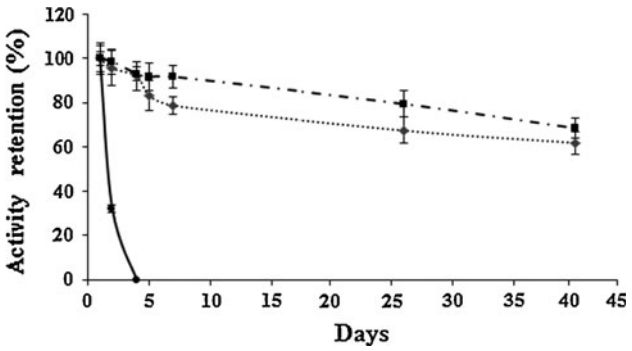

Fig. 6 Effect of different drying and aging conditions. Activity retention by pH STAT method of the immobilized PGA (Sigma) sol–gel particles (micro-emulsion method) relative to the activity after one day of immobilization. (*filled circle*) Incubation at RT (room temperature), (*filled diamond*) 2–8°C in the refrigerator, (*filled square*) RT under controlled water activity ($a_w = 0.75$)

sol–gel entrapped enzymes [10, 12, 56]. The presence of polyols has been shown to increase the thermostability of PGA from *E. coli* [57]. Within this scope, several additives were screened to assess their stabilizing effect on PGA activity (Fig. 4). None of the additives tested improved PGA activity, so in further work no additives were used. The addition of polymers, such as PEG, or of organic functionalities to the silica gels, makes it possible to modify the enzyme silica interactions [52]. As the condensation reaction proceeds, the pore size decreases as a result of the continued formation of siloxane chains (–Si–O–Si–) and the final pore size of a xerogel is less than 100 Å [48]. Given the dimensions of the PGA molecule (7.0 × 5.0 × 5.5 nm), it can be suggested that the additives tested could minimize the conformational freedom of immobilized PGA within silica matrix, reducing the enzyme mobility and therefore catalytic activity.

3.5 Effect of different aging and drying conditions

The conditions used for aging and drying the sol–gel have been shown to influence the characteristics of the final product [58]. Pore size and surface area of synthesized silica hydrogel by acid decomposition reaction were shown to depend on the silica polymerization [27]. Also, under the constant temperature and pressure conditions, pore size was changed with aging which affects enzyme activity. The results show that water content plays a key role in the stability of immobilized PGA (Figs. 5, 6). As the water activity (a_w) increases so does the retention of PGA activity (Fig. 5), but with a_w values over 0.8 it was impossible to obtain dry powders in concordance with experimental results of others authors [45, 59]. So, in further work a_w was fixed at 0.75. The best results were obtained for aging at room temperature under controlled water activity which allowed for the production of a dry powder, with activity retention of 68% after 40 days of storage. Drying at 2–8°C in the soggy environment of a refrigerator led to a moist powder as final product, which hinders manipulation and mass transfer, although displaying 62% activity retention after 40 days of storage. At room temperature, without controlled water activity, the capillary stresses that occur during evaporation are largely superior to the evaporation under controlled water activity, adding further shrinkage, and most likely pore collapse, resulting in a marked decrease in enzyme activity [37, 60]. In further work, drying and aging were carried out for 7 days.

3.6 Effect of enzyme concentration on immobilization

Different amounts of enzyme were added in the immobilization step in order to establish the capacity of the carrier for the retention of enzyme activity. The effect of PGA concentration added during the immobilization step is shown in Fig. 7. The catalytic activity of the sol–gel increased with an increasing concentration of PGA, and reached a plateau at a protein concentration of

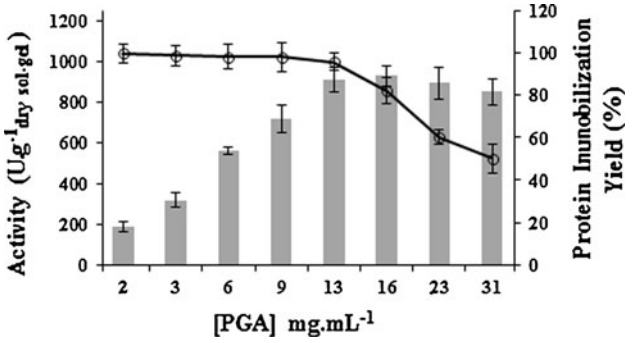

Fig. 7 Micro-emulsion method with magnetite and 7 days aging at room temperature at $a_w = 0.75$. Effect of enzyme (PGA from CPC Biotech) concentration on immobilization yield and activity (pH STAT method). (*Filled square*) specific sol–gel activity; (*continuos line*) Protein immobilization yield

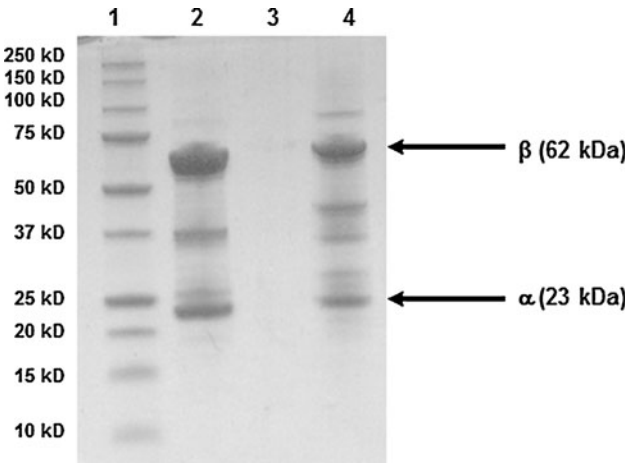

Fig. 8 SDS-PAGE 10%. Lane *1*: molecular-mass markers; *lane 2*: PGA 26.6 mg mL^{-1} (Sigma) 1:50; *lane 3*: reaction media penicillin G hydrolysis 10 times concentrated; *lane 4*: PGA 50 mg mL^{-1} (CPC Biotech) 1:100

13 mg mL^{-1}. A further increase in enzyme loading beyond a critical amount did not result in higher catalytic activity. The yield of immobilization remains approximately constant up to 13 mg mL^{-1} and for higher concentrations decays abruptly, which can be ascribed to the failure of the the gel to encapsulate the total enzyme added. A significant part of the enzyme added may remain adsorbed on the surface of the gel, being released during the wash steps, as suggested by the presence of active enzyme in the supernatants of washings. The decrease of the specific activity can be related to loss of protein for the supernatant, so there is an apparent limit of protein that can be retained in the support, which might occur at very high concentrations. In the conditions tested, the higher values for immobilized PGA obtained were 74 mg protein $g^{-1}_{dry\ sol-gel}$ and 930 $Ug^{-1}_{dry\ sol-gel}$.

3.7 Mechanical stability of immobilized PGA in micro-sol gel beads

PGA mechanical stability in sol–gel was evaluated with beads prepared under optimized conditions (Micro-emulsion method with magnetite and 7 days aging at room temperature at $a_w = 0.75$). After ten consecutive reactions of PG hydrolysis (pH STAT method) the remaining catalytic activity exceeded 70, 85 and 95%, for magnetic stirred conical reactor, mechanical four blades impeller stirred reactor and differential fixed bed reactor, respectively. Once the immobilized biocatalysts was removed, no enzyme activity was detected in the bioconversion medium after 10 min of reaction (pH STAT method), or in the 10-fold concentrated reaction media (NIPAB method), and no protein was observed in the gel electrophoresis SDS–PAGE (Fig. 8), suggesting that PGA did not leak from the sol–gels particles. The activity of sol–gel immobilized PGA was effectively retained in the beads during the reaction period, and the mechanical stability was maintained. The activity decrease could be mainly ascribed to physical losses of the particles during manipulation, namely in the transfer procedures required for the reuse of the immobilized biocatalyst. Physical degradation of the magnetic particles was significant in the magnetic stirred reactor, but this limitation could be avoided using

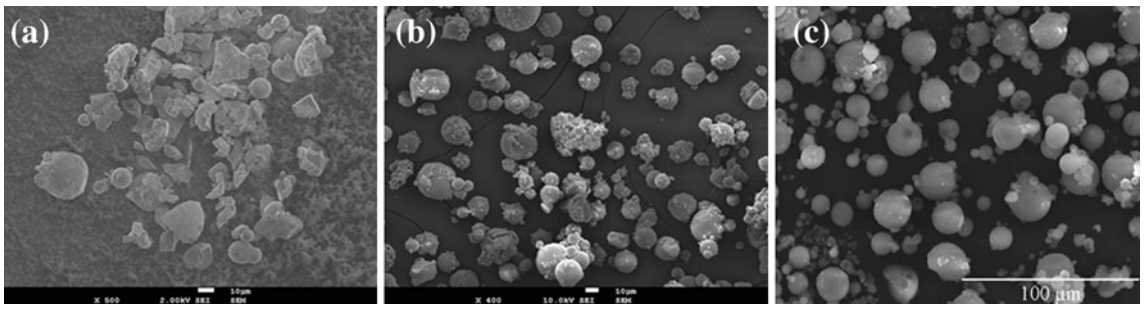

Fig. 9 SEM micrograph of PGA and magnetite entrapped on silica xerogel produced by sol–gel micro-emulsion (Micro-emulsion method) after 10 consecutive reactions of penicillin G hydrolysis. **a** magnetic stirred conical reactor (bar match 10 μm); **b** mechanical stirred reactor (bar match 10 μm); **c** differential fixed bed recycled reactor (bar match 100 μm)

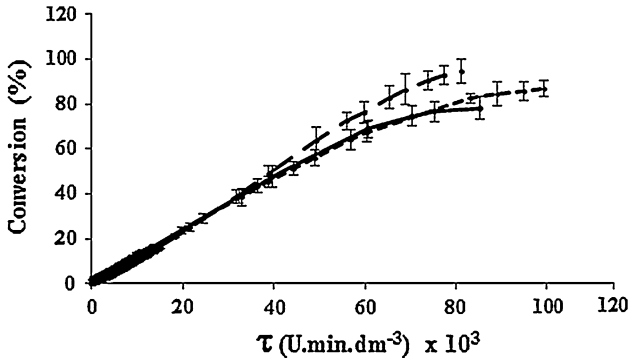

Fig. 10 Penicillin G hydrolysis (pH STAT method) with microparticles of biocatalyst. Runs were performed at 37°C and pH 8.0, 20 mM phosphate buffer, for an initial PG concentration of 4% (m/v). The variable τ represents the normalized residence time (U min dm^{-3}), (Fonseca et al. 1993; Carvalho et al., 2000). (*continuos line*) Mechanical stirred reactor; (*dotted line*) Magnetic stirred reactor; (*thick line*) Fixed bed reactor

mechanical agitation or a fixed bed reactor, where the physical degradation effects were negligible (Fig. 9). Diffusion limitations are larger in the fixed bed reactor and magnetic stirred reactor (Fig. 10), which caused a decrease in conversion yield during penicillin G hydrolysis reaction. Compaction of the bed in the fixed-bed reactor was observed, most likely as a result of a significant pressure drop across the bed, caused by the relatively small dimension of the support particles (<30 μm), and given the down-flow mode of operation. The compaction further enhances pressure drop, the whole leading to a decline in reactor performance [61]. In the mechanically stirred reactor the diffusion limitations are lower, so it was found that this configuration is more suitable for reactions with the produced sol–gel particles.

4 Conclusions

Increased operational stability of immobilized enzymes is essential in order to achieve cost benefits required for the implementation of bio-based processes in large-scale. The feasibility of silica sol–gel immobilization methods using the xerogel method has been established [22, 30, 62], however, the results published to the level of retention of activity after immobilization of PGA on sol-gel matrices were not competitive [34]. This drawback was overcome as described recently [45], however, in this work the mechanical stability of the biocatalyst was not established, a feature which was ascertained in the present work. The water-in-oil reverse micro-emulsion method is a good technique for fabricating superparamagnetic silica composite microbeads [63].

With the methodology presented in this work, PGA immobilization could be successfully carried out in silica xerogels micro-particles (<30 μm) with magnetic properties, with activity retention of 65%, upon immobilization. Scaling-up of the immobilization method could lead to improved results, namely since the physical losses upon immobilization procedures and reuses would be minimized.

Acknowledgments S. M. S. A. Bernardino, N. I. Estrela and P. Fernandes acknowledge Fundação para a Ciência e a Tecnologia (Portugal) for financial support in the form of the PhD grants SFRH/BD/30632/2006, SFRH/BD/18639/2004 and for a contract under Program Ciência 2007, respectively.

References

1. Cheetham PSJ (1995) The application of enzymes in industry. In: Wiseman A, Horwood Ellis (eds) Handbook of enzyme biotechnology. Wiley, London
2. Straathof AJJ, Panke S, Schmid A (2002) The production of fine chemicals by biotransformations. Curr Opin Biotechnol 13: 548–556
3. Giordano RC, Ribeiro MPA, Giordano RLC (2006) Kinetics of β-lactam antibiotics synthesis by penicillin G acylase (PGA) from the viewpoint of the industrial enzymatic reactor optimization. Biotecchnol Adv 24:27–41
4. Jin X, Wu Q, Chen Q, Chen C-X, Lin X-F (2008) Immobilization of penicillin G acylase on a composite carrier with a biocompatible microenvironment of chitosan. J Chem Techonol Biotechnol 83:1710–1716
5. Shewale JG, Sivaraman H (1989) Penicillin acylase: enzyme production and its application in the manufacture of 6-APA. Process Biochem 24:146–154
6. Sun H, Bao XY, Zhao XS (2009) Immobilization of penicillin G acylase on oxirane-modified mesoporous silicas. Langmuir 25:1807–1812
7. Serenovic L, Stankovic N, Spizzo P, Basso A et al (2006) Highlevel production and covalent immobilization of *Providencia rettgeri* Penicillin G Acylase (PAC) from recombinant *Pichia pastoris* for the development of a novel and stable biocatalyst of industrial applicability. Biotechnol Bioeng 93:344–354
8. Ignatova Z, Wischnewski F, Notbohm H, Kasche V (2005) Prosequence and Ca^{2+} -binding: implications for folding and maturation of Ntn-hydrolase Penicillin Amidase from *E. coli*. J Mol Biol 348:999–1014
9. Duggleby HJ, Tolley SP, Hill CP, Dodson EJ, Dodson G, Moody PCE (1995) Penicillin acylase has a single amino acid catalytic centre. Nature 373:264–268
10. Arroyo M, de la Mata I, Acebal C (2003) Biotechnological applications of penicillin acylases: state-of-the-art. Appl Microbiol Biotechnol 60:507–514
11. Illanes A (2004) Optimization of cephalexin synthesis with immobilized penicillin acylase in ethylene glycol medium at low temperatures. J Mol Catal B Enzym 30:95–103
12. Sheldon RA (2007) Enzyme immobilization: the quest for optimum performance. Adv Synth Catal 349:1289–1307
13. Mateo C, Palomo JM, Fernandez-Lorente G, Guisan JM, Fernandez-Lafuente R (2007) Improvement of enzyme activity, stability and selectivity via immobilization techniques. Enz Microb Technol 40:1451–1463
14. Cabral JMS, Kennedy JF (1993). In: Gupta MN (ed). Thermostability of enzymes. Springer, Berlin
15. Schroën CGPH, Eldin MSM, Janssen AEM, Mita GD, Tramper J (2001) Cephalexin synthesis by immobilised penicillin G acylase

under non-isothermal conditions: reduction of diffusion limitation. J Mol Catal B Enzym 15:163–172
16. Kallenberg AI, van Rantwijk F, Sheldon RA (2005) Immobilization of penicillin G acylase: the-linked enzyme aggregates (CLEAs): a novel and versatile method for enzyme immobilization (a review). Biocatal Biotrandformation 23(3/4):141–147
17. Yu HW, Chen H, Wang X, Yang YY, Ching CB (2006) Cross-linked enzyme aggregates (CLEAs) with controlled particles: application to *Candida rugosa* lipase. J Mol Catal B Enzym 43:124–127
18. Betancor L, Luckarift HR (2007) Biosilica-immobilized enzymes for biocatalysis. In: Palomo JM (ed) Recent advances in biocatalysis and biotransformation. Research Signpost, Florida
19. Sheldon RA, Schoevaart R, Van Langen LM (2005) Cross-linked enzyme aggregates (CLEAs): a novel and versatile method for enzyme immobilization (a review). Biocatal Biotransform 23(3/4):141–147
20. Wilson L, Illanes A et al (2004) Encapsulation of crosslinked penicillin G acylase aggregates in lentikats: evaluation of a novel biocatalyst in organic media. Biotechnol Bioeng 86(5):558–562
21. Avnir D, Braun S, Lev O, Ottolenghi M (1994) Enzymes and other proteins entrapped in sol-gel materials. Chem Mater 5:1605–1614
22. Livage J (1997) Sol-gel processes. Curr Opin Solid State Mater Sci 2:132–138
23. Livage J, Coradin T, Roux C (2001) Encapsulation of biomolecules in silica gels. J Phys Condens Matter 13:R673–R691
24. Shin MJ, Park JY, Park K, Song S, Yoo YJ (2007) Novel sol-gel immobilization of horseradish peroxidase employing a detergentless micro-emulsion system. Biotechnol Bioprocess Eng 12:640–645
25. O′Neill H, Angley CV, Hemery I, Evans BR, Dai S, Woodward J (2002) Properties of carbohydrate-metabolizing enzymes immobilized in sol-gel beads: stabilization of invertase and β-glucosidase by blue dextran. Biotechnol Lett 24:783–790
26. Reetz MT, Zonta A, Vijayakrishnan V, Schimossek K (1998) Entrapment of lipases in hydrophobic magnetite-containing sol-gel materials: magnetic separation of heterogeneous biocatalysts. J Mol Catal A Chem 134:251–258
27. Brinker JC, Scherer GW (1990) Sol-gel science: the physics and chemistry of sol-gel processing. Book News Inc, Portland
28. Kawakami K, Yoshida S (1995) Sol-gel entrapment of lipase using a mixture of tetramethoxisilane and methyltrimethoxysilane as the alkoxide precursor: esterification activity in organic media. Biotechnol Tech 9:701–704
29. Li B, Takahashi H (2000) New immobilization method for enzyme stabilization involving a mesoporous material and an organic/inorganic hybrid gel. Biotechnol Lett 22:1953–1958
30. Jin W, Brennan JD (2002) Properties and applications of proteins encapsulated within sol-gel derived materials. Anal Chim Acta 461:1–36
31. Zhou HX, Dill KA (2001) Stabilization of proteins in confined spaces. Biochemistry 40:11289–11293
32. Betancor L, López-Gallego F, Hidalgo A, Fuentesm M, Podrasky O, Kuncova G, Guisán JM, Fernandez-Lafuente R (2005) Advantages of the pre-immobilization of enzymes on porous supports for their entrapment in sol-gels. Biomacromolecules 6:1027–1030
33. Gill I, Ballesteros A (2000) Bioencapsulation within synthetic polymers (part 1): sol-gel encapsulated biological. Trends Biotechnol 18:282–296
34. Basso A, De Martin L, Ebert C, Gardossi L, Tomat A, Casarci M, Rosi OL (2000) A novel support for enzyme adsorption: properties and applications of aerogels in low water media. Tetrahedrom Lett 41:8627–8630
35. Avnir D, Coradin T, Lev O, Livage J (2006) Recent bio-applications of sol-gel materials. J Mater Chem 16:1013–1030
36. Lee C-H, Lin T-S, Mou C-Y (2009) Mesoporous materials for encapsulating enzymes. Nano Today 4:165–179
37. Desimone MF, Matiacevich SB, Buera MP, Diaz LE (2008) Effects of relative humidity on enzyme activity immobilizedin sol–gel-derived silica nanocomposites. Technology 42:583–588
38. Wei Y, Xu J, Fen Q, Dong H, Lin M (2000) Encapsulation of enzymes in mesoporous host materials via the nonsurfactant-templated sol-gel process. Mater Lett 44:6–11
39. Chang-won L, Yi S, Kim J, Lee Y, Kim B (2006) Improved immobilized enzyme systems using spherical micro silica sol-gel enzyme beads. Biotechnol Bioprocess Eng 11:277–281
40. Adachi K, Iwamura T, Chujo Y (2004) Novel synthesis of submicrometer silica spheres in non-alcoholic solvent by microwave-assisted sol-gel method. Chem Lett 33:1504–1505
41. Dong J, Xu Z, Wang F (2008) Engineering and characterization of mesoporous silica-coated magnetic particles for mercury removal from industrial effluents. Appl Surf Sci 254:3522–3530
42. Abu-Reziq R, Wang D, Post M, Alper H (2008) Separable catalysts in one-pot synthesis for greener chemistry. Chem Mater 20:2544–2550
43. Yilmaz E, Sezgin M and Yilmaz M (2011) Immobilization of Candida rugosa lipase on magnetic sol–gel composite supports for enzymatic resolution of (R,S)-Naproxen methyl ester. J Mol Catal B Enzym. doi:10.1016/j.molcatb.2010.12.007
44. Kobayashi Y, Saeki S, Yoshida M, Nagao D, Konno M (2008) Synthesis of spherical submicron-sized magnetite/silica nanocomposite particles. J Sol-Gel Technolol 45:35–41
45. Bernardino SMSA, Fernandes P, Fonseca LP (2009) A new biocatalyst: penicillin G acylase immobilized in sol-gel microparticles with magnetic properties. Biotechnol J 4:695–702
46. Schägger H (2006) Tricine-SDS-PAGE. Nat Protoc 1(1):16–22
47. Kim YD, Dordick JS, Clark DS (2001) Siloxane-based biocatalytic films and paints for use as reactive coatings. Biotechnol Bioeng 72(4):475–482
48. Ellerby LM, Nishida CR, Nishida F, Yamanaka SA, Dunn B, Valentine JS, Zink JI (1992) Encapsulation of proteins in transparent porous silicate glasses prepared by the sol-gel method. Science 255:1113–1115
49. Fonseca LP, Cardoso JP, Cabral JMS (1993) Immobilization studies of an industrial penicillin acylase preparation on a silica carrier. J Chem Tech Biotechnol 58:27–37
50. Smith PK et al (1985) Measurements of protein using bicinhoninic acid. Anal Biochem 150:76–85
51. Zaia DAM, Zaia CTBV, Lichtig J (1998) Determinação de Proteínas Totais via especrofotometria: vantagens e desvantagens dos métodos existentes. Quim Nova 21(6):787–793
52. Pierre AC (2004) The sol-gel encapsulation of enzymes. Biocatal Biotransform 22(3):145–170
53. Kim Y-D, Park CB, Clark DS (2001) Stable sol-gel microstructured and microfluidic networks for protein patterning. Biotechnol Bioeng 73:331–337
54. Kawakami K, Yoshida S (1996) Thermal stabilization of lipase by sol-gel entrapment modified silicates formed on kieselguhr. J Ferment Bioeng 82:239–245
55. Bergeron LM, Tokatlian T, Gomez L, Clark DS (2008) Redirecting the inactivation pathway of penicillin amidase and increasing amoxicillin production via a thermophilic molecular chaperone. Biotechnol Bioeng 102(2):417–424
56. Arroyo M, Torres-Guzmán R, de la Mata I, Castillón MP, Acebal C (2002) A kinetic examination of penicillin acylase stability in water-organic solvent systems at different temperatures. Biocatal Biotransform 20(1):53–56

57. Erarslan A (1994) Effect of polyol compounds on thermostability of penicillin G acylase obtained from a mutant of *Escherichia coli* ATCC 11105. Process Biochem 30:133–139
58. Meixner DL, Dyer PN (1999) Influence of sol-gel synthesis parameters on the microstructure of particulate silica xerogels. J Sol-Gel Sci Technol 14:223–232
59. Greenspan L (1977) Humidity fixed points of binary saturated aqueous solutions. J Res Natl Bureau Standards-A Phys Chem 81A(1):89–96
60. Pagliaro M, Ciriminna R, Palmisano G (2007) The chemical effects of molecular sol–gel entrapment. Chem Soc Rev 36: 932–940
61. Ramakrishna SV, Sreedharan VP, Prema P (1988) In: Moo-Young M (ed) Bioreactor immobilized enzymes and cells. Elsevier Publishers, Amsterdam
62. Gill I, Ballesteros A (1998) Encapsulation of biologicals within silicate, siloxane, and hybrid sol-gel polymers: an efficient and generic approach. J Am Chem Soc 120:8587–8598
63. Li L, Choo ESG, Yi J, Ding J, Tang X, Xue J (2008) Superparamagnetic silica composite nanospheres (SSCNs) with ultra-high loading of iron oxide nanoparticles via an oil-in-DEG microemulsion route. Chem Mater 20:6292–6294