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Controlling the hydration of covalently immobilised penicillin G amidase in low-water medium: properties and use of Celite R-640

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Abstract

The study describes how Celite R-640 adsorbs liquid water in toluene and vapour water from a gas phase. In toluene, Celite R-640 is able to maintain the water activity (a_w) constant within broad ranges of water concentrations. The a_w values are strongly related to the original hydration of the Celite batches, but prolonged drying confers comparable and reproducible properties to the different batches. The use of Celite R-640 in controlling the hydration and activity of covalently immobilised PGA in toluene is reported.

Keywords: Celite; Penicillin amidase; Organic solvent; Control of water activity

1. Introduction

Celite is a solid support widely used for the adsorption of biocatalysts and it is commercially available in different forms, which vary in particle size, shape and porosity. Many examples of improved enzyme performances by means of deposition onto Celite powder are reported in the literature [1–3]. Porous Celite beads and rods are used primarily as supports for the immobilisation of whole cells [4–6] and fungi [7–9], and — less frequently — of isolated enzymes. For instance, chemically derivatised Celite beads (Celite R-648) have been used for the covalent immobilisation of trypsin [10], while β-glucosidase and γ-amylase have been adsorbed onto Celite R-640 and used in solvent-free environments [11]. Celite rods (Celite R-640) have been employed in our laboratory for the adsorption of penicillin G amidase (PGA) used in biotransformations in toluene [12,13].

Celite R-640 is a chemically inert, silica-based matrix which consists of diatomaceous earth broken up and subsequently recalcined to create porous particles with controlled pore sizes [7]. This type of porous Celite differs from Celite powder in its capacity to adsorb water (more than 90% of Celite weight). The great capacity of Celite R-640 to adsorb water is determined mainly by its porosity and its surface properties [13–16].

Recently, we have demonstrated that Celite R-640 in toluene adsorbs and releases water such that water activity (a_w) is maintained constant in a reaction system within wide and defined ranges of water concentrations [12,13].

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Here, we describe how water is adsorbed onto Celite R-640 in two different conditions, namely, in air and in apolar solvent. Factors causing variations in the properties of the porous matrix are pointed out together with a method for obtaining preparations with homogeneous comparable properties.

This work reports the control of the water activity in reactions in toluene catalysed by PGA covalently immobilised onto polymers, by using Celite R-640 as an additive in the reaction system.

2. Experimental

2.1. Materials

Celite rods R-640 (surface area = 65.0 m^2/g; water adsorption: 90% by weight) are from Fluka. The saturated solutions used in the study of the adsorption isotherms in vapour phases were prepared at 25°C using ultrapure water and the following salts (analytical grade): $CaCl_2$ ($a_w = 0.29$); K_2CO_3 ($a_w = 0.45$); NH_4NO_3 ($a_w = 0.63$); NaCl ($a_w = 0.75$); KCl ($a_w = 0.84$). Tyrosine ethyl ester is from Sigma and methyl phenylacetate was synthesised according to Ref. [17]. Eupergit-PcA® (PGA from *Escherichia coli* immobilised on Eupergit-C®, enzymatic activity = 126 U/g) and PGA-polyacrylamide (PGA immobilised onto a copolymer of metacrylamide and *N,N′*-methylene-bisacrylamide, enzymatic activity = 190 U/g) are from Fluka, and batches have variable water content (40–65% w/w).

All solvents were dried over molecular sieves (4 Å). Ultrapure water was used in all experiments.

2.2. Methods

Water activity and relative humidity were measured using a hygrometer (Novasina MS1) equipped with a humidity–temperature sensor (enCR-3), calibrated at 25°C at five different a_w values (0.12, 0.33, 0.52, 0.75, 0.90) using standard salt solutions.

Porosimetry was determined by means of an AutoPore III 9420 System, Micrometrics Instruments, Norcross, GA, USA.

Adsorption isotherms in toluene were obtained by mixing 1 ml of dry toluene, three Celite rods (95 mg) and water into 5 ml vials with screw caps and Teflon-lined septa, after which the system is equilibrated for at least 24 h in a thermostatted orbital shaker. After equilibration, water activity was measured by sealing the humidity sensor into the open end of the thermostatted vials, until a constant reading was obtained. It was verified that the system reaches the equilibrium after 24 h of incubation.

Adsorption isotherms in vapour phase were obtained by keeping three rods (95 mg) in contact with a vapour phase at known humidity in chambers (500 ml) containing saturated salt solutions (200 ml) or ultrapure water, carefully capped. The samples of Celite were weighed every 24 h.

The adsorption of liquid water in air was studied by adding increasing amounts (5–150 mg) of water to 95 mg of dry Celite R-640 in 5 ml glass vials. The samples were capped and incubated for 24 h before measuring the relative humidity in the gas phase.

Enzymatic activity of native and immobilised PGA was assayed according to the following procedure: 266 mg (0.715 mmol) of benzylpenicillin (Fluka) were added to 20 ml of a solution 5 μg/ml of native PGA in phosphate buffer (0.05 M pH 7.6, containing 0.1 M NaCl) or to 20 ml of a suspension of the immobilised enzyme (0.5–1.5 mg/ml). The phenylacetic acid formed was titrated at 37°C with a solution 0.01 N NaOH containing 0.1 M of NaCl. An automated titrator (TTT80 Radiometer, Denmark) was employed. One enzymatic unit corresponds to the amount of enzyme that hydrolyses 1 μmol of benzylpenicillin in 1 min at pH 7.6 at 37°C.

The immobilised PGA was dehydrated by washing three times 150 mg of the catalyst with

1 ml of 2-propanol and by removing the solvent after centrifugation.

Enzymatic synthesis were carried out by adding 95 mg (three rods) of Celite R-640 to 1 ml of dry toluene into a 5 ml glass vial. After the addition of 25 μl of water, the system was equilibrated for 24 h at 30°C in an orbital shaker (250 rpm). The reaction was started by the addition of the immobilised PGA, 80 mM of tyrosine ethyl ester and 100 mM of methyl phenylacetate. The a_w was measured at the start of the reaction and after complete conversion of the substrates into the product. No product was observed in the absence of the catalyst.

Initial rates were determined by following the first 10% of conversion of the reaction by RP-HPLC (Pharmacia), according to a procedure previously reported [12,13].

Celite rods exposed to humidity were dried in an oven at 100°C for 16 h and then over P_2O_5 for 24 h.

3. Results and discussion

3.1. Control of water activity by means of Celite R-640: adsorption isotherms in toluene

Our recent studies concerning the adsorption of Penicillin G amidase (PGA) on Celite R-640 have demonstrated that in toluene, this porous matrix is able to control the water activity of the reaction system [12,13].

Now we describe how the adsorption properties of Celite R-640 vary from batch to batch as a direct consequence of their different original humidity content, so that the shape of the adsorption isotherms of the batches changes considerably, although their total pore volume and pore size distribution are comparable (Fig. 1).

Fig. 2 illustrates the adsorption isotherms in toluene of three batches of Celite R-640 used, without any treatment, immediately after their delivery from the supplier. In dry toluene at the equilibrium, the samples of these batches have a_w values of 0.12, 0.47 and 0.54 (28°C), respectively. The batches adsorb large amounts of water, keeping water activity reasonably constant within defined ranges of water concentrations. At water concentrations of 100–500 mg water/g Celite, batch 1, the driest one, maintains water activity close to 0.50, whereas batches 2 and 3 keep the water activity at values of 0.74 and 0.78, respectively. At higher water concentrations, the adsorption isotherm of batch 1 shows a_w values around 0.75–0.85 [13].

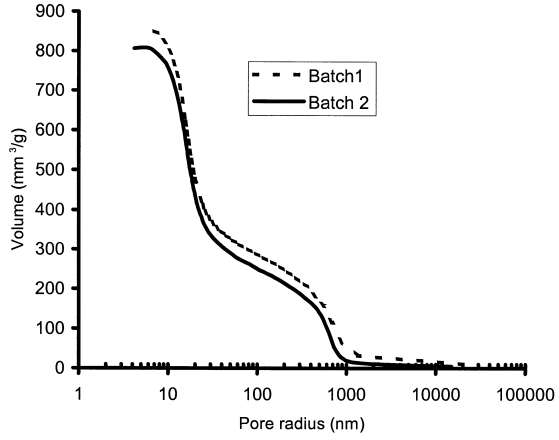

Fig. 1. Pore volume distribution in batches 1 and 2.

Experimental observations indicate that the differences in the original humidity content of the Celite batches are related to their storage. Since Celite rods are hygroscopic and adsorb moisture from the atmosphere, their properties may change during the storage. For instance, storing batch 1 over P_2O_5 has proved effective in preserving the dryness of the rods for at least 1 year, whereas the same batch of Celite after 1 year of storage, but exposed to the atmosphere humidity (batch 1*), gives in toluene an initial a_w of 0.5. The hydration causes a significant variation of the shape of the adsorption isotherm as well as of the adsorption capacity of the rods. This was estimated by evaluating the largest water concentration leading to values of water activity > 0.90. Fig. 2 indicates that batch 1 is able to adsorb about 150% of water (w/w), whereas batches 1*, 2 and 3 adsorb about 90% of water (w/w). These observations suggest

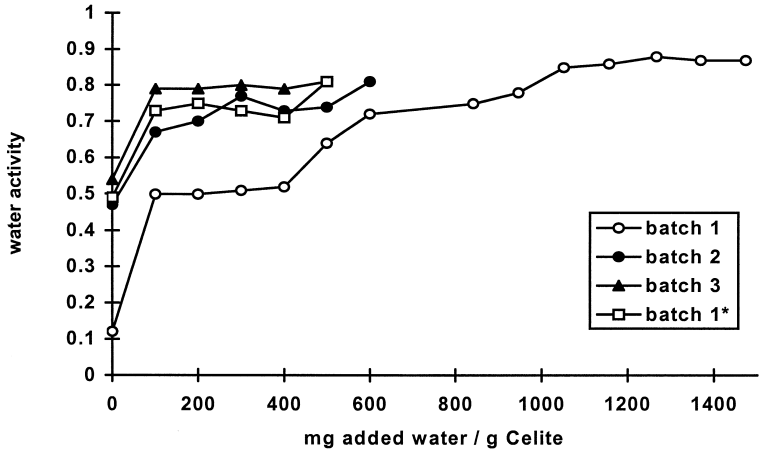

Fig. 2. Adsorption isotherms in toluene ($T = 28°C$) of different batches of Celite R-640 (equilibration time = 24 h). Batch 1* corresponds to batch 1 after prolonged exposure to atmosphere humidity.

that batch 1 is characterised by an extremely low humidity content, which was not observed for any other batch considered.

By drying the hydrated Celite batches (2, 3 and 1*), first at 100°C for 16 h in an oven and then over P_2O_5 for 24 h, part of the adsorbed moisture is removed so that the initial a_w of the three Celite batches in dry toluene decreases to 0.16. Adsorption isotherms in Fig. 3 show that after this treatment, the three batches have comparable behaviour and they are characterised by a single broad plateau of constant a_w around 0.70 ± 0.02, corresponding to a range of water concentrations of 100–700 mg water/g Celite. Therefore, by drying different batches of Celite, which have been exposed to the atmosphere humidity, it is possible to increase their adsorption capacity and to obtain Celite rods with homogeneous properties.

However, the first exposure of the rods to humidity changes irreversibly, the adsorption properties of Celite R-640, as appears from the comparison of adsorption isotherms of batch 1 (Fig. 2) and batch 1* after drying (Fig. 3). The

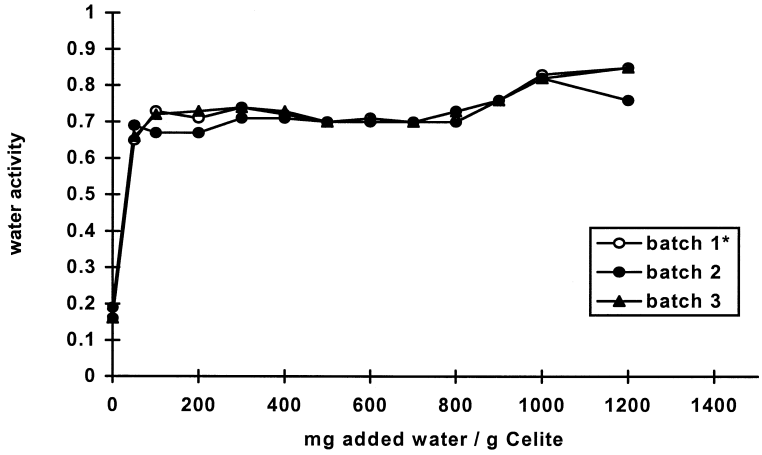

Fig. 3. Adsorption isotherms in toluene ($T = 28°C$) of batch 1*, 2 and 3, after the drying procedure described in the text (equilibration time = 24 h).

drying treatment is not sufficient to restore the original ability of the dry batch 1 to control the a_w at values around 0.5 and this can be explained by taking into account the strong interactions which occurs between water molecules and dry silica surfaces. Physico-chemical studies [14–16] indicate that the first few layers of water molecules adsorbed on silica are strongly affected by the interactions with the groups at the surface, which influence the structure of water to a distance of about 10 Å, thus limiting the mobility of water molecules. Beside high porosity, Celite R-640 has also a wide surface area (65 m^2/g), which allows water molecules to engage such strong interactions. Our hypothesis is that the silica surface of batch 1 is so poorly hydrated that upon the first exposure to the moisture, the first layers of water molecules are adsorbed onto the dry rods through interactions with the silica surface. The adsorption of such molecules corresponds to the first plateau at $a_w = 0.5$. The further water molecules adsorb onto the pores and fill them, following a behaviour similar to that observed for the other Celite batches. However, the first layers of water molecules cannot be removed by drying the rods at 100°C and then over P_2O_5 because of their strong interactions with the silica surface. Therefore, the first plateau at $a_w = 0.5$ cannot be reproduced. According to this hypothesis, the silica surface of batches 2, 3 and 1* is prevalently hydrated, so that the first plateau at $a_w = 0.5$ is not observable because it is either very narrow or it is not present at all.

The values of water activity reported in the diagrams are always related to measurements carried out in identical conditions, namely by adding three rods (95 mg) with uniform weight (32 ± 2 mg each) and, therefore, uniform external surface (1.55 mm^2/mg). It must be emphasised that also measurements carried out on larger samples of rods (1 g of Celite in 10 ml of toluene), having non-homogeneous sizes, give comparable results.

3.2. Hydration of Celite R-640 in the absence of organic solvent

A general method for performing biotransformations at known water activity consists of exposing each phase of the reaction mixture to a vapour phase characterised by a constant known relative humidity [18,19]. In principle, once the components are mixed, the phases reach the equilibrium water activity.

Figs. 4 and 5 show the rate of equilibration of dry Celite R-640 exposed to atmospheres generated by saturated salt solutions or pure water inside carefully capped vessels. The curves are obtained by keeping three rods (95 mg) in con-

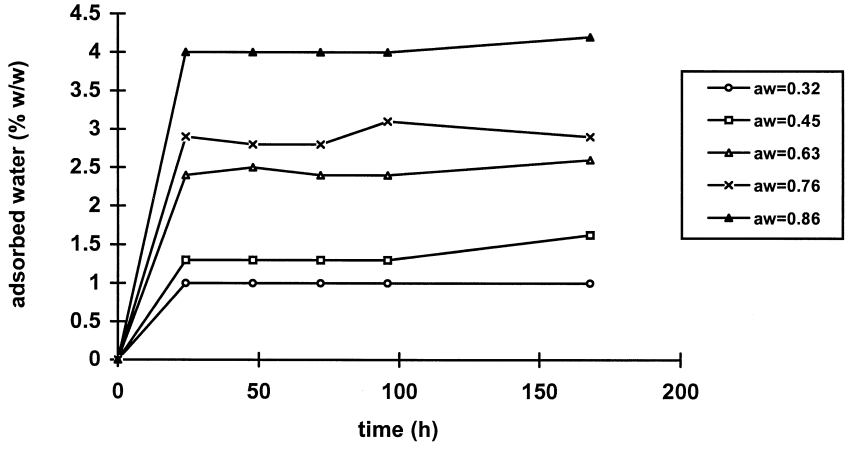

Fig. 4. Water adsorption ($T = 25$°C) of Celite R-640 vs. time at different a_w generated by saturated salt solutions.

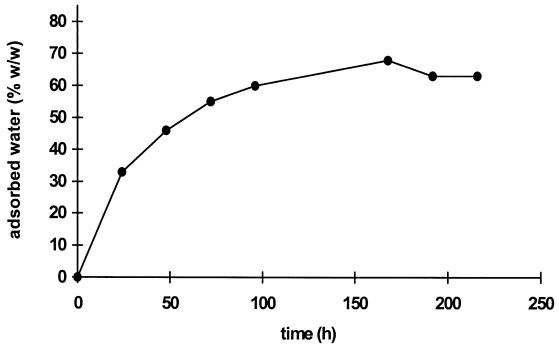

Fig. 5. Water adsorption ($T = 25$°C) of Celite R-640 vs. time at $a_w = 1$, generated by pure water.

tact with vapour phases characterised by different, known relative humidity, and then weighing the Celite every 24 h. Fig. 4 indicates that most of the moisture is adsorbed within the first 24 h of exposure of the rods to the vapour phase, while there are negligible increments of weight over subsequent days. In all cases, Celite R-640 has a limited capacity of adsorbing water from vapour phases, ranging from 1% w/w ($a_w = 0.32$) to 4% w/w ($a_w = 0.86$).

The rods equilibrated at $a_w = 1$ behave differently, as they progressively adsorb water up to 60% of their weight (Fig. 5), a result which is quite far, however, from the adsorption capacity exhibited by Celite R-640 in toluene (up to 120% w/w).

The possibility that the plateau reported in Figs. 4 and 5 represents merely an apparent equilibrium, and that the actual equilibrium requires extremely prolonged equilibration times, cannot be excluded.

The physical and chemical processes governing the adsorption of vapour water and liquid water in toluene on Celite R-640 appear very different. The difference is even clearer when Celite rods and toluene, previously equilibrated at the same value of water activity, are mixed. Fig. 6 shows how the relative humidity (RH%) of the gas phase in the headspace above these mixtures decreases with time, and no equilibrium is reached even after 300 h of incubation (Fig. 6). For instance, in the case of the Celite equilibrated at $a_w = 0.86$, the relative humidity after 24 h of incubation is 0.75 and decreases to 0.51 after 300 h of incubation.

Most probably, upon moving from the gas phase to the apolar solvent, the variation of the interfacial properties promotes a re-distribution of the water inside the rods, so that the system reaches the equilibrium in the new environment very slowly. Therefore, the equilibration of this porous matrix in vapour phase is not advisable when Celite rods have to be used in apolar medium.

On the contrary, when liquid water is added to the Celite in toluene, all the molecules immediately form a uniform coating around the rods, and the Celite surface adsorbs homogeneously, the water. Therefore, the presence of the apolar solvent promotes the fast adsorption of the water and the penetration of molecules into the inner pores of the rods. This has been confirmed by studying how Celite R-640 adsorbs liquid water in air. By adding liquid water to dry Celite R-640 in air and the addition of as little as 50 mg water/g Celite leads to a relative humidity of the gas phase already > 0.90. This result is mainly ascribable to a very slow penetration of the water molecules into the silica matrix, as demonstrated by data of Fig. 7 indicating the a_w values measured in the same

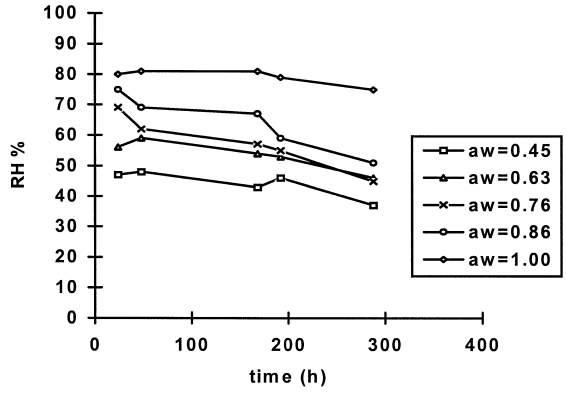

Fig. 6. Relative humidity ($T = 25$°C) of the gas phase in the headspace above toluene (1 ml) and Celite rods (95 mg, dry weight) previously equilibrated at the same value of water activity in air using saturated salt solutions and pure water (see Figs. 4 and 5).

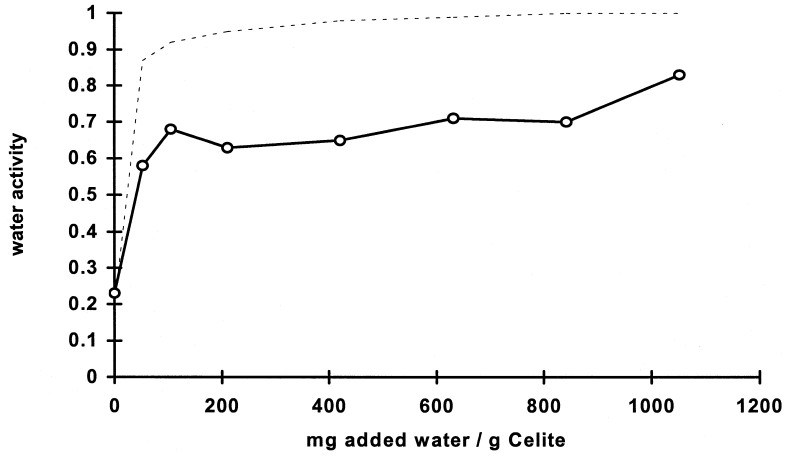

Fig. 7. a_w ($T = 30$°C) vs. water added to dry Celite R-640 in air (- - - -) (equilibration time = 24 h), compared to the a_w values (————) measured after adding 1 ml of toluene to the same samples (equilibration time = 24 h).

samples but after the addition of 1 ml of toluene and an incubation of 24 h. It has been also verified that in the presence of toluene, the equilibrium was reached within 24 h.

3.3. Use of Celite R-640 in low-water media: activity of rehydrated immobilised PGA

Eupergit-PcA® and PGA-polyacrylamide are commercially available as hydrated beads. The water content may vary significantly among batches, and in most cases, they are not suitable for biotransformations in organic media, because the excess water induces the formation of clusters and the non-homogeneous dispersion of the catalyst.

Halling and Moore recently reported that immobilised enzymes can be dehydrated by washing the biocatalyst with anhydrous organic solvents [20,21], and this technique is more efficient than lyophilization in preserving the activity of the catalyst.

We have used this method to dehydrate the two immobilised PGA, by washing the catalysts with 2-propanol. Rinsing the catalyst with the solvent does not cause any decrement of enzymatic activity in water within the first hour of

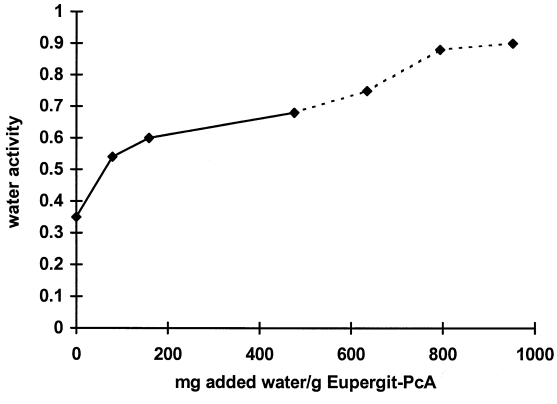

Fig. 8. a_w ($T = 30$°C) vs. water adsorbed by Eupergit-PcA® previously dehydrated by washing with 2-propanol. After water addition, the system has been equilibrated for 24 h.

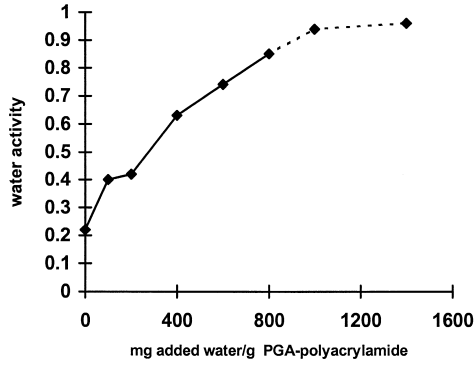

Fig. 9. a_w ($T = 25$°C) vs. water adsorbed by PGA-polyacrylamide previously dehydrated washing with 2-propanol. After water addition, the system has been equilibrated for 24 h.

Table 1
Initial rates of the acylation of tyrosine ethyl ester with methyl phenylacetate in toluene catalysed by immobilised PGA dehydrated with 2-propanol

Enzyme	Treatment	a_w ($T = 30°C$)	v_0^a (μmol/min/U)
Eupergit-PcA®	washed with 2-propanol	0.35	no reaction
	washed with 2-propanol + three Celite rods + 25 μl H_2O[b]	0.50	0.073
PGA-polyacrylamide	washed with 2-propanol	0.22	no reaction
	washed with 2-propanol + three Celite rods + 25 μl H_2O[b]	0.50	0.012

[a] Initial rates are calculated on the basis of the enzymatic units determined in water. The reactions reached 95% of conversion in 24 h with Eupergit-PcA® using eight enzymatic units, and 94% in 24 h with PGA-polyacrylamide using 9.5 enzymatic units.
[b] Reaction volume = 1 ml.

storage, whereas storing the washed enzyme for longer periods of time leads to the progressive decrease of the enzymatic power.

Figs. 8 and 9 shows the adsorption isotherms in toluene of these two enzymatic preparations rinsed with 2-propanol. The two preparations of immobilised PGA have a good capacity of adsorbing water, although they are not able to maintain the water activity constant as water concentration changes. Furthermore, at water concentrations above 300 mg/ml in the case of Eupergit-PcA®, and 800 mg/ml for PGA-polyacrylamide, the beads aggregate, so that the distribution of water between the phases is not homogeneous.

It must be mentioned that 1 g of native PGA adsorbs 300 mg of water and therefore, the low amounts of catalytic protein immobilised on the two supports (see Section 2) give a negligible contribution to the global adsorption capacity of the enzymatic preparations [13].

Figs. 8 and 9 indicate that after the dehydration with 2-propanol, the water content of the two immobilised enzymes is so low that in dry toluene, the a_w is below 0.4, thus insufficient to maintain the enzyme active in organic solvent. Nevertheless, the catalytic power can be completely restored by using hydrated Celite rods to equilibrate the reaction medium at a higher value of water activity. This is demonstrated by data in Table 1, which reports the catalytic activity of the immobilised PGA in toluene catalysing the acylation of tyrosine ethyl ester with methyl phenylacetate.

The rods were hydrated in toluene by adding amounts of water which are determined on the basis of the adsorption properties of the Celite batch employed. Reactions reported in Table 1 were carried out employing Celite from batch 1, so that after the addition of 25 μl of water and 24 h of equilibration, the system has a water activity of 0.5, consistent with the adsorption isotherm reported in Fig. 2. After the addition of the catalyst and the reactants, the rods are maintained in the reaction environment, so that they can act as an additive which is able to release or adsorb water in a controlled way.

Thanks to the buffering properties of the Celite rods, constant values of water activity were measured during the whole course of the reaction, until the complete conversion of the reactants. Kinetic data indicate that the enzymatic activity of Eupergit-PcA® is comparable to the activity of the native PGA adsorbed on Celite R-640 ($v_0 = 0.083$ μmol/min/U) as previously reported [13].

PGA-polyacrylamide has an activity about six times lower, indicating that PGA immobilised on Eupergit-C® is preferable for applications in organic media.

4. Conclusions

Celite rods cannot replace hydrated salts [22] in a large number of contexts because, at present, there is no established method for fixing the water activity at different values by using

this porous matrix. However, the features of Celite R-640 here described, make it a practical and simple tool for avoiding some of the problems occurring in biotransformations in low-water media which are related to the variation of the water activity/concentration. Because of the ability of controlling the water activity in apolar medium, Celite R-640 can be used not only as a support for the adsorption of native enzymes [12] but also as an additive in reactions catalysed by immobilised biocatalysts. The original water content of the rods strongly influence their adsorption behaviour. Nevertheless, the properties of different batches of Celite can be made homogeneous upon drying the rods.

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