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The first encapsulation of hydroxynitrile lyase from *Hevea brasiliensis* in a sol–gel matrix

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Abstract—A straightforward process for the encapsulation of *Hb*HNL under low methanol conditions has been developed. By adding a sol, prepared by hydrolysis of TMOS/MTMS at pH 2.8 with continuous removal of methanol, to a stirred solution of the enzyme in a buffer at pH 6.5, at least 65% of the activity of the free enzyme could be recovered after the encapsulation. The aquagels were successfully used in the synthesis of (*S*)-cyanohydrins.

1. Introduction

Hydroxynitrile lyases (HNL's) are a class of enzymes that can be found in a wide range of plants, such as millet and the apple, almond, rubber and plum trees.[1] When the plant material is damaged, for instance by a herbivore, HNL catalyses the breakdown of a cyanohydrin into an aldehyde/ketone and toxic HCN. More interestingly, the enzyme may also catalyse the reverse reaction, enabling the synthesis of enantiopure cyanohydrins from aldehydes/ketones and HCN with excellent yields and enantioselectivities.[2–8] These enantiopure cyanohydrins can in turn readily be converted into a wide range of compounds that are versatile building blocks for the synthesis of fine chemicals, pharmaceuticals and agrochemicals.[2,9,10]

Recently, the immobilisation of enzymes has attracted much attention, increased stability and recyclability being the main research objectives. Cross-linked crystals of HNL's and HNL's adsorbed on solid supports or encapsulated in sol–gels and PVA-gels have been used as catalysts in the synthesis of optically active cyanohydrins.[11–15] However, only the PVA-entrapped HNL from *Prunus amygdalus* and the crosslinked enzyme crystals from *Manihot Esculenta* proved to be stable upon recycling. In this context it is important to notice, that HNL's only have in common that they all catalyse the cyanogenesis. Structurally they can belong to different classes of enzymes and are therefore not always comparable. For example, HNL from *Hevea brasiliensis* is closely related to the α/β hydrolases, which also include lipases, while *Prunus amygdalus* is closely related to FAD dependant oxidoreductases.[16,17]

The (*S*)-hydroxynitrile lyase from *Hevea brasiliensis* (*Hb*HNL) has been used for the addition of HCN to a wide range of aldehydes and ketones.[18–23] In spite of its versatility, the only immobilisation reported is the adsorption of *Hb*HNL onto celite.[11] Here it was found that the maximum activity of the enzyme was only obtained when there was a discrete water layer surrounding the enzyme. This indicates that the enzyme is only active in an aqueous environment. To assure this whilst avoiding a separate aqueous phase in the reaction, the *Hb*HNL can be encapsulated in a sol–gel matrix. The pores of such a matrix can be filled with the aqueous buffer of choice or any organic phase. In this manner, the versatile *Hb*HNL will become available for an even wider range of reaction conditions.

The sol–gel technique allows the synthesis of chemically inert glasses, which can be formed into any desired shape. They have high porosity (up to 98% pore volume) and high mechanical and thermal resistance. In addition they can be produced under conditions that are relatively benign to enzymes. This technique has successfully been applied for the encapsulation of lipases into various sol–gel materials,[24–28] and as the *Hb*HNL is structurally related to lipases, we assumed that the encapsulation of *Hb*HNL should proceed in a similar manner.

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As sol–gel encapsulated CAL-B (33 kDa) which belongs to the α/β hydrolase family just like the *Hb*HNL, has been recycled up to eight times without any loss of activity,[24] it is reasonable to assume that the dimer of *Hb*HNL (58.4 kDa) is equally well encapsulated in a sol–gel matrix (*Hb*HNL is a dimer in aqueous solutions[29]). In spite of this structural similarity, the *Hb*HNL is much more susceptible towards deviations from its optimum conditions.[30–34] Due to this, newly developed methodology rather than the standard immobilisation procedures, is applied.

Here we present the first successful encapsulation of the (*S*)-selective hydroxynitrile lyase from *Hevea brasiliensis* in a sol–gel matrix.

2. Results and discussion

In preliminary experiments, we encapsulated the *Hb*HNL into a sol–gel matrix following standard procedures.[24,26] In these procedures, the methanol released during the formation of the sol was not eliminated, which caused a complete deactivation of the *Hb*HNL during the gelation process. This is in line with the previously described methanol sensitivity of *Hb*HNL.[35] In another procedure, the alkoxy silanes are partially hydrolysed and then transesterified with glycerol. The methanol is removed from the sol, and then the sol is mixed with water containing the biomolecules.[14] This method is so far the best method reported for the encapsulation of methanol sensitive enzymes, however, applied to *Hb*HNL it gave unsatisfactory results.

Therefore, a new procedure was developed, were the alkoxy silanes were almost 100% hydrolysed by acid mediated hydrolysis, and the released methanol was removed by evaporation. The enzyme, dissolved in a buffer with pH 6.5, was then added to this precursor sol. At this pH, the gelation was catalysed and at the same time the enzyme was stabilized.[34,36] As soon as the gel was formed, it was submerged in the same buffer, pH 6.5, to remove any remaining methanol, possibly formed from hydrolysis of residual methoxy groups, by dialysis.

With this procedure, the aquagels[37] showed an activity of at least 65% relative to the free enzyme in the standard aqueous activity test. The apparent decrease of activity is probably due to deactivation by residual methanol and diffusion limitations. Initial rate studies showed that the system is indeed limited by diffusion,[38] which indicates that the actual loss of activity during the encapsulation procedure is lower than 35%. Due to changes of the specific particle size of the ground aquagels under the reaction conditions, quantification of the diffusion limitations was not pursued.

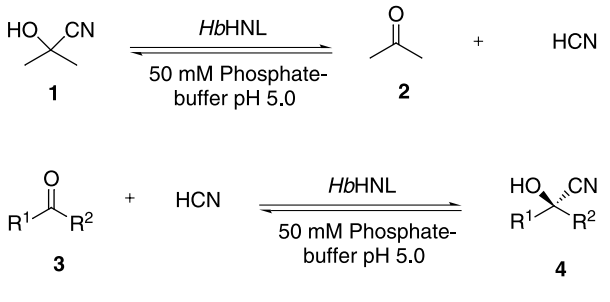
The use of poly vinyl alcohol (PVA) as an additive in the sol–gels is known to increase the activity of lipases in hydrophobic sol–gel materials.[25] Since the structure of *Hb*HNL is comparable with that of lipases, this possibility was also investigated, but, no effect of the PVA on the enzyme activity could be observed.[39] This indicates that the presence of PVA might only have an effect on the lipase activity after or during the drying of the gel.

Varying the MTMS (methyltrimethoxysilane)/TMOS (tetramethoxysilane) ratio is also known to change the activity of the sol–gel entrapped lipases.[40] Aquagels with a concentration of MTMS in TMOS varying from 0 to 50 vol% were prepared[41] and used in the addition of HCN to benzaldehyde, but, no difference in activity could be detected. No higher MTMS concentrations were examined since the gelation process then took several hours and the gels had a paste like aspect. A mixture of 20 vol% MTMS in TMOS was chosen for further studies since this mixture gave the most convenient gelation time.

Attempts to dry the aquagels as aerogels and xerogels resulted in a total loss of activity. In the case of the xerogel there are two possible reasons for deactivation. When the gel is dried by evaporation of the water phase in open air (xerogels), capillary stress will cause partial collapse of the gel structure as the liquid–gas interface moves in through the gel. As the gel shrinks, some of the enzymes will also be crushed. Secondly, it has been suggested that the exposure of *Hb*HNL to a gas–liquid interface drastically reduces the half-life of the enzyme.[34] In the preparation of the aerogels, the water in the aquagel is replaced with acetone, which again is replaced with CO_2 in an autoclave. When the supercritical conditions for CO_2 are reached, by increasing the temperature of the autoclave, the autoclave is slowly evacuated. The acetone is most likely causing the deactivation in this drying procedure. To verify whether this was the case, the buffer filling the pores of the aquagel was exchanged with acetone and then back to the buffer by dialysis. The resulting gel showed no activity, indicating that indeed acetone or the acetone water mixture did deactivate the *Hb*HNL. It has been shown that stirring *Hb*HNL in acetone containing 0.25% water over 15 h at room temperature gives only 15% loss of activity.[35] From this it can be concluded that it is the acetone–water mixture, rather than the acetone itself, that deactivates the enzyme. However, as it is known that *Hb*HNL is inactive at low water concentrations, it is not desirable to dry the gels, but rather to use them directly as aquagels. In this manner the enzyme will be completely hydrated with the buffer of choice. The buffer remains inside the pores of the aquagel and no separate macroscopic water phase is formed in the reaction mixture.

The encapsulated *Hb*HNL was applied in the enantioselective addition of HCN to benzaldehyde **3a**, furaldehyde **3b**, hexanal **3c**, *m*-phenoxybenzaldehyde **3d** and methyl isopropyl ketone **3e** to give their (*S*)-cyanohydrins **4a–e**. For safety reasons acetone cyanohydrin was used as the cyanide source, even though the liberated acetone has a negative effect on the enzyme (Scheme 1).

As both, the generation of HCN and the addition of the HCN to the carbonyl group, are reversible reactions, the maximum conversion will be determined by the equilibrium constants of the two reactions, and, can therefore not be compared to the isolated yields (typically 95–99%) that are obtained in the standard method where a five fold excess of free HCN is used.[21] When the amount of enzyme used in the literature procedure (2.5 times more) is taken into account, the reaction time that we observe, approximately 40–50 min (Fig. 1), is comparable with that found in the literature



	R^1	R^2
a	Phenyl	H
b	2-Furyl	H
c	Hexyl	H
d	*m*-Phenoxy-phenyl	H
e	Isopropyl	Me

Scheme 1. The synthesis of optically active cyanohydrins.

(15 min).[21] The overall equilibrium in Scheme 1 was established rapidly in all cases except for **3d**, a substrate known to be "difficult" for HNL's.[3] A satisfactory result for this substrate has only been described in a highly enzyme loaded emulsion, using free HCN.[21]

The enantiomeric excess of the products is in line with those reported in the literature (>98% for **4a–d**, and 75% for **4e**)[21] for the same reactions. Small differences are due to the chemical background reactions (which form the racemic cyanohydrin) under the specific reaction conditions. To establish the stability of the sol–gel encapsulated *Hb*HNL, it was recycled 3 times in the reaction of HCN with benzaldehyde, using acetone cyanohydrin as the cyanide source. When the gels were washed with diisopropyl ether between each cycle, there was a rapid loss of activity and the gels were completely deactivated after the second recycle (Fig. 2A). This result was improved when the gels were washed with a 50 mM phosphate buffer of pH 5.0 between each cycle. Then, the initial rate only dropped by around 50% for each cycle. In all cases the enantiomeric excess of the product was higher than 98% (Fig. 2B).

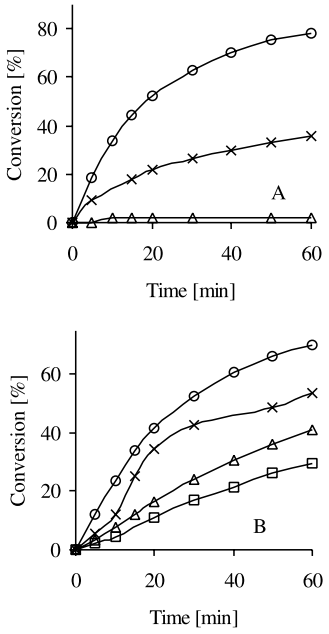


Figure 2. Recycling of the sol–gel encapsulated *Hb*HNL using benzaldehyde as the substrate. In (A) the gel was washed with diisopropyl ether between each cycle and in (B) the gel was washed with a 50 mM phosphate buffer pH 5.0. Cycle 1 (○), cycle 2 (×), cycle 3 (△) and cycle 4 (□).

A gel identical to the one used in the experiment described in Figure 2B was stirred with diisopropyl ether for four hours (which is the same time as it took to perform the first three cycles of the experiment described in Fig. 2B) before benzaldehyde and acetone cyanohydrin were added. The conversion observed for this gel was comparable with the conversion in cycle one of Figure 2B, indicating that there is no deactivation due to the buffer inside the gel or the solvent. Instead, the loss of activity must be caused by HCN,

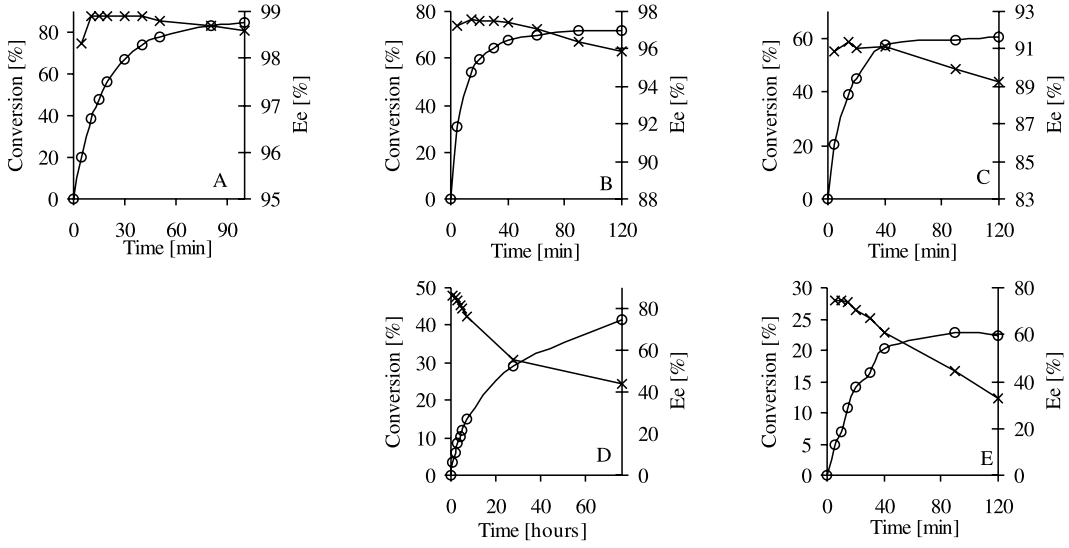


Figure 1. The conversion (○) of **3a** (A), **3b** (B), **3c** (C), **3d** (D) **3e** (E) into their respective (*S*)-cyanohydrins **4a–e**. 0.98 mM **3a–e**, diisopropyl ether, 50 mM phosphate buffer pH 5.0, 3 equiv acetone cyanohydrin and 1.8 KU *Hb*HNL. The enantiomeric excess (×) was determined by chiral GC (see materials and methods).

acetone, acetone cyanohydrin, the substrate, the product or leakage of the enzyme.

In the case of washing with diisopropyl ether, the deactivation is probably due to the acetone, which is formed during the reaction. Firstly, acetone is harmful for the enzyme, secondly, it changes the solubility of the solvent in the aqueous phase and vice versa. When the solvent is used for washing some of the water will be washed away from the gel, lowering the enzyme activity.

When the gels are washed with the buffer, the initial conditions for the enzyme are re-established and the activity is relatively higher than when washing with a solvent. The loss of activity will in this case, too, be due to the acetone formed, but also due to leakage of enzyme during the washing procedure.

In order to rule out any possible leakage of activity into the organic phase, the suspension of one reaction was filtered (taking care that no HCN could escape during the filtration) and the filtrate was monitored for activity. As expected the filtrate showed no activity (Fig. 3). However, when the reaction was performed in water, it was found that up to 17% of the observed activity derives from *Hb*HNL that has leached into the aqueous phase.[42]

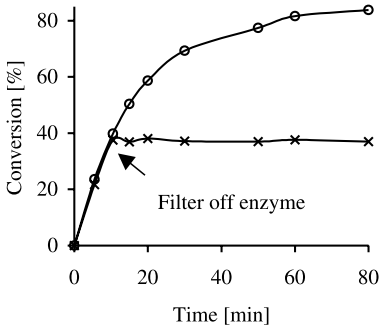


Figure 3. Test for leakage of activity from the gel into the organic phase. Unfiltered reaction (○) and filtered reaction (×).

Encapsulated enzymes are in principle not bound to the sol gel surface. This means that if the gel breaks and the 'capsule' around an enzyme opens, the enzyme will be washed away. Indeed, when the activity of an aquagel, which had been crushed into a fine powder was compared to an aquagel that had been crushed into a fine powder and then washed, a significant difference was found (Fig. 4). The

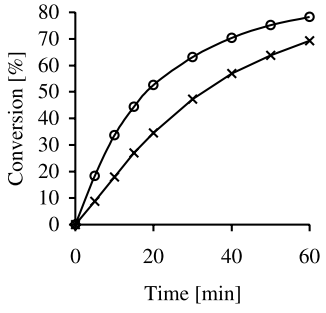


Figure 4. Test for leakage of activity during the washing procedure. Ground gel (○) and ground and washed gel (×).

washed powder showed a decrease of the initial reaction rate by 48% compared to the unwashed sol–gel powder. Even though the gels were shaken and not magnetically stirred in the recycling experiment, it is probable that some of the gels got further crushed leading to loss of activity in the subsequent washing.

In order to characterise the gels, they were dried under supercritical conditions with CO_2 to give aerogels. BET-analysis gave a surface area of 1000 m^2/g and a pore volume of 1.74 cm^3/g. The pore size distribution (Fig. 5) is relatively wide, where the maximum pore radius is 5 nm. In comparison the dimensions of the *Hb*HNL monomer is approximately $3.0\times3.8\times4.8$ nm.[43] After the formation of the gel, the aquagel is usually aged for 12–72 h in order to complete the hydrolysis and condensation. To investigate if there is an optimal ageing time for the *Hb*HNL, we tested the activity of gels aged up to 16 days. The result (Fig. 6) shows that there is a significant drop in activity during the first four to five days; then the activity stabilizes. The initial drop of activity is probably due to the formation of methanol during the beginning of the aging process and structural changes within the gel. The structural changes during the first days then level off.

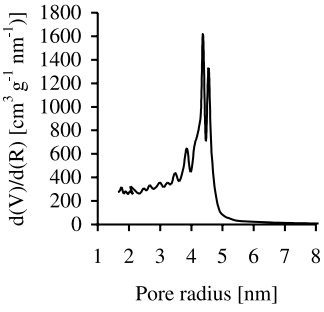


Figure 5. Pore size distribution of the sol–gel; the vertical axis is the differential d*V*/d*R*, where *V* is the adsorbed nitrogen gas volume, at standard conditions, per gram of gel (cm^3/g) and *R* is the pore radius (nm).

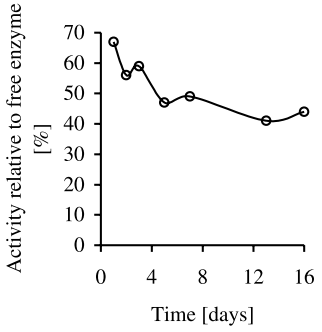


Figure 6. Aging of the gels.

3. Conclusion

*Hb*HNL, an enzyme very sensitive to organic solvents and requiring a near neutral pH, was successfully immobilized in a sol–gel. This low methanol immobilisation and first application of an aquagel holds great potential for the sol–gel encapsulation of many sensitive enzymes.

4. Experimental

4.1. General

*Hb*HNL was made available in a 25 mM potassium phosphate buffer pH 6.5 with 0.09% sodium azide (3600 IU/ml) by Roche Diagnostics (Penzberg, Germany). The activity of the homogenous enzyme was determined as described in the literature.[30] The reactants used in this study were poly vinyl alcohol with an average molar mass of $M=$ 15,000 (Fluka), methyltrimethoxysilane (MTMS, 98%, Aldrich) and tetramethoxysilane (TMOS, 98%, Aldrich). Benzaldehyde, *m*-phenoxybenzaldehyde, furaldehyde, hexanal and methyl isopropyl ketone were all of analytical grade and distilled under a nitrogen atmosphere less than two hours before use. Mandelonitrile was purified by column chromatography at most 24 h prior to use and stored under nitrogen at −4 °C. Acetone cyanohydrin was distilled and stored under nitrogen at 4 °C. Diisopropyl ether was of analytical grade and used without further purification. The derivatised samples were analysed on a β-cyclodextrin column (CP-Chirasil-Dex CB 25 m× 0.25 mm) using a Shimadzu Gas Chromatograph GC-14B equipped with a FID detector and a Shimadzu Auto-injector AOC-20i, using N_2 as the carrier gas. The conversion and the enantiomeric excess were calculated from the peak areas. The temperature programs and retention times are given in Table 1. UV measurements were performed on a UNICAM UV/Vis spectrometer. A Brunauer, Emmett and Teller (BET) analysis of a gel dried as an aerogel, desorbed at 200 °C, gave the specific surface area and the pore size distribution. Buffer A is a 25 mM potassium phosphate buffer pH 6.5 with 0.09% sodium azide, buffer B is a 50 mM citrate/potassium phosphate buffer pH 5.0. Racemic reference compounds were prepared according to standard procedures[44] and their NMR-spectra were in accordance with literature.[44–47]

4.2. General procedure A. Preparation of the sol–gel precursor

Acidic water (1.38 ml, pH adjusted to 2.85 by addition of HCl) was added to a mixture of MTMS (2.10 g, 15.4 mmol), TMOS (9.08 g, 58.5 mmol) and distilled water (10.4 ml) and stirred in a 100 ml round bottom flask until a homogenous mixture was obtained. The formed methanol was continuously removed on a rotary evaporator until the characteristic odours of MTMS, TMOS and MeOH were not detectable any more. The mixture was then cooled to 0 °C and water was added until the total volume corresponded to the initial MTMS/TMOS—volume. The precursor (sol) was used immediately for the encapsulation of *Hb*HNLGeneral procedure B. Encapsulation of *Hb*HNL in a sol–gel matrix for standard activity test.

4.3. General procedure B. Encapsulation of *Hb*HNL in a sol–gel matrix for standard activity test

The stock solution of *Hb*HNL (100 mg, 3.6 KU/ml) was diluted to 6.0 g with buffer A. This solution (40 µl) was added to a mixture of the precursor (500 µl; prepared as described in general procedure A), and buffer A (460 µl) and stirred magnetically for 20 s. The stirring bar was removed and when the mixture gelled (4–5 min), the gel was submerged in buffer A and aged at 4 °C for 24 h. Buffer A was then replaced with distilled water and the gels were aged for further 20 h at 4 °C. The aquagel was ground into a fine powder and tested for catalytic activity.

4.4. General procedure C. Encapsulation of *Hb*HNL in a sol–gel matrix for synthetic reaction

A mixture of the stock solution of *Hb*HNL (0.5 ml, 3.6 KU/ml) and the precursor mixture (500 µl; prepared according to standard procedure A) was stirred magnetically for 20 s. The stirring bar was removed and when the mixture gelled (4–5 min), the gel was submerged in buffer A and aged at 4 °C for 24 h. Then the buffer in the pores was exchanged against buffer B by dialysis over 1 h. The aquagel was ground into a fine powder and used for the synthesis of enantiopure cyanohydrins.

4.5. General procedure D. Standard aqueous activity test for the encapsulated enzyme in aqueous media

Mandelonitrile (80 µl) was dissolved in 10 ml of a citric acid/potassium phosphate buffer (3 mM, pH 3.5). This solution (1.4 ml), and all of the sol–gel encapsulated *Hb*HNL, prepared as described in general procedure B, were added to buffer B (4.9 ml) at 25 °C. The mixture was stirred magnetically for another 6 min, before the reaction was stopped by addition of concentrated HCl (2 drops). A sample was filtered through cotton and the UV-absorption of the supernatant was measured at 280 nm against a blank reaction. The activity was calculated according to the equation below.

$$\text{Activity} = \frac{V}{\varepsilon_{280} l S T} \Delta Abs/\text{min}$$

Activity. The activity of the sample (U/ml); V: total reaction volume (ml); ε_{280}: 1.376 ($\text{l}\times\text{mmol}^{-1}\times\text{cm}^{-1}$); l: path length in the UV-cell (cm); S: volume of enzyme solution added in the preparation of the gel (ml); T: reaction time (min).

Table 1. Temperature programs and retention times for **3a–e**, (*R*)-**4a–e** and (*S*)-**4a–e**

Substrate	Temperature program[a]	R_t (min) **3**	R_t (min) (*R*)-**4**	R_t (min) (*S*)-**4**
a	125 °C (3 min)–20 °C/min–200 °C (0 min)	3.11	6.35	6.64
b	100 °C (3 min)–20 °C/min–200 °C (0 min)	3.17	6.50	6.80
c	75 °C (5 min)–30 °C/min–200 °C (1 min)	5.49	9.03	9.27
d	125 °C (3 min)–20 °C/min–200 °C (13 min)	10.16	19.19	19.58
e	60 °C (5 min)–2 °C/min–98 °C (0 min)	2.80	22.85	23.07

[a] Initial temperature (holding time)–temperature gradient–final temperature (holding time).

4.6. General procedure E. Synthesis of optically active cyanohydrins

At 25 °C, all of the sol–gel encapsulated *Hb*HNL, prepared according to general procedure C (1.8 kU *Hb*HNL), was added to a magnetically stirred solution of the freshly distilled aldehyde/ketone (4.92 mmol) in diisopropyl ether (5 ml) which was saturated with buffer B. The reaction was started by the addition of acetone cyanohydrin (1.35 ml, 14.76 mmol). Samples of 10 µl were taken through the septum at different stages of conversion. The samples were added to a mixture of dichloromethane (0.5 ml), acetic anhydride (40 µl) and pyridine (40 µl). After at least 12 h at room temperature the samples were analysed by chiral GC. The conversion and the enantiomeric excess were calculated from the relative peak areas of the aldehyde and the cyanohydrin derivative. The results are given in Figure 1.

4.7. Recycling, washing with diisopropyl ether saturated with a 50 mM citrate/phosphate buffer pH 5.0

The gels were prepared according to general procedure C and used in the addition of HCN to benzaldehyde according to general procedure E. The synthetic reaction was stopped after 1h and the gel was washed with diisopropyl ether saturated with buffer B before it was reused in a new cycle. The results of three cycles are given in Figure 2A.

4.8. Recycling, washing with a 50 mM citrate/phosphate buffer pH 5.0

The gels were prepared according to general procedure C and used in the addition of HCN to benzaldehyde according to general procedure E, with the following exception: The synthetic reaction was shaken orbitally and after one hour the gel was washed on a filter with a 50 mM phosphate buffer pH 5.0 (40 ml) and then reused in a new cycle. The results of four cycles are given in Figure 2B.

4.9. Test for leakage of activity to the organic solvent

Two gels were prepared according to general procedure C and used in the addition of HCN to benzaldehyde according to general procedure E, in two separate reactions A and B. After 10 min reaction B was filtered in a closed system to avoid any leakage of HCN. No more conversion could be detected in the supernatant. The conversion curves of the two reactions are given in Figure 3.

4.10. Test for leakage of activity by washing

Two gels were prepared according to general procedure C and used in the addition of HCN to benzaldehyde according to general procedure E, in two separate reactions A and B. The crushed gel used in reaction B was washed with buffer B prior to its use in the synthetic reaction. The conversion curves for the two reactions are given in Figure 4.

4.11. The effect of ageing on the gels

Seven gels were prepared according to general procedure B and stored for 1, 2, 3, 5, 7, 13 and 16 days respectively. The activity relative to the free enzyme was then measured according to general procedure D. The results are given in Figure 6.

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37. In an aquagel, the pores of the gel are filled with water.
38. Gels prepared according to standard procedure C were cut or ground into a particle size of 3–5 mm, 1–2 mm and <0.1 and used in the addition of HCN to benzaldehyde according to standard procedure E. The gels showed relative initial rates of 0.2, 0.3 and 1, respectively.
39. Three gels were prepared according to procedure B where the 25 mM phosphate buffer pH 6.5 contained 0.0, 1.0 and 2.0% PVA respectively. The gels were tested for activity according to procedure D.
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42. Gels prepared according to standard procedure B and tested for activity according to standard procedure D. After 10 min the reaction mixtures were filtered and the activity of the filtrates were monitored.
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