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Immobilized Hydroxynitrile Lyases for Enantioselective Synthesis of Cyanohydrins: Sol-Gels and Cross-Linked Enzyme Aggregates

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Abstract: The hydroxynitrile lyases (HNLs) from *Prunus amygdalus* (*Pa*HNL), *Manihot esculenta* (*Me*HNL), and *Hevea brasiliensis* (*Hb*HNL) were successfully immobilized in sol-gels. The cross-linked enzyme aggregate (CLEA) of *Hb*HNL was also prepared. These immobilized enzymes and the commercial *Pa*HNL- and *Me*HNL-CLEAs were employed for the enantioselective synthesis of cyanohydrins. The sol-gels were highly efficient at low catalyst loading and particularly stable towards the organic solvent (diisopropyl ether) and substrate/product deactivation. The stabilization effect was inconsistent for CLEAs of different HNLs and significant deactivation of *Pa*HNL- and *Hb*HNL-CLEAs in diisopropyl ether was observed. In contrast commercial *Me*HNL-CLEA proved to be a remarkably robust and efficient biocatalyst in diisopropyl ether.

Keywords: asymmetric synthesis; CLEA; cyanohydrins; hydroxynitrile lyase; oxynitrilase; sol-gel

Introduction

The addition of hydrogen cyanide to carbonyl compounds is an important carbon-carbon bond forming reaction. The cyanohydrins generated are versatile intermediates in organic synthesis and different catalytic approaches toward enantioenriched cyanohydrins from prochiral aldehydes and ketones have been described.[1–5] Hydroxynitrile lyases (HNLs) can be isolated from many different plants and they eliminate HCN from cyanohydrins leaving the corresponding carbonyl compound. This natural defense mechanism occurs when the plant is attacked by herbivores. In reverse, this reaction serves for the enantioselective synthesis of cyanohydrins. Indeed many successful studies have been described and HNLs are today used on an industrial scale for the synthesis of enantiopure cyanohydrins.[1,5–9]

The stability of enzymes in organic solvents can be greatly enhanced by immobilization. Moreover the catalyst can be filtered off easily and no extraction step is required to recover the product of the reaction from the liquid phase. It is also particularly beneficial in the HNL-catalyzed enantioselective cyanohydrin synthesis since the racemic, non-catalyzed, addition of HCN to carbonyl compounds can reduce the enantiopurity of the product significantly. The rate of this competing process depends on the water content and the pH of the water phase in the reaction system.[10,11] By immobilizing HNLs it might become possible to avoid an organic/water biphasic reaction mixture and the racemic background reaction should ideally be completely suppressed. Although several immobilization methods have been employed for HNLs, each having their particular advantages, the reaction conditions under which they have been tested were not always identical. Here, we compare different forms of immobilized HNLs in diisopropyl ether (DIPE), a solvent used industrially for an *Hb*HNL-catalyzed reaction.[5]

In order to improve the HNLs robustness, immobilization onto supports such as celite,[10] cellulose[10,12–14] or nitrocellulose[13,14] has been studied. Our attention focuses on immobilization strategies where the HNL can be recycled efficiently. Cross-linked enzyme crystals (CLECs) of the HNL from *Manihot esculenta* (*Me*HNL) were reported earlier as stable and recyclable biocatalysts for the addition of hydrogen cyanide to carbonyl compounds.[15] However, challenges inherent to the preparation of crystals from proteins have limited the range of applications.[16] Attempts to overcome these limitations on a large scale have been reported[17] but a recently established technology based on the cross-linking of enzyme *aggregates* rather than crystals is now considered a more viable alternative for immobiliza-

tion.[18,19] Cross-linked enzyme aggregates (CLEAs) of the HNLs from *Prunus amygdalus* (*Pa*HNL) and *Manihot esculenta* (*Me*HNL) are even commercially available biocatalysts for the enantioselective synthesis of (*R*)- or (*S*)-cyanohydrins, respectively. Poly(vinyl alcohol) hydrogels (Leutikats®) of *Pa*HNL have been reported as efficient and robust catalysts for the synthesis of (*R*)-mandelonitrile.[20] Furthermore, the *Pa*HNL-CLEA-catalyzed synthesis of cyanohydrins derived from substituted aromatic aldehydes was recently described in microaqueous (2% v/v aqueous buffer in organic solvent) and biphasic systems. Under these conditions the biocatalyst could be recycled up to 10 times.[21]

Another successful immobilization technique is the encapsulation of enzymes in sol-gels. Encapsulation in a *sol-gel matrix* of the HNL from *Hevea brasiliensis* (*Hb*HNL) was reported recently and the system proved to be efficient for the synthesis of a range of cyanohydrins.[22] Capsules of the sol-gel matrix were filled with buffer solution, thereby forming an "aqua gel" with the enzyme maintained in an aqueous environment. Although some loss of activity was observed upon recycling, the "aqua gel" was still catalytically active in buffer-saturated DIPE.

Here we compare the catalytic performance of "aqua gels" and cross-linked enzyme aggregates (CLEAs) of (*S*)-selective *Hb*HNL, (*S*)-selective *Me*HNL and (*R*)-selective *Pa*HNL for HCN addition to a range of carbonyl compounds, including the industrially relevant *m*-phenoxybenzaldehyde **1d** (Figure 1).

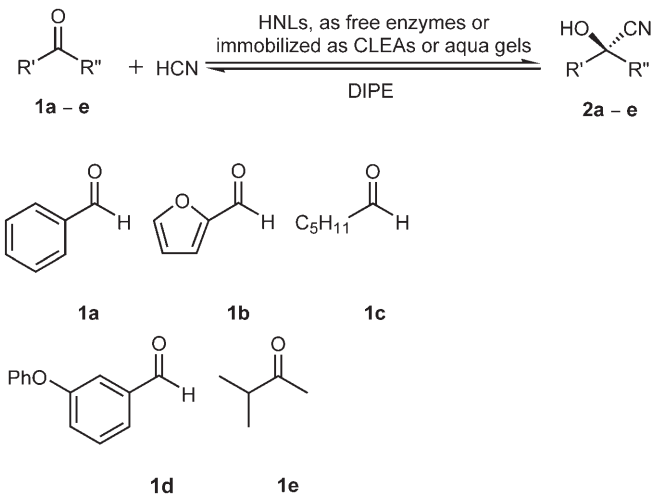

Figure 1. Substrates investigated.

Results and Discussion

Immobilized Enzymes

When immobilizing a homogeneous catalyst two targets have to be achieved: A high percentage of the catalyst has to be immobilized in its active form, and the immobilized catalyst, here the enzyme, has to be stable. Aqua gels of *Hb*HNL were first prepared according to the reported procedure[22] and 58% of the activity was recovered when starting from a 3.6 kU/mL stock solution, in line with the literature value. The residual methanol in the gel precursor and diffusion limitations are believed to be responsible for the decrease in activity.[22] When diluted *Hb*HNL was encapsulated using the same amount of precursor as in the original procedure, the recovered activity was found to be significantly lower (Table 1). However, it has earlier been observed that the supposedly low activity of an immobilized enzyme in aqueous buffers does not effectively represent its catalytic activity in organic solvents.[21] Indeed, this drop in activity when encapsulating approximately 13 times less *Hb*HNL in the aqua gel is consistent with a diffusion-limited system where the catalyst is immobilized but not accessible enough for a fast activity test such as the decomposition of dilute *rac*-mandelonitrile **2a** in aqueous buffers. Aqua gels of *Me*HNL and *Pa*HNL were prepared according to the procedure reported for *Hb*HNL, and the recovered activities were again low (Table 1).

When the CLEA of *Hb*HNL (2.61 kU/g) was prepared according to the procedure developed for *Pa*HNL[21] the activity recovered after immobilization was 16%, which is somewhat higher than the recovery value reported for *Pa*HNL-CLEA (9.6%).[21] As described for the *Pa*HNL-CLEA and proposed for the aqua gels above, the low activity is most likely due to diffusion limitations. To compare the different enzymes and the effects of the carriers on them, the aqua gels of *Hb*HNL, *Pa*HNL and *Me*HNL, as well as the CLEA of *Hb*HNL, were used at loadings of 6 U/mmol substrate in the catalytic experiments. Commercially available CLEAs from *Pa*HNL (4.61 kU/g)

Table 1. Enzyme activity recovered upon encapsulation in aqua gels according to activity test described in the Experimental Section.

	*Hb*HNL	*Hb*HNL	*Me*HNL	*Pa*HNL
Enzyme Stock Solution [U/mL]	3,600	274	230	300
Activity Recovery in Aqua Gel [%]	58	22	8.5	15

and *Me*HNL (1.02 kU/g) were also included into the study and standardized at 6 U/mmol of substrate. This amount of catalyst was relatively low when compared to loadings typically used for these enzymes.

The aqua gel-catalyzed reactions were carried out in diisopropyl ether (DIPE) saturated with 50 mM citrate/phosphate buffer (pH 5.0 to suppress the undesired racemic background reaction) to prevent a possible drying effect of the solvent on the catalyst capsules. For the same reason the hydrogen cyanide stock solution in DIPE was also saturated with aqueous buffer. This buffer (pH 5.5) was selected in order to stabilize the hydrogen cyanide solution upon storage and again to avoid the background reaction (see Experimental Section). The *Pa*HNL-CLEA-catalyzed addition of HCN to a range of aldehydes has been described in microaqueous media (2% v/v buffer in an organic solvent) but under those conditions[21] a water layer can still be observed. In the study reported here the CLEAs were suspended in commercial DIPE in order to avoid the extraction step during the reaction work-up. Under these conditions the organic solvent still contained trace amounts of buffer from the HCN stock solution but the reaction medium was a single liquid phase. Thus, in both the aqua gel and the CLEA system a single organic phase was present. In the case of the CLEAs the enzyme is surrounded by DIPE providing a true one-phase system, in the case of the aqua gel the enzyme is in the aqueous buffer inside the aqua gel and the system is comparable to a biphasic medium. For comparison the reaction was also studied with the free enzymes (6 U/mmol substrate); in this case a biphasic buffer-DIPE system had to be used (see Experimental Section).

Benzaldehyde

As a first model reaction the enantioselective addition of HCN to benzaldehyde was studied in DIPE. Almost enantiopure mandelonitrile (**2a**) was obtained at excellent conversions within 4 h when the reaction was catalyzed by free HNLs. Remarkably the aqua gels of all three HNLs catalyzed the same reaction in a much shorter period of time with the same enantioselectivity (Table 2). Under these standard reaction conditions, with relatively low catalyst loading (6 U/mmol), both the free enzymes and the HNL aqua gels operated well. However, initial rates were higher for HNL aqua gels than for the corresponding free enzyme. Clearly the diffusion limitation that indicated a low enzyme loading in the aqueous activity test (Table 1) was giving misleading results and much less enzyme was deactivated during the immobilization than indicated by this test. Furthermore, the increase in surface area of the aqueous phase for encapsulated HNLs, in comparison with the aqueous environment of homogeneous enzymes, is also believed to be responsible for this effect.

When the reaction was allowed to proceed over an extended period of time, racemization of mandelonitrile **2a** was observed in all cases. This might be due to the chemical background reaction. However, these enzymes do not only catalyze the formation of one enantiomer, they also catalyze its degradation and thus speed up racemization significantly. Rates of racemization for the free enzyme-catalyzed reactions were comparable to the rates observed in a biphasic system for the non-catalyzed racemization of (*S*)-mandelonitrile (Figure 2), demonstrating a deactivation of the enzymes. In the presence of HNL aqua gels, racemization rates were significantly higher than for the free enzymes indicating the high stability of the aqua gel immobilized enzymes. The racemization of (*S*)-mandelonitrile in the presence of enzyme-free aqua gels proceeded at rates comparable to the non-catalyzed reaction in a biphasic system. Thus the racemization was enzyme induced.

Overall the aqua gels of HNLs investigated here had very similar catalytic properties for the synthesis of **2a** although these enzymes come from different plants and are not always structurally related.[23] The performance of cross-linked enzyme aggregates of these HNLs, however, varied greatly. The synthetic activity of *Me*HNL-CLEA was comparable to the results obtained for the free enzyme and *Me*HNL aqua gel while the CLEAs from *Pa*HNL and *Hb*HNL suffered from a noticeable loss of activity under the con-

Table 2. Conversion ratios and *ee*s (parentheses) at optimum reaction times in the synthesis of mandelonitrile (**2a**) catalyzed by free HNLs and the corresponding aqua gels.

(*S*)-*Hb*HNL		(*S*)-*Me*HNL		(*R*)-*Pa*HNL	
Free[a]	Aqua Gel[b]	Free[a]	Aqua Gel[b]	Free[a]	Aqua Gel[b]
4 h: 97(97)	0.5 h: 97(99)	4 h: 97(98)	0.5 h: 96(99)	4 h: 98(97)	2 h: 97(97)

Reaction conditions: Benzaldehyde (0.5 mmol/mL DIPE), HCN (3 equivs.), and the catalyst (6 U/mmol) were shaken at room temperature and the reaction was monitored by GC.

[a] The catalyst stock solution was diluted with citrate/phosphate buffer (50 mM, pH 5.0) to a DIPE:aqueous media ratio of 5:1.

[b] DIPE saturated with citrate/phosphate buffer (50 mM, pH 5.0).

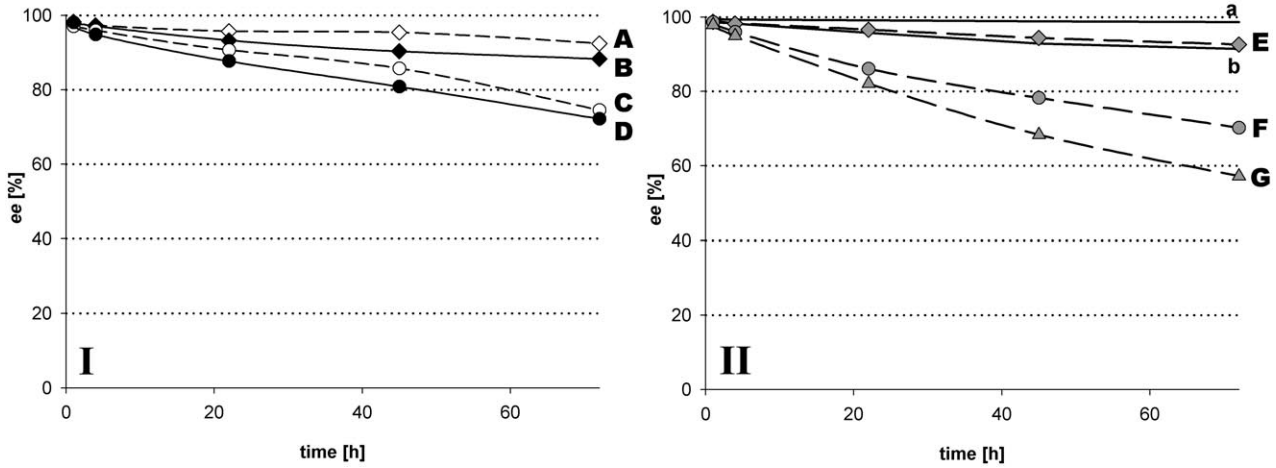

Figure 2. The *ee*s in the HNL-catalyzed synthesis of mandelonitrile. Results in **I: A**: free *Pa*HNL, **B**: free *Hb*HNL, **C**: *Pa*HNL aqua gel and **D**: *Hb*HNL aqua gel. **II**: Results of blank racemisations (no enzyme) in DIPE (**a**) and in a biphasic system (**b**); and results from *Me*HNL. **E**: free *Me*HNL, **F**: *Me*HNL aqua gel and **G**: *Me*HNL-CLEA.

Table 3. Conversion ratios and *ee*s (parentheses) in the synthesis of mandelonitrile catalyzed by CLEAs of different HNLs.

(*S*)-*Hb*HNL CLEA	(*S*)-*Me*HNL CLEA	(*R*)-*Pa*HNL CLEA
72 h: 55(67)	2 h: 96(97)	72 h: 97(99)

Reaction conditions: Benzaldehyde (0.5 mmol/mL DIPE containing traces of water from the HCN solution), HCN (3 equivs.), and the respective CLEA (6 U/mmol) were shaken at room temperature and the reaction was monitored by GC.

ditions used and both enzymes catalyzed the reaction very slowly (Table 3). *Hb*HNL and *Pa*HNL are known to be unstable in the absence of water[24,25] and the decrease in activity might be due to the "drying" effect of the reaction medium (DIPE with traces of buffer from the HCN solution) on the catalyst. The rate of racemization for *Me*HNL-CLEA was even higher than observed for the aqua gel of the same enzyme (Figure 2), indicating that the *Me*HNL-CLEA is particularly robust in this single-phase organic reaction medium.

Full conversion was achieved with *Pa*HNL-CLEA over an extended period of time (Figure 3). (*R*)-Mandelonitrile, being the natural substrate of *Pa*HNL, was obtained in high selectivity over the course of the reaction. The CLEA of *Hb*HNL was most dramatically affected by the reaction conditions. The reaction rate and catalyst selectivity were significantly lower than for all other catalysts. When it was employed under microaqueous conditions (Figure 3) its performance improved significantly, but *Hb*HNL-CLEA remained very sensitive toward deactivation by the organic solvent.

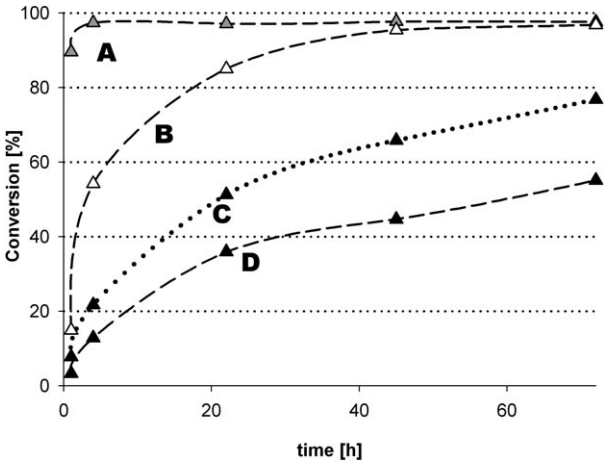

Figure 3. Conversion ratios in the HNL-CLEA catalyzed synthesis of mandelonitrile. **A**: *Me*HNL-CLEA, **B**: *Pa*HNL-CLEA, **C**: *Hb*HNL-CLEA (2% aqueous suspension: "microaqueous"), **D**: *Hb*HNL-CLEA .

The loss of activity observed for CLEAs of *Pa*HNL and *Hb*HNL in DIPE with traces of buffer from the HCN solution was consistent for all the substrates investigated and these catalysts will not be included further in this study. Cross-linked enzyme aggregates are carrier-free biocatalysts where the protein is directly exposed to the reaction media. Relative robustness of HNLs from different sources is therefore revealed to a greater extent in a CLEA than upon encapsulation in an aqua gel where the enzyme is maintained in an aqueous buffer environment. The catalytic performance of *Me*HNL-CLEA is reported in the accompanying paper.[35] The remarkable performance of this immobilized form made it a good candidate for a comparison with the aqua gels of HNLs and the free enzymes.

2-Furaldehyde

The synthesis of 2-furaldehyde cyanohydrin (**2b**) was achieved within 30 min at high conversion ratios and *ee*s when catalyzed by HNL-aqua gels, free enzymes and the CLEA of *Me*HNL (Table 4).

Enantiomeric excesses at full conversion were slightly lower for **2b** than for **2a**. Rapid racemization of **2b** accounted for the lower *ee* values obtained. Stabilization of free *Pa*HNL and *Me*HNL upon encapsulation in an aqua gel matrix was observed (Figure 4). This stabilization is much less pronounced for *Hb*HNL. While the free enzyme catalyzed the racemization more efficiently than the other two free HNLs its aqua gel displays the lowest racemization rate.

HNLs are susceptible to deactivation not only by solvents, but also by the substrate[11,26] and by the product formed. This effect could not be observed in the case of benzaldehyde (**1a**) since the enzyme loadings were standardized according to their catalytic properties in aqueous media for the decomposition of mandelonitrile (**2a**). The extent of substrate/product deactivation was therefore leveled out and accounted for in the reaction studied earlier. Using 2-furaldehyde (**1b**), the relative stability of individual enzyme toward a specific substrate became apparent. Moreover the greater solubility of this aldehyde in aqueous media is believed to enhance this effect. Indeed, whilst mandelonitrile is sparingly soluble in water,[27] the solubility of 2-furaldehyde is significant[28] (*ca.* 8.2 wt%). The results obtained suggest that the substrate/product deactivation phenomenon is significant for *Pa*HNL and *Me*HNL whereas free *Hb*HNL remained active over the course of the reaction (3 days). Furthermore, conversion ratios reported by Griengl et al. for this substrate in biphasic[29] and aqueous[30] media by *Hb*HNL were significantly different (95% and 55%, respectively). This trend seems to indicate that greater concentrations of **2b** in the aqueous phase deactivate *Hb*HNL. Aqua gel encapsulation of *Pa*HNL and *Me*HNL improved the stability of these enzymes. The stabilization effect upon immobilization observed for *Me*HNL was also noticed for the CLEA of this enzyme. The CLEA immobilization strategy seems efficacious in preventing substrate/product deactivation.

Hexanal

As a representative alkyl substrate the addition of hydrogen cyanide to hexanal was studied. The free enzyme, aqua gel and CLEA form of *Me*HNL achieved full conversion about as fast as for benzaldehyde (Table 5). Enantiomeric excesses obtained indicate a trend in selectivity for HNLs from different sources for this substrate. Selectivity in the *Hb*HNL-catalyzed reactions were as high as 94% whereas the

Table 4. Conversion ratios and *ee*s (parentheses) after 30 min in the synthesis of 2-furaldehyde cyanohydrin.

(*S*)-*Hb*HNL		(*S*)-*Me*HNL			(*R*)-*Pa*HNL	
Free	Aqua Gel	Free	Aqua Gel	CLEA	Free	Aqua Gel
89 (94)	89 (94)	90 (95)	95 (87)	94 (94)	91 (96)	95 (82)

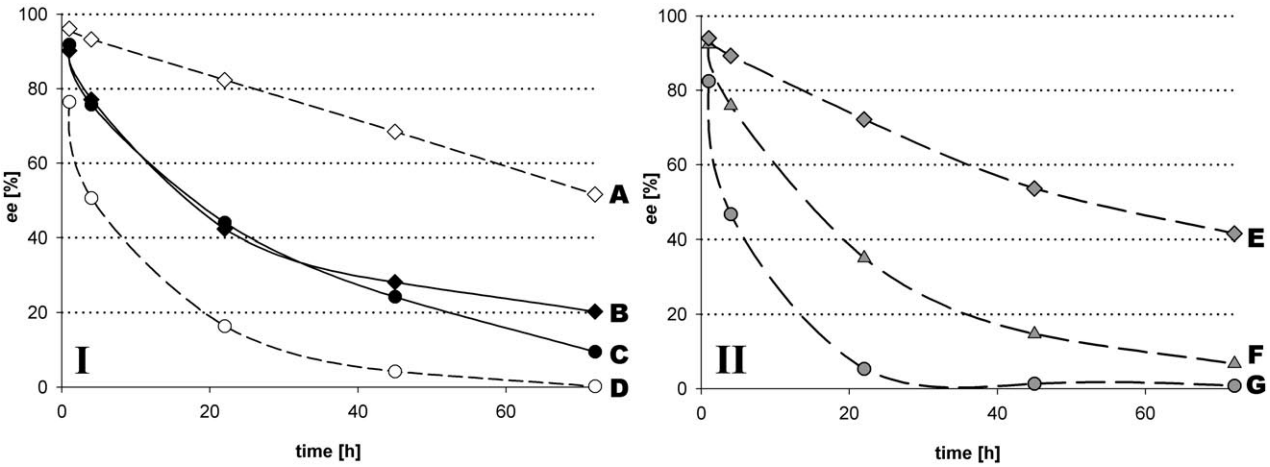

Figure 4. The *ee*s in the HNL-catalyzed synthesis of 2-furaldehyde cyanohydrin. Results in **I** are presented for *Pa*HNL and *Hb*HNL. **A**: free *Pa*HNL, **B**: free *Hb*HNL, **C**: *Hb*HNL aqua gel and **D**: *Pa*HNL aqua gel. Results for *Me*HNL are presented in **II. E**: free *Me*HNL, **F**: *Me*HNL-CLEA and **G**: *Me*HNL aqua gel.

Table 5. Conversion ratios and *ee*s (parentheses) at optimum reaction times in the synthesis of hexanal cyanohydrin.

(*S*)-*Hb*HNL		(*S*)-*Me*HNL			(*R*)-*Pa*HNL	
Free	Aqua Gel	Free	Aqua Gel	CLEA	Free	Aqua Gel
4 h: 91 (94)	2 h: 92 (94)	3 h: 93 (84)	3 h: 96 (85)	3 h: 92 (81)	3 h: 91 (87)	3 h: 88 (85)

*Pa*HNL- and *Me*HNL-catalyzed reactions reached *ee* values of only 87 % and 85 %, respectively.

The racemization of **2c** was very limited, as illustrated for *Me*HNL (Figure 5), and could not account for the lower *ee*s observed. Catalyst selectivity for the addition of hydrogen cyanide to hexanal is clearly higher for *Hb*HNL than *Pa*HNL and *Me*HNL under the conditions used. The enantioenriched mixtures of **2c** racemized at lower rates than those observed for **2a** in the same conditions and only a slight influence was observed for the immobilized enzymes *vs.* the free enzymes. The general trend of enzyme stabilization upon immobilization was consistent with the results obtained for the other substrates.

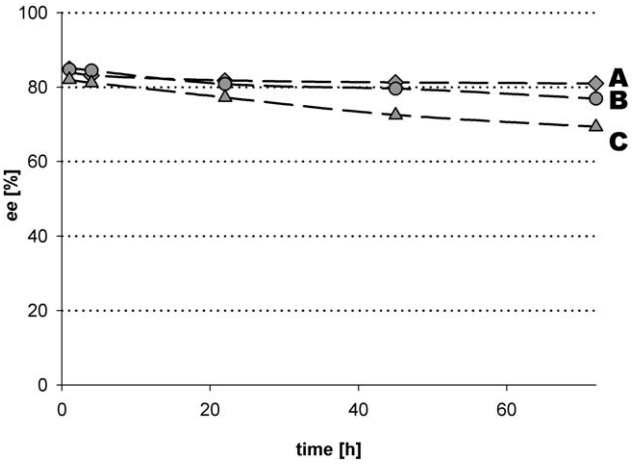

Figure 5. The *ee*s in the *Me*HNL-catalyzed synthesis of hexanal cyanohydrin. **A**: free *Me*HNL, **B**: *Me*HNL aqua gel, **C**: *Me*HNL-CLEA. Racemisation rates upon the catalysis of *Hb*HNL and *Pa*HNL (free enzyme and aqua gel) were in the range presented here for *Me*HNL.

m-Phenoxybenzaldehyde

The (*S*)-enantiomer of cyanohydrin **2d**, derived from *m*-phenoxybenzaldehyde, is of commercial interest for the preparation of pyrethroid insecticides.[31,32] High conversions and *ee*s have been reported for the addition of hydrogen cyanide to **1d** but the reaction had to be performed over 6 days[33] (50 U/mmol of HNL from *Sorghum bicolor*) or at high catalyst loadings to shorten the reaction time[29] (15 min using 1000 U/mmol of *Hb*HNL). Very good results have also been reported for (*S*)-**2d** using mutant strains of *Me*HNL.[34] In the *Hb*HNL aqua gel-catalyzed formation of **2d**, using acetone cyanohydrin as the cyanide source,[22] high conversion ratios were obtained but the *ee* of the final product was just over 40 %, most likely due to very long reaction times. The reaction time was also relatively long in our investigations (2–3 days) but encapsulated HNLs afforded the corresponding cyanohydrin in excellent conversion ratios and *ee*s (Table 6).

The use of hydrogen cyanide evidently improved the performance of the encapsulated catalyst considering that the enzyme loading was much lower than described earlier.[22] Free HNLs catalyzed the reaction but the performance of the enzymes was strongly dependent on the enzyme source (Figure 6). In parallel with the results obtained for 2-furaldehyde (**1b**) the stability of individual enzyme toward the substrate/product of the reaction became apparent. High initial rates were obtained using free *Hb*HNL, but the reaction became very sluggish upon accumulation of the product in the reaction medium, suggesting a cyanohydrin-induced deactivation. Free *Me*HNL showed very low activity under the conditions used for the synthesis of **2d**. It is noteworthy that the two oxygen-containing substrates investigated (**1b** and **1d**) gave better results for free *Hb*HNL than free-*Me*HNL. On the other hand, the catalytic performances of encapsulated *Me*HNL were in both cases greater than for *Hb*HNL. The stabilization upon encapsulation toward substrate/product deactivation for these compounds was therefore more efficient for *Me*HNL than for *Hb*HNL. CLEA immobilization also improved the stability of this enzyme but its overall performance

Table 6. Conversion ratios and *ee*s (parentheses) at optimum reaction times in the synthesis of *m*-phenoxybenzaldehyde cyanohydrin.

(*S*)-*Hb*HNL		(*S*)-*Me*HNL			(*R*)-*Pa*HNL	
Free	Aqua Gel	Free	Aqua Gel	CLEA	Free	Aqua Gel
72 h: 45 (82)	72 h: 92 (98)	72 h: 14 (75)	45 h: 98 (97)	72 h: 81 (83)	72 h: 68 (99)	72 h: 97 (99)

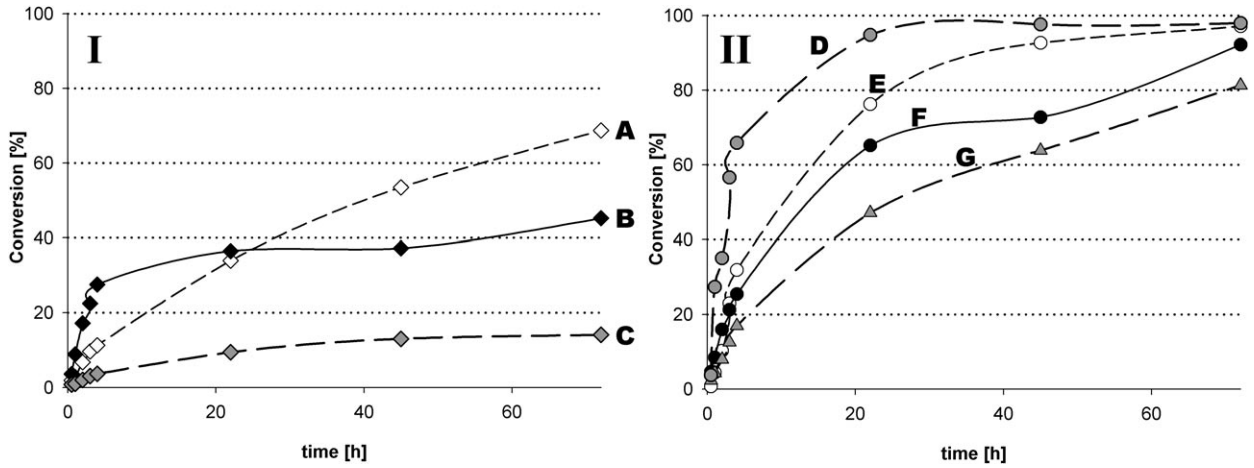

Figure 6. Conversion ratios in the HNL-catalyzed synthesis of *m*-phenoxybenzaldehyde cyanohydrin. Results in **I** are presented for free enzymes. **A**: *Pa*HNL, **B**: *Hb*HNL, **C**: *Me*HNL. The respective aqua gels and *Me*HNL-CLEA are presented in **II. D**: *Me*HNL aqua gel, **E**: *Pa*HNL aqua gel, **F**: *Hb*HNL aqua gel and **G**: *Me*HNL CLEA.

was inferior to that of the aqua gel form. Furthermore, the solubility of *m*-phenoxybenzaldehyde in aqueous media is very limited and the biphasic system used for the free-enzyme-catalyzed reaction did not favor the kinetics of the reaction when compared to the organic media used for immobilized forms. Similar results were obtained for the *Pa*HNL-catalyzed synthesis of (*R*)-**2d**.

3-Methyl-2-butanone

Conversion ratios greater than 90% were achieved in the synthesis of 3-methyl-2-butanone cyanohydrin (**2e**) but poor selectivity ($ee < 40\%$) was observed under the conditions used. Literature results for the *Hb*HNL aqua gel-catalyzed formation of **2e**, using acetone cyanohydrin as a cyanide source,[22] were significantly lower in terms of conversion (below 30%). HCN as cyanide source shifted the equilibrium favorably but catalyst selectivity in the relevant literature[27] was nonetheless higher ($>70\%$ *ee*) than in our results. The low catalyst loading was considered unsuitable for this ketone and no acceptable enantiopurity could be achieved for **2e**. Free *Me*HNL and *Hb*HNL were significantly more active than free *Pa*HNL for this substrate (Figure 7).

Aqua gel encapsulation of *Pa*HNL improved its catalytic activity, but the reaction was still sluggish *vs.* free *Me*HNL and *Hb*HNL. The latter two (*S*)-selective enzymes have ketone-derived cyanohydrins as natural substrates, while mandelonitrile is the natural substrate of *Pa*HNL. Hence, the catalytic activity of HNLs from different sources toward **2e** is in line with the natural substrate preferences of the individual enzymes.

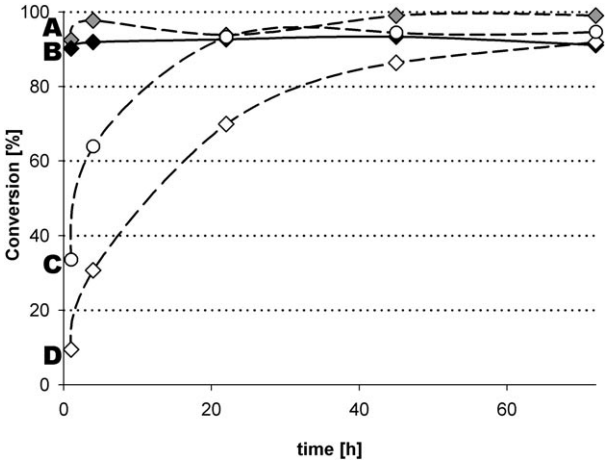

Figure 7. Conversion ratios in the HNL-catalyzed synthesis of 3-methyl-2-butanone cyanohydrin. **A**: free *Me*HNL, **B**: free *Hb*HNL, **C**: *Pa*HNL aqua gel, **D**: free *Pa*HNL.

Conclusions

The biocatalysts investigated herein were active for the addition of hydrogen cyanide, in DIPE, to five structurally different carbonyl compounds. Catalytic performance and robustness were strongly influenced by the reaction media, the substrate/product, the enzyme source and consequently the structure of the enzyme as well as the immobilization method. Encapsulation of HNLs in an aqua gel matrix stabilized them against deleterious effects of the reaction media and substrate/product interference. Moreover, it could be demonstrated that a fast and highly enantioselective formation of the desired cyanohydrins could be achieved with much lower enzyme loadings than re-

ported earlier.[1,21,22] However, the reaction conditions used here were not suitable for the preparation of 3-methyl-2-butanone cyanohydrin in high enantiomeric excess and efforts are currently being made to address this limitation.

The stability of cross-linked enzyme aggregates of HNLs in DIPE containing only traces of water was strongly dependent on the enzyme structure. The CLEA prepared from *Hb*HNL as well as the commercial CLEA from *Pa*HNL did not allow an application of these enzymes in a single organic phase. In contrast the *Me*HNL CLEA showed outstanding stability in DIPE. This opens up new opportunities for the application of this enzyme, which are described in an accompanying article.[35]

Experimental Section

General Remarks

Enzymes: The hydroxynitrile lyase (HNL) from *Prunus amygdalus* (PaHNL, [EC 4.1.2.10], Jűlich Fine Chemicals, 300 U/mL) was commercially available in 50% glycerol. More concentrated enzyme solutions from *Hevea brasiliensis* (HbHNL, [EC 4.1.2.39], DSM, 3.6k U/mL), and *Manihot esculenta* (MeHNL, [EC 4.1.2.39], Jűlich Fine Chemicals, 2.3 kU/mL) were diluted to 274 U/mL and 230 U/mL, respectively, using 25 mM potassium phosphate buffer (pH 6.5). CLEAs of PaHNL (4.61 kU/g) and MeHNL (1.02 kU/g) were prepared according to commercial procedures (CLEA Technologies).

Chemicals: Diethyl ether solutions of benzaldehyde (Fluka, 99%+), (±)-mandelonitrile (Acros Organics, technical grade, distilled before use), 2-furaldehyde (Acros Organics, 99%), hexanal (Aldrich, 98%), and *m*-phenoxybenzaldehyde (Acros Organics, 97%) were treated with saturated sodium bicarbonate solution prior to each use to remove traces of acid. The organic phase was dried and the solvent was removed under reduced pressure. (*S*)-Mandelonitrile (96% purity, containing 4 mol% benzaldehyde; 99.3% *ee*) was prepared according to a literature procedure.[35] Ethylene glycol dimethyl ether (glyme, Aldrich, 99.5%), dichloromethane (Aldrich, Anhydrous 99.8%), pyridine (Aldrich, Anhydrous, 99.8%), acetic anhydride (Acros Organics, 99%+), dodecane (Acros Organics, 99%), methyltrimethoxysilane (MTMS, Aldrich, 98%), tetramethoxysilane (TMOS, Fluka, 99%+), glutaraldehyde (Fluka, 25% in water, *ca.* 2. 6M), and 3-methyl-2-butanone (MIPK, Aldrich, 99%) were used as supplied, without further purification. Diisopropyl ether (DIPE, Acros Organics, 98%+, stabilized with 2,6-di-*tert*-butyl-*p*-cresol) was used without further treatment unless otherwise specified. Aqueous buffers were prepared from analytical grade salts and stabilized with 0.09% sodium azide.

Analytical Methods: The course of the reaction was followed by chiral gas chromatography on a Shimadzu Gas Chromatograph GC-14B equipped with a FID detector and a beta-cyclodextrin column (CP-Chirasil-Dex CB 25 m × 0.25 mm). Derivatization of reaction samples (20 μL aliquots) into cyanohydrin acetates, and GC analysis were performed as reported in the literature.[22] Shorter retention times are obtained with helium as carrier gas as compared to nitrogen (Table 7). Depending on the reaction scale, 200 μL or 40 μL additions of *n*-dodecane were used as internal standards to determine conversions and yields. Enantiomeric excess values were calculated from the areas of the respective cyanohydrin acetate peaks. UV measurements were carried out at 25.0 °C on a Varian CARY 3 spectrophotometer.

Table 7. GC retention times with helium as carrier gas.

Substrate	R_t [min]	Dodecane R_t [min]	(*R*)-Acetate R_t [min]	(*S*)-Acetate R_t [min]
1a	1.16	1.74	4.04	4.52
1b[a]	1.21	3.84	4.91	4.46
1c	1.67	6.92	7.28	7.61
1d	6.74	1.73	9.84	10.06
1e	1.18	18.85	15.37	15.59

[a] CIP rules invert the designation of the respective enantiomers for 2-furaldehyde cyanohydrin. Temperature as ref.[27]

Enzyme Activity Measurements

Enzymatic activity of free enzymes[26] and aqua gels[22] was measured according to reported literature procedures. CLEAs were suspended in 25 mM potassium phosphate buffer (pH 6.5) and samples of this suspension were used to calculate the activity according to the procedure reported for the free enzyme.[26] UV measurements were performed with continuous stirring in order to keep the CLEA suspended.

Preparation of *Hb*HNL-CLEA

*Hb*HNL-CLEA was prepared based on the reported procedure for *Pa*HNL.[21] *Hb*HNL stock solution (frozen at −20 °C) was diluted to 1.8 kU/mL in 25 mM potassium phosphate buffer (pH 6.5). The dilute sample (4.5 mL) was allowed to warm up slowly from −20 °C to 0 °C (ice bath) and glyme (4.5 mL) was then added. Precipitation was allowed to proceed for 15 min while stirring at 0 °C and glutaraldehyde (1.5 mL, 25% in water) was then added. The mixture was stirred at 0 °C for 17 h. The CLEA was filtered and rinsed thoroughly with acetonitrile and diethyl ether. After vacuum drying for 4 h, *Hb*HNL-CLEA (496 mg, 2.61 kU/g as determined by the activity test described above) was stored at −20 °C.

Hydrogen Cyanide (HCN): 2M Solution in DIPE

Caution: Due to the toxicity of hydrogen cyanide, all procedures involving this stock solution were performed in a well-ventilated fume hood equipped with an HCN detector. HCN-containing wastes were neutralized using commercial bleach and stored independently for disposal.

Sodium cyanide (49 g, 1.0 mol) was dissolved in a mixture of water (100 mL) and diisopropyl ether (DIPE) (250 mL) at 0 °C. The biphasic system was stirred vigorously for 15 min and 30 % aqueous HCl (100 mL) was added slowly. This mixture was allowed to warm slowly to room temperature (at least 25 min). The phases were separated and 150 mL of DIPE was added to the organic layer. The combined organic phases were stirred and residual water was separated. This procedure was repeated with another 100 mL of DIPE. The 2 M standard HCN solution[36] was kept over citric acid buffer (pH 5.5) in the dark.

Free HNL-Catalyzed Synthesis of Cyanohydrins

The HNL (30 units) from *Prunus amygdalus* (300 U/mL), *Manihot esculenta* (230 U/mL) or *Hevea brasiliensis* (274 U/mL) was diluted in 50 mM citrate/potassium phosphate buffer (pH 5.0) to a total volume of 2 mL. Diisopropyl ether (2.5 mL) was then added followed by the carbonyl compound of interest (5 mmol) and *n*-dodecane (200 μL). An analytical sample representative of initial conditions (5 μL) was drawn from the organic layer and diluted in DIPE (1 mL) for GC analysis. The reaction was initiated by addition of 2 M HCN in DIPE (7.5 mL, 3 equivs.) and monitored by chiral GC over three days while shaking the sealed flask at room temperature.

HNL Aqua Gel-Catalyzed Synthesis of Cyanohydrins

500 μL of *Pa*HNL (300 U/mL), *Me*HNL (230 U/mL) and *Hb*HNL (274 U/mL) were encapsulated into aqua gels using 500 μL of precursor prepared according to the literature.[22] The exchange of aqueous buffers in the gels was allowed to proceed over two days. Gels were then ground into a fine powder and used immediately. The scale of the reaction (Table 8) was adapted from the activity recovery (Table 1) to suit 6 U/mmol catalyst loading.

The *Hb*HNL sol gel reaction is given as a representative procedure. The aqua gels were ground into a fine powder and DIPE (2.5 mL) saturated with 50 mM citrate/potassium phosphate buffer (pH 5.0) was added. The substrate (5 mmol) and *n*-dodecane (200 μL) were dissolved into the mixture. A GC sample was taken to determine initial conditions and the reaction was initiated by addition of 2 M HCN in DIPE (7.5 mL, 3 equivs.). The reaction was monitored by chiral GC over three days while shaking the sealed flask at room temperature.

Table 8. Reaction conditions for the synthesis of cyanohydrins catalyzed by aqua gels.

	HbHNL	MeHNL	PaHNL
Residual activity in aqua gel[a] [units]	30	9.8	22.5
Reaction Scale[b] [mmol substrate]	5	1.6	3.75
Catalyst Loading [U/mmol]	6	6.1	6

[a] Calculated for a gel prepared from 500 μL of precursor and 500 μL of HNL dilute solution.
[b] All other reaction parameters were scaled accordingly.

HNL-CLEA-Catalyzed Synthesis of Cyanohydrins

The procedure given here for *Hb*HNL-CLEA was scaled accordingly for individual CLEAs to suit 6 U/mmol loading of catalyst. *Hb*HNL-CLEA (30 units, 2610 U/g) was suspended in commercial DIPE (2.5 mL). The starting material (5 mmol) and dodecane (200 μL) were added to the mixture and a GC sample was taken to determine initial conditions. The reaction was initiated by addition of 2 M HCN in DIPE (7.5 mL, 3 equivs.) and monitored by chiral GC over 3 days while shaking in a sealed flask at room temperature. The *Hb*HNL-CLEA-catalyzed synthesis of mandelonitrile in microaqueous media was performed by first suspending the catalyst (30 units) in 200 μL of 50 mM citrate/potassium phosphate buffer (pH 5.0) and the procedure was followed accordingly.

Racemization of (*S*)-Mandelonitrile

The non-enzymatic racemization of (*S*)-mandelonitrile was based on the conditions at full conversion of the synthetic reaction. Conditions in the biphasic system used for the free enzyme were reproduced by adding HCN (2 equivs., 5 mL) to a mixture of DIPE (4 mL) and 2 mL of 50 mM citrate/potassium phosphate buffer (pH 5.0). The reaction was initiated by addition of (*S*)-mandelonitrile (1 mL) from a 5 mmol mL^{-1} stock solution in DIPE:dodecane (4:1) and monitored by chiral GC. The racemization reaction described above was repeated in commercial DIPE in order to model the conditions of the CLEA-catalyzed reaction.

The influence of the carrier in aqua gels was evaluated from the rate of racemization of (*S*)-mandelonitrile in the presence of enzyme-free aqua gels prepared from 500 μL of precursor and 500 μL of 50 mM citrate/potassium phosphate (pH 5.0). Enzyme free gels were ground into a fine powder and DIPE (4 mL) saturated with 50 mM citrate/potassium phosphate buffer (pH 5.0) was added followed by HCN (5 mL, 2 equivs.). The reaction was initiated by addition of the (*S*)-mandelonitrile stock solution described above (1 mL) and monitored by chiral GC over three days while shaking in a sealed flask at room temperature.

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