ChemPubSoc Europe

DOI: 10.1002/cctc.201300892

Immobilized Hydroxynitrile Lyase: A Comparative Study of Recyclability

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Hydroxynitrile lyase from cassava, *Manihot esculenta*, (*Me*HNL) catalyzes the formation of (*S*)-cyanohydrins from HCN and aldehydes or ketones. Four differently immobilized *Me*HNLs were prepared: by noncovalent immobilization (celite R-633), covalent immobilization (cross-linked enzyme aggregates, CLEA), encapsulation [in a poly(vinyl alcohol) hydrogel Lentikats] and a combination of the above, an unusual immobilization, CLEA encapsulated in a poly(vinyl alcohol) hydrogel. A comparative study of the recyclability of each immobilized *Me*HNL was performed. Particular attention was paid to the stability and activity of the new immobilized *Me*HNL–CLEA–Lentikats and to the minimum enzyme loading required to achieve high yields and enantiomeric excesses. *Me*HNL–CLEA stability was slightly improved by encapsulation into Lentikats and good recyclability rates at low enzyme loading were obtained. However, *Me*HNL immobilized on celite R-633 exhibited the best recyclability, giving $>95\,\%$ conversion and an enantiomeric excess of 99% during 12 cycles.

Introduction

Chiral cyanohydrins are important synthetic intermediates for many industrial products, owing to the fact that both functional groups of the cyanohydrins, the hydroxyl and cyanide moiety attached to the same carbon, can easily be converted into a wide range of other chiral products such as α-hydroxy aldehydes and ketones, β-amino alcohols, α-fluoro cyanides, etc.[1] One possibility of synthesizing chiral cyanohydrins is the enantioselective addition of HCN to a prochiral aldehyde or ketone with hydroxynitrile lyases (HNLs) as biocatalysts.[2]

The enantiomeric purity of cyanohydrins obtained from the single-aqueous-phase reaction catalyzed by HNLs is disturbed by the nonenzymatic reaction that forms the undesired racemic products. The rate of this competing process depends on the water content and the pH of the water phase in the reaction system.[3] Different approaches were introduced to solve this problem: 1) biphasic systems of buffer and immiscible organic solvent, which provides many advantages such as high efficiency, prevention of substrate or product inhibitions, cost effectiveness, and easy downstream processing because the product is extracted into the organic phase while the enzyme remains in the aqueous phase; 2) organic solvents (dry or containing a small amount of water), in which the racemic background reaction is suppressed.

The stability of enzymes in organic solvents can be strongly 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. Although several immobilization methods have been employed for HNLs,[4] each having their particular advantages, the reaction conditions under which they have been tested were not always identical. In addition, if immobilized HNLs are reused, enzyme recycling protocols are diverse,[5] including washing steps that can clearly effect enzyme activity and/or make the process unviable on an industrial scale. This is to say often high enzyme loadings are used masking enzyme deactivation and/or enzyme deactivation is not mentioned.

Previous studies on the syntheses of chiral cyanohydrins in biphasic systems and organic solvents were performed on the HNL from *Manihot esculenta* (*Me*HNL),[6] but information on enzyme recyclability is scarce. Moreover, the reaction conditions are not comparable, that is, it is not easy to ascertain which immobilization method is more plausible on an industrial scale. This HNL is particularly interesting because of its stability with respect to higher temperatures and low pH values making it superior to the other HNLs with α/β-hydrolase fold.[2b,7]

Cross-linked enzyme aggregates (CLEAs) are considered a more viable alternative for industrial immobilization[8] and CLEAs of the HNL from *Manihot esculenta* (*Me*HNL) are commercially available biocatalysts for the enantioselective synthesis of (*S*)-cyanohydrins. Though a *Pa*HNL–CLEA exhibited good recyclability, no recycling studies using *Me*HNL–CLEA can be found in the literature. On the other hand, poly(vinyl alcohol) hydrogels (Lentikats) of *Pa*HNL have been reported as efficient and robust catalysts for the synthesis of (*R*)-mandelonitrile,[5a]

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but the encapsulation of a *Me*HNL into Lentikats has not been tested yet.

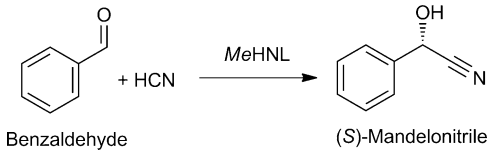
Another successful immobilization technique is the immobilization of enzymes on Celite. A comparative study of a *Me*HNL immobilized on Celite and cross-linked crystals of *Me*HNL was reported.[5b] The *Me*HNL–Celite exhibited low operational stability, losing all the activity after 5 batches, although the use of dry acetone to wash the enzyme several times between batches could explain these results, because it could remove the entrapped water in the immobilized enzyme and successive studies have shown the importance of this water in enzyme stability.[6c, 9]

Herein, to compare the stability and recyclability in the enantioselective synthesis of (*S*)-mandelonitrile by *Me*HNL, four differently immobilized *Me*HNLs were prepared: by noncovalent immobilization (Celite R-633), covalent immobilization (CLEA), encapsulation (in Lentikats), and a combination of the above, an unusual immobilization, CLEA encapsulated in a poly(vinyl alcohol) hydrogel. Our attention focused on immobilization strategies and reaction protocols by which the HNL can be recycled efficiently.

Results and Discussion

Recyclability of *Me*HNL–CLEA

The synthetic potential of the *Me*HNL–CLEA in organic solvents was previously described,[5c, 10] although the reuse was not studied thoroughly. Recyclability of the *Me*HNL–CLEA was, therefore, studied herein on a laboratory scale by reusing the catalyst in seven successive reactions of benzaldehyde (1 mmol) and HCN (3 equiv., Scheme 1) with a low enzyme



Scheme 1. *Me*HNL-catalyzed hydrocyanation of benzaldehyde.

loading (5 mg, 15 U) in buffer-saturated methyl *tert*-butyl ether (MTBE, citrate phosphate buffer 50 mM, pH 5.5). The enzyme loading was chosen such that full conversion was reached but a possible loss of activity would also be immediately visible. The successive reactions were performed by allowing each reaction cycle to proceed for 4 h, after which the reaction solution was replaced with a fresh solution of the reagents in buffer-saturated MTBE. The reactions were shaken because we found that magnetic stirring can damage the CLEAs. The *Me*HNL–CLEA was not washed between cycles. The first three cycles reached 98–95% conversion (Figure 1). In the next cycles, however, a successively decreasing reactivity of approximately 10% was observed. In addition to deactivation, a possible reason for the decrease is catalyst loss rather than actual loss in reactivity (centrifugation and removal the reaction solution before the addition of new reagents). Furthermore, the catalyst loss is likely to accumulate with each cycle. In all cases, the enantiomeric excess of the product was higher than 98%.

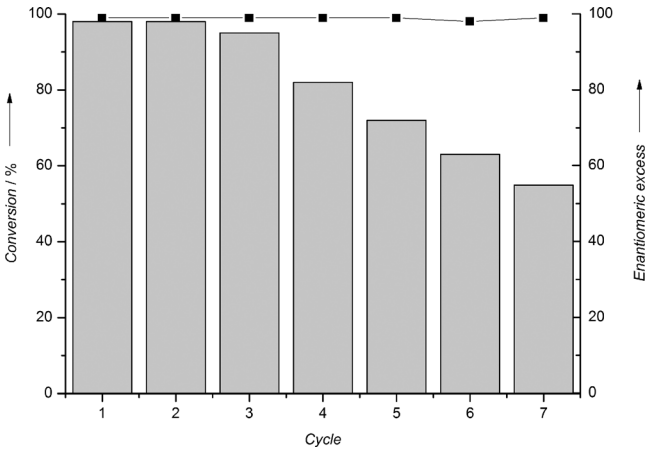


Figure 1. Recycling of the *Me*HNL–CLEA (5 mg, 15 U) in seven successive mandelonitrile synthesis reactions. Conditions: benzaldehyde (1 mmol) in 1.5 M HCN solution in buffer-saturated MTBE (2 mL); reaction time 4 h.

Three different enzyme washing protocols were tested to evaluate the effect on enzyme activity over 5 cycles. If the *Me*HNL–CLEA was washed with pure MTBE and dried between each cycle, a rapid loss of activity was observed and the *Me*HNL–CLEA gave only 44% conversion after the fourth cycle and 32% conversion after the fifth (Figure 2). In this case, the

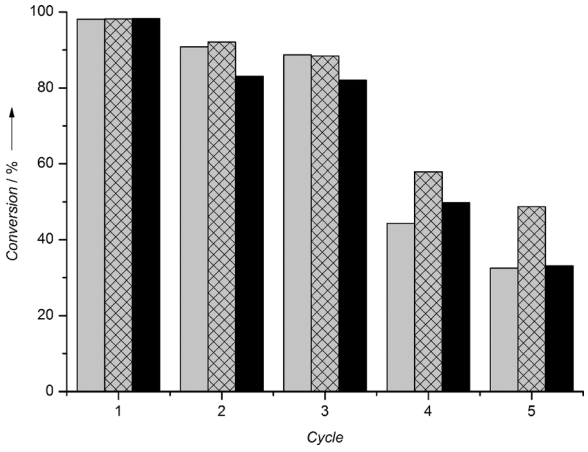


Figure 2. Recycling of the *Me*HNL–CLEA (5 mg, 15 U) in five successive mandelonitrile synthesis reactions, including washing of the enzyme between cycles. Gray: wash with pure MTBE (1 mL); gray cross-hatched: wash with buffer-saturated MTBE (1 mL); black: wash with 50 mM citrate phosphate buffer (1 mL), pH 5.5. Conditions: benzaldehyde (1 mmol) in 1.5 M HCN solution in buffer-saturated MTBE (2 mL); reaction time 4 h.

deactivation is probably owing to the fact that if solvent is used for washing, some of the entrapped water will be washed away from the *Me*HNL–CLEA, lowering the enzyme activity. If the *Me*HNL–CLEA was washed with buffer-saturated MTBE (citrate phosphate buffer 50 mM, pH 5.5) the loss of activity was similar to that with the washing steps using pure

MTBE during the first three cycles. However, the *Me*HNL–CLEA gave 58% conversion after the fourth cycle and 49% after the fifth. Compared to the first protocol, this lower deactivation probably owes to suppression of the loss of the essential water entrapped in the CLEA by use of a buffer-saturated solvent. Finally, the *Me*HNL–CLEA was washed with citrate phosphate buffer 50 mM, pH 5.5. As the CLEA is not entirely stable in buffer, a small amount of enzyme was withdrawn during each washing step. Moreover, the CLEA took up part of the buffer, making it difficult to obtain the CLEA with low water content before starting a new cycle. Owing to this, the successive reactions revealed that the CLEA is surrounded by water and the reaction conditions are similar to those of a biphasic system. The loss of activity was similar to that with the washing step using pure MTBE, even slightly higher in the cycles 2 and 3.

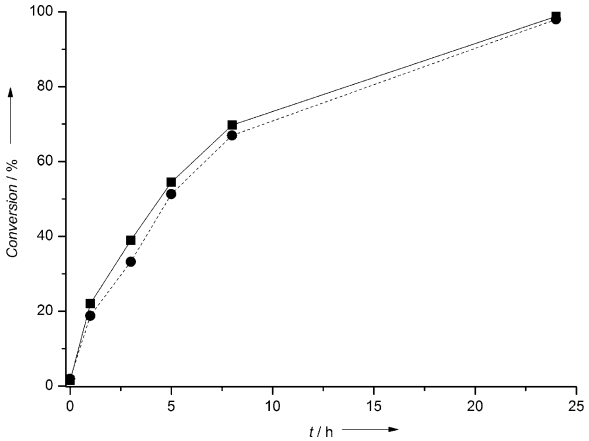


Figure 3. Mandelonitrile synthesis reaction by *Pa*HNL–Lentikats (1 g, 5 U) after 144 h stirring (■) and by fresh PaHNL–Lentikats (●). Conditions: citrate phosphate buffer 50 mM (5 mL) pH 4.5, MTBE (3 mL), 1.5 M HCN solution in MTBE (2 mL), benzaldehyde (1 mmol).

Preparation of *Me*HNL–CLEA–Lentikats

The first encapsulation of a nonpurified *Pa*HNL into Lentikats was described by Gröger et al.[5a] The protocol to prepare an efficiently entrapped HNL included a cross-linking process to increase the molecular weight of the enzyme, because, according to the authors, enzymes with a molecular mass lower than 50000 Da are not restrained in the hydrogels. A combination of glutaraldehyde and chitosan gave the best results. Then, the cross-linked enzyme was entrapped in a hydrogel matrix based on poly(vinyl alcohol) and used for the synthesis of mandelonitrile in a biphasic system.

As *Pa*HNL has a molecular mass 61 kDa, we reasoned that the cross-linking process could be omitted if using the correct poly(ethylene glycol)/poly(vinyl alcohol) ratio. Purified *Pa*HNL was encapsulated in a poly(vinyl alcohol) hydrogel and a leaching experiment was performed. The *Pa*HNL–Lentikats was stirred for 144 h in a 50 mM citrate phosphate buffer at pH 4.5. The buffer phase was used to conduct a standard activity test for HNLs to check the presence of leached enzyme. No activity was found. The *Pa*HNL–Lentikats were stirred for 144 h and fresh *Pa*HNL–Lentikats were used separately to perform mandelonitrile synthesis reactions in a biphasic system. Results of these experiments are shown in Figure 3. Clearly, the *Pa*HNL maintained its original activity after 144 h of stirring.

A biphasic system with low-pH buffer combined with MTBE was chosen to perform the synthesis of mandelonitrile, because the Lentikats tend to form aggregates in pure organic solvents, resulting in a poor conversion of the substrates. The organic layer in which most of the starting material and product reside helps to suppress the racemic reaction, since no reaction occurs in it. It does, however, introduce diffusion limitations, which have to be overcome by rapid stirring.

According to the results obtained with the *Pa*HNL, we proceeded to encapsulate the *Me*HNL in a poly(vinyl alcohol) hydrogel. At first glance, the low molecular mass of the *Me*HNL (28–30 kDa) seems to require the use of an initial cross-linking step to increase the enzyme size and avoid leaching, but this enzyme forms trimers and tetramers in solution, giving molecular masses higher than 60 kDa.[2b] For that reason, purified *Me*HNL was encapsulated in a first trial without a previous cross-linking step. 13% of the original activity was recovered after the encapsulation in Lentikats (Table 1). This result is in accordance with a previous study that demonstrated that immobilization by this method yielded very little attached enzyme.[11] The same leaching experiment described previously for the *Pa*HNL was then performed with the *Me*HNL-Lentikats. In this case, in contrast, the activity test using the reaction buffer gave positive results. Similarly, the *Me*HNL–Lentikats storage buffer was checked and enzyme activity was observed. Sodium dodecyl sulfate (SDS) gel electrophoresis confirmed the presence of free *Me*HNL outside the poly(vinyl alcohol).

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

	*Me*HNL	CLEA–*Me*HNL
Enzyme stock solution [U mL^{-1}; U mg^{-1}]	453	3
Activity after purification [U mg^{-1}]	207	–
Activity recovery in Lentikats [%]	13	50

Once leaching was confirmed, a cross-linking step to avoid this leaching was introduced. An unusual immobilization, *Me*HNL–CLEA encapsulated in a poly(vinyl alcohol) hydrogel, was prepared. Three different amounts of *Me*HNL–CLEA were encapsulated: 5, 10, and 15 milligrams per gram of poly(vinyl alcohol). Approximately 50% of the original activity was recovered in the Lentikats with *Me*HNL–CLEA contents of 5 and 10 mg g^{-1}, respectively (Table 1), but only a 22% in the Lentikats with *Me*HNL–CLEA content of 15 mg g^{-1}. In the last case, the *Me*HNL–CLEA–Lentikats obtained were spindle-shaped and tended to form aggregates. For that reason, the 5 and 10 mg g^{-1} *Me*HNL–CLEA–Lentikats were chosen for further experiments. Owing to the diffusion limitations of the biphasic system, the reaction time necessary to reach full conversion was 8 h. This was also the case if the same number of enzymatic units (15 U) and amount of substrate as in the experi-

ments with the *Me*HNL–CLEA were used. Clearly the extra immobilization layer hindered diffusion.

Recyclability of *Me*HNL–CLEA–Lentikats

As was previously mentioned, a tightly attached nonpurified *Pa*HNL in a poly(vinyl alcohol) hydrogel was prepared by Gröger et al.,[5a] and the enzyme could be recycled 20 times without loss of activity or enantioselectivity. However, the inability to see enzyme deactivation can be attributed to the high enzyme loading (11 grams of immobilized enzyme, 440 U) used in the recyclability study.

For the recyclability of the *Me*HNL–CLEA–Lentikats, our attention focused on the minimum enzyme loading required to achieve high yields and enantiomeric excesses, and at the same time, the amount of enzyme that allows monitoring the enzyme deactivation. Seven successive reactions of benzaldehyde (1 mM) and HCN (3 equiv.) were performed. The successive reactions were performed by allowing each reaction cycle to proceed for 8 h, after which the organic phase was replaced with a fresh solution of the reagents in MTBE, and the water phase containing the Lentikats was reused. The first three cycles reached almost full conversion (Figure 4) with $>98\%$ *ee*.

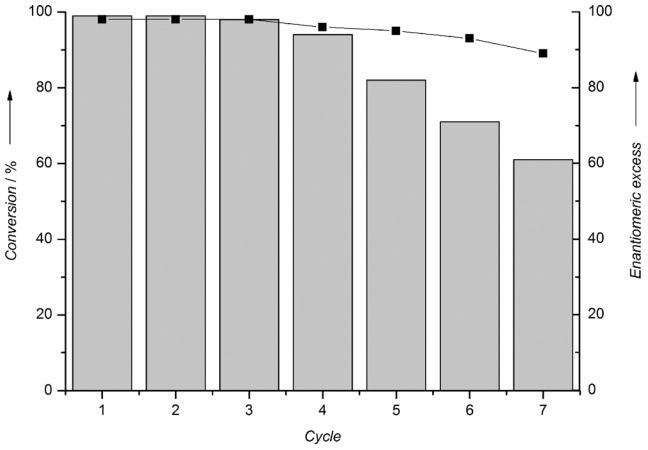


Figure 4. Recycling of the *Me*HNL–CLEA–Lentikats (15 U) in seven successive mandelonitrile synthesis reactions. Conditions: 50 mM citrate phosphate buffer (5 mL) pH 4.5, MTBE (3 mL), benzaldehyde (1 mmol), and 1.5 M HCN solution in MTBE (2 mL); reaction time 8 h.

In the fourth cycle, a slight decrease of the conversion and *ee* value was observed. As in the case of the *Me*HNL–CLEA, in the next cycles a decreasing reactivity of approximately 10% was observed successively, and, additionally, the *ee* dropped by approximately 2–4%. Clearly, if the conversion is low, the *ee* value drops, owing to the fact that the racemic-base-catalyzed reaction takes place in the water phase. Notably, similar results were obtained if the aqueous phase was replaced after each cycle.

Preparation of *Me*HNL–Celite (R-633)

Previous studies have demonstrated the moderate hydrophobicity of celite supports, which lead to a good water transport to the adsorbed enzymes and correspondingly excellent activities inside the microaqueous reaction systems.[12] To determine an appropriate enzyme/support ratio, several ratios from 1:50 to 1:4 were tested. To obtain a high conversion (99%) and *ee* value (99%) in 180 min, an enzyme/support ratio of 1:4 was used in buffer-saturated MTBE for the enantioselective addition of HCN to benzaldehyde. Prolonged reaction times (300 min) did not reduce the *ee* of the mandelonitrile (data not shown).

Generally, enzyme immobilization facilitates filtration and recycling of the biocatalyst. Therefore, a sufficiently strong binding of the biocatalyst to the support under the respective reaction conditions is required. To analyze the potential leaching of active *Me*HNL from the celite into the reaction medium, two identical samples of celite–*Me*HNL were prepared and packed into fine-woven nylon-mesh "tea bags". Such tea bags allow the simple removal of the biocatalyst from the reaction medium. Subsequently, the synthesis of (*S*)-mandelonitrile was monitored over 60 min in two parallel reactions; in one reaction setup the *Me*HNL-celite was removed from the reaction medium after 15 min. However, the conversion in both reactions was monitored over 60 min. As demonstrated in Figure 5, the continuous *Me*HNL-celite reaction displayed the expected course of conversion, whereas the aborted reaction stopped

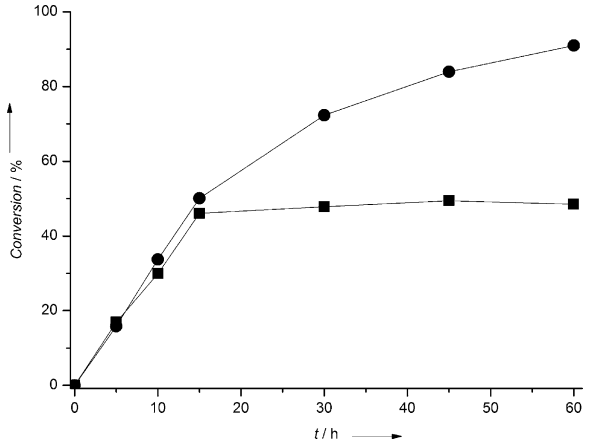


Figure 5. Test for leaching of *Me*HNL–celite into the reaction medium (buffer-saturated MTBE). Standard reaction (●) and reaction in which the immobilized *Me*HNL was removed from the reaction medium (■).

directly after removing the immobilized catalyst from the reaction medium. This observation demonstrates that no leaching of active catalyst occurs in the reaction system.

Recyclability of *Me*HNL–Celite (R-633)

The same setup using *Me*HNL–celite packed into a tea bag was applied to study the recyclability of the immobilized enzyme preparation.[13] These *Me*HNL tea bags were applied in fifteen consecutive hydrocyanations of benzaldehyde, including intermediate washing steps with pure MTBE. As demonstrated in Figure 6, *Me*HNL-celite displayed excellent recyclability. A moderate decrease in enzyme activity was observed after 12 cycles. Importantly, the stability of the enzyme was highly

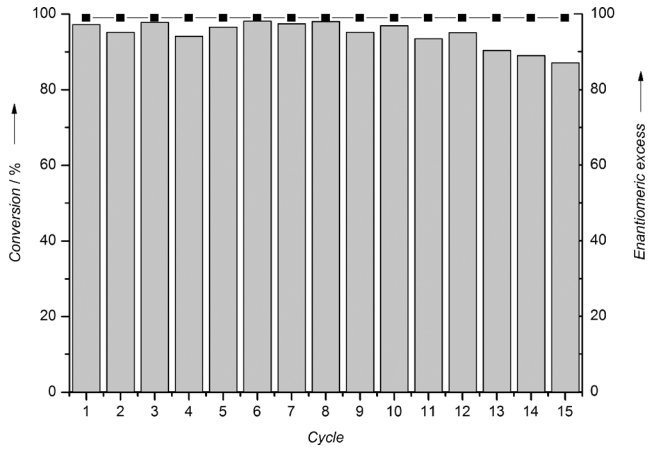


Figure 6. Recycling of the *Me*HNL–Celite (50 mg, 14 U) in fifteen successive mandelonitrile synthesis reactions. Conditions: benzaldehyde (1 mmol) in 1.5 M HCN solution in buffer-saturated MTBE (2 mL); reaction time 3 h.

dependent on the treatment between the cycles. The enzyme had to remain in the organic phase and drying needed to be avoided. Rapid deactivation was observed if the enzyme was allowed to dry, most likely owing to the phase change. However, the enzyme remained active for a long time if stored in organic solvent, in line with earlier observations.[4]

Conclusions

The use of hydroxynitrile lyase from cassava, *Manihot esculenta*, covalently immobilized as cross-linked enzyme aggregates (*Me*HNL–CLEA) for the hydrocyanation of benzaldehyde in monophasic buffer-saturated methyl *tert*-butyl ether is an efficient alternative to the hydrocyanation reaction in aqueous media at low pH. The biotransformation of benzaldehyde resulted in almost complete enzymatic conversion with excellent stereoselectivity. A further advantage of reaction systems with minimal water content is the efficient suppression of the non-catalyzed formation of racemic cyanohydrins and the better solubility of aromatic substrates and products. *Me*HNL–CLEA exhibits reasonable recyclability with low enzyme loading. Washing steps between batches proved to be unnecessary.

*Me*HNL encapsulated in poly(vinyl alcohol) hydrogels (Lentikats) was not stable without a previous cross-linking step. However, if the *Me*HNL–CLEA was encapsulated, good stability and storability were observed. Owing to diffusion limitations of the biphasic system (although the same enzymatic units, 15 U, and substrate amounts as in the experiments with the *Me*HNL–CLEA were used), longer reaction times were necessary. A decrease in the *ee* values was observed if the conversion was lower than 95%, as a result of the racemic-base-catalyzed reaction that takes place in water. The encapsulation of the *Me*HNL–CLEA in a poly(vinyl alcohol) hydrogel improved its stability slightly. However, diffusion limitations of both reaction systems differed significantly and prevented a direct comparison of the relative enzyme activities.

*Me*HNL–celite exhibited excellent recyclability and storability; together with the cheap, rapid, and simple preparation method, these features make the *Me*HNL–celite extremely attractive for biocatalytic applications, in contrast with the results found in the literature that mentioned a poor operational stability. It is important to perform the reactions in solvents with a minimum amount of water, because the entrapped water has a significant influence on the enzyme activity. Thus, almost as important as the immobilization technique used is the use of less aggressive washing protocols to keep this water, and therefore, the enzymatic activity.

Experimental Section

Caution: All procedures involving hydrogen cyanide were performed in a well-ventilated fume hood equipped with a HCN detector. HCN-containing wastes were neutralized by using commercial bleach and stored independently over a large excess of bleach for disposal.

Enzymes

*Me*HNL was supplied by Jülich Chemical Company (Codexis). The enzymatic activities of commercial *Me*HNL was measured according to reported literature procedures[14] and was found to be 453 U ml^{-1}. The commercial enzyme was purified to remove the salts to achieve a final enzyme in a well-known solution, by using the following procedure: 15 mL volumes of the enzyme solution were added up to an Amicon Ultra Filter Device and placed capped into a centrifuge rotor (3000 × *g* for several hours). The concentrated solutes were recovered with a pipette and few microliters of 0.1 M citrate phosphate buffer (pH 5.0) containing 0.15% of NaCl were added. The crude, brown/yellow-colored, enzyme suspensions (2.5 mL) were applied to the top of a PD-10 desalting column (Sephadex G-25 medium) and eluted with 10 mM potassium phosphate buffer (pH 6.0) and subsequently lyophilized. The enzyme activity after the purification step was found to be 207 U mg^{-1}. CLEAs of *Me*HNL (66 U g^{-1}) were supplied by CLEA Technologies.

Preparation of Lentikats

Poly(ethylene glycol) (PEG, 3 g) was dissolved in demineralized water (10 g). Then, poly(vinyl alcohol) (5 g) and demineralized water (30 g) was added. The mixture was heated and stirred during 20 min to reach the boiling point (98–98.5 °C). When the solution became transparent, it was cooled down to 35 °C. The enzyme solution [enzyme (75–225 mg) + 0.5 M sodium phosphate buffer (3 g), pH 6.5] was added to poly(vinyl alcohol) gel (12 g). A low-capacity laboratory device LentiPrinter was used for manufacturing of Lentikats biocatalyst. The poly(vinyl alcohol) and the enzyme mixture were poured into a Petri plate. Delivery system was first sunken into the poly(vinyl alcohol) mixture. Secondly, drops of the poly(vinyl alcohol) and biomass mixture were printed on a polyethylene drop plate. The drops of poly(vinyl alcohol) and biomass mixture were dried at 30 to 35 °C to reduce the initial weight of the biocatalyst by 25 to 30%. Collection and re-swelling of the biocatalyst was performed in 0.5 M phosphate buffer pH 6.5 within 20–40 min. After the production and collection, Lentikats biocatalysts were placed into a fresh 0.5 M phosphate buffer pH 6.5 (stabilized with 0.09% sodium azide) to be further stored in a refrigerator (4 °C) until the use.

Preparation of Celite-immobilized HNL

Lyophilized *Me*HNL was re-dissolved in 50 mM potassium phosphate buffer (pH 6.0). Enzyme solution with an appropriate enzyme concentration (enzyme/support ratio 1:4–1:100) was added to the Celite support (Celite R-633). The preparation was dried for at least 12 h under vacuum (20 mbar) in a desiccator over silica gel and molecular sieves. HNL–Celite was transferred into screw cap vials and stored at 4 °C.

Chemicals

(±)-Mandelonitrile (Sigma–Aldrich) was purified through column chromatography (PE/EtOAc 9:1/3:7) prior to use. Acetone cyanohydrin (Sigma–Aldrich) was distilled in the presence of 2% phosphoric acid prior to use and was stored under nitrogen at 4 °C. Benzaldehyde (Acros Organics) was always distilled prior to use and was stored under nitrogen at 4 °C. Isopropanol and heptane (HPLC grade) were purchased from Sigma–Aldrich. Petroleum ether and ethyl acetate (technical grade) were purchased from VWR International. Methyl *tert*-butyl ether (MTBE, Acros Organics, 99.9% extra pure) was used without further treatment unless otherwise specified. Aqueous buffers were prepared from analytical grade salts and stabilized with 0.09% sodium azide.

Hydrogen cyanide 1.5–2 M solution in MTBE: Sodium cyanide (4.9 g, 0.1 mol) was dissolved in a magnetically stirred mixture of water (10 mL) and MTBE (25 mL) at 0 °C. The biphasic system was stirred vigorously for 15 min and 30% aqueous HCl (10 mL) was added slowly. This mixture was allowed to warm slowly to RT (at least 25 min). The phases were separated and MTBE (7 mL) was added to the organic layer. The combined organic phases were stirred and residual water was separated. This procedure was repeated with another 7 mL volume of MTBE. The standard HCN solution was kept over citrate phosphate buffer (50 mM, pH 5.5) in the dark. Determination of HCN concentration was performed as described in the literature.[15]

Activity test for Lentikats biocatalyst

Activity test for Lentikats biocatalyst was conducted in 10 mL final reaction volume. The standard activity test procedure for HNLs[14] was modified to increase the final volume to 10 mL. Lentikats were added to a 50 mL three-neck flask containing 0.05 M citrate/phosphate buffer pH 5.0 (7 mL) and 0.005 M phosphate buffer pH 6.5 (1 mL). The reaction medium was stirred with a magnetic stirrer in a temperature-controlled silicone bath until the lentils separated (20 °C). Mandelonitrile solution (2 mL, 0.06 mol L^{-1}) in 0.003 M citrate phosphate buffer pH 3.5 was added to start the reaction. Samples were taken over 7 min and the absorbance was measured at 280 nm.

Synthesis of (*S*)-mandelonitrile by *Me*HNL–CLEA and *Me*HNL–Celite

*Me*HNL–CLEA (5–10 mg) or *Me*HNL–Celite (30–100 mg) were added to 1.5 M HCN solution in MTBE (2 mL, saturated with buffer, see above) containing benzaldehyde (1 mmol) and 1,3,5-triisopropylbenzene (0.01 mmol, as an internal standard) previously mixed under a nitrogen atmosphere. The reaction was monitored by chiral HPLC over 120–240 min while the reaction flask was stirred at RT.

Synthesis of (*S*)-mandelonitrile by *Me*HNL–Lentikats

Reactions were performed in a 25 mL two-neck flask with magnetic stirring. Lentikats were added to 50 mM citrate phosphate buffer (5 mL) pH 4.5. Then, a 3 mL volume of MTBE containing benzaldehyde (1 mmol) and 1,3,5-triisopropylbenzene (0.02 mmol, as an internal standard) was added and the reaction was started with 1.5 M HCN solution in MTBE (2 mL). Reaction was monitored by chiral HPLC over 120–1440 min while the reaction flask was stirred at RT.

Recycling studies

*Me*HNL–CLEA: Seven consecutive hydrocyanation reactions were performed over 240 min with 5 mg amounts of *Me*HNL–CLEA (activity of the immobilized HNL = 15 U), as described for the synthesis of (*S*)-mandelonitrile by *Me*HNL–CLEA. The reactions were stopped and the mixture was centrifuged 2 min at 13000 rpm. The solvent was removed and the *Me*HNL–CLEA pellet was resuspended with fresh MTBE containing the substrates. Chiral HPLC was used to monitor the reactions. Experiments were performed in duplicate.

*Me*HNL–CLEA-Lentikats: Lentikats (activity of the immobilized HNL = 15 U) were placed in a 25 mL two-neck flask. Seven consecutive hydrocyanation reactions were performed over 480 min with the *Me*HNL–CLEA-Lentikats, as described for synthesis of (*S*)-mandelonitrile by Lentikats-*Me*HNL. The organic phase was removed after each cycle and the aqueous phase containing the Lentikats was reused. Fresh MTBE containing the substrates was added to start the new cycle. Chiral HPLC was used to monitor the reactions. Experiments were performed in duplicate.

*Me*HNL-Celite: Celite containing *Me*HNL (activity of the immobilized HNL = 14 U) was sealed into an organic solvent-resistant, fine-woven nylon mesh "tea bag" (nylon net, pore size 0.4 mm) for easy removal from the reaction medium by filtration. Fifteen consecutive hydrocyanation reactions were performed over 180 min with the *Me*HNL–Celite tea bag, as described for the synthesis of (*S*)-mandelonitrile by *Me*HNL-Celite. The tea bags were washed between each reaction cycle with pure MTBE without buffer to remove remaining product and refresh the immobilizate. Care had to be taken to ensure that the enzyme preparation did not dry out. Chiral HPLC was used to monitor the reactions. Experiments were performed in duplicate.

HPLC method

Samples from the reaction mixtures were mixed with heptane/isopropanol (95:5), filtered, and dried with anhydrous $MgSO_4$. Volumes of 10 μL of the final clear solution were injected into an HPLC (Waters). Analyses were performed on a column (Daicel 4.6× 250 mm 5μ Chiralpak AD-H) coupled to a SpH 99 column thermostat (Chrompack), a 515 HPLC pump (Waters), an autosampler (717, Waters), and a UV/Vis detector SPD-10 A (Shimadzu). The column temperature was maintained at 40 °C. HPLC methodology: mobile phase: heptane/isopropanol = 95:5 (0.1% trifluoroacetic acid); flow rate: 1 mL min^{-1}; detection UV wavelength: 254 nm. Retention times: 5.0 min (benzaldehyde), 11.3 min ((*S*)-mandelonitrile), 12.6 min ((*R*)-mandelonitrile).

Investigation of potential leaching of active *Me*HNL from immobilizates

1) Two identical *Me*HNL–Celite samples, shrink-wrapped in a nylon-mesh tea bag, were prepared. Each sample was used for addition of HCN to benzaldehyde as described in the synthesis of mandelonitrile by *Me*HNL–Celite. In one reaction, the tea bag containing the immobilized *Me*HNL was inside the reaction medium for the whole reaction time, whereas it was removed from the reaction medium after 15 min in the parallel reaction. Both samples were monitored over 60 min by chiral HPLC. After removal of the tea bag, the reaction should be aborted if no active enzyme leaches into reaction medium. 2) *Me*HNL–CLEA–Lentikats (1 g) was stirred for 144 h under the same reaction conditions as described for synthesis of mandelonitrile by *Me*HNL–Lentikats but without the substrates. After removal of the organic phase, samples of the buffer phase were withdrawn and concentrated under vacuum to remove MTBE traces. Activity assays were performed according to reported literature procedures[14] by using the concentrated buffer samples. SDS gel electrophoresis was performed to detect enzyme presence and size (see "SDS polyacrylamide gel electrophoresis" below) and protein concentration was checked (see "Protein quantification" below).

SDS polyacrylamide gel electrophoresis

Sample volumes of 10 μL were mixed with SDS gel loading buffer (10 μL) and heated up to 95 °C for 10 min. After 10 min samples were allowed to cool to RT. 10 μL volumes of the samples and the standard, respectively, were applied in the gel sample pockets. A gel Criterion Precast XT Bis-Tris/Tris-Acetate (4–12%) (Biorad) was run at 180 V (constant V) for 45 min. 2-morpholinoethanesulfonic acid (Biorad) was used as the running buffer. After complete run, gel was stained (SimplyBlue SafeStain) for 1–2 h and then was destained using water. Precision Plus Protein Prestained Standards was used as protein size standard.

Protein quantification

A BC assay protein quantification kit (Uptima) was used to measure the protein concentration in buffer following the enhanced protocol (detection limits: 2–500 $\mu g\,mL^{-1}$).

Acknowledgements

G.T. thanks the Fundación Alfonso Martín Escudero (Spain) for a postdoctoral fellowship. The authors thank LentiKat's a.s. (Czech Republic) for hosting G.T. for immobilization experiments.

Keywords: aldehydes · cyanides · enzyme catalysis · gels · immobilization

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Received: October 21, 2013
Published online on January 21, 2014