DOI: 10.1002/adsc.201100199

Hydroxynitrile Lyase from *Arabidopsis thaliana*: Identification of Reaction Parameters for Enantiopure Cyanohydrin Synthesis by Pure and Immobilized Catalyst

Daniel Okrob,[a,b] Monica Paravidino,[b] Romano V. A. Orru,[c] Wolfgang Wiechert,[a] Ulf Hanefeld,[b,*] and Martina Pohl[a,*]

[a] Institute of Bio- and Geosciences, IBG-1: Biotechnology, Forschungszentrum Jülich GmbH, 52425 Jülich, Germany
Fax: (+49)-2461-613870; phone: (+49)-2461-614388; e-mail: ma.pohl@fz-juelich.de
[b] Laboratory of Biocatalysis and Organic Chemistry, Department of Biotechnology, Delft University of Technology, Julianalaan 136, 2628 BL Delft, The Netherlands
Fax: (+31)-15-278-1415; phone: (+31)-15-27-89304; e-mail: u.hanefeld@tudelft.nl
[c] Department of Chemistry and Pharmaceutical Sciences, Vrije Universiteit Amsterdam, De Boelelaan 1083, 1081 HV Amsterdam

Received: March 18, 2011; Revised: May 24, 2011; Published online: August 30, 2011

Abstract: The (*R*)-selective hydroxynitrile lyase from *Arabidopsis thaliana* (*At*HNL) is a promising biocatalyst for the synthesis of a broad range of chiral cyanohydrins. However, the enantiomeric excess of the reaction is strongly compromised by a non-catalyzed side reaction resulting in racemic cyanohydrins besides the chiral product obtained by enzymatic catalysis. This reaction is influenced by the pH value, the temperature and the water content of the reaction medium. In aqueous media this side reaction can be suppressed at low pH (4–5) and by lowering the temperature. However both approaches are not possible with *At*HNL, since the enzyme is rapidly inactivated below pH 5.4, which prevents its use in aqueous media or two-phasic aqueous-organic reaction systems. Alternatively the side reaction can be suppressed by lowering the water concentration in the reaction system as far as possible. This approach was successfully tested for *At*HNL using buffer-saturated methyl *tert*-butyl ether (MTBE) as a reaction medium for the hydrocyanation of aromatic, heteroaromatic and aliphatic aldehydes. Here we compare the activity and stability of *At*HNL immobilized on celite (celite-*At*HNL) and in solgel (solgel-*At*HNL) relative to the precipitated enzyme, which was directly used in the organic solvent. Surprisingly, *At*HNL was activated (up to 10-fold) upon solgel immobilization, an effect that was up to now only described for solgel-immobilized lipases. In contrast to lipases, *At*HNL is not stabilized by the solgel. Best results were obtained with *At*HNL adsorbed on celite, which is an easy and efficient way of immobilization and shows good recyclability (>5 cycles), storability ($\tau_{1/2}$ = 71 days, 4 °C) and excellent catalytic properties upon adjustment of a relative water content of 26% water per g celite-*At*HNL as determined by thermogravimetry.

Keywords: asymmetric carboligation; cyanohydrins; enzyme catalysis; hydroxynitrile lyase; immobilization; oxynitrilase

Introduction

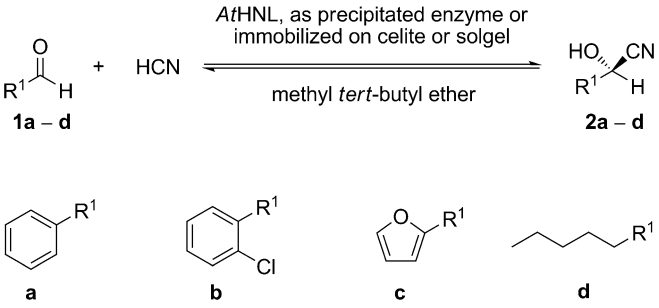
In organic chemistry the hydrocyanation of carbonyl compounds is an important carbon-carbon bond forming reaction. The chemical synthesis of enantio-enriched cyanohydrins as versatile intermediates for pharmaceuticals, fine chemicals and agrochemical products often requires harsh reaction conditions.[1] For more than 100 years the production of chiral cyanohydrins is performed also using biocatalysis.[2] Hydroxynitrile lyases (HNLs) were first identified in cyanogenic plants, where they catalyze the cleavage of HCN from cyanohydrins during herbivore attacks as part of the plant defence system. In biocatalysis the reverse reaction is of interest, where HNLs catalyze the stereoselective formation of chiral cyanohydrins from hydrogen cyanide (HCN) and aldehydes or ketones under mild reaction conditions. Meanwhile, several studies have successfully demonstrated the potential of HNLs as industrial biocatalysts for the synthesis of enantiopure cyanohydrins.[3]

Recently, a novel HNL from *Arabidopsis thaliana* (*At*HNL) was identified. *At*HNL is the first (*R*)-selective HNL with an α/β hydrolase fold and structurally

related to the (*S*)-selective HNLs from *Hevea brasiliensis* (*Hb*HNL) and *Manihot esculenta* (*Me*HNL).[4] The characterization of the broad substrate range and of key parameters, which influence the catalytic activity of *At*HNL, has recently been described.[5] Furthermore, the crystal structure of *At*HNL was solved (pdb-code 3dqz). Whereas *At*HNL and *Me*HNL are very similar concerning substrate range and kinetic parameters, their stability especially toward weak acidic aqueous solvents is quite different and *At*HNL is rapidly deactivated below pH 5.[5] However, the stability of HNLs toward acidic pH (≤ 4) is a prerequisite for their application in the formation of enantiopure cyanohydrins in aqueous media or aqueous-organic two-phase systems, since the non-catalyzed hydrocyanation, which results in racemic products, can efficiently be suppressed at low pH.[6] An alternative approach to suppress this side reaction is to minimize the water concentration in the reaction system.[6] As *At*HNL is irreversibly inactivated at pH <5.4 within a few minutes, the pH-control of this chemical background reaction is impossible in this case. An attractive alternative to aqueous reaction media and aqueous-organic two-phase systems is the application of HNLs in monophasic organic solvents with a minimum of water to ensure HNL activity. In such micro-aqueous reaction systems the aromatic substrates and products are highly soluble and the spontaneous formation of racemic cyanohydrins is strongly reduced due to the low water concentration in the reaction medium.[7] Nevertheless, the enzymatic activity and the enantiomeric excess (*ee*) of the reaction are affected by the pH of the aqueous buffer, the buffer concentration, the organic solvent, the enzyme stability and the enzyme concentration.[6,8]

In pure organic solvents HNLs can be applied directly as precipitated aggregates or immobilized on supports. Whereas precipitated *Hb*HNL was described to be significantly inactivated in organic solvents and at high substrate concentrations,[9] the enzyme's stability and performance benefit much from immobilization such as adsorption on mesoporous supports or entrapment in solgels. Furthermore, immobilized HNLs from *Manihot esculenta*, *Sorgum bicolor, Linum usitatissimum* and *Prunus amygdalus* have shown almost excellent substrate conversions and stereoselectivities, as well as improved stabilities compared to the precipitated enzyme in organic solvents.[8a,10] Furthermore, immobilization simplifies separation of product and recycling of the biocatalyst, which can easily be achieved by for example, filtration.

Immobilization experiments with lipases, which also belong to the α/β hydrolase-superfamily have demonstrated a stabilizing and activity increasing effect by adsorption on mesoporous celite or by solgel entrapment caused by protection of the lipase from aggregation or denaturing effects and a "hyperactivated" structural change.[11–13]



Scheme 1. *At*HNL-catalyzed hydrocyanation of model substrates (**1a–d**) in buffer-saturated mono-phasic methyl *tert*-butyl ether. Reactions were performed on a 1-mL scale using a solution of HCN (2 M) in MTBE with 0.5 mmol **1a–d**, respectively.

In order to enable the synthetic application of *At*HNL we have investigated the hydrocyanation of benzaldehyde, 2-chlorobenzaldehyde, 2-furaldehyde and hexanal in buffer saturated mono-phasic MTBE with the direct use of precipitated *At*HNL (Scheme 1). Furthermore, we have studied these test reactions with *At*HNL adsorbed on celite and entrapped in solgels; as well as factors affecting the immobilization and the influence of the support on the enzyme's activity, stability and recyclability. Additionally, the influence of the support on the non-catalyzed racemic hydrocyanation was investigated.

Results and Discussion

Activity of Immobilized *vs.* Precipitated *At*HNL

Initial studies on the suitability of different organic solvents for *At*HNL-catalyzed reactions yielded buffer-saturated methyl *tert*-butyl ether (MTBE) as a preferred micro-aqueous reaction system (data not shown). Using this reaction system the enantioselective addition of HCN to benzaldehyde **1a**, 2-chlorobenzaldehyde **1b**, 2-furaldehyde **1c**, and hexanal **1d** was comparatively studied yielding optically pure cyanohydrins **2a–d** (Scheme 1). Purified *At*HNL was used either dissolved in a small volume of buffer (5 mg in 50 μL) or immobilized, either adsorbed on celite R-633 particles (celite-*At*HNL) or entrapped in a methyltrimethoxysilane/tetramethoxysilane (MTMS/TMOS) solgel (solgel-*At*HNL).

First, purified *At*HNL (dissolved in phosphate buffer, 5 mg/50 μL) was added to the reaction medium containing HCN and **1a**. Although *At*HNL precipitated within 3–5 min after addition to the buffer-saturated MTBE, the reaction was almost complete after 45 min. (conversion 97.9%) and **2a** was ob-

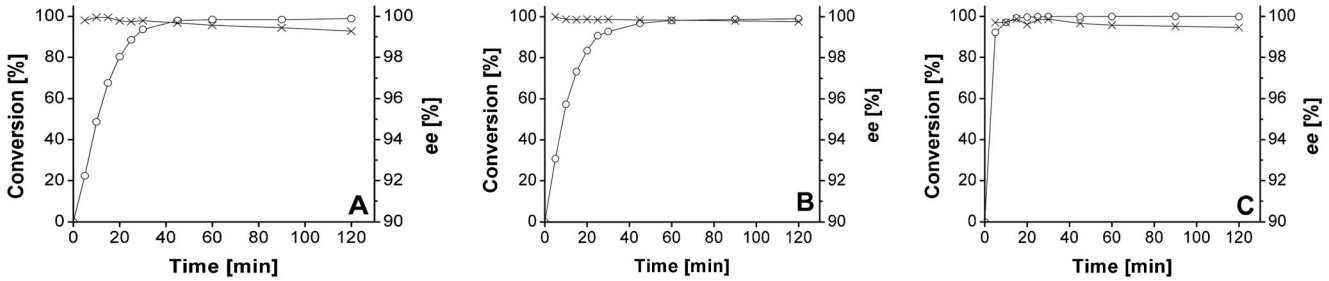


Figure 1. The conversion (○) of benzaldehyde (**1a**) to (*R*)-mandelonitrile (**2a**) by precipitated *At*HNL (**A**), celite-*At*HNL (**B**) and solgel-*At*HNL (**C**). 0.5 M **1a**, 1 mL 1.5–2 M HCN dissolved in MTBE. Conversion (○) and the enantiomeric excess (×) were determined by chiral GC (see Experimental Section).

tained with excellent enantiomeric excess (*ee*) of 99.7% [Figure 1 (**A**)]. Prolonged reaction times did not increase the yield further, but decreased the *ee* of **2a** to 99.3%. Although precipitated *At*HNL shows a very good activity and stereoselectivity in monophasic buffer-saturated MTBE, separation of the biocatalyst by filtration and recycling was almost impossible. Therefore, *At*HNL was immobilized on celite (celite-*At*HNL) and in a solgel matrix (solgel-*At*HNL), respectively.

Previous studies have demonstrated the moderate hydrophobicity of celite supports, which led to a good water transport to the adsorbed enzymes and correspondingly excellent activities inside the micro-aqueous reaction systems.[7b,14] To determine an appropriate enzyme/support ratio, several ratios starting from 1:50 to 1:4 were tested. To obtain a measureable reaction rate as demonstrated in Figure 1 (**A**) an enzyme/support ratio of 1:4 was used. In order to compensate a potential loss of activity due to the immobilization step, in reactions with celite-*At*HNL a 1.5-fold concentration of *At*HNL (compared to precipitated *At*HNL) was used. With this preparation a conversion of 96.8% of **1a** was reached in 45 min with an *ee* of 99.8% [Figure 1 (**B**)]. Prolonged reaction times (120 min) did not reduce the *ee* of the product **2a**. Thus, the application of celite-*At*HNL increased the enantiopurity of the product **2a** although the reaction rate was slightly reduced (78%) compared to the precipitated enzyme.

Solgel-*At*HNL containing an enzyme amount equal to the one applied with precipitated *At*HNL [Figure 1 (**A**)] catalyzed the addition of HCN to **1a** significantly faster compared to precipitated *At*HNL and celite-*At*HNL and conversion of **1a** was almost complete (97%) within 10 min [Figure 1 (**C**)]. Monitoring the reaction over 120 min gave 99.9% conversion of **1a** with an *ee* of 99.5% for **2a**. As with precipitated *At*HNL [Figure 1 (**A**)] a slight decrease of the *ee* of **2a** was observed after prolonged reaction times (99.7% after 10 min, 99.5% after 120 min) which might be due to a higher water concentration in this system compared to celite-*At*HNL [Figure 1 (**B**)].

Due to the fast reaction rates, initial rates (< 10% conversion) could not reliably be calculated. Thus, the relative enzyme activities of precipitated *At*HNL, celite-*At*HNL and solgel-*At*HNL were estimated at 50% conversion of **1a** and normalized to the enzyme concentration in the reaction system. As demonstrated in Table 1 (**2a**), celite-*At*HNL displays 78% residual activity compared to precipitated *At*HNL, whereas the solgel-*At*HNL preparation shows a clearly enhanced relative reaction velocity of 671%. Similar effects were also observed using **1b** or **1c** as carbonyl substrates. Whereas the conversion of 2-chlorobenzaldehyde (**1b**) to **2b** was more than 10-fold faster catalyzed by the solgel-*At*HNL compared to precipitated *At*HNL, this effect was much less pronounced (1.76-fold) with 2-furaldehyde (**1c**). While all aromatic products (**2a–c**) were obtained with very good stereoselectivity using all three preparations of *At*HNL, the enantioselectivity for **2d** was impaired with both immobilized *At*HNL-preparations. The reduced enzymatic activity of celite-*At*HNL with respect to the conversion of substrates **1a–c** might be caused by enzyme inactivation during immobilization, reduced enzyme flexibility and diffusion limitations inside the highly loaded celite particles, as was reported previously in similar studies.[8a,15] In contrast, the strongly

Table 1. Relative activity of precipitated *At*HNL, celite-*At*HNL and solgel-*At*HNL at 50% conversion of **1a–d** to their respective chiral cyanohydrins **2a–d**.

50% Enzymatic formation of	Relative *At*HNL activity in %[a] Precipitated *At*HNL (*ee*)	Celite-*At*HNL (*ee*)	Solgel-*At*HNL (*ee*)
2a (*R*)	100 (> 99)	78 (> 99)	671 (> 99)
2b (*R*)	100 (> 99)	86 (> 99)	1036 (> 99)
2c (*S*)	100 (> 99)	76 (> 98)	176 (> 99)
2d (*R*)	100 (> 95)	57 (> 86)	83 (> 58)

[a] *At*HNL concentration was adapted in all reactions. Standard reaction rate was defined as reaction rate of 5 mg precipitated *At*HNL by hydrocyanation of 0.5 mmol of **1a–d** to **2a–d**.

enhanced activity observed with solgel-*At*HNL seems not to be influenced by these factors. Similar enhanced enzyme activities upon solgel-entrapment were reported for HNLs from *Hevea brasiliensis*, *Manihot esculenta* and *Prunus amygdalus*[10a] and various lipases by Reetz et al.[12a] HNLs from the α/β hydrolase superfamily (*Me*HNL, *Hb*HNL) were most active and displayed an up to 8-fold enhanced conversion rate toward **1a** compared to the free enzyme in a biphasic reaction system.[10a] However, diffusion limitations of both reaction systems differ extremely and prevented a direct comparison of the relative enzyme activities.

Our results with solgel-*At*HNL demonstrate similar enhanced activities under identical reaction conditions as reported for lipases, which underlines the suggested protection of the enzyme from aggregation, but contradicts the "lid-opened" theory caused by the "alkyl-effect" in solgels.[12] An alternative explanation for the activation of *At*HNL could be that the entrapped buffer inside the solgel matrix supports a native and flexible form of the enzyme, which results in a highly active biocatalyst. Nevertheless, the local water concentration in the solgel is low enough to prevent the non-catalyzed side reaction at least in the case of the aromatic cyanohydrins **2a–c** (Table 1).

However, the concentration of aqueous buffer inside the solgel is most probably the reason why the hydrocyanation of hexanal (**1d**) yielded **2d** with an *ee* of only 58% (Table 1). As *At*HNL prefers aromatic aldehydes, aliphatic aldehydes are only slowly converted.[4a] As a consequence, the velocity of the non-catalyzed reaction becomes competitive to biocatalysis and the resulting stereoselectivity of the product is significantly lower. Precipitated *At*HNL displays the highest enzymatic activity and thus the lowest non-enzymatic background reaction (95% *ee*) for the hydrocyanation of **1d** to **2d**. In contrast, the reaction rate with celite-*At*HNL was clearly reduced to 57% relative activity and yielded only 86% *ee* for **2d**. As the solgel is assumed to contain by far the highest water content among the tested reaction systems, the very low stereoselectivity for **2d** obtained with solgel-*At*HNL can most probably be explained therewith. In order to investigate the impact of the carrier, the non-catalyzed reaction using substrate **1d** was monitored in the presence of celite and solgel without *At*HNL under identical reaction conditions. With pure celite 18% racemic **2d** were formed within 120 min, whereas pure solgel converted 50% of **1d** to racemic **2d** in the same time. These results clearly demonstrate a significant impact of the water concentration in the carrier on the hydrocyantion of aldehydes, which is most pronounced if the enzymatic reaction is slow. In conclusion, immobilization of *At*HNL on celite and in solgel resulted in highly active enzyme preparations. In order to further evaluate the potential of both methods for the application in organic syntheses the recyclability and the stability of the immobilized enzyme preparations were studied.

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 leakage of active *At*HNL from the carrier material into the reaction medium two identical samples of celite-*At*HNL and solgel-*At*HNL were prepared and packed into fine woven nylon mesh "tea-bags", respectively. Such "tea-bags" allow the simple removal of the biocatalyst from the reaction medium. Subsequently, the synthesis of **2b** was followed over 60 min in four parallel reactions; in one reaction set-up the celite-*At*HNL or solgel-*At*HNL, respectively, was removed from the reaction medium after 8 min. But the conversion in all four reactions was monitored over 60 min. As demonstrated in Figure 2 (**A**)and (**B**), the continuous celite-

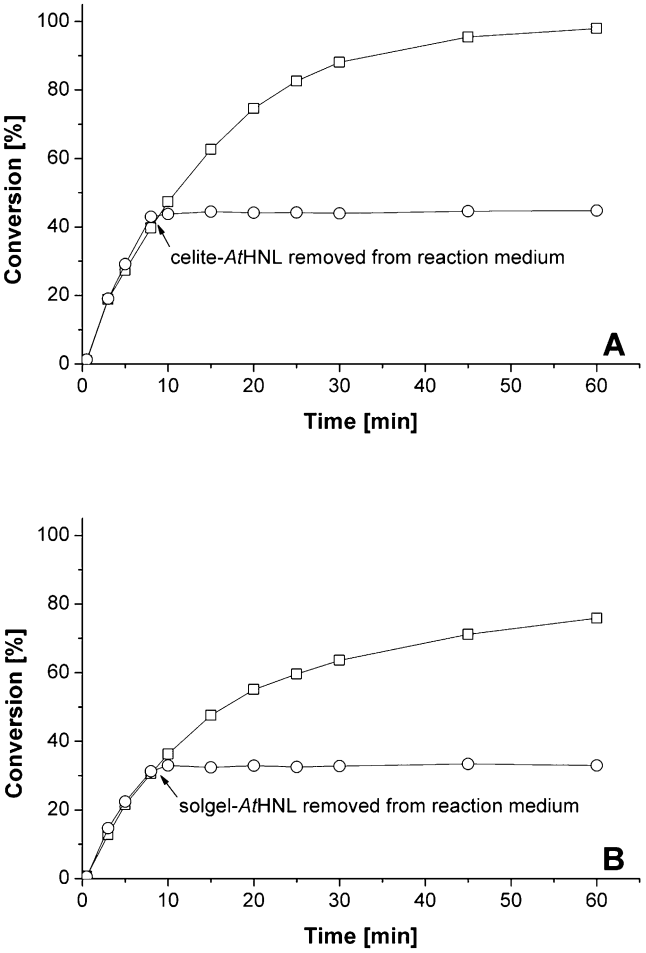


Figure 2. Test for leakage of active *At*HNL from the celite (**A**) and solgel (**B**) support into the reaction medium (buffer saturated MTBE). Standard reaction (□) and reaction where the immobilized *At*HNL were removed from the reaction medium (○).

*At*HNL and solgel-*At*HNL reaction displayed the expected course of conversion, whereas the aborted reactions of both, celite-*At*HNL and solgel-*At*HNL, stopped directly after removing the immobilized catalyst from the reaction medium. This observation demonstrates that no leakage of active catalyst occurs in either reaction systems.

Recyclability of Immobilized *At*HNL

The same set-up using celite-*At*HNL and solgel-*At*HNL packed into "tea-bags" was applied to study the recyclability of the immobilized enzyme preparations. These *At*HNL "tea-bags" were applied in five consecutive hydrocyanations of **1a**, including intermediate washing steps with pure MTBE. As demonstrated in Figure 3 (**A**), celite-*At*HNL displayed excellent recyclability. A moderate decrease in enzyme activity was observed, but in all reaction cycles almost complete conversion (>95%) was reached with a constant *ee*>98% for **2a**. Similar recycling studies with solgel-*At*HNL demonstrated complete conversion of **1a** to **2b** only in the first cycle, whereas a rapid loss of activity was observed in the next reaction cycles. Nevertheless, the stereoselectivity of cycles 1–3 was very good, >98% *ee* [Figure 3 (**B**)].

However, the stereoselectivity was progressively reduced with every further recycling step. Efforts to reduce the inactivation rate by washing solgel-*At*HNL with phosphate buffer, pH 6.5, instead of MTBE between each cycle resulted in improved reaction rates only for the 4th and 5th cycles. However, this was accompanied by a significant drop of *ee* (cycles 4 and 5: 82% *ee*), probably caused by the incremental deactivation of *At*HNL, and an increased water concentration in the reaction medium [Figure 3 (**C**)] due to the washing steps with phosphate buffer.

To verify this hypothesis, two identical solgel-*At*HNL samples were prepared in "tea-bags" and preincubated in MTBE for 60 and 120 min, respectively. This preincubation time is equivalent to 1 or 2 reaction cycles as described in the recycling studies. Addition of HCN to **1a** was performed after preincubation and 80% relative activity was observed for the 60 min preincubated sample, whereas only 52% residual activity was found for the 120 min preincubated sample. In conclusion, the loss of activity is similar to the 2nd and 3rd reaction cycles of Figure 3 (**B**/**C**) and is related to the enzyme inactivation caused by MTBE, which permeates into the solgel over time. Thus, *At*HNL is not stable in solgels, which is in contrast to related studies with lipases.

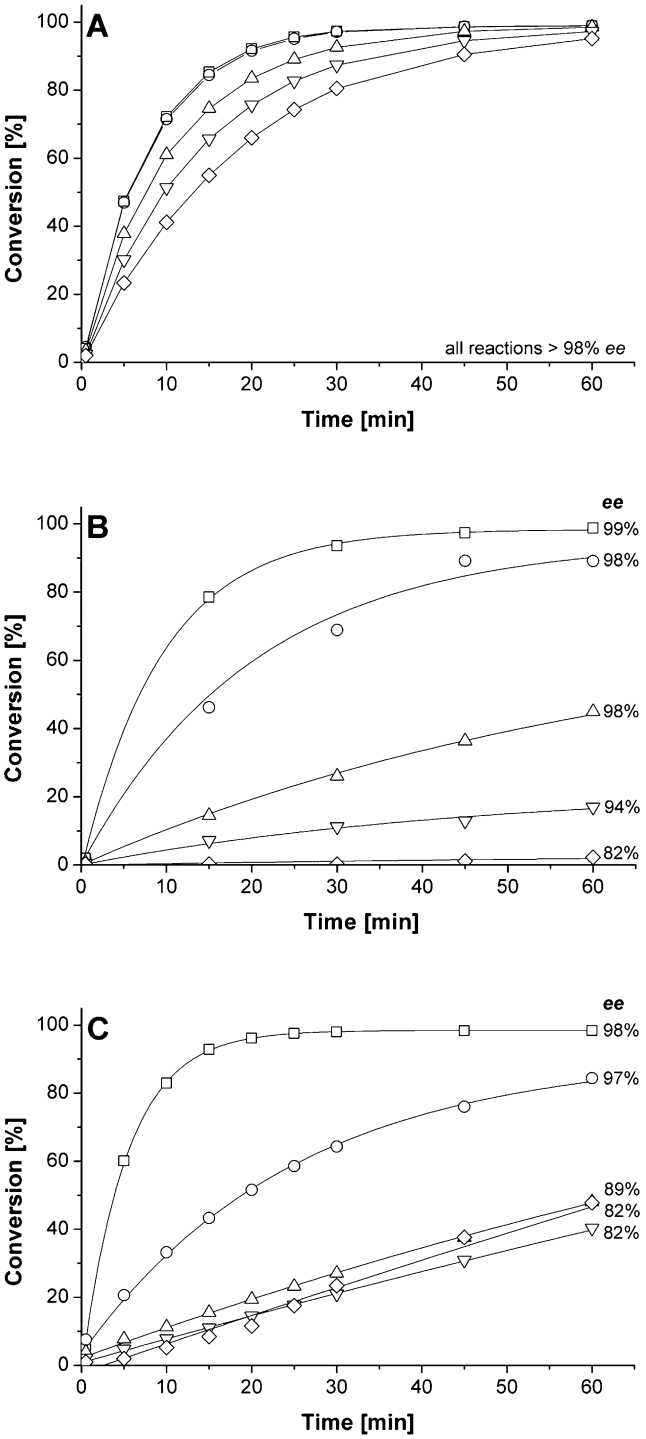


Figure 3. Recycling of celite-*At*HNL and solgel-*At*HNL using benzaldehyde and HCN as substrates. In (**A**) celite-*At*HNL was washed with pure MTBE after each cycle. In (**B**) solgel-*At*HNL was washed with MTBE and in (**C**) solgel-*At*HNL was washed with 50 mM phosphate buffer pH 6.5. Cycle 1 (□), cycle 2 (○), cycle 3 (△), cycle 4 (▽), cycle 5 (◇).

Storability of Immobilized *At*HNL

In order to characterize the storability of the immobilized enzyme, adequate amounts of celite-*At*HNL and solgel-*At*HNL were prepared and transferred into a screw cap glass vial and stored at 4 °C. Over a period of 31 days equal amounts of immobilized *At*HNL were withdrawn and residual activity was assayed by following the synthesis of **2a** under identical reaction conditions. The relative activity was determined by comparing the substrate conversion of **1a** to **2a** for a defined reaction time. For celite-*At*HNL an unexpected doubling of enzyme activity was observed over the first 7 days, which decreased slightly again in the following 14 days. Extrapolation of this slow inactivation process led to a theoretical half-life of 71 days [Figure 4 (**A**)]. The initial rise in activity observed in the first days might be related to the water concentration inside the reaction medium. As the experimental set-up was based on only one storage sample of celite-*At*HNL, the frequent opening and closing of the screw cap vial during sampling might have progressively increased the water content in the celite-*At*HNL preparation, which in turn might have enhanced the enzyme activity. After about 14 days celite-*At*HNL particles were probably saturated by air moisture and enzyme activity reached a plateau.

In order to test this hypothesis, activities of celite-*At*HNL prepared as described before [dried over silica gel; 3% (w/w) water] and of four celite-*At*HNL preparations containing increasing weight percentages of water [5–26% (w/w)] were compared [Figure 4 (**B**)]. Thereby, the sample with the lowest water content [also used for the storage test, Figure 4 (**A**)] showed the lowest relative activity in the hydrocyanation of **1a**, compared to the samples with higher water concentrations. Interestingly, the observed increase of activity with increased water concentration did not lead to a decrease of enantioselectivity. This indicates a limitation of enzyme activity by a lack of water inside the reaction medium. Celite-*At*HNL samples containing 10% or 26% water displayed the highest activities, whereas a water content of 26% (w/w) led to an only 3% higher activity compared to the sample containing 10% (w/w) water. This indicates that 10% (w/w) water inside celite-*At*HNL is close to the minimal water amount, which is necessary for optimal enzymatic activity.

In contrast to celite-*At*HNL, solgel-*At*HNL displayed a clearly visible loss of activity starting from the first day of incubation at 4 °C with a half-life of

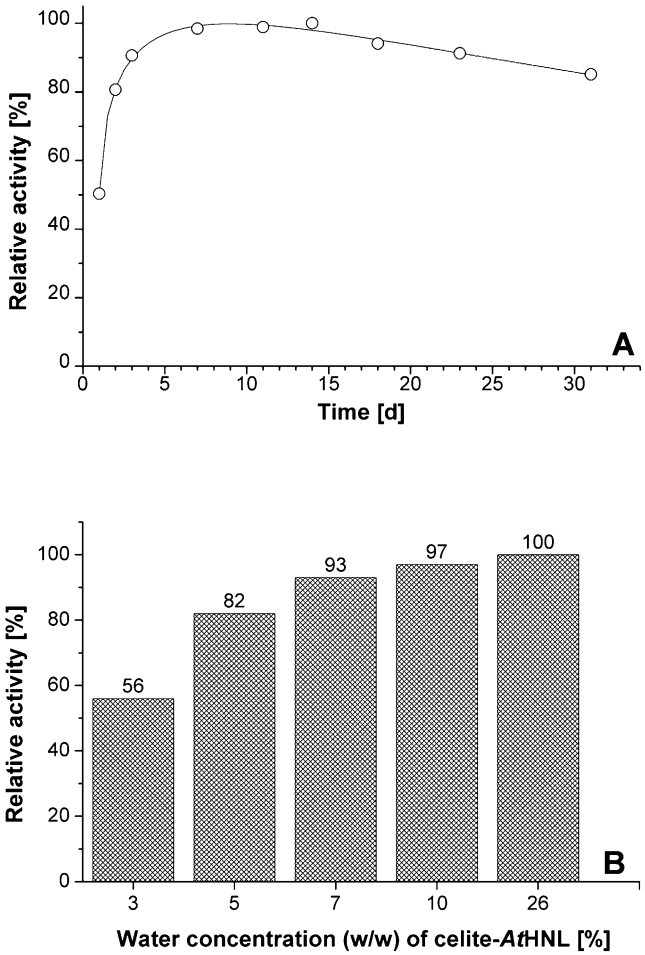


Figure 4. (**A**) Storability of dry celite-*At*HNL at 4 °C. Comparison of conversion rates of the hydrocyanation of **1a** using different samples of the same batch over 31 days. (**B**) Influence of weight percentage (w/w) of water on enzyme activity of celite-*At*HNL. Preparations of celite-*At*HNL were incubated over silica gel [3% (w/w) water] and over different saturated salt solutions [5–26% (w/w) water] for 16 h prior to activity measurements. Activity was assayed by following the hydrocyanation of **1a.** Relative enzyme activities were calculated by comparing substrate conversion at a defined point of time (all reactions yielded > 98% *ee*). The final water content was determined using TGA.

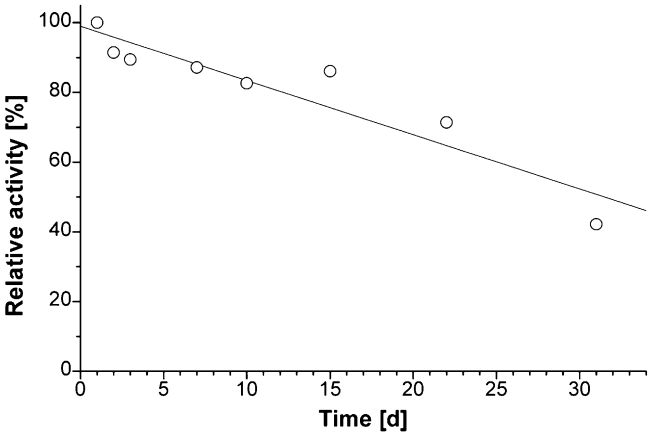


Figure 5. Storability of solgel-*At*HNL at 4 °C. Residual activity (velocity of hydrocyanation of **1a)** was assayed from samples taken at the indicated time points.

about 29 days (Figure 5). This rapid loss of activity seems to be due to the progressive inactivation of *At*HNL inside the moist environment and is probably further accelerated by the methanol remaining from the gelling process of the solgel.[10b]

Conclusions

The use of precipitated *At*HNL for the hydrocyanation of aromatic, heteroaromatic and aliphatic aldehydes in monophasic buffer-saturated MTBE is an efficient alternative to the hydrocyanation reaction in aqueous media at low pH. Using this approach *At*HNL, which is not stable even under weakly acidic conditions ($<$ pH 5), can efficiently be used. The biotransformations of the tested substrates 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. To improve the recyclability of *At*HNL, the enzyme was entrapped in a solgel matrix and immobilized on celite.

Solgel-entrapped *At*HNL displayed an up to 10-fold increase in activity with respect to the conversion of aromatic aldehydes; this is most probably due to the aqueous environment of the enzyme in the solgel, which results in a higher flexibility and turnover frequency. However, we observed a rapid loss of activity, probably caused by permeation of MTBE into the solgel, which led to reduced recyclability. Furthermore, the laborious preparation and poor storability make solgel-entrapped *At*HNL less attractive.

With celite-adsorbed *At*HNL a significant influence of the water content in the celite-*At*HNL preparation on the enzyme activity was observed. With optimal humidity, the enzymatic activity was only slightly reduced relative to precipitated *At*HNL (76–86%) for the aromatic substrates (Table 1). Furthermore, celite-*At*HNL shows excellent recyclability and storability; together with the cheap, rapid and simple preparation method, these features make the celite-*At*HNL extremely attractive for biocatalytic applications. The advantage of both non-covalent immobilization methods is the high preserved activity since no side chains essential for activity are negatively influenced. However, non-covalent immobilization methods are less stable than multipoint covalent support attachment of the enzyme. Therefore, further studies concerning covalent immobilization as well as stabilization by enzyme engineering are currently performed.

Experimental Section

CAUTION: Sodium cyanide and HCN are highly poisonous. Procedures involving sodium cyanide or HCN were performed in a well-ventilated lab-hood equipped with a calibrated HCN detector. Neutralization of HCN-containing wastes was performed with commercial bleach (14% sodium hypochlorite solution). The wastes were then stored over a large excess of bleach for disposal. For safety reasons reactions were performed in closed reaction vessels on a 1-mL scale by using a solution of HCN (2 M) in MTBE with 0.5 mmol **1a–d**, respectively.

Chemicals

Celite (0.2–0.5 mm) was purchased from World Minerals Inc. (Santa Barbara, USA). Methyltrimethoxysilane (MTMS, 98%), tetramethoxysilane (TMOS, 98%), benzaldehyde (99%), furaldehyde (99%), hexanal (98%), 2-chlorobenzaldehyde (99%), dodecane (anhydrous, $\geq$ 99%), pyridine (99%), magnesium nitrate hexahydrate and sodium cyanide (97%) were purchased from Aldrich (St. Louis, USA). Acetic anhydride (p.a.), lithium chloride and magnesium chloride hexahydrate were purchased from Fluka (St. Louis, USA). Methyl *tert*-butyl ether (MTBE, p.a.), potassium sulfate and sodium chloride were purchased from Merck (Darmstadt, Germany). Dichloromethane was purchased from KMF (St. Augustin, Germany). Purified mandelonitrile was purchased from Jülich Fine Chemicals (Jülich, Germany) and stored at -20 °C. Benzaldehyde, 2-chlorobenzaldehyde, 2-furaldehyde and hexanal were freshly distilled and stored under argon atmospheres.

Analytical Methods

Synthesis reactions were followed by chiral gas chromatography on a Shimadzu gas chromatograph GC-14B or on an Agilent Technologies gas chromatograph 6890N, both equipped with an FID detector and a beta-cyclodextrin column (CP-Chirasil-Dex CB 25 m $\times$ 0.25 mm). Identical GC methods as described in the literature were used.[10b] Samples (30 μL aliquots) of the reaction were taken at defined time points, dissolved in 1.7 mL dichloromethane and derivatized by addition of 40 μL pyridine and 40 μL acetic anhydride for at least 3 h. Conversion rate and *ee* were calculated from the relative peak areas of the aldehyde and the cyanohydrin derivative.

Preparation of 2 M HCN 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 room temperature (at least 25 min). The phases were separated and 7 mL MTBE were added to the organic layer. The combined organic phases were stirred and residual water was separated. This procedure was repeated with another 7 mL of MTBE. The 2 M 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 literature.[16]

Cultivation and Purification of *At*HNL

BL21(DE3) p*At*HNL[5] were cultivated using a standard fed-batch fermentation protocol.[17] From a 15 L cultivation 1.75 kg cells were harvested, containing a total activity of 2 GU (mandelonitrile cleavage assay). 15 g BL21-(DE3)_p*At*HNL cells were slowly suspended in 50 mM potassium phosphate buffer (pH 7.5) at 0 °C and disrupted by sonication [4×5 min at 70W/cm^2 on ice with an ultrasonic processor UP200S and a sonotrode S14D (Dr. Hielscher GmbH)]. After ultracentrifugation (35,000×*g*, 4 °C, 45 min), the resulting crude extract (*ca.* 50 mL) was desalted by gel-filtration on Sephadex G-25 [1 L bed volume, 10 mM potassium phosphate buffer (pH 7.5)]. Subsequently, anion exchange chromatography on a Q-Sepharose column (90 mL bed volume) was performed, which was equilibrated with 50 mM potassium phosphate buffer (pH 7.5). After elution of non-bound proteins, *At*HNL containing fractions were eluted with a linear NaCl gradient at 150 mM. Combined fractions with HNL activity were desalted using a Sephadex G-25 column [1 L batch volume, 10 mM potassium phosphate buffer (pH 7.5)] and subsequently lyophilized. Protein determination was performed according to Bradford (1976).[18] Purified *At*HNL (90% purity) exhibits a specific cleavage activity of 70–90 U/mg toward mandelonitrile (pH 5.8) according the activity assay (see below).

Standard Preparation of Celite-Immobilized *At*HNL

Lyophilized *At*HNL was re-dissolved in 10 mM potassium phosphate buffer (pH 6.0) and protein determination was performed (Bradford). Enzyme solution with an appropriate enzyme concentration (enzyme/support ratio 1:4–1:50) was added to the celite support (celite R-633). The preparation was dried for at least 12 h under vacuum (20 mbar) in a desicator over silica gel and molecular sieves. The standard celite-*At*HNL preparation contained 0.25 mg *At*HNL/mg celite. Celite-*At*HNL transferred into dense screw cap vials and stored at 4 °C.

Preparation of Solgel-Entrapped *At*HNL

For solgel entrapment MTMS/TMOS was chosen. A precursor solution was prepared under mild conditions, where alkoxysilanes were almost completely hydrolyzed by acid-mediated hydrolysis and the released methanol was removed by evaporation.[10b]

Solgel precursor preparation: Acidic water (690 μL, pH adjusted to 2.85 by addition of HCl) was added to a mixture of MTMS (1.05 g, 7.7 mmol), TMOS (4.54 g, 28.25 mmol) and distilled water (5.2 mL) and stirred in a 50-mL round bottom flask until a homogenous solution was obtained. Formed methanol was removed using a rotary evaporator (20–30 mbar, 40 °C) until the characteristic odours of MTMS, TMOS and methanol were no longer detectable. The solution was cooled to 0 °C and used immediately for the entrapment of *At*HNL.

Entrapment of AtHNL: Lyophilized *At*HNL was dissolved in 50 mM potassium phosphate buffer (pH 6.35) and protein concentration was adjusted to 50 mg *At*HNL mL^{-1}. The same volume of cooled solgel precursor solution and *At*HNL solution were mixed by magnetic stirring until the mixture gelled completely. The prepared solgel-*At*HNL was submerged in 3 equiv. 50 mM potassium phosphate buffer (pH 6.0) and aged for at least 16 h at 4 °C. Afterwards, the solgel-*At*HNL was filtered, washed with distilled water, ground into fine powder and stored in a dense closable screw cap vial at 4 °C.

*At*HNL Activity Assay (Mandelonitrile Cleavage)

The increase of the benzaldehyde concentration was measured continuously at 280 nm in quartz glass cuvettes. 700 μL citrate phosphate buffer (50 mM, pH 5.8) was mixed with 100 μL enzyme in potassium phosphate buffer (10 mM, pH 6.0) at 25 °C. The reaction was started by addition of 200 μL mandelonitrile solution (67 mM mandelonitrile in citrate phosphate buffer, pH 3.5) and monitored for 1 min. Subsequently, the activity was calculated using the molar extinction coefficient of benzaldehyde (ε_{280nm} = 1376 L mmol^{-1} cm^{-1}). One unit of HNL activity is defined as the amount of enzyme, which converts 1 μmol mandelonitrile per minute in citrate phosphate buffer, pH 5.8, 25 °C. All measurements were performed at least as triplicates; References with all components except *At*HNL were always determined twice.

Synthesis of Cyanohydrins by Precipitated *At*HNL

5 mg *At*HNL were dissolved in 50 μL potassium phosphate buffer (50 mM, pH 6.35). Under an argon atmosphere 1 mL 1.5–2 M HCN solution in MTBE, 0.5 mmol aldehyde, 0.1 mmol dodecane (internal standard) were mixed thoroughly by magnetic stirring. A sample for the point of origin (30 μL) was taken. Synthesis was started by addition of 50 μL *At*HNL in potassium phosphate buffer (pH 6.5). Reaction was monitored by chiral GC over 60–120 min while the reaction flask was stirred at room temperature.

Synthesis of Cyanohydrins by Celite-*At*HNL or Solgel-*At*HNL

Celite or solgel containing 5–7.5 mg *At*HNL, respectively, was transferred into the closed reaction vessel and aerated with argon. 1 mL HCN (1.5–2 M) solution in MTBE saturated with citrate phosphate buffer (pH 5.5) (see preparation of 2 M HCN solution in MTBE) and 0.1 mmol dodecane (internal standard) were added and mixed by strong magnetic stirring. The reaction was started by addition of 0.5 mmol aldehyde under continuous stirring. Aliquots were taken at defined time points and the continuous reaction was monitored by chiral GC over 60–120 min while the reaction mixture was stirred at room temperature.

Comparison of Relative Activity at 50% Substrate Conversion for Precipitated *At*HNL and Immobilized *At*HNL

Hydrocyanation of **1a–d** was catalyzed by precipitated *At*HNL, celite-*At*HNL and solgel-*At*HNL, respectively, and monitored by chiral GC. The *At*HNL concentration in each experiment was adjusted such that ≥95% substrate conversion was observed within 90 min. The time when 50% conversion was reached by 5 mg precipitated *At*HNL was set as 100% activity. Relative activities of immobilized *At*HNL were calculated relative to the activity with precipitated

*At*HNL and considering the enzyme concentration in the respective immobilisate.

Example: When the same amount of immobilized enzyme achieved 50% substrate conversion within 1/5 of time, relative to the precipitated enzyme, its relative activity is 500%.

Recycling of Celite-*At*HNL or Solgel-*At*HNL

Celite or solgel containing 3–5 mg *At*HNL was sealed into an organic solvent-resistant, fine-woven nylon mesh "tea-bag" [nylon net, pore size 0.4 μm; sealing unit: Polystar 100 GE-GS (Rische + Herfurth, Germany)] for easy removal from the reaction medium by filtration. Five consecutive hydrocyanation reactions (substrate: **1a**) were performed over 60 min with the celite-*At*HNL "tea-bag" and the solgel-*At*HNL "tea-bag", respectively, as described for immobilized *At*HNL. The "tea-bags" were washed between each reaction cycle with pure MTBE or potassium phosphate buffer (50 mM, pH 6.0) to remove remaining product and refresh the immobilisate. Chiral GC was used to monitor the reactions.

Investigation of Potential Leakage of Active *At*HNL from Immobilisates

Two identical celite-*At*HNL or solgel-*At*HNL samples, shrink-warped in a nylon mesh "tea-bag", were prepared. Each sample was used for addition of HCN to benzaldehyde **1a** as described in the synthesis protocol for immobilized *At*HNL. In one reaction the "tea-bag" containing the immobilized *At*HNL was inside the reaction medium for the whole reaction time while it was removed from the reaction medium after 8 min in the parallel reaction. Both samples were monitored over 60 min by chiral GC. After removal of the "tea-bag" the reaction should be aborted, when no active enzyme leaks into reaction medium.

Investigation of Storability of Celite-*At*HNL and Solgel-*At*HNL

One batch containing an adequate amount of celite- or solgel-immobilized *At*HNL was prepared as described in the standard protocols and transferred into a dense closable screw cap vial. Over a period of 31 days the vial was stored at 4 °C. From time to time equal amounts of immobilized *At*HNL were taken from the batch and residual activity was assayed by measuring the hydrocyanation of **1a** under identical reaction conditions. All reactions were monitored by chiral GC. Relative activity was calculated by comparing the substrate conversion at a defined reaction time (celite-*At*HNL: 25 min; solgel-*At*HNL: 5 min).

Investigation of the Influence of Water Concentration (w/w) on the Activity of Celite-*At*HNL

One adequate batch of celite-*At*HNL was prepared as described above. The batch was split into 5 aliquots (A–E). Sample A was directly transferred into a dense closable screw cap vial and stored at 4 °C. Samples B–E were transferred into four desiccators and incubated over a saturated salt solution of $Mg(NO_3)_2$ $a_w = 0.54$, $MgCl_2$ $a_w = 0.33$, NaCl $a_w = 0.75$ or K_2SO_4 $a_w = 0.95$ to adjust a specific humidity (water activity a_w) in each desiccator for at least 16 h (room temperature), before they were transferred into a dense closable screw cap vial and stored at 4 °C. The activity was finally assayed by following the hydrocyanation of **1a** under identical reaction conditions. All reactions were monitored by chiral GC. Relative activities were calculated by comparing the substrate conversion at a defined reaction time (30 min). Water bound to celite-*At*HNL was measured by thermogravimetry using a Perkin–Elmer TGA7 thermogravimetric analyzer. The measurements were performed under nitrogen atmosphere in the range of 25–625 °C at a heating rate of 10 °C min^{-1}. The initial sample mass was always in the range 4–12 mg.

Acknowledgements

D.O. thanks Roger A. Sheldon for establishing contact with the TU Delft – Biocatalysis & Organic Chemistry Group. This work was financial supported by the Deutsche Forschungsgemeinschaft in frame of the research training group "BioNoCo" GK 1166 and NWO/ACTS (project no. 053.63.304). M.P. thanks Dr. Menno Sorgedrager for fruitful discussion and the IBOS program of NWO/ACTS (project no. 053.63.304) for financial support.

References

[1] a) M. Breuer, K. Ditrich, T. Habicher, B. Hauer, M. Kesseler, R. Stürmer, T. Zelinski, *Angew. Chem.* **2004**, *116*, 806–843; *Angew. Chem. Int. Ed.* **2004**, *43*, 788–824; b) R. Gregory, *Chem. Rev.* **1999**, *99*, 3649–3682; c) J. Wang, W. Wang, W. Li, X. Hu, K. Shen, C. Tan, X. Liu, X. Feng, *Chem. Eur. J.* **2009**, *15*, 11642–11659; d) M. North, D. Usanov, C. Young, *Chem. Rev.* **2008**, *108*, 5146–5226.

[2] a) J. Holt, U. Hanefeld, *Curr. Org. Synth.* **2009**, *6*, 15–37; b) L. Rosenthaler, *Biochem. Z.* **1908**, *14*, 238–253.

[3] a) J. N. Andexer, J. V. Langermann, U. Kragl, M. Pohl, *Trends Biotechnol.* **2009**, *27*, 599–607; b) R. Hatti-Kaul, U. Törnvall, L. Gustafsson, P. Börjesson, *Trends Biotechnol.* **2007**, *25*, 119–124; c) D. Pollard, J. Woodley, *Trends Biotechnol.* **2007**, *25*, 66–73.

[4] a) J. Andexer, J. von Langermann, A. Mell, M. Bocola, U. Kragl, T. Eggert, M. Pohl, *Angew. Chem.* **2007**, *119*, 8833–8835; *Angew. Chem. Int. Ed.* **2007**, *46*, 8679–8681; b) P. Carr, D. Ollis, *Protein Pept. Lett.* **2009**, *16*, 1137–1148.

[5] J. Guterl, J. Andexer, T. Sehl, J. von Langermann, I. Frindi-Wosch, T. Rosenkranz, J. Fitter, K. Gruber, U. Kragl, T. Eggert, *J. Biotechnol.* **2009**, *141*, 166–173.

[6] J. von Langermann, J. Guterl, M. Pohl, H. Wajant, U. Kragl, *Bioprocess Biosyst. Eng.* **2008**, *31*, 155–161.

[7] a) G. Lin, S. Han, Z. Li, *Tetrahedron* **1999**, *55*, 3531–3540; b) E. Wehtje, P. Adlercreutz, B. Mattiasson, *Biotechnol. Bioeng.* **1990**, *36*, 39–46.

[8] a) D. Costes, E. Wehtje, P. Adlercreutz, *Enzyme Microb. Technol.* **1999**, *25*, 384–391; b) M. Paravidino, M. Sorgedrager, R. Orru, U. Hanefeld, *Chem. Eur. J.* **2010**, *16*, 7596–7604.

[9] D. Costes, G. Rotcenkovs, E. Wehtje, P. Adlercreutz, *Biocatal. Biotransform.* **2001**, *19*, 119–130.

[10] a) F. L. Cabirol, U. Hanefeld, R. A. Sheldon, *Adv. Synth. Catal.* **2006**, *348*, 1645–1654; b) L. Veum, U. Hanefeld, A. Pierre, *Tetrahedron* **2004**, *60*, 10419–10425; c) F. L. Cabirol, P. L. Tan, B. Tay, S. Cheng, U. Hanefeld, R. A. Sheldon, *Adv. Synth. Catal.* **2008**, *350*, 2329–2338.
[11] a) M. Hartmann, D. Jung, *J. Mater. Chem.* **2010**, *20*, 844–857; b) U. Hanefeld, L. Gardossi, E. Magner, *Chem. Soc. Rev.* **2009**, *38*, 453–468.
[12] a) M. T. Reetz, *Adv. Mater.* **1997**, *9*, 943–954; b) M. Reetz, P. Tielmann, W. Wiesenhöfer, W. Könen, A. Zonta, *Adv. Synth. Catal.* **2003**, *345*, 717–728.
[13] a) R. Fernandez-Lafuente, P. Armisen, P. Sabuquillo, G. Fernandez-Lorente, J. M. Guisan, *Chem. Phys. Lipids* **1998**, *93*, 185–197; b) C. Mateo, J. M. Palomo, G. Fernandez-Lorente, J. M. Guisan, R. Fernandez-Lafuente, *Enzyme Microb. Technol.* **2007**, *40*, 1451–1463.
[14] E. Wehtje, P. Adlercreutz, B. Mattiasson, *Biotechnol. Bioeng.* **1993**, *41*, 171–178.
[15] R. Barros, E. Wehtje, P. Adlercreutz, *Biotechnol. Bioeng.* **1998**, *59*, 364–373.
[16] L. M. van Langen, F. van Rantwijk, R. A. Sheldon, *Org. Process Res. Dev.* **2003**, *7*, 828–831.
[17] D. J. Korz, U. Rinas, K. Hellmuth, E. A. Sanders, W. D. Deckwer, *J. Biotechnol.* **1995**, *39*, 59–65.
[18] M. M. Bradford, *Anal. Biochem.* **1976**, *72*, 248–254.

