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Linum usitatissimum Hydroxynitrile Lyase Cross-Linked Enzyme Aggregates: A Recyclable Enantioselective Catalyst

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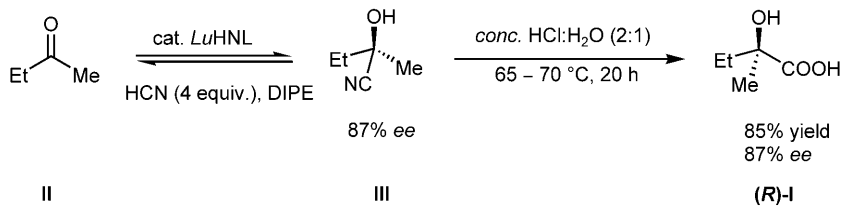
Abstract: An immobilized form of the hydroxynitrile lyase from *Linum usitatissimum* (*Lu*HNL) as cross-linked enzyme aggregate (CLEA) with high specific activity (303.5 U/g) and recovery (33%) was developed. Molecular imprinting using 2-butanone as additive in the immobilization process improved the synthetic activity of the biocatalyst. *Lu*CLEA could be partially recycled for the synthesis of (*R*)-2-butanone cyanohydrin on a preparative scale over two batches. The enantioenriched cyanohydrin obtained was further hydrolyzed to give (*R*)-2-hydroxy-2-methylbutyric acid in 85% yield (from 2-butanone) and 87% *ee*.

Keywords: biocatalysis; cross-linked enzyme aggregate (CLEA); cyanohydrins; hydroxynitrile lyase; oxynitrilase; quaternary stereogenic carbon

Introduction

The enantioselective synthesis of quaternary stereogenic carbon atoms is a major challenge in organic chemistry. In particular, chiral tertiary alcohols are at the centre of attention. Due to their importance in the pharmaceutical industry and the drive towards environmentally benign synthesis,[1,2] catalytic routes are the focus of current research.[3] 2-Hydroxy-2-methylbutyric acid (**I**) is such a building block. It is, for two reasons, a particularly interesting synthetic challenge. (1) Its *S*-enantiomer is used for the preparation of a COX-2 specific inhibitor,[4] while its *R*-enantiomer forms part of several biologically active natural products, such as the clerodendrins,[5,6,7] protoveratrine A[8,9] and germinalinine.[10] (2) The synthesis of **I** *via* the enantioselective addition of cyanide to the prochiral butanone and subsequent hydrolysis involves a daunting stereodifferentiation between a methyl and an ethyl group (Scheme 1). While chemical catalysis has so far proven rather unsuccessful for this challenge,[11,12,13,14] enantioselective biocatalysis using hydroxynitrile lyases (HNL) is the way forward to prepare both the *R*- and *S*-enantiomers of **I**.[11,12,15]

Recently, an HNL-based pathway to *S*-**I** was described in which butanone was "disguised", that is, an auxiliary was introduced in order to ease stereodifferentiation.[16] This elegant approach has the drawback that its atom-economy is poor,[1,2] comparable to the chemical chiral auxiliary route.[17] For the *R*-enan-



Scheme 1. Synthetic approach towards (*R*)-enantioenriched 2-hydroxy-2-methylbutyric acid (*R*)-**I**.

tiomer of **I**, *Prunus amygdalus*, *Prunus mume*, and *Linum usitatissimum* HNL have been employed. However, *Prunus amygdalus* and *Prunus mume* enzymes displayed moderate enantioselectivity[18,19] while the *Linum usitatissimum* HNL (*Lu*HNL) was reported to give good to very good enantiopurities of the cyanohydrin intermediate.[20,21] We recently described that immobilizing the HNLs from *Prunus amygdalus*, *Hevea brasiliensis* and *Manihot esculenta* as cross-linked enzyme aggregates (CLEAs) can improve their stability and ease their recycling.[22,23,24] In particular, their application in organic solvents became possible. All these parameters are essential for the repeated and sustainable application of enzymes in a green process.[1,2,25,26,27] Here we describe the preparation of a CLEA from *Lu*HNL (*Lu*CLEA) and its application in the synthesis of *R*-**I**.

Results and Discussion

Development of *Lu*CLEA

Enzyme Precipitation

The selection of precipitation parameters (nature and amount of cosolvent, duration, and temperature) is a critical step in the preparation of active CLEAs with a high recovery of enzyme activity.[24,28] A rapid evaluation of *Lu*HNL sensitivity towards temperature and length of exposure to a cosolvent prompted us to select conditions that allow fast precipitation at 0 °C (data not shown). A screening procedure could then be developed to determine the best cosolvents from a range of alcohols and saturated aqueous salt solutions in various amounts (Figure 1).

Ideally, the cosolvent should provide 100% activity recovery (no deactivation) in the form of an aggregate (no enzyme left in solution). One particular solvent, *tert*-butanol, in amounts greater than twice (v/v) the volume of commercial *Lu*HNL solution gave very satisfying results in this regard. Saturated aqueous ammonium sulfate [sat. $(NH_4)_2SO_4$] also provided a fair amount of precipitate with little deactivation and soluble enzyme left when it was used in amounts greater than twice the initial volume of enzyme solution. The other alcohols screened here did not provide efficient precipitation since a large amount of enzyme was left in the solution. Brine had a severe deleterious effect on the enzyme activity. We continued this screening of cosolvents with a range of water-miscible organic solvents and PEG (Figure 2). Glyme and diglyme stood out as relatively good candidates for precipitation in this screening. The extent of deactivation was particularly significant with *N*,*N*-dialkylamide-type solvents such as dimethylformamide (DMF) and dimethylacetamide (DMAc) while PEG, DMSO and acetonitrile left a significant amount of active enzyme in solution.

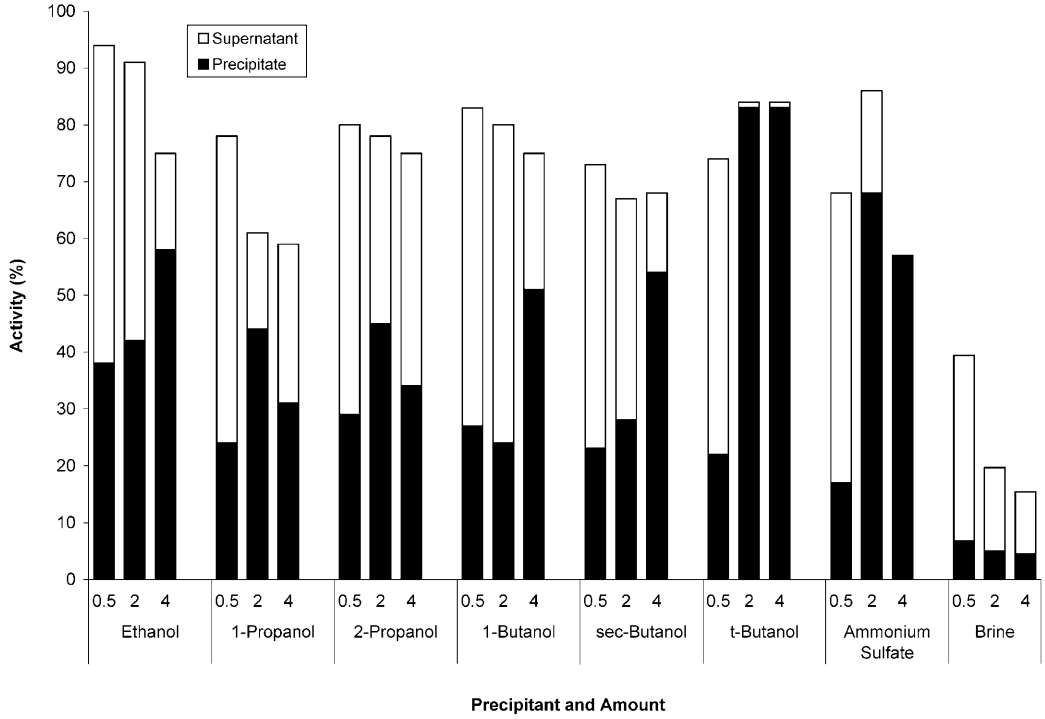


Figure 1. Activity recovery (% of commercial enzyme solution used) in the precipitate (black) and supernatant (white) after enzyme precipitation using alcohols and saturated saline solutions [the amount of precipitant is expressed as a factor (in volumes) of the commercial enzyme solution used].

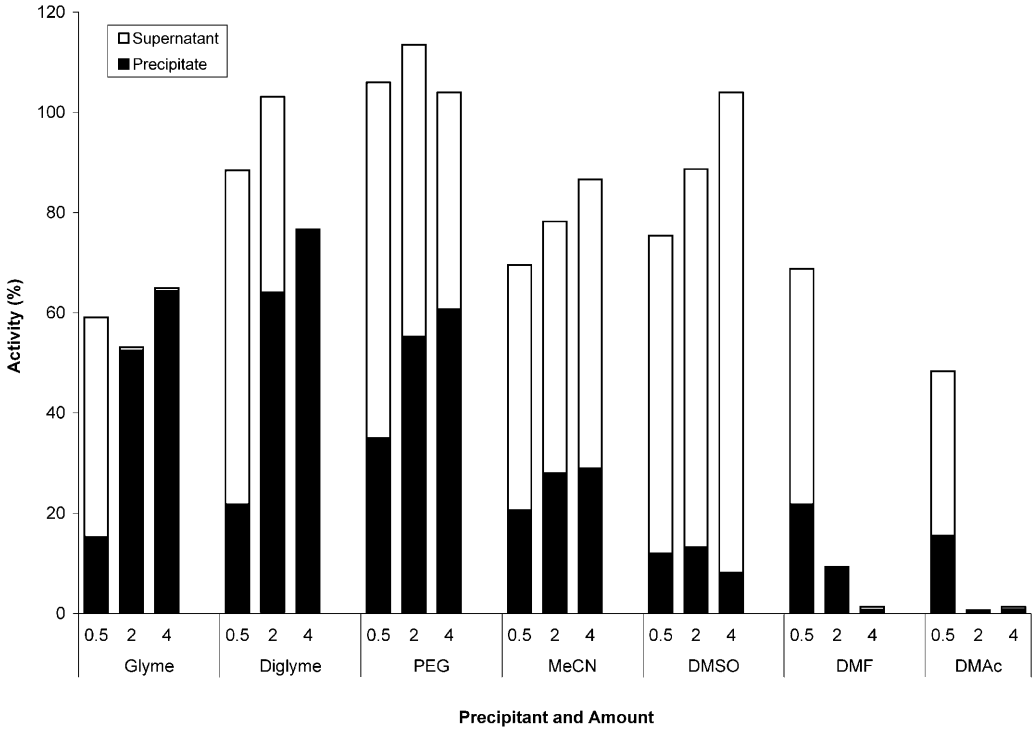


Figure 2. Activity recovery (%) in the aggregate and the supernatant after enzyme precipitation using organic solvents and PEG [the amount of precipitant is expressed as a factor of the volume of commercial enzyme solution used].

Interestingly the influence of precipitants on the activity of *Lu*HNL was consistent with earlier investigations on suitable water-miscible cosolvents in the *Lu*HNL-catalyzed conversion of 2-butanone into its corresponding cyanohydrin.[20] Based on the results obtained in the precipitation study, four cosolvents [*tert*-butanol, sat. $(NH_4)_2SO_4$, glyme and diglyme] were selected for the cross-linking study.

Cross-Linking of Aggregates

Although precipitation aims at shaping the physical state of the aggregates while maintaining the enzyme activity,[29] the cross-linking step is no less important to the successful preparation of an active CLEA. Efficient cross-linking will indeed "lock" the enzyme into its active state and prevent redissolution (leaching) during reaction.[26] Moreover, cross-linking determines the particle size and contributes to making the biocatalyst more robust toward deleterious effects such as substrate/product inhibition or organic solvent deactivation during the reaction.[23] Traditionally, glutaraldehyde is preferred as a cross-linking agent as it is commercially available and inexpensive. We investigated the activity recovery in the CLEA after cross-linking using 5%, 10%, 20% and 30% (v/v of the commercial enzyme solution used) of glutaraldehyde for each of the four cosolvents selected in the precipitation study. The amount of each precipitant used was also screened in order to fine tune this parameter when combined with the cross-linking step.

When glyme was used as a precipitant, no satisfactory results could be obtained and this cosolvent was not considered further. The optimized conditions using the other three cosolvents (Table 1) also indicated that diglyme was the least preferable and we did not consider this precipitant further.

The CLEAs prepared using optimized amounts of *tert*-butanol and sat. $(NH_4)_2SO_4$ as precipitants and glutaraldehyde as cross-linker (Table 1) were selected for further study. We also noticed that although

Table 1. Cross-linking study: optimized results for each precipitant.

Co-solvent[a]	Cross-linker[b]	Activity Recovery[c]
tert-Butanol (2)	10	19.5 (0)
Sat. $(NH_4)_2SO_4$ (4)	10	53 (8)
Diglyme (3)	5	17 (3)

[a] Precipitant and amount (parentheses) as a factor of the volume of commercial enzyme solution used.
[b] Amount of glutaraldehyde 25% aqueous solutions as a percentage (v/v) of the volume of commercial enzyme solution used.
[c] Activity recovery (%) in the CLEA based on the initial amount of enzyme used (in units). The number in parentheses indicates the percentage of activity present in the buffer used to wash the CLEA (enzyme not immobilized).

*Lu*HNL shares several structural homologies with the Zn^{2+}-containing alcohol dehydrogenases (ADHs)[21,30] efficient cross-linking could be achieved using glutaraldehyde while dextran polyaldehyde had to be used to prepare CLEAs of the ADH from *Lactobacillus brevis* since glutaraldehyde deactivated the enzyme.[31] This observation further highlights the need to optimize the conditions for CLEA preparation independently of results obtained for similar enzymes.

Enzyme Aggregates (EAs) and other HNLs

The results obtained so far in the study seem to indicate that the relatively low activity recovery in the CLEA is mostly due to the cross-linking step. For instance, when *tert*-butanol was used as precipitant the recovered activity was greater than 80% after precipitation and below 20% after cross-linking. Since we aimed at developing an immobilized version of *Lu*HNL that can perform in buffer-saturated organic solvent as we reported earlier for *Me*CLEA,[23] a simple aggregate of *Lu*HNL (without cross-linking) could also be considered as an immobilized catalyst as it would be insoluble in the reaction media. Enzyme aggregates of *Lu*HNL (*Lu*EA) were therefore prepared using *tert*-butanol and sat. $(NH_4)_2SO_4$ in a scale-up (factor 10) of the optimized conditions for precipitation. The preparation of CLEAs from these two precipitants was also scaled-up accordingly and a significant drop in activity recovery was observed (Table 2) when compared to the results obtained on a smaller scale as described in the cross-linking study.

Table 2. Specific activity and activity recovery of CLEAs and EAs.

Biocatalyst	Precipitant	X-linking	Activity[a]	Recovery[b]
*Lu*CLEA (Am.Sulf)	Sat. $(NH_4)_2SO_4$	Yes	180.5 U/g	20%
*Lu*EA (Am.Sulf)	Sat. $(NH_4)_2SO_4$	No	9.9 U/g	5%
*Lu*CLEA (*t*-Bu)	*tert*-Butanol	Yes	110.9 U/g	6%
*Lu*EA (*t*-Bu)	*tert*-Butanol	No	221.9 U/g	9%
*Pa*CLEA[c]	Glyme	Yes	Not active	*nd*[d]
*Me*CLEA[c]	Sat. $(NH_4)_2SO_4$	Yes	847.1 U/g	36%

[a] Specific activity per g of solid measured according to the standard procedure for *Lu*HNL.
[b] Activity recovery (%) in the catalyst based on the initial amount of enzyme used (in units).
[c] Prepared according to literature procedure.[22,24]
[d] *nd*: not determined.

The specific activity and activity recovery for CLEAs of *Pa*HNL and *Me*HNL were also measured for comparative purposes (Table 2).

Better recovery was obtained when the CLEA was prepared from sat. $(NH_4)_2SO_4$ than from *tert*-butanol as a cosolvent as observed on a smaller scale. *Lu*EA(Am.Sulf) was poorly active which was attributed to a large excess of salt $[(NH_4)_2SO_4]$ in the catalyst. The EA was indeed washed with acetonitrile since an aqueous buffer would redissolve the enzyme aggregate. As a result some of the salt precipitated upon addition of MeCN resulting in low specific activity (<10 U/g) and overall recovery (5%). To the contrary, the specific activity of *Lu*EA(*t*-Bu) was twice as high as for *Lu*CLEA(*t*-Bu) and the activity recovery was also improved. *Pa*CLEA was not active in the conditions of the standard activity test which was attributed to the relatively low pH (4.1). On the other hand, the very high specific activity and activity recovery measured for *Me*CLEA are consistent with an earlier report on the robustness and high activity of this biocatalyst.[23]

Molecular Imprinting

The use of surfactants and crown ethers as additives in the preparation of CLEAs has been reported[28] and very significant improvements of the biocatalyst activity could be observed in some instances. These additives were selected in order to stabilize the enzyme leading to a more robust biocatalyst and to modify the enzyme conformation into a more active state that could be "locked" by immobilization. Our approach to additives for the development of immobilized *Lu*HNL was closer to the concept of molecular imprinting[32,33,34] where the enzyme could potentially be immobilized in its enzyme-substrate complex conformation. A cyanohydrin as substrate for this purpose would be problematic since its decomposition into the corresponding carbonyl compound and HCN would be difficult to prevent without complete inhibition of the enzyme. However, in the absence of HCN, a carbonyl compound that can bind to the active site of *Lu*HNL in solution and be washed away easily after immobilization would be suitable. Moreover, the additive should be water-soluble, preferably inexpensive and a low boiling point would be an advantage in order to remove traces under vacuum. Considering these requirements, we selected 2-butanone as an additive and studied its influence on the preparation of CLEAs and EAs (Table 3).

We used a standard 1 μL of 2-butanone per unit of *Lu*HNL as a reference and observed significant differences in the specific activities of *Lu*EA(*t*-Bu) and *Lu*CLEA(*t*-Bu). A drop in activity was indeed observed for the EA whereas the CLEA specific activity

Table 3. Influence of 2-butanone as additive on the immobilization process.

Biocatalyst	Activity difference[a]
*Lu*CLEA(Am.Sulf)	+8%
*Lu*EA(Am.Sulf)	*nd*[b]
*Lu*CLEA(*t*-Bu)	+44%
*Lu*EA(*t*-Bu)	−68%

[a] Results are presented as the difference in specific activity between the biocatalyst prepared with and without 2-butanone as additive (see experimental section).

[b] *nd*: not determined (catalyst not active when 2-butanone was used as additive).

increased very significantly. Since the EA redissolves in the aqueous buffer of the standard activity test thereby restoring the free enzyme conformation (non-complexed) one would not expect a significant difference in activity. In contrast, a CLEA retains the structural features imposed during immobilization even in an aqueous buffer. Hence, we conclude that the enzyme-substrate complex was indeed formed but the benefits of the additive could only be retained in the biocatalyst after "locking" the structure by cross-linking. The additive had little effect on the activity of *Lu*CLEA(Am.Sulf) which suggests that *tert*-butanol also plays a role in coordinating to the active site during immobilization but the enzyme conformation obtained with *tert*-butanol can be modified further to the enzyme-2-butanone complex allowing immobilization in an even more active form. The deleterious effect of the additive was observed on *Lu*EA-(Am.Sulf) that was rendered totally inactive.

These investigations on the parameters that influence immobilization of *Lu*HNL in the form of an EA or CLEA led us to select five immobilized forms for synthetic applications: *Lu*CLEA(Am.Sulf) (no additive), *Lu*CLEA(*t*-Bu) (with and without additive), and *Lu*EA(*t*-Bu) (with and without additive). *Me*CLEA was also selected as a standard for a robust and active biocatalyst.

Synthetic Application

Catalyst Selection

The stereodifferentiation in 2-butanone is a challenge, in particular since the racemic non-catalyzed addition of HCN to the ketone strongly depends on the reaction conditions. In an uncatalyzed control experiment using a biphasic system (DIPE:buffer pH 4.1) the conversion of 2-butanone into its racemic cyanohydrin was indeed 16% in 2 h. When the control experiment was performed in buffer-saturated DIPE the conversion of 2-butanone was still 9% after 48 h. We therefore decided to screen the immobilized forms of *Lu*HNL for the synthesis of 2-butanone cyanohydrin in buffer-saturated DIPE as suggested in Scheme 1. The conversion of 2-butanone (**II**) and the *ee* of the cyanohydrin formed (**III**) were monitored to evaluate the synthetic activity and selectivity of the catalyst, respectively (Figure 3).

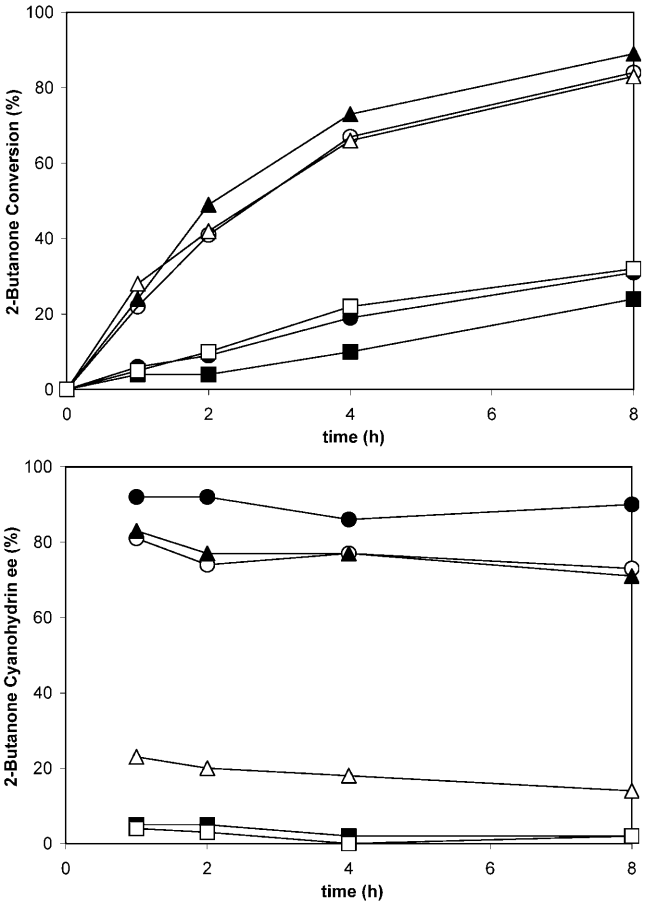


Figure 3. *Lu*HNL- and *Me*CLEA-catalyzed conversions of 2-butanone (**A**) and *ee* of the formed cyanohydrin (**B**). *Reaction conditions*: 2-butanone (0.50 mmol), HCN (4 equiv.), biocatalyst (2.2 U), room temperature, in buffer-saturated DIPE (1.00 mL). ●: *Lu*CLEA(Am.Sulf), ○: *Lu*CLEA(*t*-Bu), ▲: *Lu*CLEA(*t*-Bu) prepared using 2-butanone as additive, △: *Me*CLEA, ■: *Lu*EA(*t*-Bu), □: *Lu*EA(*t*-Bu) prepared using 2-butanone as additive.

The catalysts could be clearly divided into two categories based on their synthetic activity (Figure 3, **A**). *Lu*CLEA(*t*-Bu) (with and without additive) performed exceptionally well under the above selected conditions, matching the 2-butanone conversion profile obtained with *Me*CLEA. *Lu*CLEA(*t*-Bu) prepared in the presence of 2-butanone performed even better than the standard *Me*CLEA (Figure 3, **A**). *Lu*CLEA(Am.Sulf) and the EAs performed poorly under these conditions, the EA prepared with 2-buta-

none being slightly more active. We investigated the effect of organic solvents on *Lu*CLEA(Am.Sulf) by washing the freeze-dried catalyst with acetonitrile, ethyl acetate and then diethyl ether and only 31% of the activity could be recovered. The specific activity of *Lu*CLEA(Am.Sulf) had also decreased greatly to 39.8 U/g after washing. These results indicated a severe deleterious effect of the media for this catalyst.

Next to the excellent activity, *Lu*CLEA(*t*-Bu) (with and without additive) also showed good selectivities (Figure 3, **B**). 2-butanone as additive did not seem to affect the *ee* profile. This indicates that the imprinting strategy only affected the catalyst activity but not its selectivity. Difficult stereodifferentiation for this substrate was reflected in the low selectivity observed for *Me*CLEA (*ee* around 20% under the reaction conditions) which was consistent with earlier reports for this enzyme-substrate combination.[35]

This evaluation of the synthetic activity highlighted the importance of selecting an immobilized biocatalyst not only from the results in the standard activity test but from its performance under actual reaction conditions. The standard activity test proved to be a fast and efficient method to narrow the range of suitable conditions for the development of immobilized *Lu*HNL but only the evaluation of the synthetic activity allowed us to select *Lu*CLEA(*t*-Bu) prepared using 2-butanone as additive (referred to as *Lu*CLEA from here on) as the best candidate for synthetic application in buffer saturated organic solvent.

SEM analysis of *Lu*CLEA (Figure 4) revealed relatively large particles of *ca.* 100 μm (Figure 4, **A**). The internal structure of *Lu*CLEA is organized as a network of "branches" (Figure 4, **B**) separated by pores of about 10 μm. Enlargement of these "branches" showed aggregates in the range of 500 nm (Figure 4, **C**). The amalgams of these aggregates created a second level of pores of about 1 μm.

This arrangement is typical of CLEAs of HNLs such as the highly active *Me*HNL.[24] The structure maximizes the catalyst surface available for reaction (amalgams of small aggregates separated by pores) while minimizing the diffusion effect within the catalyst (branch-like sub-structure).

Catalyst Recycling

We investigated the recycling ability of *Lu*CLEA on a suitable scale to minimize catalyst loss during filtration between cycles. The catalyst loading was increased to 8 U/mmol and the temperature set at 30 °C. The reaction was monitored over 6 h under these conditions for four consecutive cycles (Figure 5).

When the reaction was catalyzed by fresh catalyst, good conversion and selectivity was achieved in a relatively short time (6 h). *Lu*CLEA activity decreased with each cycle and therefore it could not be reused directly for repeated batches. In contrast, the selectivity was maintained upon recycling (Table 4) consistent with racemization not being significant at 30 °C (Figure 5). In an attempt to further improve the overall selectivity, the reaction was performed with fresh catalyst at 5 °C under otherwise identical conditions (data not shown) but the reaction became extremely slow (7% conversion after 2 h) while the selectivity improved only very slightly (84% *ee* after 2 h).

A straightforward filtration and evaporation under reduced pressure yielded the crude product which contained less then 1% of residual substrate (Table 4.)

The loss of activity upon recycling without decrease in selectivity was low enough to consider adding a fraction of fresh catalyst between batches in order to compensate for this effect. This approach is particu-

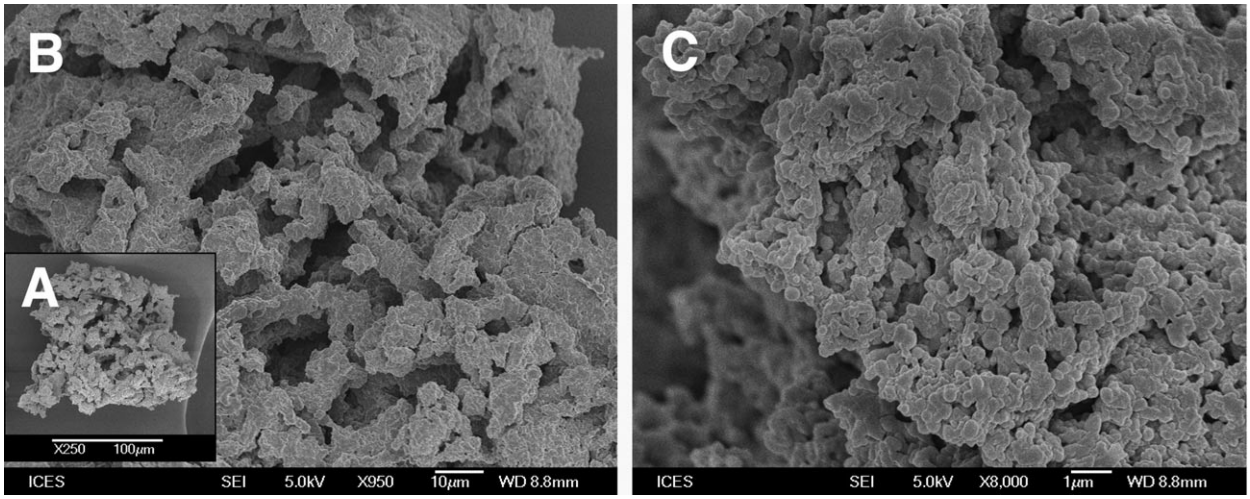


Figure 4. SEM photographs of *Lu*CLEA; **A**: ×250, **B**: ×950, and **C**: ×8,000.

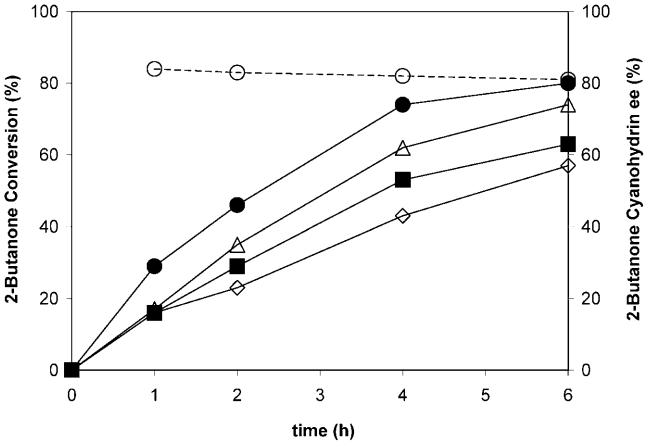


Figure 5. *Lu*CLEA recycling experiment in the preparation of 2-butanone cyanohydrin (5 mmol scale). *Reaction conditions*: 2-butanone (5.0 mmol), HCN (4 equiv.), *Lu*CLEA (40 U), 30 °C, in buffer-saturated DIPE (10 mL). ●: conversion of 2-butanone (fresh catalyst), ○: *ee* of 2-butanone cyanohydrin (fresh catalyst). △: conversion (cycle 1), ■: conversion (cycle 2), ◇: conversion (cycle 3).

Table 4. 2-Butanone cyanohydrin obtained upon recycling: isolated yield after 6 h, *ee*, and residual substrate content.

Catalyst	Yield[a]	*ee*[b]	Residual Substrate[c]
Fresh CLEA	84	81	<1%
Cycle 1	76	80	<1%
Cycle 2	64	81	<1%
Cycle 3	56	78	<1%

[a] Isolated yield (%).
[b] *ee* of the isolated product.
[c] In mol% as determined by ^{1}H NMR.

larly suitable for a carrier-free immobilized catalyst with very high volumetric activity such as CLEAs since the catalyst accounts for a small percentage of the overall reaction volume.

Preparative Scale Synthesis of (*R*)-2-Hydroxy-2-methylbutyric Acid

Our approach for the preparation of (*R*)-2-hydroxy-2-methylbutyric acid [(*R*)-**I**] in high enantiopurity (Scheme 1) involved the direct synthesis of the cyanohydrin (**III**) from 2-butanone (**II**) catalyzed by *Lu*CLEA followed by acid hydrolysis to the corresponding α-hydroxy acid (**I**) as reported for *Pa*HNL.[18]

We first optimized *Lu*CLEA preparation on a 90-mL scale and noticed that freeze drying affected the biocatalyst activity. From results obtained in earlier samples it was clear that the CLEA could not be freeze-dried overnight and should be promptly stored at −20 °C when the biocatalyst is sufficiently dry. We investigated this effect by measuring the specific activity of the CLEA as a function of the freeze drying duration and established that a ratio of 1 g CLEA obtained per 12 mL of commercial *Lu*HNL solution gave a sufficiently dry catalyst with a very high specific activity (303.5 U/g) and recovery (33%).

*Lu*CLEA was then used in the synthesis of (*R*)-2-butanone cyanohydrin on an 80-mmol scale and the reaction was complete in 3 h at 30 °C. Following the conclusions of the recycling experiments, *Lu*CLEA was recycled for a second batch and a portion of fresh catalyst (20% of the original loading) was added to compensate for the loss of activity. The second batch was equally fast, selective and high yielding thereby allowing the preparation of (*R*)-2-butanone cyanohydrin in a 160-mmol scale over two 3 h batches using an overall catalyst loading of 4.8 U/mmol. The *ee* of the cyanohydrin formed was also improved significantly to 87% which was attributed to the optimized catalyst used here. This *ee* value comes close to a literature report for *Lu*HNL immobilized on nitrocellulose on an analytical scale (*ee* = 95%). Due to the analytical conditions, a ten times higher catalyst loading (50 U/mmol) could be employed. This helped to suppress the fast racemic chemical reaction even better than under the conditions described here.[21] The crude cyanohydrin was easily obtained by filtration of the catalyst and evaporation of the volatiles under reduced pressure and was subjected to acid hydrolysis to give (*R*)-2-hydroxy-2-methylbutyric acid in 85% isolated yield (from 2-butanone) and 87% *ee*. The optical purity of the acid can readily be improved by a very efficient crystallization using enantiopure 1-(1-naphthyl)ethylamine as a resolving reagent.[4]

The *Lu*CLEA-based approach towards enantiopure acid **I** described here is highly atom-efficient and generates little waste, even when a recrystallization is taken into account. Moreover, *Lu*CLEA can be recycled without loss of enantioselectivity and modest loss of activity. When compared to the substrate engineering approach described earlier[16] where overall yields of 45% and 51% were reported for the preparation of (*S*)-**I** in 99% *ee*, the direct synthesis of the cyanohydrin from 2-butanone catalyzed by *Lu*CLEA towards (*R*)-**I** proved to be high yielding with a good atom-economy. Moreover, relatively high catalyst loadings were required in the substrate engineering approach (>150 U/mmol) to achieve good conversion while our approach required less than 5 U/mmol of *Lu*CLEA, which corresponds to less than 15 U/mmol of initial commercial *Lu*HNL solution.

Conclusions

The CLEA immobilization strategy was applied successfully to the sole representative of the zinc-depen-

dent alcohol dehydrogenases group among HNLs: *Lu*HNL. Various strategies were attempted to develop this catalyst including the first report of molecular imprinting as a tool to improve the activity of a CLEA. *Lu*CLEA with high specific activity (303.5 U/g) could be prepared in good activity recovery (33%) on a multigram scale.

(*R*)-2-Butanone cyanohydrin was synthesized on a preparative scale over two batches upon the catalysis of *Lu*CLEA using only a small portion of fresh catalyst (20%) between batches to compensate for the loss of activity upon recycling. After hydrolysis, (*R*)-2-hydroxy-2-methylbutyric acid was obtained in 85% isolated yield (from 2-butanone) and 87% *ee*.

Experimental Section

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

General Remarks

Enzymes: Solutions of the hydroxynitrile lyases from *Prunus amygdalus* (*Pa*HNL, 690 U/mL), *Manihot esculenta* (*Me*HNL, 2.23 kU/mL), and *Linum usitatissimum* (*Lu*HNL, 114 U/mL or 76.9 U/mL) were purchased from Codexis. The CLEAs from *Pa*HNL[22] and *Me*HNL[24] were prepared according to literature procedures.

Chemicals: A standard solution of HCN (2 M in DIPE) was prepared as previously reported.[23] Acetone cyanohydrin was prepared according to a literature procedure.[36] Racemic 2-butanone cyanohydrin was prepared *via* its TMS derivative according to a literature procedure.[37] Other chemicals and reagents were purchased from commercial sources and used without further purification unless otherwise specified.

Analytical Methods: ^{1}H and ^{13}C NMR spectra were recorded on a Bruker 400 Avance Ultrashield spectrometer. Absorbance was measured using a Shimadzu Biospec-1601 UV spectrometer. For SEM analysis, the sample was coated with gold and the pictures were taken on a Jeol JSM-6700M field emission scanning electron microscope. Chiral gas chromatography (GC) was performed using an Agilent Technologies 6890N chromatograph equipped with a β-Dex 225 column and an FID detector. High performance liquid chromatography (HPLC) was performed on an Agilent Technologies 1100 series chromatograph equipped with a diode array detector. Specific conditions for chromatography and retention times are given in the experimental section for the respective compounds.

Standard Enzyme Activity Test

The activity of soluble enzyme was measured according to a modified version of the literature procedure.[20] The reaction was performed in a screw-cap vial using a dilute solution of commercial *Lu*HNL in phosphate buffer (5 mM, pH 6.5). The dilute solution (50 μL) was added to citrate/phosphate buffer (438 μL, 0.2 M Na_2HPO_4, 0.1 M citric acid, pH 4.1) followed by 12 μL of a solution of acetone cyanohydrin (10% v/v) in 0.1 M citric acid. The reaction mixture was incubated for 10 min at 30 °C and quenched with 0.01 M aqueous HCl (500 μL). A sample (10 μL) was then diluted in water (9.99 mL) and the cyanide concentration was measured using a commercially available cyanide test (Merck, Spectroquant cyanide test). The test was calibrated using standard aqueous solutions of sodium cyanide. A reference reaction (without enzyme) was performed in parallel and the activity of the soluble enzyme was determined in μmol of acetone cyanohydrin hydrolyzed per min. The CLEA and EA activities were measured according to the same procedure.

Precipitation Study

Phosphate buffer (75 μL, 0.1 M, pH 7) was added to a commercial solution of *Lu*HNL (225 μL, 114 U/mL) and the mixture was kept shaking at 0 °C for 5 min. The cosolvent (113 μL, 450 μL, or 900 μL depending on the experiment) was then added and the mixture was kept shaking at 0 °C for an additional 10 min. The aggregates were separated from the supernatant by centrifugation (10 000 rpm, 5 min) and the supernatant was diluted in 500 μL of phosphate buffer (5 mM, pH 6.5) before measuring the activity. The aggregates were dissolved in 1.50 mL of phosphate buffer (5 mM, pH 6.5) and the activity was measured.

Cross-Linking Study

The cross-linking experiments were conducted as described for the precipitation and after 10 min at 0 °C a 25% aqueous glutaraldehyde solution (11 μL, 22.5 μL, 45 μL, 67.5 μL depending on the experiments) was added to the mixture. The suspension was kept shaking at 0 °C for 1 h and the CLEAs were separated by centrifugation (10,000 rpm; 5 min). The CLEAs were resuspended in 1.50 mL of phosphate buffer (5 mM, pH 6.5) and after centrifugation the activity of the supernatant was measured to determine the efficiency of the cross-linking. The CLEAs were then suspended in 1.50 mL of fresh phosphate buffer (5 mM, pH 6.5) and the activity was measured accordingly.

EAs and CLEAs Specific Activity and Recovery

Enzyme Aggregates (EAs): Commercial *Lu*HNL (900 μL, 69.2 U) was diluted in phosphate buffer (300 μL, 0.1 M, pH 7) and the solution was shaken in an ice/water bath. After 5 min, saturated aqueous $(NH_4)_2SO_4$ (3.6 mL) for *Lu*EA(Am.Sulf) or *tert*-butanol (1.8 mL) for *Lu*EA(*t*-Bu) was added and shaking in ice was continued for 10 min. The suspension was then centrifuged (10,000 rpm, 5 min) washed with acetonitrile and centrifuged again. The enzyme aggregates were freeze dried and the activity was measured according to the standard activity test.

Cross-Linked Enzyme Aggregates (CLEAs): Precipitation of the enzyme was performed as described for the EAs. A 25% aqueous glutaraldehyde solution (90 μL) was added to the suspension and shaking in ice was continued for 1 h. The CLEAs were centrifuged (10,000 rpm, 5 min), washed with phosphate buffer (5 mM, pH 6.5), and freeze dried. The CLEA activity was measured according to the standard activity test.

PaCLEA and MeCLEA: The biocatalysts were prepared as reported.[22,24] The activity of commercial *Pa*HNL, *Me*HNL and the corresponding CLEA was measured according to the standard activity test for *Lu*HNL.

Molecular Imprinting Study

EAs and CLEAs with 2-butanone as additive were prepared as describe above from a solution of commercial *Lu*HNL solution (900 μL, 69.2 U) in phosphate buffer (300 μL, 0.1 M, pH 7) and 2-butanone (69.2 μL). The activity of the biocatalysts obtained was measured according to the standard activity test and the results were expressed as the difference of specific activity (in percentage) between the catalyst prepared with and without additive [Eq. (1)].

$$\text{Activity Difference (\%)} = \frac{100 \times [\text{Activity}_{(\text{with additive})} - \text{Activity}_{(\text{without additive})}]}{\text{Activity}_{(\text{with additive})}} \quad (1)$$

Influence of molecular imprinting on immobilization.

Synthetic Activity

Selected CLEAs and EAs were loaded (2.2 U) in a screw-cap vial and a solution of HCN 2 M in DIPE (1.00 mL) was added. The HPLC internal standard, biphenyl (2 crystals), was added to the mixture upon stirring to ensure complete dissolution. The reaction was started by addition of 2-butanone (44.8 μL, 0.50 mmol) and an analytical sample was taken immediately after addition to determine the initial conditions in HPLC. The conversion ratios were determined by HPLC [10 μL reaction samples in 1.00 mL hexane; Chiralpak AD; mobile phase: hexanes:2-propanol (99:1); flow: 1.5 mL min^{-1}; UV detection at 280 nm; R_t(biphenyl) = 2.64 min, R_t(ketone) = 3.45 min]. The *ee* of the cyanohydrin formed was monitored by chiral GC of the trifluoroacetate derivative in a modified version of the literature procedure[20] [20 μL reaction samples in 1.00 mL anhydrous dichloromethane, 10 μL trifluoroacetic acid anhydride, 10 μL anhydrous pyridine; β-Dex 225 column; 80 °C; 10 psi; R_t(*R*) = 13.77 min , R_t(*S*) = 14.20 min].

Recycling Experiments

Commercial *Lu*HNL (9.00 mL, 692 U) was diluted in phosphate buffer (3.00 mL, 0.1 M, pH 7) at 0 °C and 2-butanone (692 μL) was added. The solution was shaken at 0 °C for 5 min and *tert*-butanol (18 mL) was added. Shaking at 0 °C was continued for 10 min and a 25% aqueous glutaraldehyde solution (900 μL) was added to the suspension. After shaking at 0 °C for 1 h *Lu*CLEA was centrifuged (10,000 rpm, 5 min), washed with phosphate buffer (5 mM, pH 6.5), and freeze dried to give 392.5 mg of immobilized biocatalyst (125.9 U/g, 7% recovery). *Lu*CLEA (318.0 mg, 40 U) was then loaded in a jacketed flask and a 2 M HCN solution in DIPE (10 mL) was added. The temperature was kept at 30 °C and biphenyl (*ca.* 2 mg) was added to the suspension. 2-Butanone (448 μL, 5 mmol) was then added and the reaction was monitored as described above. After 6 h of reaction at 30 °C *Lu*CLEA was filtered off, rinsed with fresh DIPE (30 mL) and loaded back into the jacketed flask for the next cycle. The combined DIPE phases were evaporated under reduced pressure to give the crude 2-butanone cyanohydrin as a colourless liquid. ^{1}H NMR ($CDCl_3$, 400 MHz): δ = 1.10 (t, 3 H, J = 7.5 Hz), 1.59 (s, 3 H), 1.81 (m_c, 2 H), 3.77 (bs, 1 H); ^{13}C NMR ($CDCl_3$, 100.65 MHz): δ = 8.4, 26.8, 34.6, 69.1, 121.8.

(*R*)-2-Hydroxy-2-methylbutyric Acid

Commercial *Lu*HNL (90 mL, 6.92 kU) was diluted in phosphate buffer (30 mL, 0.1 M, pH 7) at 0 °C and 2-butanone (6.92 mL) was added. The solution was shaken at 0 °C for 5 min and *tert*-butanol (180 mL) was added. Shaking at 0 °C was continued for 10 min and a 25% aqueous glutaraldehyde solution (9.00 mL) was added to the suspension. After shaking at 0 °C for 1 h *Lu*CLEA was centrifuged (10,000 rpm, 5 min), washed with phosphate buffer (5 mM, pH 6.5), and freeze dried until the weight of immobilized biocatalyst was 7.59 g (303.5 U/g, 33% recovery). *Lu*CLEA (2.11 g, 640 U, 8.0 U/mmol) was then loaded in a jacketed flask and a 2 M HCN solution in DIPE (160 mL) was added. The temperature was kept at 30 °C and biphenyl (*ca.* 10 mg) was added to the suspension. 2-Butanone (7.2 mL, 80 mmol) was then added and the reaction was monitored as described above. After 3 h at 30 °C *Lu*CLEA was filtered off, rinsed with diethyl ether (3 × 100 mL), loaded back into the jacketed flask, and resuspended in 2 M HCN solution in DIPE (160 mL). An additional loading of fresh *Lu*CLEA (421.9 mg, 128 U, 2.0 U/mmol) was added and a second batch of 2-butanone cyanohydrin was prepared accordingly. The combined ethereal phases from the first batch were evaporated under reduced pressure to give the crude (*R*)-2-butanone cyanohydrin (yield: 8.69 g, 86% *ee*). This crude cyanohydrin was stirred at room temperature in a mixture (1:2) of water:conc. HCl while the enzymatic reaction for the second batch proceeded (3 h). The crude (*R*)-2-butanone cyanohydrin from the second batch (yield: 7.92 g, 87% *ee*) was diluted in 60 mL of water:conc. HCl (1:2) and combined with the reaction mixture of the first batch. The hydrolysis was allowed to proceed upon vigorous stirring at 65–70 °C and monitored by ^{1}H NMR of reaction samples (100 μL) extracted in ether (1.00 mL). After 20 h the reaction mixture was cooled to room temperature, diluted with water (80 mL) and ether (300 mL) was added. After stirring for 10 min, the phases were separated and the aqueous layer was extracted with ether (2 × 300 mL). The combined ethereal layers were dried ($MgSO_4$) and the solvent was removed under reduced pressure to give (*R*)-2-hydroxy-2-methylbutyric acid as a white solid; yield: 16.08 g (136 mmol, 85% yield, 87% *ee*). ^{1}H NMR ($CDCl_3$, 400 MHz): δ = 0.94 (t, 3 H, J = 7.5 Hz), 1.47 (s, 3 H), 1.73 (dq, J = 7.5 Hz, 15.0 Hz, 1 H), 1.86 (dq, J = 7.5 Hz, 15.0 Hz, 1 H), 5.79 (bs, 2 H); ^{13}C NMR

($CDCl_3$, 100.65 MHz): $\delta = 7.8, 25.4, 32.9, 75.1, 181.4$. The *ee* of the product was determined as described previously[4] [*ca.* 1 mg product dissolved in 2-propanol (100 μL) and diluted in hexane (1.00 mL); Chiralpak AD; mobile phase: hexanes:2-propanol:TFA (96:4:0.1); flow: 1.5 mL min^{-1}; UV detection at 210 nm; $R_t(R) = 8.22$ min, $R_t(S) = 9.10$ min].

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