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Cross-Linked Amorphous Nitrilase Aggregates for Enantioselective Nitrile Hydrolysis

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Abstract: A recombinant *Escherichia coli* strain was constructed which efficiently expressed the enantioselective nitrilase from *Alcaligenes faecalis* DSMZ 30030 as a hisitidine-tagged enzyme variant under the control of a rhamnose inducible promoter. The enzyme was purified from cell extracts and used for the preparation of cross-linked enzyme aggregates (CLEAs). The conditions for the preparation of the CLEAs were optimized using various organic solvents and cross-linking agents and a procedure was developed which combined a precipitation with 85% (v/v) isopropyl alcohol and a cross-linking with 30 mM glutaraldehyde. Thus, about 80% of the initial nitrilase activity could be incorporated into the CLEAs. The hydrolysis of racemic mandelonitrile to (*R*)-mandelic acid was compared between the soluble nitrilase preparations and their CLEAs (nit-CLEAs). The nitrilase activity in the CLEAs was at 30 °C and 60 °C about 5 times more stable than in the soluble preparations. The CLEAs could be reused 5 times with only about 10% reduction in activity. The enantioselectivity of the nitrilase for the formation of (*R*)-mandelic acid from racemic mandelonitrile decreased for both preparations with increasing temperatures (10 °C to 50 °C), but this effect was significantly less pronounced for the CLEAs. A detailed analysis of solvent effects on nitrilase enantioselectivity allowed thermodynamic insights into contributions from free energy component (activation enthalpy and entropy) to chiral preference of nitrilase in such non conventional media.

Keywords: cross-linked enzyme aggregates; enantioselectivity; nitrilase; solvent engineering

Introduction

Nitrilases have attracted tremendous attention and interest in the past decade owing to the desirability and ease of conducting nitrile hydrolysis at ambient conditions with precise chemo-, regio- and enantioselectivity.[1] The search for alternatives to conventional harsh chemical methods has been the major driving force behind the development of these biotransformations. In spite of their significant potential for production of high-value chiral acids, there are still only very few industrial applications of these enzymes described. Thus, there are some examples for industrial conversions of pyridine- and pyrazine-nitriles and the regioselective transformation of aliphatic dinitriles by nitrilases. The quantitatively most important industrial application of nitrilases appears to be the transformation of racemic mandelonitrile to (*R*)-mandelic acid which is catalyzed by several nitrilases from *Alcaligenes (faecalis)* strains.[2–5] (*R*)-(−)-Mandelic acid is a key intermediate for the production of semi-synthetic cephalosporins[6] and penicillins.[7] It is also used as a chiral resolving agent[8–10] and chiral synthon for the production of antitumor agents[11–15] and antiobesity agents.[16] It has been repeatedly reported that nitrilases are rather sensitive enzymes. This observation is generally correlated with the presence of a cysteine residue in the catalytic center.[17,18] Therefore, the development of a robust, highly active and easily reusable formulation of nitrilases that is stable under a range of deleterious conditions remains a challenge. We have earlier demonstrated that the entrapment of whole cells of *Alcaligenes faecalis* which actively produce an (*R*)-specific mandelonitrile converting nitrilase in polymer matrices (such as alginate) leads to a significant improvement in the handling of the catalyst but at the cost of mass-transfer limitations and dilution of enzyme activity owing to the presence of a large proportion of non-catalytic mass.[19] In addition, the diffusion restricted system was also found to result in decreased enantioselectivity of the immobilized biocatalyst. One possible strategy to circumvent the problems inevitably connected with immobilized whole cell catalysts is to work with *in vitro* systems. In

this area, methods for immobilizing enzymes without a support are gaining importance because of advantages like high volumetric productivity, lower production costs and greater stability.[20] This class of biocatalyst formulations includes cross-linked enzyme crystals (CLECs), cross-linked enzymes (CLEs) and cross-linked enzyme aggregates (CLEAs). Cross-linking of a soluble enzyme results in a gelatinous mass that is weakly aggregated and hence results in handling problems. Preparation of CLECs necessitates the use of pure enzyme and laborious screening for crystallization conditions. These problems may be overcome by use of CLEAs. The procedure involves precipitation of the enzyme (need not be pure) from its aqueous solution (by using solvents, salts etc.) and its subsequent cross-linking by using a bifunctional agent (like glutaraldehyde). Recently, it was reported that a highly active but only modestly enantioselective nitrilase from *Pseudomonas fluorescens* EBC191 could be efficiently formulated as CLEAs.[18] Therefore, in the present investigation it was attempted to develop a similar highly active, reusable biocatalyst formulation from a nitrilase which converts mandelonitrile with a higher enantioselectivity that may not suffer from any diffusion restrictions. Furthermore, this catalyst was used for the first detailed analysis of the influence of temperature and organic solvents on enantioselectivity of a nitrilase.

Results and Discussion

Cloning, Over-Expression and Purification of the Nitrilase from *Alcaligenes faecalis* DSMZ 30030

The nitrilase gene was amplified by polymerase chain reaction (PCR) from the genomic DNA of *A. faecalis* DSMZ 30030 (ATCC 8750) by using oligonucleotides derived from the literature (see Experimental Section). Thus, a 1071 bp fragment was obtained that was digested with *Nde*I/*Bam*HI and ligated into the expression vector pJOE2775[21] digested with the same restriction enzymes. In this construct the nitrilase gene was placed under the control of a rhamnose inducible rha*BAD* promoter. Furthermore, a C-terminal histidine tag was added to the nitrilase, which allowed easy purification of the enzyme by using an Ni-NTA column (see Experimental Section). The resulting expression plasmid (pAFN) was used to transform *E. coli* JM109 cells and nitrilase was induced by the addition of rhamnose (0.2% w/v). A comparison of SDS-PAGE gels from induced and uninduced cells of *E. coli* JM109 (pAFN) and the vector control *E. coli* JM109 (pJOE2775) demonstrated the induction of an additional protein with a molecular mass of about 32 kDa (Figure 1). The cell extracts from the induced cells of *E. coli* JM109 (pAFN) converted mandelonitrile with a specific activity of 0.64 U/mg of protein. In contrast, no nitrilase activity was found in the cell extracts prepared from the vector control. The recombinant His-tagged nitrilase was purified from rhamnose induced cells of *E. coli* JM109 (pAFN) by affinity chromatography (see Experimental Section) (Figure 1). This resulted in enzyme preparations that were almost 100% pure according to SDS-PAGE and that converted mandelonitrile with a specific activity of about 1.96 U/mg of protein.

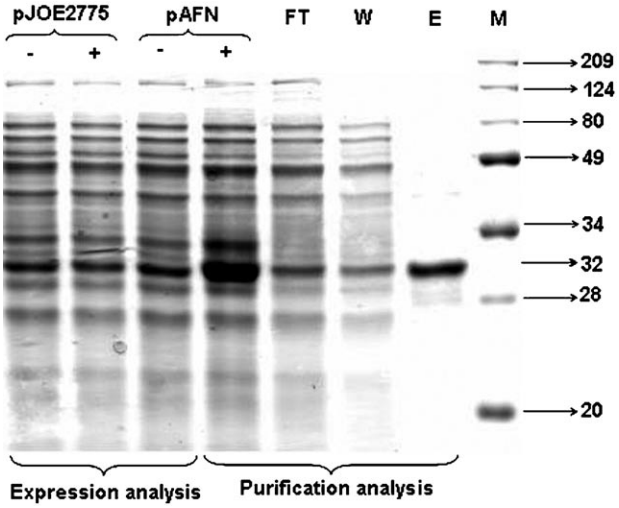

Figure 1. Expression analysis of the nitrilase in cell extracts from rhamose induced (+) or uninduced (−) cells of *E. coli* JM109 (pJOE2775) as control or *E. coli* JM109 (pAFN) (10 μg of protein were added per lane). Following lanes represent purification analysis of fractions over an Ni-NTA column; FT (flow through), W (wash) and E (eluate). M represents molecular weight markers.

Enzyme Precipitation

The preparation of CLEAs generally requires (1) choice and optimization of precipitant concentration, (2) choice and concentration optimization of an appropriate cross-linker and (3) optimization of protein loading.[20] Therefore, first the activity recoveries of the protein aggregates which were obtained after the addition of different solvents were examined before cross-linking. The precipitation reactions were performed by using various solvents up to 90% (v/v). The formation of aggregates was in most cases indicated by a precipitate at the bottom of the reaction tubes or increased turbidity in the medium. The aggregates obtained were centrifuged ($6000 \times g$, 4 °C, 30 min) to remove the solvent and unrecovered proteins before they were used for determination of recovered nitrilase activity. Maximum nitrilase recovery (95%) was achieved with isopropyl alcohol (85% v/v) (Figure 2).

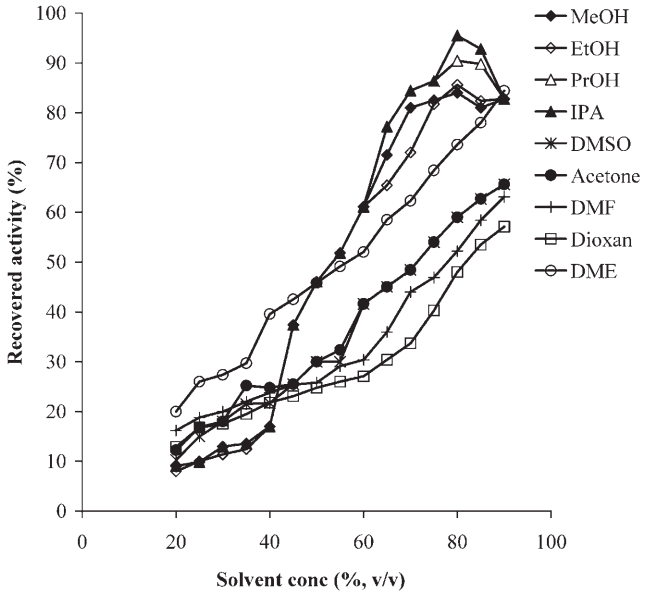

Figure 2. Recovery of enzyme activity in aggregates upon precipitation with various organic solvents.

Usually solvent concentrations below 50% (v/v) were insufficient for efficient enzyme recovery. As reported previously for other enzymes, it was observed that adding the enzyme solution to a concentrated solution of precipitant resulted in greater recovery of activity, in comparison to the addition of precipitant to enzyme solution.[22]

Cross-Linking of Aggregates

Various cross-linkers like glutaraldehyde (GA), poly(ethyleneimine) (PEI), GA+PEI or dextran polyaldehyde (DPA) were used to obtain CLEAs from precipitates obtained by addition of isopropyl alcohol and the activities of the preparations were compared (Figure 3 a–d).

It was observed that maximum recovery of activity was obtained with GA (81%) as compared to PEI (44%), GA+PEI (61%) and DPA (56%). Total activity represents mass balance of the unrecovered activity (in supernatant) and the recovered activity (in CLEAs). As expected, the total activity approached 100% when cross-linking was performed with GA, PEI or DPA (Figure 3 a, b, d). However, when a combination of 30 mM GA and varying concentrations of PEI were used, the total activity declined to 80% (Figure 3 c). This loss in total activity may have resulted from excessive cross-linking of the aggregates leading to diffusion restrictions for interaction of substrate with the active site.

CLEAs prepared with GA were chosen for further studies since they retained highest fraction of recovered nitrilase activity. The surface structure of CLEAs was also visualized through scanning electron microscopy (SEM) and appeared as an aggregated lump of particles with sizes around 40 μm (Figure 4).

Table 1. Estimated kinetic constants for soluble nitrilase and its CLEAs.

Biocatalyst form	V_{max} [mM min^{-1}]	K_m [mM]	k_{cat} [min^{-1}]	k_{cat}/K_m [min^{-1} мм$^{-1}$]
Nit-soluble	0.13	6.4	4.33	0.67
Nit-CLEAs	0.11	8.34	4.95	0.59

Comparison of the Conversion of Mandelonitrile by the Soluble and Immobilized Nitrilase

The soluble nitrilase preparation and the CLEAs converted racemic mandelonitrile to almost pure (*R*)-mandelic acid. The immobilization resulted in an approximately 40% reduction of the reaction rates (Figure 5).

In order to further analyze the observed restrictions, the soluble and the immobilized preparation were incubated with different mandelonitrile concentrations. As compared to the soluble nitrilase, the procedure of making CLEAs resulted in a greater decrease in specificity constant (k_{cat}/K_m, indicates the efficiency with which an enzyme converts substrate to product) rather than the turnover number (k_{cat}). This decrease in efficiency suggested that the effect seemed to be more dominant on the apparent K_m than on V_{max} (Figure 6, Table 1).

Biocatalyst Stability

The stability of the biocatalysts was studied under operational conditions (30°C) and under denaturing conditions (50°C). These experiments demonstrated a significant stabilization of the nitrilase by the CLEA formation under both conditions (Figure 7).

In order to mathematically model the different inactivation reactions it was assumed that the native nitrilase from *A. faecalis* is converted during the early stage of the inactivation process into a more labile but still active intermediate form. This inactivation is schematized in Eq. (1), where $(Nitrilase)_{native}$ is the native active form, $(Nitrilase)_{labile}$ is the labile intermediate and the $(Nitrilase)_d$ is the deactivated form.

$$(Nitrilase)_{native} \xrightarrow{k_i} (Nitrilase)_{labile} \xrightarrow{k_d} (Nitrilase)_d \quad (1)$$

Assuming that at time zero only the native enzyme is present, then the total activity α, may be expressed

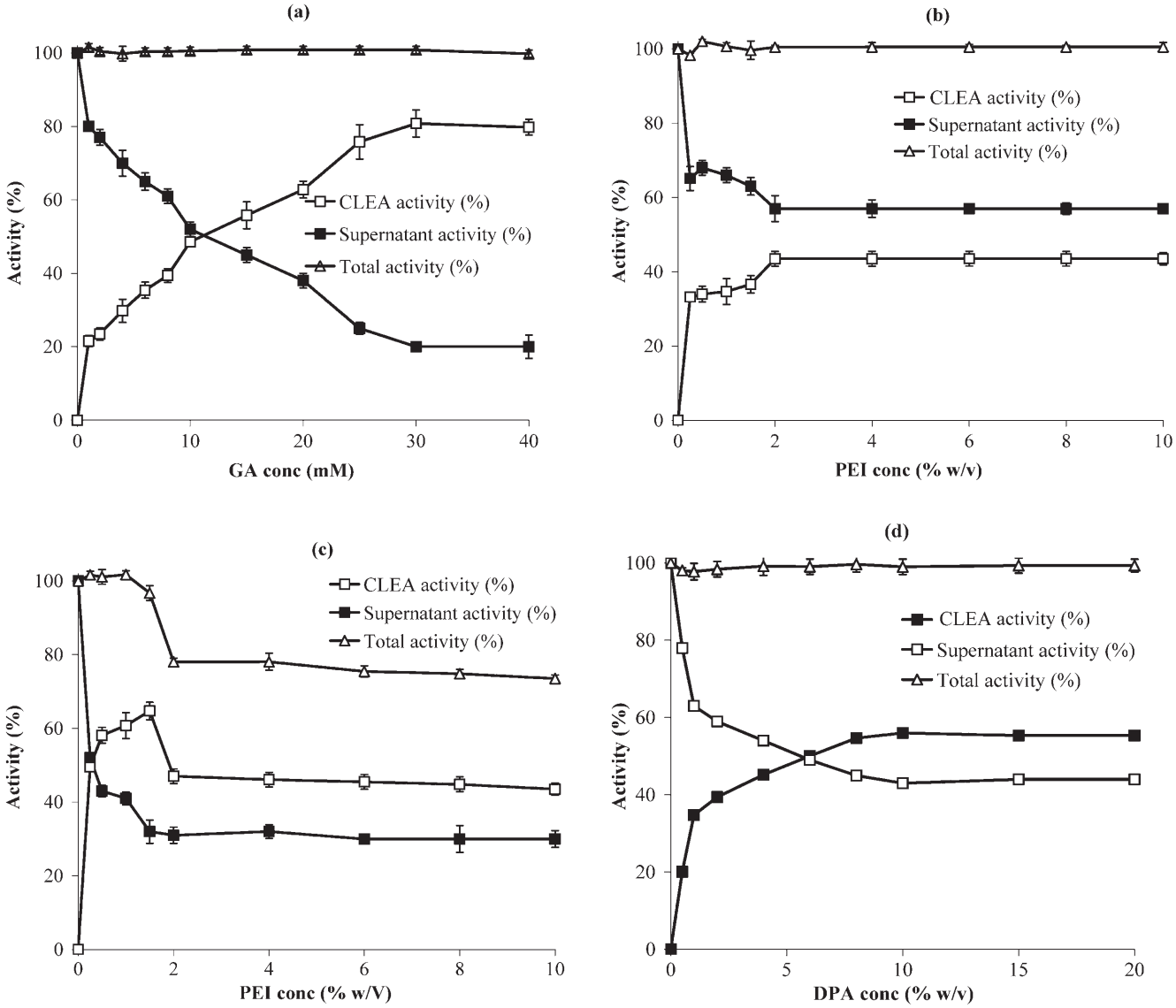

Figure 3. Recovery of enzyme activity in CLEAs upon cross-linking with different agents; (**a**) GA, (**b**) PEI, (**c**) 30 mM GA + PEI and (**d**) DPA.

by Eq. (2), where k_i and k_d are first-order rate constants and α_i and α_d the relative activities of the species $(\text{Nitrilase})_{\text{labile}}$ and $(\text{Nitrilase})_d$.

The equation yields the classical first-order deactivation rate expression for $\alpha_i = 0$, $a = \exp(-k_i t)$. The experimentally determined deactivation profiles were fitted using the above two-step model and also with a first-order deactivation model[23] (Table 2 and Table 3).

As the results indicate, the two-step model resulted in better fitting than the latter and afforded accurate estimation of the half-life ($t_{1/2}$). Both models clearly demonstrated that the nitrilase showed about 5 times higher half-lives in the CLEAs at both temperatures. Furthermore, the two-step model suggested that at both temperatures $k_i > k_d$. This would suggest that the formation of the labile but still active intermediate would be the rate-determining step in the inactivation process.

$$a = \alpha_d + \left[1 + \frac{\alpha_i k_i}{k_d - k_i} - \frac{\alpha_d k_d}{k_d - k_i}\right] \exp(-k_i t) - \left[\frac{\alpha_i k_i}{k_d - k_i} - \frac{\alpha_d k_i}{k_d - k_i}\right] \exp(-k_d t) \quad (2)$$

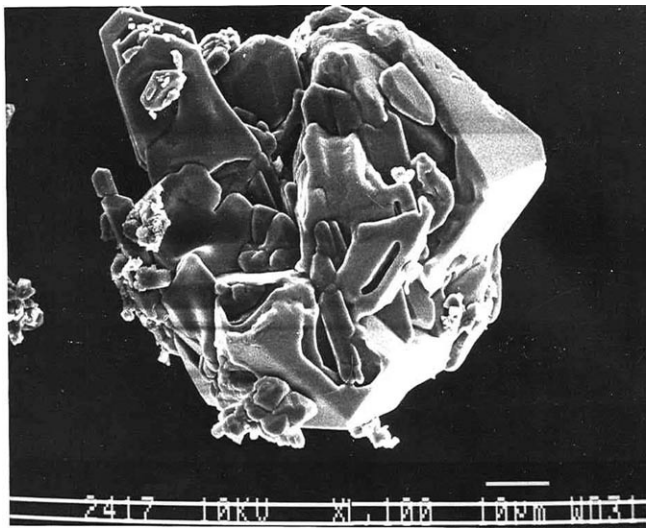

Figure 4. Physical appearance of CLEAs through SEM.

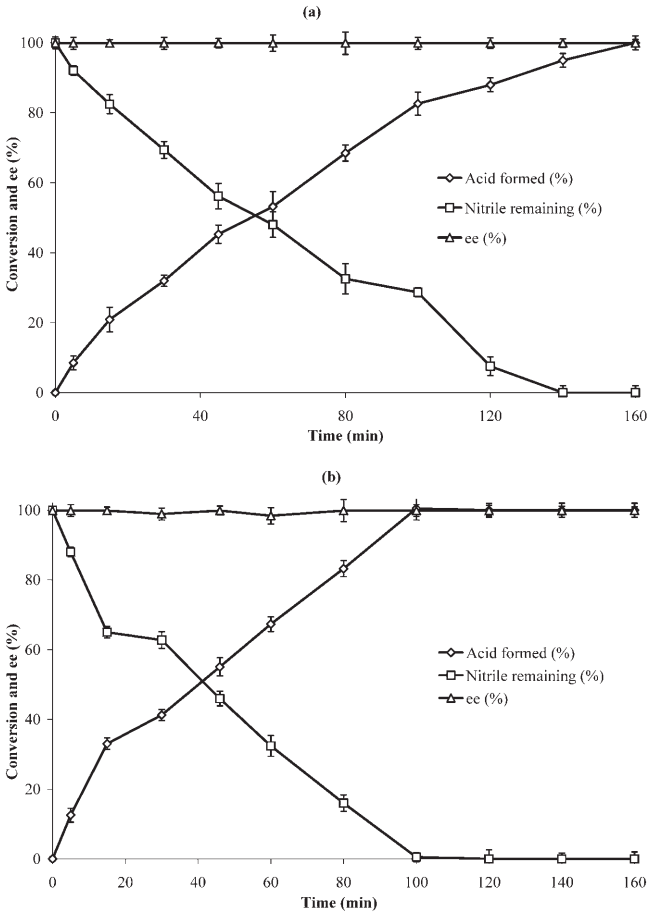

Figure 5. Course of reaction with (**a**) Nit-CLEAs and (**b**) Nit-soluble.

Biocatalyst Reusability

One of the most important characteristics of a biocatalyst for its process implementation is the easy reusa-

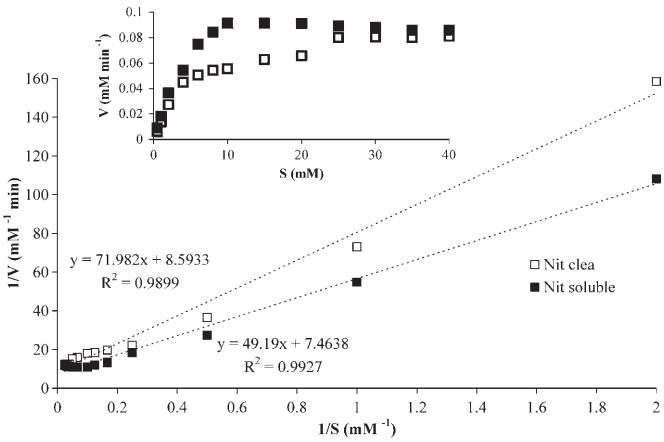

Figure 6. Lineweaver–Burk plot for estimation of kinetic constants of the free and immobilized nitrilase preparations (Inset shows Michaelis–Menten plot).

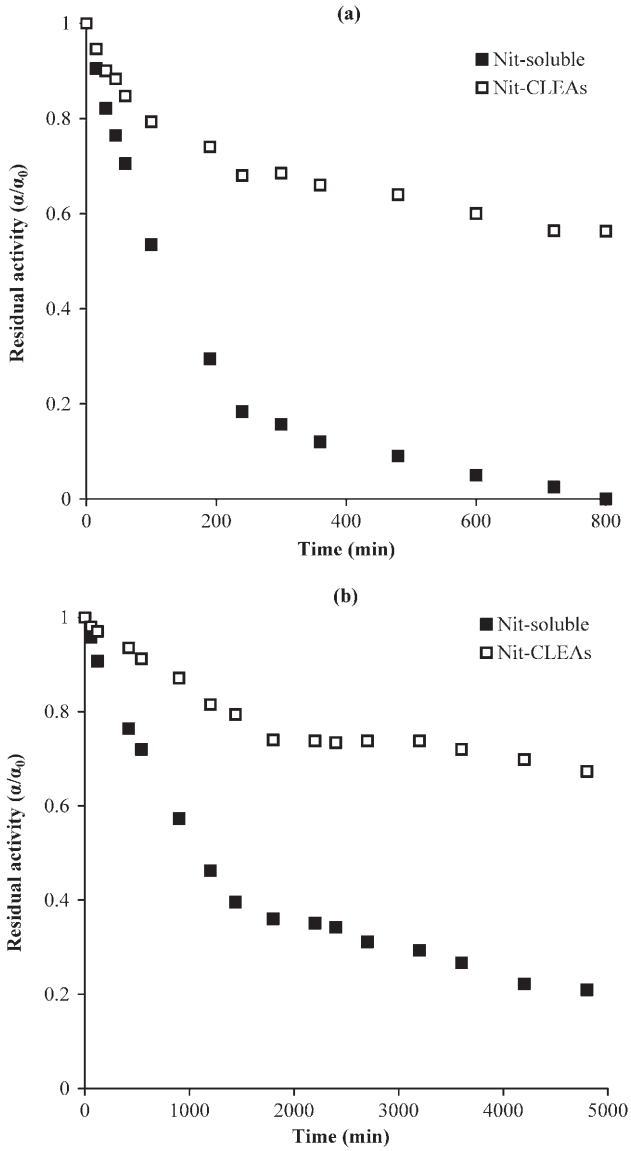

Figure 7. Thermal inactivation profiles of Nit-soluble and Nit-CLEAs at (**a**) 50 and (**b**) 30 °C.

Table 2. First order inactivation rate constant (k_d), correlation coefficient (r) and half life ($t_{1/2}$) of soluble nitrilase and its CLEAs.

Temperature [°C]	Biocatalyst form	k_d [min^{-1}]	r	$t_{1/2}$ [min]
30	Nit-soluble	0.0004	0.860	1732.5
	Nit-CLEAs	0.0001	0.930	6930
50	Nit-soluble	0.0053	0.964	128.3
	Nit-CLEAs	0.0007	0.894	990

bility of the active enzyme. Therefore, the reusability of the soluble nitrilase and its CLEAs was determined in a batch mode. This demonstrated almost 90% retention of activity after 5 reaction cycles (Figure 8).

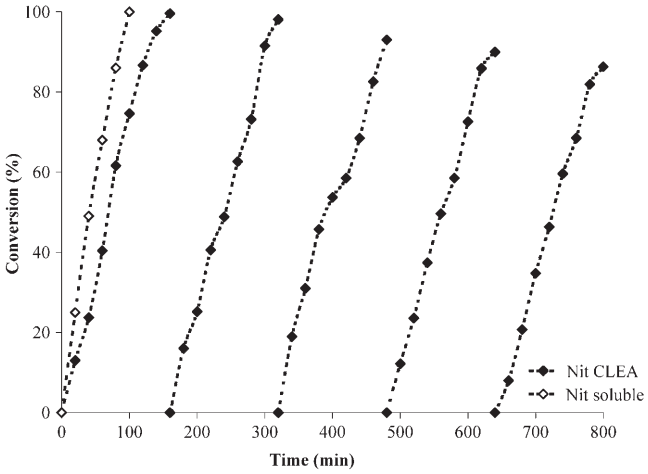

Figure 8. Comparison of reusability of Nit-soluble *vs.* Nit-CLEAs.

Temperature Effects on Enzyme Enantioselectivity

Enantioselectivity is the result of difference between activation free energies of the enantiomers ($\Delta\Delta G^{\neq}_{RS}$) which is related to enantiomeric ratio (E) as $-RT \ln E$.[24] Furthermore, $\Delta\Delta G^{\neq}_{RS}$ is related to activation enthalpy and activation entropy as $\Delta\Delta G^{\neq}_{RS} = \Delta\Delta H^{\neq}_{RS} - T\Delta\Delta S^{\neq}_{RS}$. Another relevant parameter is the racemic temperature ($T_r = \Delta\Delta H^{\neq}_{RS}/\Delta\Delta S^{\neq}_{RS}$), at which there is no discrimination of the enantiomers. The effect of temperature on enantioselectivity (E) was analyzed for soluble and immobilized nitrilase. We have demonstrated earlier for whole cells of *Alcaligenes faecalis* that entrapment of the cells resulted in a decreased enantioselectivity of the nitrilase activity due to diffusion restrictions imposed by the matrix.[19] Preparations of the soluble nitrilase and the CLEAs were incubated with (*R,S*)-mandelonitrile in the temperature range from 10–50 °C and the reactions were terminated after different time intervals. For exact determinations several data points were determined at the beginning of the reactions and only data with constant E-values during this time period were used for the calculations. The plots of $\ln E$ versus T^{-1} (Figure 9) demonstrated the linear correlation to the above equation, which is required for a meaningful determination of the thermodynamic components. The slope in this kind of presentation corresponds to $-\Delta\Delta H^{\neq}_{RS}/R$ and the intercept to $\Delta\Delta S^{\neq}_{RS}/R$. It was observed that lowering the temperature resulted in an enhancement of enantioselectivity (Figure 9). However, it is also accompanied by an unavoidable increase in turnover time resulting from the decreased reaction rate. Thermodynamic analysis allowed separation of contributions from activation enthalpy ($\Delta\Delta H^{\neq}_{RS}$) and activation entropy ($\Delta\Delta S^{\neq}_{RS}$) to nitrilase enantioselectivity.

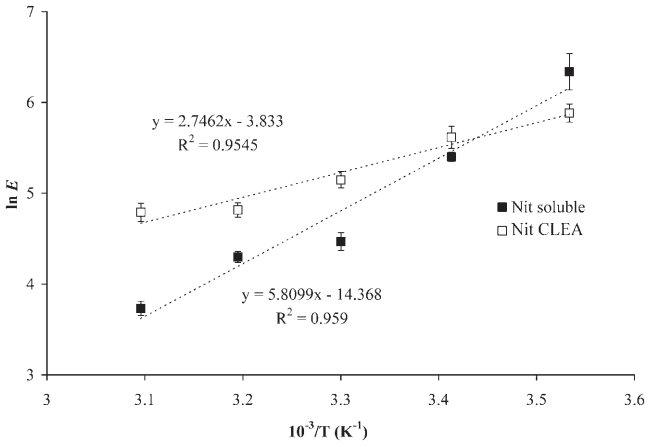

Figure 9. Correlation between $\ln E$ *vs.* 1/T for nitrile hydrolysis by soluble nitrilase and its CLEAs. Enantioselectivity (E) was calculated using the following formula $E = \ln[1-c(1+ee_p)]/\ln[1-c(1-ee_p)]$, where c = conversion and ee_p = enantiomeric excess of product.

Table 3. Model parameters for series type inactivation deactivation study of soluble nitrilase and its CLEAs.

Temperature [°C]	Biocatalyst form	α_i	α_d	k_i [min^{-1}]	k_d [min^{-1}]	r	$t_{1/2}$ [min]
30	Nit-soluble	0.36	0.0	0.00051	0.00006	0.980	2597
	Nit-CLEAs	0.73	0.04	0.0002	0.00005	0.977	14160
50	Nit-soluble	0.18	0.0	0.0054	0.0003	0.977	172
	Nit-CLEAs	0.66	0.02	0.0025	0.0004	0.971	1200

Table 4. Differential thermodynamic parameters for mandelonitrile hydrolysis.

Biocatalyst form	$\Delta\Delta H^{\neq}_{RS}$ [kcal mol^{-1}]	$\Delta\Delta S^{\neq}_{RS}$ [cal mol^{-1}/K]	T_r [°C]
Nit-soluble	−12.57	−32.17	117.73
Nit-CLEAs	−5.14	−7.48	414.16

The results indicated that complexation of the preferred (*R*)-enantiomer of mandelonitrile with the active site is enthalpically favored ($\Delta\Delta H^{\neq}_{RS}$ is negative) but entropically unfavored ($\Delta\Delta S^{\neq}_{RS}$ is negative) (Table 4).

The entropic component (of activation free energy) works against the enthalpic component to yield a lower enantioselectivity. Surprisingly, the soluble nitrilase preparations showed higher *E*-values than CLEAs in the temperature range from 10–30 °C and the situation was opposite at higher temperature. Immobilization resulted in a 3.75 % change in $\Delta\Delta S^{\neq}_{RS}$ (a greater negative value would decrease $\Delta\Delta G^{\neq}_{RS}$) in favor of enantioselectivity and also a 2.11 % change in $\Delta\Delta H^{\neq}_{RS}$ to oppose the enantioselectivity. It seems quite probable that the process of preparing CLEAs (precipitation and cross-linking of aggregates) results in 'locking' of a slightly altered enzyme conformation that prevents a favorable interaction of the fast reacting (*R*)-enantiomer with the active site. However this altered conformation of the nitrilase in CLEAs is sufficiently 'frozen' to prevent any further substantial conformational perturbation that might result in reduced enantioselectivity at higher temperatures. At low temperatures, the contest between the substrate enantiomers to bind with the nitrilase active site appears to be weak in case of soluble nitrilase, since the (*R*)-enantiomer has a greater enthalpic advantage. The altered conformation of nitrilase in CLEAs presents slightly distorted binding pocket for the substrate, leading to a greater competition for interaction and loss of enthalpic advantage for the fast reacting (*R*)-enantiomer. This scenario of competition is altered at higher temperatures in favor of CLEAs.

Solvent Effects on Enzyme Enantioselectivity

In many cases, organic solvents have been shown to influence enzyme enantioselectivity.[25,26] Therefore, the effect of various organic solvents (10 % v/v) was analyzed on the enantioselectivity of Nit-CLEAs. Two different classes of solvents were used, alcohols (MeOH, EtOH, IPA, PrOH) and aprotic solvents (DMSO, 1,4-dioxan, acetone and DMF). The CLEAs showed highest enantioselectivity in DMSO and lowest in PrOH and acetone (Figure 10).

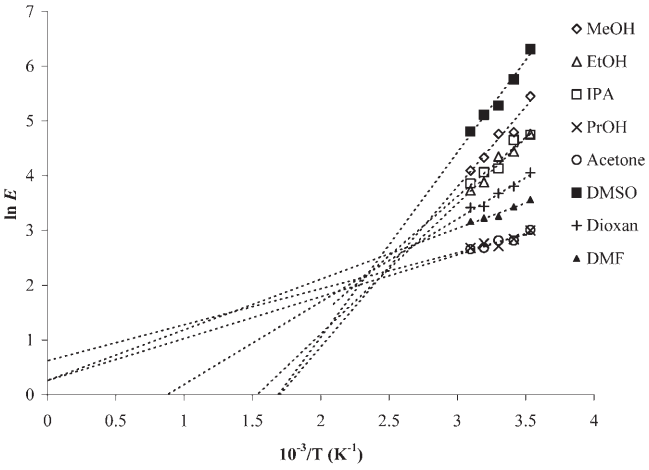

Figure 10. Correlation between ln *E* *vs.* 1/T for nitrile hydrolysis by Nit-CLEAs in various organic solvents.

Amongst all solvents used, only DMSO resulted in a minor enhancement of enantioselectivity compared to the situation without added solvents. The selectivity between the conversion of two enantiomers depends on difference in activation free energy ($\Delta\Delta G^{\neq}_{RS}$), which has an enthalpic ($\Delta\Delta H^{\neq}_{RS}$) and an entropic ($\Delta\Delta S^{\neq}_{RS}$) component. The value of these thermodynamic components was determined by estimating enantioselectivity (*E*) at different temperatures in various solvents. Variation of enthalpic component as a function of entropic component was studied in different solvents and it resulted in a strikingly good correlation ($R^2 = 0.9957$) (Figure 11).

This type of enthalpy-entropy compensations (EEC) phenomenon has been observed on several occasions in other enzyme-catalyzed reactions.[27–30] The molecular mechanism behind this effect is most likely that a change that favors substrate binding to the active site reduces both, enthalpy as well as entropy.

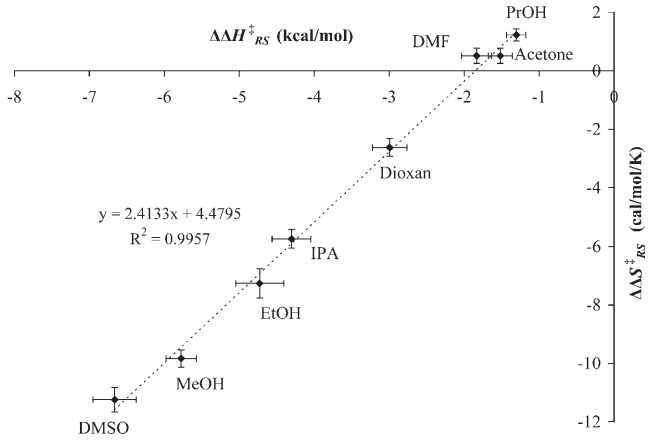

Figure 11. Enthalpy-entropy compensation plot for resolution of racemic mandelonitrile by nit-CLEAs in various solvents.

In case of a real chemical compensation, a plot of $\ln E$ *vs.* $1/T$ should yield a point of single intersection.[31] However, in the experiments described here no common intersection was found for the solvents in the plot. The outcome on the tussle between enthalpic and entropic components of free energy depends to some extent on the position of data points in the quadrants of an EEC plot. For the 8 solvents tested, 5 of the data points lie in the third quadrant of the EEC plot. This implies that $\Delta\Delta H^{\neq}_{RS}$ and $\Delta\Delta S^{\neq}_{RS}$ oppose each other and former outweighs the latter, resulting in decreased enantioselectivity as compared to the reaction without any solvent. However, for the three other solvents (DMF, PrOH and acetone) the points lie in the fourth quadrant of the graph. This means that both enthalpic and entropic components work together in the same direction for enhanced enantioselectivity. Although the Nit-CLEAs showed highest enantioselectivity in the presence of DMSO, the corresponding point lies in the third quadrant. Since the points in the fourth quadrant lie too close to the origin, the absolute magnitude of enthalpic and entropic components is too small to result in a significant enhancement in enantioselectivity. Therefore it will be a task of future research to access the area of the fourth quadrant with solvent systems for which the magnitude of both the components is high enough to significantly enhance enantioselectivity.

Conclusions

Successful implementation of a bioprocess demands on the availability of a highly active, stable and easily reusable biocatalyst formulation. Immobilization offers a means to address this issue by providing a biocatalyst in an insoluble form to apply benefits of heterogeneous catalysis to homogenous enzyme preparations. Nitrilases are often assumed to be rather labile enzymes and this is generally correlated with the presence of an autoxidable cysteine-residue in the catalytic center of these enzymes. The labile nature of nitrilases in combination with the great industrial potential of this class of enzymes explains the many attempts that have been performed in order to immobilize the enzyme themselves or also nitrilases producing microorganisms. Thus, the immobilization of whole cells of *Alcaligenes faecalis* in various entrapment matrices has been previously compared.[19] However conventional polymers used for immobilization lead to escalation of mass transfer problems resulting from dilution of enzyme activity due to the addition of a large proportion of non-catalytic mass. Development of a carrier-free immobilized preparation resulted in a highly active and reusable biocatalyst that was free from diffusion restriction problems and highly stable under deleterious conditions. Thermodynamic analysis allowed explaining the difference in enantioselectivity of CLEAs from the soluble nitrilase. The study also allowed segregation of enthalpic and entropic components to the binding of substrate with the nitrilase active site in different solvents. To the best of our knowledge, this is the first attempt to study in detail the solvent effects on nitrilase enantioselectivity from a thermodynamic perspective. It may be regarded that an enzyme that is highly enantioselective for the (*R*)-enantiomer of mandelonitrile can in certain different reaction conditions accommodate and hydrolyze the mirror image of the preferred substrate enantiomer, thus providing a means to regulate enantiomer 'traffic' at the active site Similar studies on variation of enantioselectivity with temperatures in different solvents at various concentrations may permit a thermodynamic insight into the source of enantioselectivity of the enzyme. Assessing nitrile biotransformations in other solvents may afford admittance to the fourth quadrant of the EEC plot for enhanced enantioselectivity. Work on these lines is in progress in our laboratory.

Experimental Section

Chemicals

Racemic mandelonitrile and enantiomers of mandelic acid were obtained from Sigma Aldrich Chemical Company (Milawukee, USA). Different restriction enzymes and DNA ligase were procured from Roche Diagnostics (Mannheim, Germany). Growth media components and antibiotics were obtained from Roth Chemical Co. (Mumbai, India). All solvents used were of HPLC grade and were procured from Mallinckrodt Baker Inc. (Phillipsburg, USA). All other reagents or chemicals used were of analytical grade and obtained from standard companies.

Bacterial Strains and Culture Conditions

Escherichia coli JM109 was used for plasmid preparation, cloning experiments and heterologous expression of the nitrilase. *E. coli* strains were grown at 37 °C in 2× yeast tryptone medium (YT) of following composition (g/L): tryptone, 10; yeast extract, 5; NaCl, 10; agar, 15; and ampicillin at a final concentration of 0.1 mg mL^{-1}. Solid media were prepared by the addition of 1.5 % (w/v) agar.

Source of the DNA and Amplification of the Nitrilase Gene by PCR

Genomic DNA of *Alcaligenes faecalis* DSMZ 30030 was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) in Braunschweig (Germany). The primers for the amplification of the nitrilase gene were derived from previously described sequence informations for the nitrilase(s) from *A. faecalis* ATCC 8750 and *A. faecalis* JM3.[3,32,33] The forward primer Nit-Nde-(5′-AAGGAGGACATATGCAGACAAGAAAAATCGTCCGGG-3′) and the

reverse primer Nit-Bam-CHT (5′-AGGATCCG-GACGGTTCTTGCACCAGTAG-3′) were used to amplify a 1071 bp fragment containing the nitrilase gene. To facilitate cloning of the PCR product, *Nde*I and *Bam*HI sites were added to the primers (underlined sequences in the primers represent the restriction site for the respective enzyme). All PCR reactions contained 10–100 ng genomic DNA, 0.5 μM of each forward and reverse primer (Operon Biotechnologies, Cologne, Germany), 10 mM Tris/HCl (pH 9), 50 mM KCl, 2.5 mM MgSO4, 0.2 mM dNTPs (Pharmacia Biotech) and 2.5 U *Pwo* DNA-polymerase (Boehringer, Mannheim, Germany) in a volume of 25 μL. The PCR reactions were performed in a PTC 200 thermal cycler (MJ Research Inc, Waltham, MA). The PCR protocol comprised an initial denaturation step (96 °C, 3 min), followed by 30 cycles consisting of a denaturation step at 94 °C for 30 sec, an annealing step at 65 °C for 30 sec and an elongation step at 72 °C for 1 min. The PCR products were separated by electrophoresis using 1 % (w/v) agarose gel at 10 $V\,cm^{-1}$ and DNA bands were stained with ethidium bromide, eluted from the gel and purified using a Nucleospin Extract Kit (Macherey–Nagel, GmbH, Germany). The PCR product was digested with *Nde*I and *Bam*HI and inserted into *Nde*I/*Bam*HI cleaved plasmid vector pJOE2775[21] under the control of a rhamnose inducible promoter, *rha*BAD to give pAFN. The native stop codon was replaced by $(CAT)_6TGA$ which encodes a C-terminal His_6 tag.

Purification of Nitrilase

Cells of *E. coli* JM109 (pAFN) were grown overnight in 2× YT medium with ampicillin (100 $\mu g\,mL^{-1}$). The cells were subsequently diluted (1:100 v/v) in 600 mL fresh medium. Rhamnose (0.2 % w/v) was added to induce the nitrilase gene when the optical density at 600 nm (OD_{600nm}) of the bacterial cultures reached about 0.25. The cells were harvested after 5 h by centrifugation (15 min, 4 °C, 10,000 × *g*), washed twice with 20 mL sodium phosphate buffer (10 mM, pH 7.5) and resuspended in 10 mL of the same buffer. The cells were disrupted using a French Pressure Cell (16,000 psi) and the homogenate was centrifuged (45 min, 4 °C, 50,000 × *g*) to obtain the crude cell extract. The supernatant (soluble protein) was used for further experiments. Since the recombinant nitrilase carried a C-terminal affinity tag of six consecutive histidine residues, it was purified using the QIAexpress nickel-nitrilotriacetic acid (Ni-NTA) protein purification system (Qiagen, Hilden, Germany). Spin columns were packed with 1 mL of Ni-NTA agarose matrix. The protein purification was done with about 30–40 mg of protein per ml Ni-NTA agarose. The columns were subsequently washed with sodium phosphate buffer (20 mM, pH 7.5) containing 150 mM NaCl, 1 mM β-mercaptoethanol and 50 mM imidazole. Finally, the nitrilase was eluted with sodium-phosphate buffer (20 mM, pH 7.5) containing 150 mM NaCl, 1 mM β-mercaptoethanol and 250 mM imidazole. The imidazole was removed from the active fraction on a PD-10 column (Amersham Biosciences, Uppsala, Sweden) using sodium-phosphate buffer (10 mM, pH 7.5) containing 1 mM β-mercaptoethanol as eluent buffer.

SDS-PAGE

Denaturing polyacrylamide gel electrophoresis was performed on 12 % SDS polyacrylamidegels with a Tris-glycine buffer (pH 8.8) system.[34] The gels were routinely stained with Coomassie blue R250.

Oxidation of Dextran

66 mg dextran (100–200 kDa) were dissolved in 2 mL water and 150 mg sodium metaperiodate were added. The resulting solution was stirred at room temperature for 90 min and then transferred to dialysis tubes with a molecular weight cut-off of 12 kDa. The dialysis was performed for 3 h under stirring against water which was changed 5 times.

Preparation of Aggregates

A preparation of the purified nitrilase (10 μL, 5 $mg\,mL^{-1}$) was added to a solution of sodium-phosphate buffer (50 mM, pH 7.5) containing an appropriate concentration of precipitant (organic solvent, 20–90 % v/v) in a total volume of 100 μL. The mixtures were shaken intermittently for 5 min at 4 °C. The suspensions were centrifuged at 6000 × *g* (4 °C, 20 min) and the precipitated nitrilase aggregates collected. Finally, the recovered enzyme activity in the aggregates was measured by transferring them in the aqueous buffer media.[22] The solvents used for precipitation were methanol (MeOH), ethanol (EtOH), isopropyl alcohol (IPA), *n*-propanol (PrOH), dimethyl sulfoxide (DMSO), 1,4- dioxan, acetone, dimethylformamide (DMF), and 1,2-dimethoxyethane (DME).

Preparation of CLEAs

CLEAs were obtained by adding 10 μL of a purified preparation of the nitrilase (5 $mg\,mL^{-1}$) to a solution of sodium phosphate buffer (50 mM, pH 7.5) containing an appropriate concentration of cross-linking agent [glutaraldehyde (GA), poyethyleneimine (PEI) or dextran polyaldehyde (DPA)] (in a total volume of 100 μL). Finally, precipitant in the form of an organic solvent was added and the total mixture was shaken (300 rpm) at 4 °C for 2 h after which the volume of solution was made up to 1 mL with buffer to stop cross-linking. The insoluble CLEAs were collected by centrifugation (6000 × *g*, 4 °C, 30 min) and washed twice with sodium phosphate buffer (50 mM, pH 7.5) before use.

Biotransformation Conditions

Reactions were performed in sodium phosphate buffer (50 mM, pH 7.5) in a volume of 1 mL with purified nitrilase, Nit-aggregates or Nit-CLEAs. The nitrilase preparations (10 μl, 5 $mg\,mL^{-1}$) or the resulting CLEAs/aggregates prepared from enzyme preparation of the same concentration were added to the buffer. For the activity determinations with the supernatants obtained after preparation of CLEAs, the unrecovered nitrilase activity was estimated after diluting the sample sufficiently to obtain less than 1 % solvent content. The reactions were initiated by addition of mandelonitrile in a final concentration of 10 mM (stock concentration of 1.2 M in methanol) and incubated at 30 °C. Samples were taken at different time intervals and 1 M HCl was added to

stop the reaction. The samples were centrifuged (6000 × *g*, 4 °C, 30 min) and the supernatants analyzed by HPLC.

Scanning Electron Microscopy (SEM)

The CLEA preparations were dehydrated on a lyophilizer (Heto Dry Winer, Model DW-1–110, Denmark) and mounted on aluminum SEM stubs using a double sticky tape. The mounted samples were sputter coated with a thin layer of gold at 10 Torr vacuum (Jeol fine coat, ion sputter, JFC 1100, Japan) and examined by electron microscopy (Jeol Electron Microscope, JSM 1600, Japan) at an acceleration potential of 1.2 kV.

Analytical Methods

Nitrile hydrolysis was analysed by HPLC (Millenium Chromatography Manager 3.2, equipped with a diode array detector 996 and HPLC pump M510; Waters Associates, Milford, MA, USA). For the achiral analysis, a reversed phase column [250 mm × 4 mm (internal diameter); GROM] filled with 5 μm diameter particles of Licrospher RP18 (E. Merck, Darmstadt, Germany) was used to identify the individual compounds which were detected spectrophotometrically at 210 nm. Methanol (40 % v/v) and phosphoric acid (0.1 % v/v) in water was used as mobile phase to analyze the biotransformation. Separation of the enantiomers of mandelic acid was achieved on a chiral-HSA column (ChromTech, Hägersten, Sweden). The mobile phase consisted of sodium phosphate buffer (100 mM, pH 7) containing 4.5 % (v/v) acetonitrile.

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