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Mass transfer limitation as a tool to enhance the enantiomeric excess in the enzymatic synthesis of chiral cyanohydrins

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Abstract

The enantioselective synthesis of cyanohydrins catalyzed by *R*-hydroxynitrile lyase in an aqueous-organic liquid two-phase system using, mass transfer limitation to enhance enantiomeric excess at 5°C and pH 5.5 is described. Benzaldehyde, a good substrate, and cinnamaldehyde, a notoriously difficult substrate, were used as model substrates and compared in order to establish the mass transfer limitation concept in a two-liquid phase system, where the non-enzymatic-racemic reaction competes. Enzyme concentration and phase volume ratio between organic and buffer phase were geared to one another to enhance the enantiomeric excess for each substrate. In both cases, after optimization, excellent chemical conversion (>99% on a 60 mmol scale), high throughput and high enantiomeric excess (benzaldehyde >99% and cinnamaldehyde >96%) were achieved.

Keywords: Mass transfer limitation model; Mass transfer limiting enzyme concentration; *R*-hydroxynitrile lyase; Cyanohydrins; HCN

1. Introduction

The enantiospecific formation of a carbon–carbon bond by enzyme-catalyzed addition of hydrogen cyanide to aldehydes constitutes an efficient route to chiral cyanohydrins. These cyanohydrins are versatile chiral building blocks for fine-chemistry. Recent examples of their use include conversions into α-hydroxy acids [1], β-amino alcohols [2] and β-hydroxy nitrones [3].

The synthesis of cyanohydrins catalyzed by almond (*Prunus amygdalus*) *R*-hydroxynitrile (*R*-Hnl) lyase (EC 4.1.2.10) was first described in 1908 [4]. Its application has been rediscovered and expanded to various substrates in the second part of the last century [5]. Ground, defatted almonds are a source of *R*-Hnl lyase, an excellent catalyst for the synthesis of large quantities of chiral (*R*)-cyanohydrins starting from prochiral aldehydes [6]. Earlier studies from our laboratories on these defatted almonds containing the enzyme were devoted to optimizing the reaction conditions to improve the enantiomeric excess (EE), notably for croton-aldehyde (from 69 to 99% EE) [7]. The use of *S*-Hnl from different sources allowed the formation of the corresponding (*S*)-cyanohydrins [8]. In contrast to *R*-Hnl, *S*-Hnl contains no FAD group [9].

Effenberger et al. [10] described the enantiospecific addition of hydrogen cyanide to aldehydes in the presence of immobilized *R*-Hnl in an organic solvent

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system to give optically active (*R*)-cyanohydrins. Such systems have several advantages over biocatalysis in aqueous media [11]. The use of organic solvents may greatly enhance the solubility of the substrate for a given system and suppress the non-enzymatic reaction. Recovery of product may be easier and substrate and/or product inhibition may be reduced. However, there are limitations to these systems, in particular, enzyme activity and stability may be limited in the organic phase. A detailed study on the preparation of enantiomerically pure cyanohydrins, avoiding the problem of the spontaneous non-enzymatic-racemic reaction, was published by Kyler et al. [12]. Their synthesis was carried out by enzymatic transcyanation of several aliphatic and aromatic aldehydes with acetone cyanohydrin in an ether-aqueous biphasic solvent system. Such a two-phase system is of interest, where the enzyme is present in the aqueous buffer phase and where the bulk of the substrate and the product is maintained in the organic phase, and was also described by Loos et al. [13], using HCN. It was expected that by using the purified enzyme, activity and stability could be combined with suppression of the non-enzymatic-racemic reaction, thus optimizing the reaction parameters by using a statistic method, the so-called D-optimal design. Loos et al. concluded that methyl *t*-butyl ether (MTBE) in combination with an aqueous citric acid buffer forms a suitable biphasic solvent system for the synthesis of (*R*)-cyanohydrins. At pH 5.0 and 20°C, a combination of high conversion and high EE was achieved and changes, both in pH and in temperature, showed a large effect on the EE. An intrinsic drawback of this approach is the large number of experiments necessary to optimize the conversions and EE for each substrate aldehyde. The pH optimum is 5.5 for *R*-Hnl [19]. Recently, Griengl et al. [14] reported the use of an aqueous biphasic solvent system for an *S*-Hnl. It was found that the transformation proceeded most efficiently at temperatures between 5 and 15°C with hydrogen cyanide present in molar excess.

In the present study, we describe the synthesis of chiral cyanohydrins of high enantiomeric purity catalyzed by *R*-Hnl lyase in a similar liquid-two phase system, where the non-enzymatic-racemic reaction competes with the enzymatic reaction. Mass transfer rates as well as reaction rates and thereby enzyme concentration are important parameters in two-liquid phase biocatalysis, both contributing to the overall process kinetics [15]. The concept of mass transfer limitation in enhancing the EE, should be taken into account to optimize the reaction. This will be shown for two substrates: benzaldehyde, the product of the enzyme's natural reaction, and cinnamaldehyde, a notoriously difficult substrate [16].

2. Theory

2.1. Competition of the enzymatic and the parallel non-enzymatic-racemic reaction

The rates of the enzymatic and parallel non-enzymatic-racemic reactions are a function of the concentration of both substrates. It is assumed that the enzyme enantiomeric ratio (E) [17] is at least 100, implying that the enzyme is almost completely selective for the *R*-enantiomer and that formation of the *S*-enantiomer by *R*-Hnl can be neglected and occurs only because of the spontaneous non-enzymatic-racemic reaction. Simplified rate equations, neglecting reversibility (because at pH 5.5 and in the presence of an excess of HCN the reaction may in first approximation be regarded as irreversible) and inhibition, are shown in Eqs. (1) and (2), where r_C and r_E represent the rates of the parallel non-enzymatic-racemic reaction and the enzymatic reaction, respectively.

$$r_C = k' c_{Ald} \tag{1}$$

$$r_E = k'_{cat} \frac{c_{Ald}}{K_{m,Ald} + c_{Ald}} c_E \tag{2}$$

Assuming that we are able to keep c_{Ald} low by mass transfer limitation, $c_{Ald} \ll K_{m,Ald}$ and Eq. (2) simplifies Eq. (3), where $k'' = k'_{cat}/K_{m,Ald}$.

$$r_E = k'' c_{Ald} c_E \tag{3}$$

The non-enzymatic-racemic reaction results in a racemic product and should therefore be suppressed. In our model for the cyanohydrin synthesis (see below), it is assumed that the reactions only take place in the aqueous buffer phase and that, under the conditions used, no reaction occurs in the organic layer. As $K_{m,Ald}$ often is very low (e.g. 16 μM for benzaldehyde [18]), working at aldehyde concentrations that are much larger than $K_{m,Ald}$ only favors the

non-enzymatic-racemic reaction. Thus, a potentially effective way to suppress the non-enzymatic-racemic reaction is to keep the aldehyde concentration in the buffer phase low. Combining Eqs. (1) and (3), the ratio r_E/r_C is only dependent on the concentration of enzyme (c_E), as shown in Eq. (4).

$$\frac{r_E}{r_C} = \frac{k''}{k'} c_E \tag{4}$$

The EE increases if r_E/r_C increases, therefore high enzyme concentrations are beneficial to enhance the EE. If Eq. (2) cannot be simplified to Eq. (3), because the aldehyde concentration is too high, the ratio r_E/r_C will be smaller than the maximum value given by Eq. (4).

2.2. Mass transfer limitation in a two-liquid phase system and the mass transfer rate limiting enzyme concentration

Mass transfer limitation in a two-liquid phase system can be used as a tool to keep the substrate (aldehyde) concentration low. Fig. 1 shows a model for the enzymatic and non-enzymatic-racemic modus of cyanohydrin formation in an aqueous buffer (b)/organic (o) two-phase system.

In this figure substrates dissolved in the organic phase diffuse into the aqueous phase until a dynamic equilibrium, steady state, is reached. The substrate concentrations at equilibrium in each phase, buffer phase (c_b^*), and organic phase (c_o^*) are related by an equilibrium partition coefficient (m_s), as defined in Eq. (5).

$$m_s = \frac{c_o^*}{c_b^*} \tag{5}$$

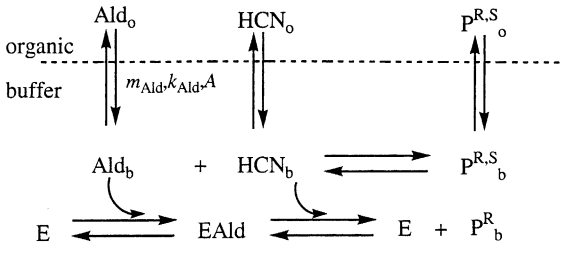


Fig. 1. Enzyme (E), enzyme aldehyde complex (EAld), concentration *R*-product in buffer layer (P_b^R), concentration aldehyde in buffer layer (Ald_b), concentration HCN in buffer layer (HCN_b), partition coefficients (m_{Ald}), mass transfer coefficients (k_{Ald}), interfacial area (A), concentration aldehyde in organic layer (Ald_o), concentration HCN in organic layer (HCN_o), concentration product in organic layer ($P_o^{R,S}$).

Because an excess of HCN is used, the concentration in the buffer phase is in excess due to its partition coefficient (2.6), we assume that it is sufficient to study only the aldehyde transfer process. The substrate transfer rate (STR) can be expressed as a function of the difference in concentration between the organic phase ($c_{Ald,o}$) and the buffer phase ($c_{Ald,b}$) multiplied by the partition coefficient (m_{Ald}), which gives the aldehyde equilibrium concentration in the buffer ($m_{Ald}c_{Ald,b}$) [15], in Eq. (6).

$$\mathrm{STR} = k_{Ald} A (c_{Ald,o} - m_{Ald} c_{Ald,b}) \tag{6}$$

In this equation, A ($m^2\,m^{-3}$) is related to the buffer volume (V_b), the organic volume (V_o), thereby the phase volume ratio (PVR) and the interfacial area in the reactor. The abbreviation k_{Ald} is the mass transfer coefficient through this liquid–liquid interface. The mass transfer coefficient (k_{Ald}) is characteristic for the temperature, the organic solvent, the geometry of the reaction vessel, the type of stirrer and the stirring speed.

Increasing the enzyme concentration in the V_b will result in a mass transfer rate limiting enzyme concentration ($c_{E,MTL}$), which is the enzyme concentration, where mass transfer of aldehyde becomes a limiting factor and the aldehyde concentration in the buffer phase decreases (see Fig. 2a). The rate of conversion than becomes equal to the maximum STR = $k_{Ald}Ac_{Ald,o}$ and independent of c_E (see Fig. 2b).

Below this mass transport rate limiting enzyme concentration ($c_{E,MTL}$) the EE will not reach its maximum value. When the ratio r_E/r_C is fixed, the EE progression in time will turn into a horizontal line. Increasing the mass transfer area by increasing the buffer phase volume V_b, while keeping the enzyme concentration constant at $c_{E,MTL}$, will result in higher process rates. Therefore, it is important to determine $c_{E,MTL}$. Increasing the productivity, while maintaining the maximum EE in time can be achieved by increasing V_b with the same $c_{E,MTL}$. Finally, to reach the highest EE, the benzaldehyde concentration must be kept low, it is important to start the reaction by adding benzaldehyde, after first adding the enzyme.

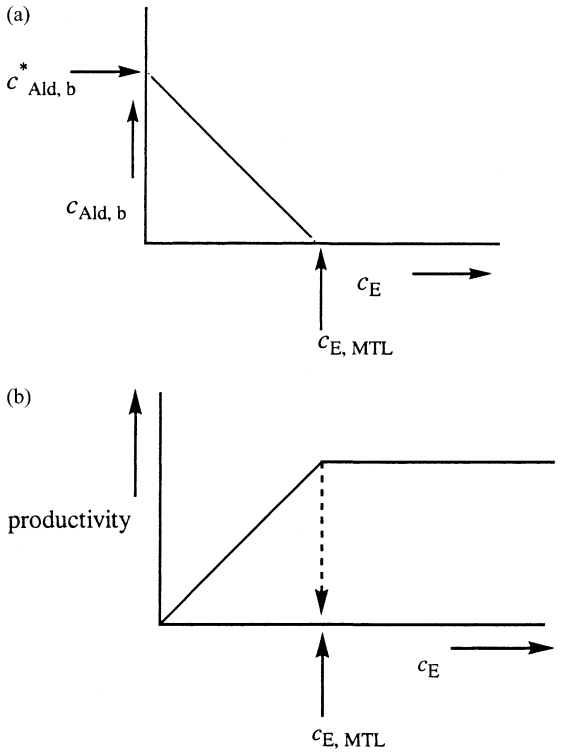
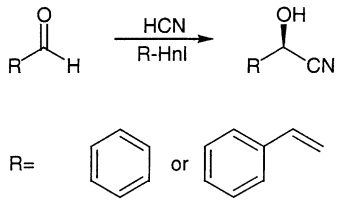


Fig. 2. Simplified schematic representation of the effect of (a) increasing the enzyme concentration c_E at the mass transfer rate limiting enzyme concentration ($c_{E,MTL}$), where the aldehyde concentration in the buffer phase becomes very low and (b) mass transfer becomes a limiting factor and the productivity becomes equal to the maximum STR.



Scheme 1. Synthesis of chiral cyanohydrins.

3. Results and discussion

The formation and the EE of the cyanohydrins of benzaldehyde (mandelonitrile) and cinnamaldehyde, using *R*-Hnl lyase were monitored in time (reaction shown in Scheme 1). The reaction conditions were chosen on the basis of preliminary results. For the *R*-Hnl from almonds the pH value of 5.5 is the optimal value [19]. Using citrate as the buffer, the enzyme was found to be stable. The reaction temperature, pH, stirrer type (so-called clock stirrer), reaction vessel and solvent system (MTBE/aqueous buffer) were not varied. To determine $c_{E,MTL}$ for the two substrates (benzaldehyde and cinnamaldehyde), c_E was varied, while the buffer phase volume V_b was kept constant. Slight deviations in some other variables are considered to be insignificant. The results are presented in Tables 1 and 2 and in Figs. 3 and 4.

The reaction rate expressed as $\tau_{1/2}$, increases until a limiting enzyme of concentration is reached. In the case of benzaldehyde using more than $1\,g\,l^{-1}$ enzyme/buffer showed no significant additional increase in the reaction rate and EE. In the case of cinnamaldehyde, this situation is reached when more

Table 1
Formation of mandelonitrile using *R*-Hnl at different values of c_E

Entry	c_E[a] ($g\,l^{-1}$)	V_b (ml)	V_o (ml)	Ald[b] (mmol)	HCN (mmol)	$c_{HCN,b}$[c] (M)	$c_{HCN,b}$[d] (M)	$\tau_{1/2}$[e] (min)	Conversion (%)	EE[f]
1a	0.12	15	120	65.8	207	0.63	0.43	1260	69	0.67
1b	0.48	11	120	61.4	176	0.54	0.36	261	>98	0.98
1c	1	11	120	56.8	173	0.53	0.36	110	99	0.99
1d	3	11	120	59.4	173	0.53	0.35	95	>99	0.99

[a] The amount of enzyme is expressed by concentration in the buffer volume.
[b] Initial amount of aldehyde or HCN.
[c] Calculated concentration of HCN in the buffer phase at the start of the reaction. Partition coefficient 2.6.
[d] Calculated concentration of HCN in the buffer phase at the end of the reaction.
[e] Time to 50% conversion ($\tau_{1/2}$).
[f] Reaction is approaching completion after 3 days and the EE did not change during the reaction.

Table 2
Formation of cyanohydrin of cinnamaldehyde using *R*-Hnl at different values of c_E

Entry	c_E[a] ($g\,l^{-1}$)	V_b (ml)	V_o (ml)	Ald[b] (mmol)	HCN (mmol)	$c_{HCN,b}$[c] (M)	$c_{HCN,b}$[d] (M)	$\tau_{1/2}$[e] (h)	Conversion (%)	EE[f]
2e	3	2.5	120	40	180	0.56	0.44	90	82	0.85
2f	5	2.5	120	40	180	0.56	0.44	75	93	0.96
2g	7	2.5	120	43	180	0.56	0.43	80	95	0.96

[a] The amount of enzyme is expressed by concentration in the buffer volume.
[b] Initial amount of aldehyde or HCN.
[c] Calculated concentration of HCN in the buffer phase at the start of the reaction. Partition coefficient 2.6.
[d] Calculated concentration of HCN in the buffer phase at the end of the reaction.
[e] Time to 50% conversion ($\tau_{1/2}$).
[f] Reaction is approaching completion after 8 days and the EE did not change during the reaction.

than $5\,g\,l^{-1}$ enzyme/buffer is used. Thus, depending on the aldehyde, the so-called $c_{E,MTL}$ can be found in a straightforward manner. Clearly, there exists a $c_{E,MTL}$, and under constant stirring conditions, the $c_{E,MTL}$ is $1\,g\,l^{-1}$ for benzaldehyde and $5\,g\,l^{-1}$ for cinnamaldehyde. Above these values, there is no significant increase in rate or in the maximum EE-value that is reached. To determine the influence of the STR on the total reaction rate (productivity) and EE, for both substrates (benzaldehyde and cinnamaldehyde), c_E was kept constant at $c_{E,MTL}$, using different buffer volumes and a constant MTBE volume (120 ml). Assuming that the buffer droplets keep the same size, increasing the buffer volume means that the interfacial area and the STR increase. The results are presented in Tables 3 and 4 and in Figs. 5 and 6.

Figs. 5 and 6 show the conversion of benzaldehyde and cinnamaldehyde versus time when the buffer volume was varied, with c_E fixed at $c_{E,MTL}$. Using more buffer, implying increase of the interfacial

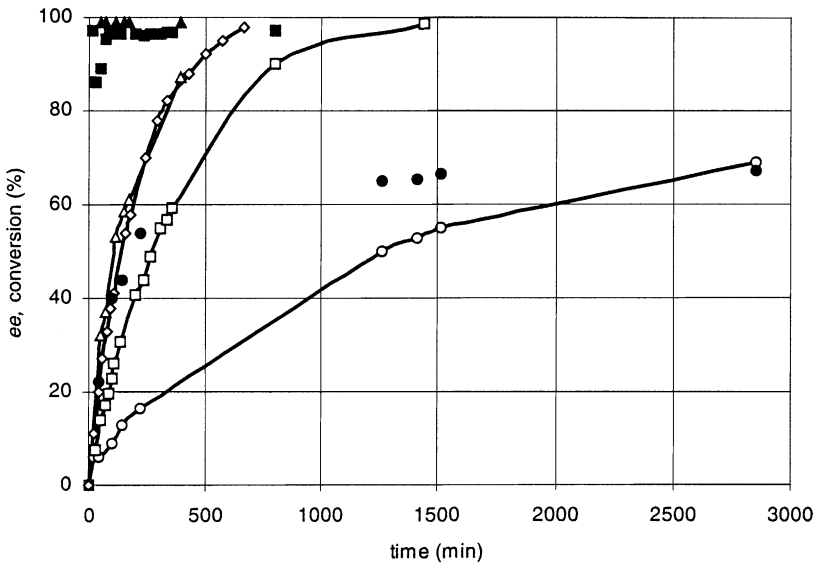


Fig. 3. Conversion of benzaldehyde and HCN into its corresponding chiral cyanohydrin using different enzyme concentrations. Curves are trend lines, open markers the extent of conversion, closed markers the EE of the cyanohydrin; (○, ●): 0.12 mg enzyme per ml buffer (1a); (□, ■): 0.48 mg enzyme per ml buffer (1b); (△, ▲): 1 mg enzyme per ml buffer (1c); (◇): 3 mg enzyme per ml buffer (EE > 0.99, for clarity not shown) (1d).

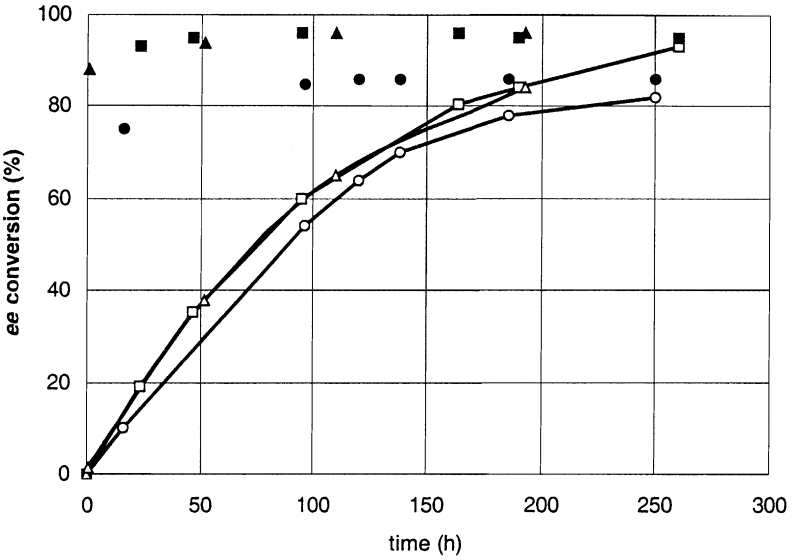


Fig. 4. Conversion of cinnamaldehyde and HCN into chiral cyanohydrin using different enzyme concentrations. Curves are trend lines, open markers the extent of conversion, closed markers the EE of the cyanohydrin; (○, ●): 3 mg enzyme per ml buffer (2e); (□, ■): 5 mg enzyme per ml buffer (2f); (△, ▲): 7 mg enzyme per ml buffer (2g).

Table 3
Formation of mandelonitrile using *R*-Hnl with variation of the buffer volume using mass transfer limitation and at a fixed enzyme concentration $c_{E,MTL}$

Entry	c_E[a] ($g\,l^{-1}$)	V_b (ml)	V_o (ml)	Ald[b] (mmol)	HCN[b] (mmol)	c_{HCN}[c] (M)	c_{HCN}[d] (M)	$\tau_{1/2}$[e] (min)	Conversion (%)	EE[f]
1c	1	11	120	56.8	173	0.53	0.36	110	>99	0.99
1h	1	20	120	54.7	173	0.52	0.35	90	>98	0.99
1i	1	105	120	62.7	180	0.43	0.28	15	>98	0.98

[a] The amount of enzyme is expressed by concentration in the buffer volume.
[b] Initial amount of aldehyde or HCN.
[c] Calculated concentration of HCN in the buffer phase at the start of the reaction. Partition coefficient 2.6.
[d] Calculated concentration of HCN in the buffer phase at the end of the reaction.
[e] Time to 50% conversion ($\tau_{1/2}$).
[f] Reaction is approaching completion after 8 days and the EE did not change during the reaction.

Table 4
Formation of cyanohydrin from cinnamaldehyde using *R*-Hnl with variation of the buffer volume, using mass transfer limitation and at a fixed enzyme concentration $c_{E,MTL}$

Entry	c_E[a] ($g\,l^{-1}$)	V_b (ml)	V_o (ml)	Ald[b] (mmol)	HCN[b] (mmol)	c_{HCN}[c] (M)	c_{HCN}[d] (M)	$\tau_{1/2}$[e] (h)	Conversion (%)	EE[f]
2f	5	2.5	120	40	180	0.57	0.44	75	93	0.96
2j	5	12	120	40	180	0.56	0.43	25	93	0.96
2k	5	120	120	52	207	0.43	0.31	19	97	0.97

[a] The amount of enzyme is expressed by concentration in the buffer volume.
[b] Initial amount of aldehyde or HCN.
[c] Calculated concentration of HCN in the buffer phase at the start of the reaction. Partition coefficient 2.6.
[d] Calculated concentration of HCN in the buffer phase at the end of the reaction.
[e] Time to 50% conversion ($\tau_{1/2}$).
[f] Reaction is approaching completion after 3 days and the EE did not change during the reaction.

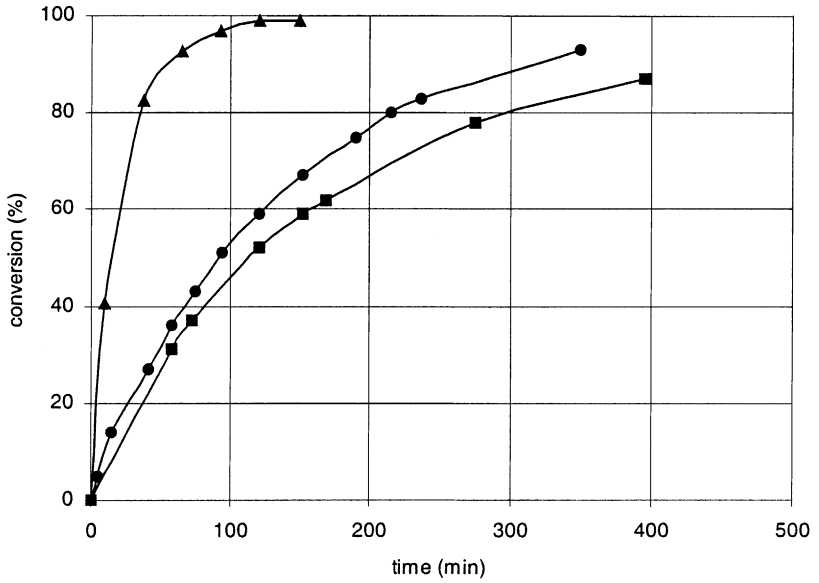


Fig. 5. Conversion of benzaldehyde and into chiral mandelonitrile using $1\,mg\,ml^{-1}$ enzyme and different volumes of buffer. Curves are trend lines, markers are the extent of conversion; (■): $V_b = 11$ ml (1c); (●): $V_b = 20$ ml (1h); (▲): $V_b = 105$ ml (1i).

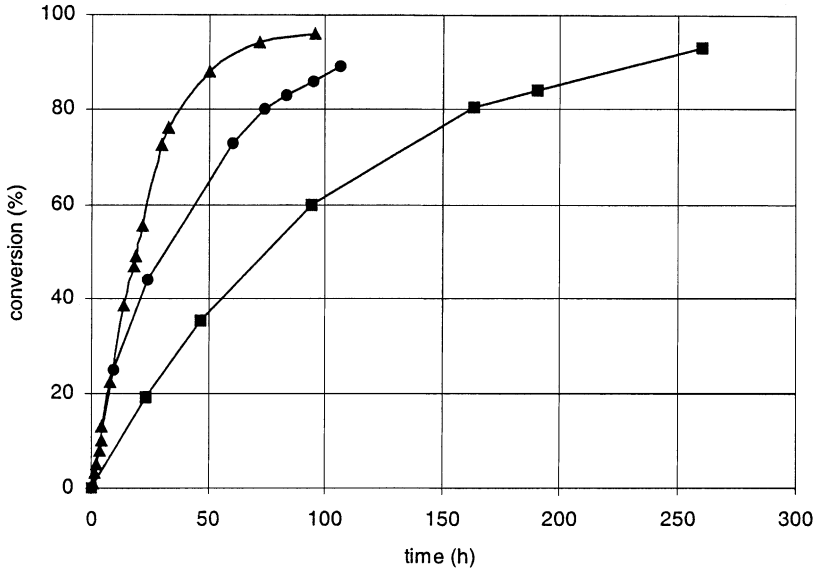


Fig. 6. Conversion of cinnamaldehyde into the corresponding chiral cyanohydrin using $5\,mg\,ml^{-1}$ enzyme and different volumes of buffer. Curves are trend lines, markers are the extent of conversion; (■): $V_b = 2.5$ ml (2f); (●): $V_b = 12$ ml (2j); (▲): $V_b = 120$ (2k).

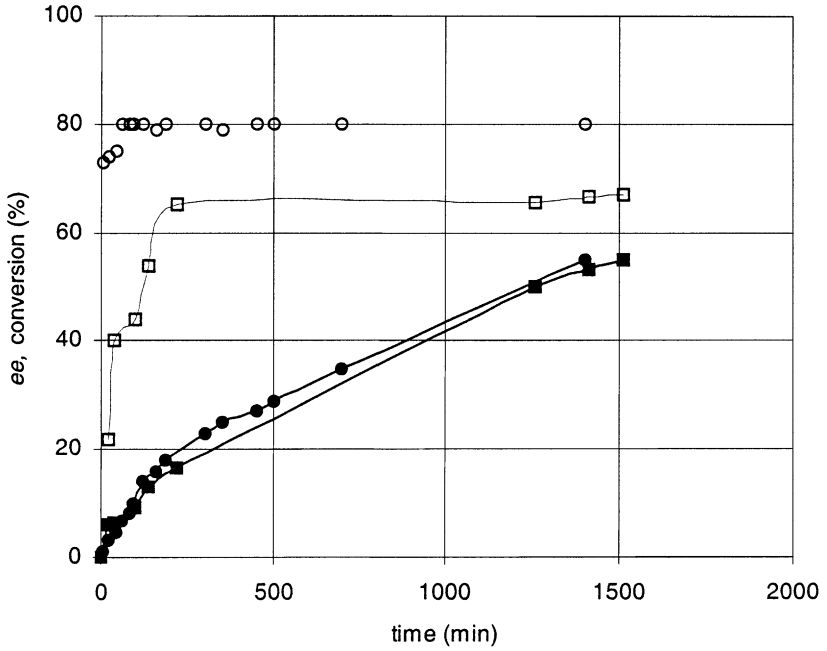


Fig. 7. Conversion of benzaldehyde into mandelonitrile vs. time using $c_E = 0.12\,mg\,ml^{-1}$ buffer, which is below the $c_{E,MTL}$ and two different start-up policies; (□, ■): (1a) start the reaction through adding pure benzaldehyde in one portion; (○, ●): start the reaction through adding benzaldehyde in MTBE slowly (5 min); (●, ■): conversion; (○, □): EE.

area, increases the mass transfer and thereby the total reaction rate (productivity), where the EE remains constant at its maximum value.

It is apparent from Fig. 3 that the initial EE is low, which leads to a sub-optimal EE at the end. To determine the origin of this phenomenon two sets of reactions beneath the $c_{E,MTL}$ were followed in time. Two alternative start-up policies were used for benzaldehyde, either adding the pure aldehyde in one portion or adding it dissolved in MTBE over a short period of time. The hypothesis is that the second approach more easily leads to coalescence of aldehyde-containing and pure MTBE droplets in the reactor, so that the aldehyde levels in the buffer phase are minimized. The results are presented in Fig. 7.

Clearly, the starting method influences the initial EE and is a crucial factor for EE progression of the process. In the case where the reaction was started by slowly adding benzaldehyde, dissolved in MTBE, the initial EE started relatively high compared to the experiment, where the pure aldehyde was added in one portion. So, the reaction should be started by adding the dissolved aldehyde to the reaction vessel in the course of a few minutes.

4. Conclusions

Benzaldehyde, the product of the natural reaction for the enzyme (*R*-Hnl lyase), and cinnamaldehyde, a notoriously difficult substrate for this enzyme, were used as model substrates and compared in order to investigate the effect of mass transfer limitation on the EE. The importance of mass transfer limitation and of the start-up procedure were proven. The EE during the formation of cyanohydrins can be described by the ratio of the reaction rates of the enzymatic and the parallel non-enzymatic-racemic reactions. Mass transfer limitation results in a low substrate concentration in the aqueous buffer phase and is therefore a powerful tool to increase the EE in the above described situation. Increasing the enzyme concentration at a constant interfacial area, accelerated the enzymatic reaction until a maximum was reached at the

so-called mass transfer limiting enzyme concentration ($c_{E,MTL}$). Increasing the interface area by increasing the buffer volume, at a constant enzyme concentration $c_{E,MTL}$, accelerated the process, while maintaining the maximum EE. In both cases, upon mass transfer optimization, excellent chemical conversion (99% on a 60 mmol scale), high throughput and high EE (benzaldehyde >99% and cinnamaldehyde >96%) were achieved. Using enzyme concentrations below the $c_{E,MTL}$ resulted in loss of EE. The start-up policy is also important, because it controls the initial substrate concentration in the buffer layer.

5. Experimental section

5.1. General methods and materials

The ^{1}H spectra were recorded on a JEOL FX-200 instrument. Samples were measured in $CDCl_3$, with Me_4Si as an internal standard for ^{1}H NMR; δ in ppm, J in Hz. Enantiomeric purities and conversions were determined by HPLC using a Chiralcel OD column (250 mm × 4.6 mm). As eluent, mixtures of hexane (H), isopropyl alcohol (I) and acetic acid (HAc), which are specified in each case, were applied. Flow = 1 ml min^{-1}. All cyanohydrins were also prepared in racemic form to optimize the conditions for peak separation. Optical rotations were measured using a propol automatic polarimeter. Melting points were measured with a Büchi melting-point apparatus and are uncorrected. Spectrophotometer: Varian DMS 200 UV–VIS. Acetic acid (99–100%) and isopropyl alcohol (99.5%) were purchased from Baker, hexane (HPLC grade) from Biosolve, MTBE (99%) from Acros, benzaldehyde (99%) from Merck, cinnamaldehyde (99%) from Aldrich and NaCN (p.a.) from Baker. The enzyme *R-Pa*Hnl was kindly supplied by Solvay Pharmaceuticals (purity 65%, specific activity 185,000 IU g^{-1}).

5.1.1. Reaction vessel and stirrer

The reaction vessel was a cylindrical double-walled glass vessel (diameter of 60 mm, volume of 225 ml) in which the liquid phases were mixed by a top-driven, liquid induced impeller (a so-called clock stirrer), shaft diameter of 8.0 mm and in the extension of the axis a cylindrical cavity (high × diameter: 30.0 mm × 15.9 mm), four hollow cylinders (diameter of 9.0 mm) form the blades with a total length of 37.5 mm in diameter.

5.1.2. Stability measurements

Preliminary studies were conducted to select buffers suitable for kinetic studies in a two phase system. With cinnamaldehyde as the substrate, no effect on enzyme activity was observed by varying the concentration of citrate buffer at pH 5.5 between 1 mM and 0.1 M. Rates of cyanohydrin decomposition were measured spectrophotometrically by monitoring the carbonyl absorption band of the free aldehyde using a UV–VIS spectrophotometer. All measurements involving benzaldehyde were made at its absorption maximum (220 nm), for cinnamaldehyde a wavelength of 261.5 nm was used.

5.1.3. Determination of the concentration and partition coefficients for benzaldehyde and HCN

The partition coefficient of HCN between MTBE and buffer (sodium citrate pH 5.5, 0.1 M) was determined by titration with sodium hydroxide. The partition coefficient of HCN was 2.6 at 20°C. The partition coefficient of benzaldehyde between MTBE and buffer (sodium citrate pH 5.5, 0.1 M) was determined gravimetrically. Benzaldehyde (5.8 g) dissolved in 50 ml MTBE was mixed with 50 ml buffer and equilibrated in a round bottom flask for 30 min at 20°C. The two layers were separated. The MTBE layer was dried ($MgSO_4$) and evaporated. The yield was 5.74 g benzaldehyde. The buffer phase was extracted with MTBE (3 × 40 ml). The combined MTBE layers were dried ($MgSO_4$) and evaporated. The yield was 0.06 g benzaldehyde. The partition coefficient of benzaldehyde was therefore 96 at 20°C.

5.1.3.1. (R,S)-mandelonitrile (2-hydroxybenzeneacetonitrile). To a round bottom flask was added 10 ml of a 0.1 M sodium citrate buffer (pH 6.8) at 5°C. Meanwhile, 3 g of NaCN were dissolved in 35 ml of cold water. The pH of the solution was adjusted to 5.5 by addition of citric acid (caution: formation of toxic hydrogen cyanide). The hydrogen cyanide solution was extracted with MTBE (3 × 15 ml). The combined MTBE layers were transferred into a dropping funnel that was placed on the round bottom flask. Freshly distilled aldehyde (1.5 g) was added to the solution. After 24 h the reaction was complete, after separation

of the two layers, the organic layer was dried ($MgSO_4$) and evaporated. Pure *R*,*S*-mandelonitrile was obtained as a clear oil which solidified at 5°C. Yield: 98%; ^{1}H NMR: 3.15 (br, 1H, OH), 5.52 (s, 1H, H-C(2)), 7.49 (m, 5H-arom); HPLC: eluent H:I:HAc = 97:3:0.1, λ 220 nm.

5.1.3.2. (R,S)-3E-2-hydroxy-4-phenylbutenenitrile. It was prepared from (*E*)-3-phenyl-2-propenal 2.5 g (20 mmol) by the procedure described above. Conversion: 98%. The crude cyanohydrin was crystallized from CH_2Cl_2/*n*-hexane to give 3 g of light yellow colored crystals: mp 78°C; ^{1}H NMR: 3.96 (br, OH), 5.16 (d, 1H, J = 6.2, H-C(2)), 6.27 (dd, 1H, J_1 = 6.2, J_2 = 15.9, H-C(3)), 6.89 (d, 1H, J 15.9, H-C(4)), 7.39 (m, 5H-arom); HPLC: eluent H:I:HAc = 87:13:0.1, λ 261.5 nm.

5.1.3.3. (R)-mandelonitrile. In a thermostatically cooled double-walled reaction vessel at 5°C, 1 mg enzyme per ml buffer was added to 10–100 ml of a 0.1 M citrate buffer (pH 5.5). Meanwhile, 10 g of NaCN was dissolved in 100 ml of cold water. The pH of this solution was adjusted to 5.5 by addition of citric acid (caution: formation of toxic hydrogen cyanide). The hydrogen cyanide solution was extracted with MTBE (3 × 40 ml). The combined MTBE layers were transferred into a dropping funnel that was placed on the double-walled reaction vessel and the MTBE was added. After stirring for 10 min, freshly distilled benzaldehyde (50 mmol) was added in one portion or dissolved in 10 ml MTBE and added via drippings (2 s^{-1}). The enzyme mixture was stirred and the conversion of the reaction was monitored till the reaction had gone to completion. Sample were taken from the organic layer after separation by stopping stirring, dried ($MgSO_4$) and analyzed by HPLC. Finally, after separating the two layers, the organic layer was dried ($MgSO_4$) and concentrated in vacuo. The pure cyanohydrin was obtained as a colorless oil, which crystallized upon standing at 10°C. EE > 99% (HPLC: eluent H:I:HAc = 97:3:0.1, λ 220 nm); mp 20°C; $[\alpha]_D^{20}$ = +44.9 (c = 1, $CHCl_3$); ^{1}H NMR: 3.15 (br, OH), 5.52 (s, H-C(2)), 7.49 (m, 5H-arom).

5.1.3.4. (R)-3E-2-hydroxy-4-phenylbutenenitrile. In a thermostatically cooled double-walled reaction-vessel at 5 °C, 1–5 ml enzyme stock solution (200 mg/10 ml) was added to 5–100 ml of a 0.1 M citrate buffer (pH 5.5). Meanwhile, 10–15 g of NaCN was dissolved in 100 ml of cold water. The pH of this solution was adjusted to 5.5 by addition of citric acid (caution: formation of toxic hydrogen cyanide). The hydrogen cyanide solution was extracted with MTBE (3 × 40 ml). The combined MTBE layers were transferred into a dropping funnel that was placed on the double-walled reaction vessel and the MTBE was added in one portion. After 10 min, the (*E*)-3-phenyl-2-propenal (50 mmol) was added to start the reaction. The enzyme mixture was kept stirring for the next 8 days. The conversion of the reaction was followed by taking samples from the organic layer, after separation by stopping the stirring, and analyzed by HPLC till the reaction almost stopped. After separating the two layers the organic layer was dried ($MgSO_4$) and concentrated in vacuo. The pure cyanohydrin was obtained as a yellow oil. The crude cyanohydrin was crystallized from CH_2Cl_2/*n*-hexane (eutecticum EE = 0.58), the yield was 4 g of light yellow crystals: mp 67.5°C; $[\alpha]_D^{20}$ = +30.5 (c = 1, $CHCl_3$) and the EE > 99% (HPLC: eluent H:I:HAc = 87:13:0.1, λ 261.5 nm); ^{1}H NMR: 3.96 (br, OH), 5.16 (d, J = 6.2, H-C(2)), 6.27 (dd, J_1 = 6.2, J_2 = 15.9, H-C(3)), 6.89 (d, J 15.9, H-C(4)), 7.39 (m, 5H-arom).

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References

[1] N. Ziegler, B. Hörsch, F. Effenberger, Synthesis (1990) 575.
[2] P. Zandbergen, J. Brussee, A. van der Gen, C.G. Kruse, Tetrahedron: Asymmetry 3 (1992) 769.
[3] J. Marcus, J. Brussee, A. van der Gen, Eur. J. Org. Chem. (1998) 2513.
[4] L. Rosenthaler, Z. Biochem. 14 (1908) 238.
[5] W. Becker, E. Pfeil, J. Am. Chem. Soc. 88 (1966) 4299.

[6] J. Brussee, E.C. Roos, A. van der Gen, Tetrahedron Lett. 29 (1988) 4485.
[7] P. Zandbergen, J. van der Linden, J. Brussee, A. van der Gen, Synth. Commun. 21 (1991) 1387.
[8] F. Effenberger, B. Hörsch, T. Förster, S. Ziegler, Tetrahedron Lett. 31 (1990) 1249.
[9] U. Hanefeld, A.J.J. Straathof, J.J. Heijnen, Biochim. Biophys. Acta 1432 (1999) 185.
[10] F. Effenberger, Th. Ziegler, S. Förster, Angew. Chem. Int. Ed. Engl. 26 (1987) 458.
[11] L.E.S. Brink, J. Tramper, K.Ch.A.M. Luyben, K. van't Riet, Enzyme Microb. Technol. 10 (1988) 736.
[12] V.I. Ognyanov, V.K. Datcheva, K.S. Kyler, J. Am. Chem. Soc. 113 (1991) 6992.
[13] W.T. Loos, H.W. Geluk, M.M.A. Ruijken, C.G. Kruse, J. Brussee, A. van der Gen, Biocatal. Biotrans. 12 (1995) 255.
[14] H. Griengl, N. Klempier, P. Pöchlauer, M. Schmidt, N. Shi, A.A. Zabelinskaja-Machova, Tetrahedron 54 (1998) 14477.
[15] J.M. Woodley, A.J. Brazier, M.D. Lilly, Biotechnol. Bioeng. 37 (1991) 133.
[16] S. Han, G. Lin, Z. Li, Tetrahedron: Asymmetry 9 (1998) 1835.
[17] A.J.J. Straathof, J.A. Jongejan, Enzyme Microb. Technol. 7 (1997) 556.
[18] W.F. Willeman, U. Hanefeld, A.J.J. Straathof, J.J. Heijnen, Enzyme Microb. Technol. 27 (2000) 423–433.
[19] E. Kiljunen, Biocatalysis for the preparation of enantiopure cyanohydrins, Ph.D. thesis, University of Turku, Turku, Finland, 1999.