UDC 577.152.4:547.49:633.93
ISSN 1330–9862 *original scientific paper*

(FTB-1098)

The Use of Lewis Cell to Investigate the Enzyme Kinetics of an *(S)*-Hydroxynitrile Lyase in Two-Phase Systems

Michael Bauer[1], *Roland Geyer*[1], *Herfried Griengl*[2] *and Walter Steiner*[1]*

[1]Institute of Biotechnology, Enzyme-Technology Group, SFB Biocatalysis
Graz University of Technology, Petersgasse 12, A-8010 Graz, Austria

[2]Institute of Organic Chemistry, SFB Biocatalysis
Graz University of Technology, Stremayrgasse 16, A-8010 Graz, Austria

Received: June 29, 2001
Accepted: November 8, 2001

Summary

(S)-Hydroxynitrile lyase from *Hevea brasiliensis* (HbHnl) (EC 4.1.2.39) catalyzes the reversible synthesis of chiral cyanohydrins from aldehydes or ketones and HCN. The enzymatic formation of *(S)*-mandelonitrile (MN) from benzaldehyde (BA) and HCN was studied in two-phase systems of buffer and organic solvents (diisopropyl ether, methyl-*t*-butyl ether) using a Lewis cell to investigate the interaction between mass transfer and the biocatalytic reaction. The enzymatic reaction rate in the aqueous phase saturated with organic solvents is drastically reduced in comparison to pure buffer due to the increase of the Michaelis-Menten constants of the substrates. Mass transfer of the substrates from the organic to the aqueous phase and mass transfer of the product in the opposite direction could be described by the two-film theory. The formation of *(S)*-mandelonitrile in the Lewis cell follows an aqueous phase distributed reaction model, which means that the enzymatic reaction takes place in the bulk of the aqueous phase and in the thin film close to the interface and/or directly at the interface. Using the Hatta number it could be shown that the mass transfer of benzaldehyde from the organic to the aqueous phase is enhanced by the biocatalytic reaction of the *(S)*-hydroxynitrile lyase from *Hevea brasiliensis*.

Key words: Lewis cell, two-phase system, mass transfer, enzyme kinetics, hydroxynitrile lyase, *Hevea brasiliensis*

Introduction

In their natural environment hydroxynitrile lyases from plants catalyze the cleavage of cyanohydrins from cyanogenic glycosides into the corresponding aldehydes or ketones and HCN (*1*). In the reverse reaction hydroxynitrile lyases can be used for the synthesis of enantiomerically pure cyanohydrins (*2–7*). Cyanohydrins can be converted into a wide range of chiral compounds that are widely used for fine chemicals, pharmaceuticals and agrochemicals. Therefore hydroxynitrile lyases have become of growing industrial interest.

A problem during the synthesis of enantiopure cyanohydrins presents the formation of racemats from aldehydes or ketones and HCN by pure chemical reaction in aqueous systems at higher pH values (*8*). Therefore this parallel chemical reaction is responsible for the reduced enantiomeric excess of the cyanohydrin in an enzymatic process. Because hydroxynitrile lyase loses its activity below pH = 5 very quickly (*9,10*), it is necessary for organic synthesis to find proper reaction conditions where the enzyme is relatively stable and the chemical reaction

* Corresponding author; Phone: ++43-316-873-8420; Fax: ++43-316-873-8434; E-mail: steiner@biote.tu-graz.ac.at

can be suppressed almost completely. This can be achieved by the use of two-phase systems of aqueous buffer and organic solvent where the concentration of the substrates in the aqueous phase is low causing a reduced chemical reaction rate.

In recent years much attention has been given to water – water-immiscible organic solvent two-phase systems (*11–14*). Such systems offer the possibility to use high concentrations of poorly water-soluble substrates and/or products. Substrate and product inhibition is reduced due to their lower concentration in the aqueous environment where the enzymatic reaction takes place. Possible disadvantages are enzyme denaturation at the liquid-liquid interface and enzyme denaturation or inhibition by the organic solvent dissolved in the aqueous phase.

In our earlier papers we described the enzyme kinetics in aqueous solution in detail and studied parameters influencing stability and activity in two-phase systems of buffer and organic solvents (*15,16*). To get a deeper insight into two-phase systems we used a Lewis cell (*17*), which consists of a cylindrical vessel in which each phase is carefully stirred without disturbing the well defined interface between the two phases. A modified Lewis cell was first used by Woodley (*18–22*) to perform an analysis of the interaction between substrate transfer and a biocatalytic reaction. In this work we performed a Lewis cell study for HbHnl using the synthesis of *(S)*-mandelonitrile from benzaldehyde and HCN as a model reaction. This analysis yielded data concerning the location of the reaction, which is critical in determining the reaction kinetics.

Models for Mass Transfer and the Enzymatic Reaction

Kinetic models describing the behavior in a two-liquid phase biocatalytic reactor are strongly dependent on the location of the reaction. Three different cases can be identified (*21*): reactions which take place in the bulk of the aqueous phase, reactions which occur only at the liquid-liquid interface and reactions which are non-uniformly distributed between the aqueous phase and the interface. Consequently three different models were proposed: the aqueous phase bulk reaction model, the aqueous phase interfacial reaction model and the aqueous phase distributed reaction model.

In contrast to the Lewis cell studies performed by Woodley *et al.* (*21*) who used a graphic method to determine the location of the reaction we have developed a mathematical model to elucidate the overall biocatalytic reaction. We used this approach due to our complex reversible two substrate kinetics where the parallel chemical reaction and enzyme inactivation cannot be neglected.

Reactant mass transfer, enzyme kinetics in the aqueous phase saturated with organic solvent and the overall biocatalytic reaction in the Lewis cell were studied separately to determine the location of the reaction.

Model for the enzymatic reaction in the aqueous phase saturated with organic solvent

The enzymatic reaction in buffer saturated with organic solvent was described by a Bi Uni mechanism including enzyme inactivation and the parallel chemical reaction (Fig. 1) (*15*).

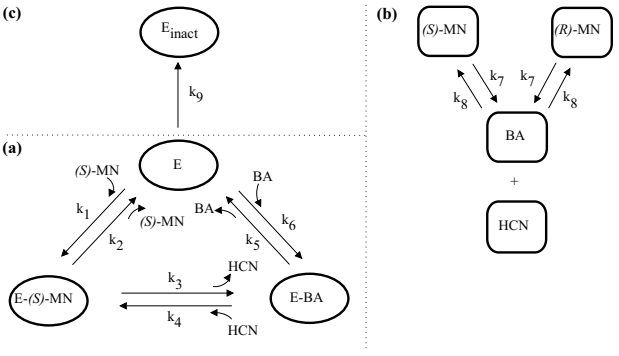

Fig. 1. Model of an Ordered Bi Uni mechanism (a), including enzyme inactivation (c) and the chemical parallel reaction (b)

As the kinetic constants are significantly different if organic solvent is dissolved in buffer the kinetic constants in buffer saturated with solvent were determined by progress curve analysis using the program Simusolv® (*15*).

Model for the enzymatic reaction in the Lewis cell

For the biocatalytic reaction in the Lewis cell an aqueous phase bulk reaction model was assumed (Fig. 2). The following steps take place during the biocatalytic conversion:

a) Substrate transfer of benzaldehyde and HCN from the organic phase to the aqueous phase.
b) Reversible stereoselective enzymatic reaction and reversible unspecific chemical reaction in the aqueous phase.
c) Product transfer of mandelonitrile to the organic phase.

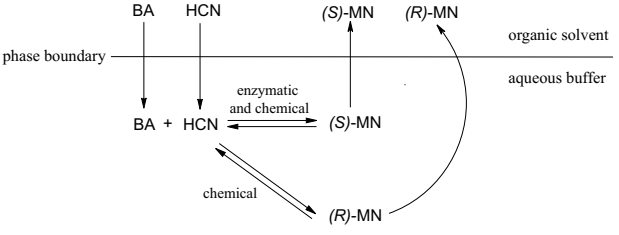

Fig. 2. Aqueous phase bulk reaction model for the formation of mandelonitrile in a two-phase system

To describe the overall reaction rate in the two-phase system terms for the reactant mass transfer have to be added to the model of the enzymatic reaction (Fig. 1). The differential equations for the resulting kinetic model

can be found in the appendix. Simulations were performed with the program Simusolv®.

Materials and Methods

Chemicals and enzyme

Benzaldehyde was obtained from Aldrich (Steinheim, Germany) and distilled under nitrogen before use to remove benzoic acid. Freshly distilled HCN was provided by the Institute of Organic Chemistry, TU-Graz. Purified racemic mandelonitrile was a gift from DSM-Chemie Linz (Linz, Austria). Diisopropyl ether and methyl-*t*-butyl ether were obtained from Fluka (Buchs, Switzerland). The Spectroquant® cyanide test was purchased from Merck (Darmstadt, Germany). All other chemicals were of p.a. quality.

The recombinant HbHnl expressed in *Pichia pastoris* was provided by Roche Molecular Biochemicals (Penzberg, Germany). The preparation contained protein in mass concentration 49.6 mg·mL^{-1} and had a specific activity of 98 IU·mg^{-1} of protein. About 70 % of the total protein in the preparation was the enzyme HbHnl. The clear yellow enzyme solution was stored at 4 °C (25 mM potassium phosphate, pH = 5.5, 0.3 mg·mL^{-1} sodium azide).

Hydroxynitrile lyase activity test

The standard activity test was performed as described by Bauer *et al.* (*15*) except that citrate-phosphate buffer was used instead of glutamate buffer.

Determination of HCN

HCN was determined in the aqueous phase using the Spectroquant® cyanide test. The test was performed in completely filled 4 mL screw cap vials to avoid the evaporation of HCN. It is based on the formation of a polymethine dye that can be measured spectrophotometrically at 585 nm. The molar absorption coefficient was determined for a concentration range of 2.5–10 μM HCN (ε = 199 057 M^{-1} cm^{-1}).

Enzyme stability

The decrease of the enzyme activity at a protein concentration of 31 μg·mL^{-1} was monitored in the Lewis cell in different media at 20 °C and pH = 5.0 : 20 mM citrate-phosphate buffer, buffer saturated with DIPE or MTBE and in two-phase systems of buffer and DIPE or MTBE. In addition, enzyme inactivation at a protein concentration of 6.2 and 93 μg·mL^{-1} was investigated in the two-phase systems. The inactivation constants were calculated by non-linear least squares fitting of a first order inactivation kinetics to the experimental values.

Specifications of the Lewis cell

The Lewis cell consisted of a thermostated glass cylinder and the bottom and lid of stainless steel and was closed gas tight to avoid the evaporation of HCN (Table 1). Each phase was well mixed without disturbing the flat interface between the two phases. Samples were taken with a gas tight syringe through a septum.

Table 1. Specifications of the Lewis cell

Aqueous phase	citrate-phosphate buffer, 20 mM, pH = 5.0	
Organic phase	diisopropyl ether (DIPE) or methyl-*t*-butyl ether (MTBE)	
Temperature / °C		20
Dimensions:	Diameter / mm	72
	Height / mm	48
Specific interfacial area / m^{-1}		50.9
Phase volumes:	Aqueous / mL	80
	Organic / mL	80
Stirrer		Four blade stirrer in each phase
	Diameter / mm	25
	Height / mm	7
Stirrer speed in both phases / min^{-1}		100

Mass transfer studies in the Lewis cell

For both substrates benzaldehyde and HCN mass transfer was studied from the organic to the aqueous phase, for the product mandelonitrile mass transfer was studied in the opposite direction. The organic solvents (DIPE or MTBE) and citrate-phosphate buffer 20 mM (pH = 5.0 for benzaldehyde and HCN, pH = 3.5 for mandelonitrile) were saturated with each other before use. Benzaldehyde dissolved in organic solvent (100, 300, 600 and 1000 mM) was carefully placed on the top of the aqueous phase in the Lewis cell. After starting the stirring the benzaldehyde concentration was followed spectrophotometrically at 280 nm in the aqueous phase as a function of time. From the time course of the benzaldehyde diffusion the mass transfer coefficient was determined for each starting concentration and for both solvents. For HCN and mandelonitrile the same method was applied. Solution of HCN (100, 300, 450, 650 and 900 mM) was added as a liquid through a septum to the organic phase that was already placed on top of the aqueous phase. The concentration of HCN in the aqueous phase was determined with the Spectroquant® cyanide test. Mandelonitrile solutions were prepared in buffer (5, 10 and 20 mM) and its concentration in the organic phase was determined by gas chromatography of the acetylated derivatives (*15*).

Determination of the mass transfer coefficients

Assuming two film theory the substrate transfer from the organic to the aqueous phase may be described by the following expressions (*21*):

$$\frac{d[BA_a]}{dt} = K_L a_{BA} \cdot ([BA_{eq}] - [BA_a]) \qquad /1/$$

$$\frac{d[HCN_a]}{dt} = K_L a_{HCN} \cdot ([HCN_{eq}] - [HCN_a]) \qquad /2/$$

$[BA_a]$ and $[HCN_a]$ represent the concentration of the substrates in the aqueous phase, $K_L a_{BA}$ and $K_L a_{HCN}$ are the mass transfer coefficients and $[BA_{eq}]$ and $[HCN_{eq}]$ are the substrate equilibrium concentrations in the aqueous phase in dependence on the substrate concentration in the organic phase.

The product transfer process from the aqueous to the organic phase can be described in a similar way (*19*):

$$\frac{d[MN_o]}{dt} = K_L a_{MN} \cdot ([MN_{eq}] - [MN_o]) \qquad /3/$$

$[MN_o]$ represents the concentration of mandelonitrile in the organic phase, $K_L a_{MN}$ is the mass transfer coefficient and $[MN_{eq}]$ is the product equilibrium concentration in the organic phase in dependence on the product concentration in the aqueous phase.

Integration of these equations leads to the following expressions:

$$\ln\frac{[BA_{eq}]}{[BA_{eq}] - [BA_a]} = K_L a_{BA} \cdot t \qquad /4/$$

$$\ln\frac{[HCN_{eq}]}{[HCN_{eq}] - [HCN_a]} = K_L a_{HCN} \cdot t \qquad /5/$$

$$\ln\frac{[MN_{eq}]}{[MN_{eq}] - [MN_0]} = K_L a_{MN} \cdot t \qquad /6/$$

From a linear plot of the logarithmic expression against time the mass transfer coefficients for each reactant were determined separately and used as starting values for modeling the chemical and the biocatalytic reaction in the Lewis cell.

Enzyme kinetics in the aqueous phase saturated with organic solvents

The kinetic constants were determined in buffer saturated with organic solvents (DIPE or MTBE) by progress curve analysis following the same procedure as described earlier (*15*). All experiments were performed in 20 mM citrate-phosphate buffer, pH = 5.0, at 20 °C. All buffers except the enzyme solution were saturated with organic solvent prior to use. For the enzymatic reaction a protein concentration of 0.62 $\mu g \cdot mL^{-1}$ was used which corresponds to the enzymatic activity used for progress curve analysis in pure buffer (*15*). The reaction rates of the Ordered Bi Uni mechanism (Fig. 1) were determined from a simultaneous fit of the model parameters to the experimental data. From these reaction rates the kinetic constants were calculated and compared with the ones obtained in pure buffer (*15*).

Chemical and enzymatic synthesis of mandelonitrile in the Lewis cell

For studying the chemical and enzymatic synthesis of mandelonitrile in two-phase systems with DIPE or MTBE a 1.5–fold excess of HCN was used (benzaldehyde 300 mM, HCN 450 mM). The protein concentration was varied 6.2 $\mu g \cdot mL^{-1}$ (2 $IU \cdot mmol^{-1}$ benzaldehyde), 31 $\mu g \cdot mL^{-1}$ (10 $IU \cdot mmol^{-1}$) and 93 $\mu g \cdot mL^{-1}$ (30 $IU \cdot mmol^{-1}$). Benzaldehyde dissolved in organic solvent was carefully placed on the top of the aqueous phase. Then enzyme was injected into the aqueous phase and liquid HCN was added to the upper organic phase. The reaction was started by stirring and monitored for 3 hours. At distinct time intervals samples were taken from both phases. The mandelonitrile content and the enantiomeric excess of *(S)*-mandelonitrile were determined in the organic phase by gas chromatography (*15*). The samples from the aqueous phase were diluted with cold (0 °C) citrate-phosphate buffer, 20 mM, pH = 3.5 to stop the enzymatic and the chemical reaction. Benzaldehyde concentrations were determined immediately spectrophotometrically at 249 nm. HCN was determined by the Spectroquant® cyanide test. The chemical synthesis of mandelonitrile was determined in the same way except that the enzyme was not added.

Results

Enzyme stability

Stability of HbHnl decreases significantly if the buffer is saturated with organic solvent (Fig. 3).

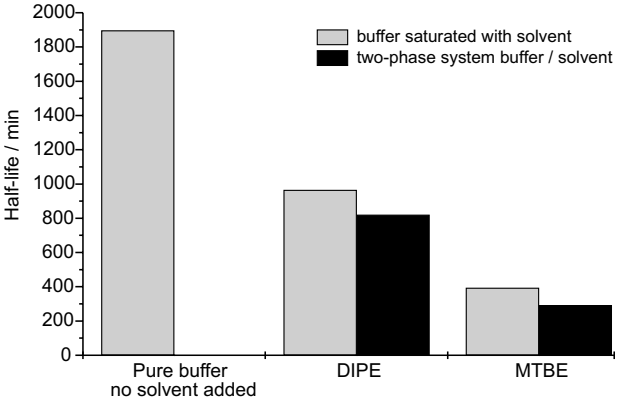

Fig. 3. Enzyme stability in the Lewis cell in different systems; buffer: citrate-phosphate, 20 mM, pH = 5.0; protein concentration: 31 $\mu g \cdot mL^{-1}$

Additionally the enzyme is inactivated at the organic solvent – buffer interface where precipitation of the protein can be observed. Regarding enzyme stability in the Lewis cell DIPE is a better solvent than MTBE. Enzyme inactivation by dissolved solvent (molecular toxicity) and by interfacial effects (phase toxicity) has already been studied in detail by Ghatorae *et al.* (*23,24*). No simple relationship between solvent polarity and the rates of inactivation by the dissolved solvent or the interfacial mechanism could be found. Moreover the inactivation characteristics were found to be enzyme specific (*23,24*).

Mass transfer studies in the Lewis cell

Mass transfer of benzaldehyde and HCN was studied from the organic to the aqueous phase whereas mass transfer of mandelonitrile was studied in the opposite direction. For both organic solvents tested (DIPE and MTBE) the mass transfer rates are similar.

From the saturation profile of the reactants (Fig. 4) the mass transfer coefficients can be determined according to Equations /4/–/6/.

The mass transfer coefficients are independent of the starting concentrations of the reactants (Fig. 5). Consequently they are constant during the course of mandelonitrile formation when the concentrations of benz-

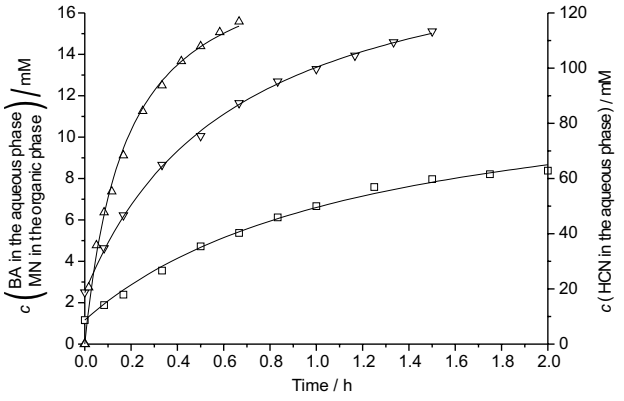

Fig. 4. Reactant transfer in the Lewis cell; benzaldehyde (300 mM, ❐) and HCN (450 mM, Δ) transport from the organic to the aqueous phase, mandelonitrile (20 mM, ∇) transport from the aqueous to the organic phase; organic solvent: DIPE

aldehyde and HCN in the organic phase decline. The mass transfer coefficient for HCN ($K_L a_{HCN}$ = 2.76 h^{-1}) is high in comparison to the ones of benzaldehyde ($K_L a_{BA}$ = 1.17 h^{-1}) and mandelonitrile ($K_L a_{MN}$ = 1.67 h^{-1}) as the solubility of HCN in buffer is rather high favoring mass transfer of HCN from the organic to the aqueous phase (Fig. 5).

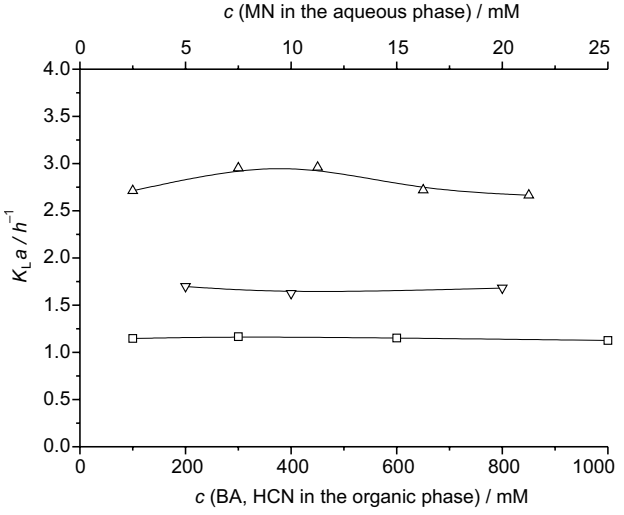

Fig. 5. Mass transfer coefficient ($K_L a$) for the mass transfer of benzaldehyde (❐) and HCN (Δ) from the organic to the aqueous phase, for the mass transfer of mandelonitrile (∇) from the aqueous to the organic phase; organic solvent: DIPE

When the concentration of benzaldehyde in the organic phase decreases during the reaction its concentration in the aqueous phase also decreases (Fig. 6). This can be expressed for both two-phase systems in analogy to a Langmuir adsorption isotherm. The numerical terms in the equation have been determined by non-linear least squares fitting of the isotherm to the experimental values (Fig. 6):

$$\text{For DIPE: } [BA_{eq}] = f([BA_o]) = \frac{[BA_o] \cdot 52.66}{[BA_o] + 1444} \qquad /7/$$

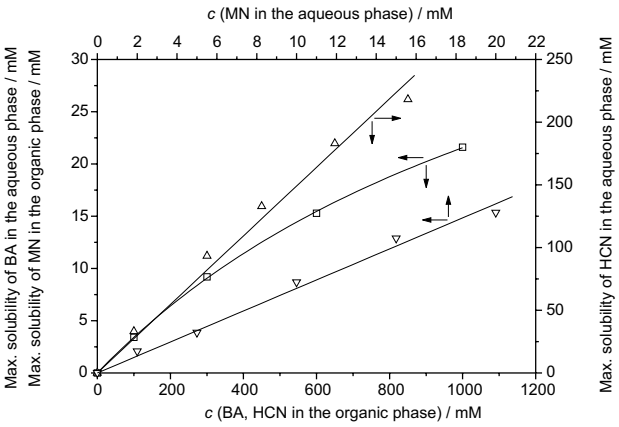

Fig. 6. Solubility isotherms in a two-phase system of buffer and DIPE for benzaldehyde (❐), HCN (Δ) and mandelonitrile (∇)

$$\text{For MTBE: } [BA_{eq}] = f([BA_o]) = \frac{[BA_o] \cdot 44.43}{[BA_o] + 1899} \qquad /8/$$

Consequently the partition coefficient of benzaldehyde between the two phases is not constant and increases with increasing concentrations of benzaldehyde in the organic phase.

For HCN and mandelonitrile the partitioning between the two phases can be described in a linear form for both organic solvents, which indicates that the partition coefficient is constant over the investigated concentration range. The following expressions have been determined using a linear fit for the experimental values (Fig. 6):

For DIPE:

$$[HCN_{eq}] = f([HCN_o]) = 0.290 \cdot [HCN_o] \qquad /9/$$

$$[MN_{eq}] = f([MN_t]) = 0.809 \cdot [MN_t] \qquad /10/$$

For MTBE:

$$[HCN_{eq}] = f([HCN_o]) = 0.178 \cdot [HCN_o] \qquad /11/$$

$$[MN_{eq}] = f([MN_t]) = 0.819 \cdot [MN_t] \qquad /12/$$

$[MN_t]$ represents the total amount of mandelonitrile produced. Equations /7/ to /12/ were used for simulating the synthesis of mandelonitrile in the Lewis cell.

Enzyme kinetics in the aqueous phase saturated with organic solvents

For the Ordered Bi Uni mechanism (Fig. 1) the kinetic constants in buffer saturated with DIPE or MTBE were determined with progress curve analysis (*15*).

For both solvents good correspondence between the experimental values and the simulated curves could be achieved (data not shown). The conversion in buffer saturated with DIPE is much faster than the conversion in buffer saturated with MTBE. In both systems saturated with organic solvent the rate of conversion is drastically reduced in comparison to pure buffer, which is in accordance with the reduced reaction rate constants for the formation of enzyme-substrate complexes (k_1, k_4, k_6) in the presence of solvents (Table 2).

Table 2. Reaction rates obtained from progress curve analysis

Reaction rates	Buffer[a]	Buffer[b] saturated with DIPE	Buffer[b] saturated with MTBE
k_1/L·mmol^{-1}·s^{-1}	504.3 ± 0.4	81.9 ± 0.1	8.1 ± 0.1
k_2/s^{-1}	2122 ± 2	2344 ± 7	1403 ± 25
k_3/s^{-1}	207.1 ± 0.2	197.9 ± 0.5	96.7 ± 0.7
k_4/L·mmol^{-1}·s^{-1}	7.91 ± 0.01	4.68 ± 0.01	2.92 ± 0.03
k_5/s^{-1}	357.9 ± 0.5	459 ± 1	227.2 ± 3.3
k_6/L·mmol^{-1}·s^{-1}	470.8 ± 0.1	223.1 ± 0.9	18.80 ± 0.09
k_7/s^{-1}	(2.40 ± 0.01)10^{-5}	(1.82 ± 0.01)10^{-5}	(1.43 ±0.01)10^{-5}
k_8/L·mmol^{-1}·s^{-1}	(9.34 ± 0.02)10^{-6}	(4.69 ± 0.01)10^{-5}	(4.63 ± 0.02)10^{-6}
k_9/s^{-1}	(3.52 ± 0.15)10^{-5}	(1.37 ± 0.04)10^{-4}	(1.75 ± 0.05)10^{-4}

[a] 20 mM glutamate buffer, pH = 5.0, 25 °C; [b] 20 mM citrate-phosphate buffer, pH = 5.0, 20 °C

Table 3. Kinetic constants calculated from the reaction rates obtained with progress curve analysis

Buffer [a]	Buffer [b] saturated with DIPE	Buffer [b] saturated with MTBE
$V_{max,f}$ = 271 ± 6 IU·mg^{-1}	$V_{max,f}$ = 276 ± 3 IU·mg^{-1}	$V_{max,f}$ = 135 ± 4 IU·mg^{-1}
$k_{cat,f}$ = 131± 1 s^{-1}	$k_{cat,f}$ = 138± 1 s^{-1}	$k_{cat,f}$ = 68 ± 2 s^{-1}
$V_{max,r}$ = 4386 ± 3 IU·mg^{-1}	$V_{max,r}$ = 4688 ± 12 IU·mg^{-1}	$V_{max,r}$ = 2831 ± 42 IU·mg^{-1}
$k_{cat,r}$ = 2111 ± 2 s^{-1}	$k_{cat,r}$ = 2341 ± 6 s^{-1}	$k_{cat,r}$ = 1414 ± 21 s^{-1}
$K_{m,MN}$ = 2.93 ± 0.01 mM	$K_{m,MN}$ = 21.7 ± 0.2 mM	$K_{m,MN}$ = 131 ± 8 mM
$K_{i,MN}$ = 4.21 ± 0.01 mM	$K_{i,MN}$ = 28.6 ± 0.1 mM	$K_{i,MN}$ = 176 ± 6 mM
$K_{m,BA}$ = 4.51 ± 0.01 mM	$K_{m,BA}$ = 10.5 ± 0.1 mM	$K_{m,BA}$ = 76 ± 2 mM
$K_{i,BA}$ = 0.76 ± 0.001 mM	$K_{i,BA}$ = 2.06 ± 0.01 mM	$K_{i,BA}$ = 12.1 ± 0.2 mM
$K_{m,HCN}$ = 294.8 ± 0.5 mM	$K_{m,HCN}$ = 543 ± 2 mM	$K_{m,HCN}$ = 531 ± 15 mM
$K_{i,HCN}$ = 71.5 ± 0.2 mM	$K_{i,HCN}$ = 140.4 ± 0.6 mM	$K_{i,HCN}$ = 113 ± 3 mM
K_{eq} = 4.73 ± 0.02 mmol·L^{-1}	K_{eq} = 3.04 ± 0.04 mmol·L^{-1}	K_{eq} = 2.3 ± 0.1 mmol·L^{-1}

[a] 20 mM glutamate buffer, pH = 5.0, 25 °C; [b] 20 mM citrate-phosphate buffer, pH = 5.0, 20 °C

The K_m values for benzaldehyde and mandelonitrile are drastically increased in buffer saturated with organic solvents (Table 3).

In the case of buffer saturated with DIPE the maximum reaction rates for synthesis and cleavage of mandelonitrile are comparable with the ones obtained in pure buffer. This indicates that DIPE behaves as a competitive inhibitor towards the enzyme. On the contrary, the maximum reaction rate in buffer saturated with MTBE is drastically reduced. As the V_{max} and K_m values are both influenced, MTBE acts as a mixed-type inhibitor. In both systems also the K_i values for the substrates and products are increased indicating that the inhibition gets less effective because the solvent acts as a second inhibitor.

Chemical and enzymatic synthesis of mandelonitrile in the Lewis cell

The chemical formation of racemic mandelonitrile in the Lewis cell could be described very well with the proposed aqueous phase bulk reaction model (data not shown). After one hour the mass transfer rate of the substrates from the organic to the aqueous phase equals the product formation rate resulting in a steady-state concentration of the substrates in the aqueous phase and a linear increase in product amount in the organic phase.

For the simulation of the enzymatic synthesis of *(S)*-mandelonitrile in the Lewis cell the mass transfer coefficients and the rate constants determined in the previous experiments were used. For the optimization of the model parameters the kinetic constants describing the enzymatic (k_1–k_6) and the chemical reaction (k_7, k_8) as well as enzyme inactivation (k_9) were kept constant whereas the mass transfer coefficients were varied to obtain good correspondence between the measured values and the simulated curves. The concentrations of the substrates benzaldehyde and HCN were determined in the aqueous phase whereas the product mandelonitrile and the enantiomeric excess of *(S)*-mandelonitrile were detected in the organic phase.

For the synthesis of *(S)*-mandelonitrile in a two-phase system with DIPE good correspondence between the simulated and measured values could be obtained for HCN, mandelonitrile and the enantiomeric excess. For benzaldehyde the simulated values do not fit to the measured concentrations (Figs. 7a and 7b).

The model used for the simulations is based on an aqueous phase bulk reaction model where the biocatalytic reaction takes place only in the bulk of the aqueous phase (*21*). This model does not correspond properly to the investigated system. The mass transfer coefficients, which should be independent of the enzyme concentration in this model, increase significantly with increasing enzyme concentration in the case of benzaldehyde and mandelonitrile (Table 4).

At the beginning of the reaction the concentrations of benzaldehyde and HCN in the aqueous phase are not zero as the filling of the reactor takes some minutes allowing substrate transfer to the aqueous phase (Fig. 7a). Within the first ten minutes the concentration of benzaldehyde decreases as the reaction is faster than the substrate transport. This decrease reduces the rate of the re-

Table 4. Optimized mass transfer rates for the enzymatic synthesis of *(S)*-mandelonitrile in the Lewis cell

HbHnl	$K_L a_{BA}/h^{-1}$		$K_L a_{HCN}/h^{-1}$		$K_L a_{MN}/h^{-1}$	
IU·mmol^{-1}	DIPE	MTBE	DIPE	MTBE	DIPE	MTBE
2	1.45 ± 0.04	1.16 ± 0.03	1.73 ± 0.05	2.61 ± 0.04	1.80 ± 0.13	2.96 ± 0.29
10	2.19 ± 0.04	1.49 ± 0.04	2.13 ± 0.13	2.20 ± 0.10	3.29 ± 0.25	1.64 ± 0.10
30	2.92 ± 0.06	1.57 ± 0.04	1.90 ± 0.08	1.88 ± 0.10	6.26 ± 0.67	2.18 ± 0.18

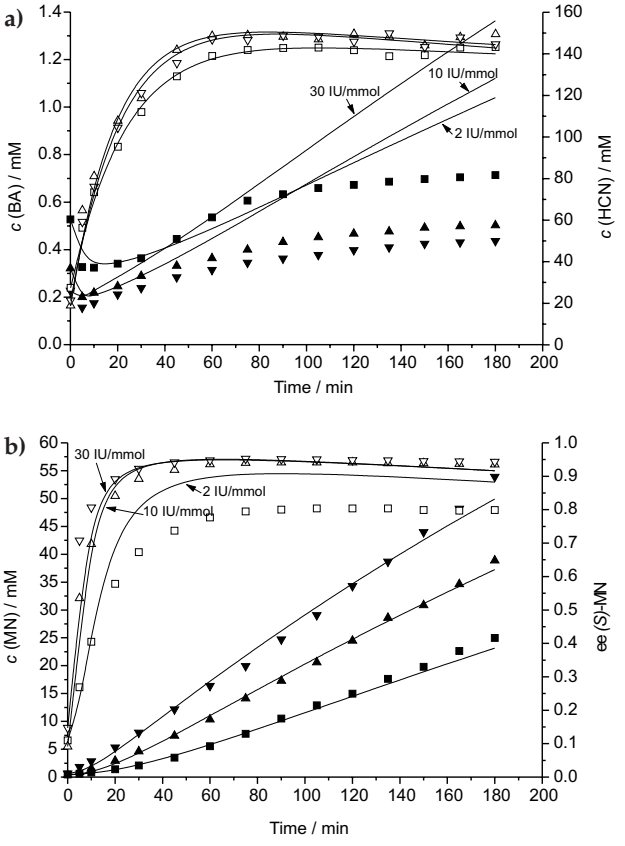

Fig. 7. Enzymatic synthesis of (*S*)-mandelonitrile in the Lewis cell at different enzyme concentrations: ■, ❐: 2 IU·mmol^{-1} BA ▲, Δ: 10 IU·mmol^{-1} BA ▼, ∇: 30 IU·mmol^{-1} BA; organic solvent: DIPE; starting concentrations in the organic phase: benzaldehyde 300 mM, HCN 450 mM; —: simulated concentrations. (a): BA (■, ▲, ▼) and HCN (❐, Δ, ∇) in the aqueous phase. (b): MN (■, ▲, ▼) and enantiomeric excess (❐, Δ, ∇) in the organic phase.

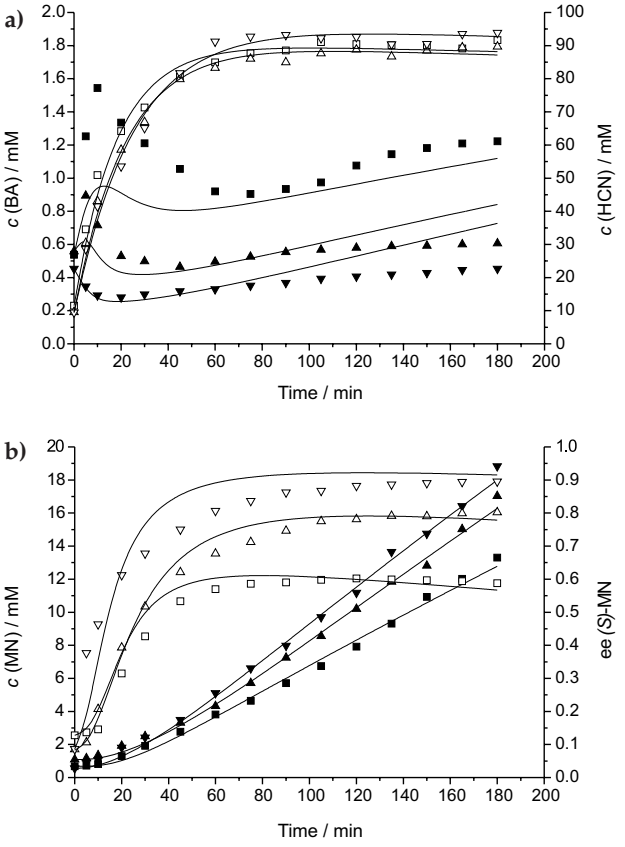

Fig. 8. Enzymatic synthesis of *(S)*-mandelonitrile in the Lewis cell at different enzyme concentrations: ■, ❐: 2 IU·mmol^{-1} BA ▲, Δ: 10 IU·mmol^{-1} BA ▼, ∇: 30 IU·mmol^{-1} BA; organic solvent: MTBE; starting concentrations in the organic phase: benzaldehyde 300 mM, HCN 450 mM; —: simulated concentrations. (a): BA (■, ▲, ▼) and HCN (❐, Δ, ∇) in the aqueous phase. (b): MN (■, ▲, ▼) and enantiomeric excess (❐, Δ, ∇) in the organic phase.

action and increases the driving force of the substrate transport. Consequently mass transport becomes faster than the reaction leading to an increase in the benzaldehyde concentration. This trend can be observed for both the measured and the simulated concentrations. As the reaction proceeds the simulated concentrations for benzaldehyde increase with increasing enzyme concentrations due to the higher $K_L a_{BA}$ values. The measured concentrations of benzaldehyde increase only slowly and approach a steady-state concentration after three hours which is lower at higher enzyme concentrations and corresponds to a linear product formation rate. The reduced steady-state benzaldehyde concentration with increasing enzyme concentration is due to the faster enzymatic reaction in the aqueous phase if more enzyme is present.

As the reaction is mass transfer limited mandelonitrile formation does not increase linearly with the enzyme concentration. The enantiomeric excess of *(S)*-mandelonitrile increases with increasing enzyme concentration as the benzaldehyde concentration in the aqueous phase is lower resulting in a slower chemical reaction rate (Fig. 7b).

The behavior of this system can be explained by an aqueous phase distributed reaction model in which the enzymatic reaction takes place in a non-uniform manner between the interface and the bulk of the aqueous phase (*21*). This means that the enzyme does not only act in the bulk of the aqueous phase but also in the thin film close to the interface and/or directly at the liquid-liquid interface.

The biocatalytic formation of *(S)*-mandelonitrile in a two-phase system of buffer and MTBE can be described better by the aqueous phase bulk reaction model because in this case the simulated concentrations of benzaldehyde fit better to the measured values (Fig. 8a) and the mass transfer coefficients for benzaldehyde and mandelonitrile do not increase significantly with increasing enzyme concentration (Table 4). This fact is due to the much slower enzymatic reaction in the aqueous phase saturated with MTBE. Using 30 IU·mmol^{-1} of enzyme results in less products than using 2 IU·mmol^{-1} of enzyme in a two-phase system with DIPE (Figs. 7b and 8b). Consequently the steady-state concentration of benzaldehyde is higher in the former case and the effect of enzymatic catalysis at or close to the interface is not so pronounced. At low enzyme concentrations the concentration of benzaldehyde increases within the first ten minutes of the reaction because the substrate transfer rate is faster than the reaction rate. Then the biocatalytic reaction rate becomes faster than mass transport due to the increased substrate concentrations in the aqueous phase.

After three hours the benzaldehyde concentration approaches a steady-state concentration resulting in a linear product formation rate. Due to the reduced enzymatic activity and the higher benzaldehyde concentration in the aqueous phase in a two-phase system with MTBE the enantiomeric excess is lower than in the DIPE system (Fig. 8b).

Discussion

Two organic solvents (DIPE and MTBE) were chosen for the production of *(S)*-mandelonitrile in the Lewis cell. DIPE because of the fast product formation and the high enantiomeric excess that could be achieved in the well mixed two-phase system and MTBE because it is a commonly used solvent for biotransformations (*16*). Using DIPE instead of MTBE in this two-phase system results in a much higher product formation rate and in a higher enantiomeric excess of *(S)*-mandelonitrile.

The hypothesis that the formation of *(S)*-mandelonitrile in the Lewis cell follows an aqueous phase distributed reaction model is strongly supported by the comparison of the measured and calculated product formation rates (Table 5) for the bulk reaction model.

Table 5. Product formation rate of mandelonitrile in the Lewis cell

HbHnl	Product formation rate in DIPE mmol·h^{-1}·L^{-1}		Product formation rate in MTBE mmol·h^{-1}·L^{-1}	
IU·mmol^{-1}	measured	predicted	measured	predicted
2	9.66	8.68	5.28	4.46
10	14.62	8.98	6.78	4.86
30	19.87	9.11	7.30	4.99

measured: rates determined from the measured values in the organic phase
predicted: rates expected for an aqueous phase bulk reaction model

Table 6. Hatta number (*Ha*) and enhancement factor (*E*) for the mass transfer of benzaldehyde in the Lewis cell

HbHnl	DIPE		MTBE	
IU·mmol^{-1}	*Ha*	*E*	*Ha*	*E*
2	0.42	1.08	0.12	1.01
10	0.62	1.18	0.21	1.02
30	0.81	1.29	0.35	1.06

The »measured« rates were determined from the measured concentrations of mandelonitrile in the organic phase (Figs. 7b and 8b). For the calculation of the »predicted« product formation rates an aqueous phase bulk reaction model was assumed. The optimized mass transfer coefficients at an enzyme concentration of 2 IU·mmol^{-1} (Table 4, line 1) were also used for simulating product formation at higher enzyme concentrations. If the aqueous phase bulk reaction model is valid the mass transfer coefficients will be constant resulting in a very slight increase in the product formation rate with enzyme concentration (Table 5). However, the measured product formation rate at an enzyme concentration of 30 IU·mmol^{-1} is more than double of the predicted one in the case of DIPE and rises about 50 % when using MTBE. This observation can be explained by the aqueous phase distributed reaction model where the enzyme acts not only in the bulk of the aqueous phase but also in the film close to the interface and/or directly at the liquid-liquid interface.

Using the dimensions of HbHnl determined by the crystal structure (*25*), 11 μg of pure enzyme are necessary to cover the interface in the Lewis cell in a monolayer. This is easily possible as the excess of the enzyme in the aqueous phase is 50-fold for 2 IU·mmol^{-1} or even 700-fold in the case of 30 IU·mmol^{-1}. But the diffusion of the enzyme into the film and its adsorption at the interface is also a time dependent process. Moreover, it is not known if and how long HbHnl stays catalytically active at the interface. Consequently the portion of the reaction that takes place at the interface cannot be determined exactly but the difference between the measured and the predicted product formation rate (Table 5) can be attributed to enzyme activity at or close to the interface. Protein adsorption at liquid-liquid interfaces has already been observed for Hnl from *Prunus amygdalus* (*26*).

The increase of the mass transfer rates for benzaldehyde and mandelonitrile with increasing enzyme concentration (Table 4) can be interpreted with enhanced mass transfer of benzaldehyde from the organic to the aqueous phase (*27,28*). In analogy to oxygen transfer at the gas-liquid interface by oxygen-consuming microbial cells or enzymes, the mass transfer of benzaldehyde from the organic to the aqueous phase for a system with simultaneous diffusion and reaction can be defined as:

$$\frac{d[BA_a]}{dt} = a \cdot ([BA_{eq}] - [BA_a]) \sqrt{\frac{2}{n+1} D_{BA} \cdot [BA_a]^{*n-1} \cdot k_r} \cdot \sqrt{1+C} \qquad /13/$$

The specific interfacial area '*a*' can be easily calculated from the dimensions of the Lewis cell (a = 50.9

m^{-1}). '*n*' represents the reaction order, k_r the reaction rate constant and D_{BA} the diffusion coefficient for benzaldehyde. In the literature the diffusion coefficient of benzoic acid in water at 25 °C is reported ($D = 0.9 \cdot 10^{-9}$ $m^2 \cdot s^{-1}$) (*29*). As structure and molecular weight of benzaldehyde and benzoic acid are similar the diffusion coefficient of benzoic acid was used as an approximation. The integration constant *C* is a function of the Hatta number (*Ha*) and can be approximated as:

$$C = 1/Ha^2 \qquad /14/$$

The Hatta number compares the transfer rate caused by the biocatalytic reaction with pure diffusion and is defined as:

$$Ha = \frac{1}{K_{L,BA}} \cdot \sqrt{\frac{2}{n+1} \cdot D_{BA} \cdot [BA_{eq}]^{*n-1} \cdot k_r} \qquad /15/$$

Calculating the Hatta number, reactions can be classified according to their velocity (*28*): »rapid reactions« ($Ha \geq 3$) and »slow reactions« ($Ha \leq 0.3$). Rapid reactions occur in the film at the interface and slow reactions in the liquid bulk.

For the mass transfer of benzaldehyde Equation /13/ can be transformed using Equation /15/:

$$\frac{d[BA_a]}{dt} = K_L a_{BA} \cdot ([BA_{eq}] - [BA_a]) \cdot E \qquad /16/$$

with the enhancement factor *E*:

$$E = \sqrt{1 + Ha^2} \qquad /17/$$

which is unity if $Ha \leq 0.3$.

The rate equation for the formation of mandelonitrile can be approximated using the general form of a double substrate limitation (*15*):

$$v = V_{max,f} \cdot \frac{[BA]}{K_{m,BA} + [BA]} \cdot \frac{[HCN]}{K_{m,HCN} + [HCN]} \qquad /18/$$

In the aqueous phase of the two-phase system the concentrations of benzaldehyde and HCN are far below the K_m values (Table 3); consequently the reaction is of first order with respect to both substrates and Equation /18/ can be simplified to:

$$v = V_{max,f} \cdot \frac{[BA]}{K_{m,BA}} \cdot \frac{[HCN]}{K_{m,HCN}} = k_r \cdot [BA] \qquad /19/$$

with the reaction rate constant k_r for benzaldehyde consumption:

$$k_r = \frac{V_{max,f}}{K_{m,BA}} \cdot \frac{[HCN]}{K_{m,HCN}} \qquad /20/$$

Substitution into Equation /15/ leads to a new expression for the Hatta number:

$$Ha = \frac{1}{K_{L,BA}} \cdot \sqrt{D_{BA} \frac{V_{max,f}}{K_{m,BA}} \cdot \frac{[HCN]}{K_{m,HCN}}} \qquad /21/$$

In the case of a two-phase system using DIPE the Hatta number for benzaldehyde is $3 \geq Ha \geq 0.3$ and increases significantly with the enzyme concentration resulting in an enhancement factor $E > 1$ (Table 6).

Therefore the mass transfer rate is enhanced by the biocatalytic reaction and the reaction takes place not only in the bulk of the aqueous phase but also in the film close to the interface and/or directly at the interface. Consequently the studied system can be described by an aqueous phase distributed reaction model. Using MTBE as the organic phase the Hatta numbers are much lower and the enhancement factor *E* is close to one. The biocatalytic reaction in this two-phase system is much slower and therefore influences the mass transfer of benzaldehyde only to a small extent.

By changing to a well-mixed two-phase system the interfacial area may be as high as 10^5-fold that in the Lewis cell which increases the mass transfer coefficients drastically (*19*). For simulating a well mixed two-phase system mass transfer coefficients resulting from a 10^5-fold increase in the specific interfacial area were used. In these simulations product formation becomes very fast and mass transfer limitations are reduced.

Due to the complexity of the investigated system up to now no reactor design and scale up was done on the basis of the Lewis cell studies as it has been shown for a much simpler system that can be described by an aqueous phase bulk reaction model (*22*). Therefore further investigations with HbHnl in two-phase systems should concentrate on the characterization of highly dispersed systems. It should be tested if the model developed in this work for defined two-phase systems can be basically used to describe well dispersed two-phase systems which are used for the industrial production of chiral cyanohydrins.

Acknowledgements

The authors wish to thank the SFB Biocatalysis project F001/D13 for the financial support and all partners from this project and from the EU-project BIO4 CT 960112 for the good collaboration. In addition, we gratefully acknowledge Antonina Zabelinskaja-Mackova for the preparation of HCN.

Symbols

a	specific interfacial area [m^{-1}]
$[BA_a]$, $[HCN_a]$, $[MN_a]$	reactant concentration in the aqueous phase [mM]
$[BA_{eq}]$, $[HCN_{eq}]$, $[MN_{eq}]$	reactant equilibrium concentration [mM]
$[BA_o]$, $[HCN_o]$, $[MN_o]$	reactant concentration in the organic phase [mM]
D	diffusion coefficient [$m^2 \cdot s^{-1}$]
E	enhancement factor
[E]	enzyme concentration [mM]
Ha	Hatta number
$k_{cat,f}$	turnover number for the cleavage of mandelonitrile [s^{-1}]
$k_{cat,r}$	turnover number for the synthesis of mandelonitrile [s^{-1}]

K_{eq} equilibrium constant

K_L liquid-liquid substrate mass transfer coefficient [$m \cdot h^{-1}$]

$K_{m,BA} - K_{i,BA}$ Michaelis-Menten – inhibition constant for benzaldehyde [mM]

$K_{m,HCN} - K_{i,HCN}$ Michaelis-Menten – inhibition constant for prussic acid [mM]

$K_{m,MN} - K_{i,MN}$ Michaelis-Menten – inhibition constant for mandelonitrile [mM]

k_r reaction rate constant [s^{-1}]

$[MN_t]$ total concentration of mandelonitrile [mM]

n reaction order

$V_{max,f}$ maximum rate for the cleavage of mandelonitrile [$IU \cdot mg^{-1}$]

$V_{max,r}$ maximum rate for the synthesis of mandelonitrile [$IU \cdot mg^{-1}$]

Appendix

The aqueous phase bulk reaction model in the Lewis cell can be described by the following equations:

$$\frac{d[E]}{dt} = k_2 \cdot [E] - [(S)MN] - k_1 \cdot [E] \cdot [(S)MN_a] - k_6 \cdot ([E] \cdot [BA_a] + k_5 \cdot [E - BA_a] - k_9 \cdot [E] \quad /A1/$$

$$\frac{d[E - (S)MN]}{dt} = -k_2 \cdot [E - (S)MN] + k_1 \cdot [E] \cdot [(S)MN_a] + k_4 \cdot [E - BA] \cdot [HCN_a] - k_3 \cdot [E - (S)MN_a] \quad /A2/$$

$$\frac{d[E - BA]}{dt} = k_3 \cdot [E - (S)MN] - k_4 \cdot [E - BA] \cdot [HCN_a] + k_6 \cdot [E] \cdot [BA_a] - k_5 \cdot [E - BA] \quad /A3/$$

$$\frac{d[(S)MN_t]}{dt} = k_2 \cdot [E - (S)MN] - k_1 \cdot [E] \cdot [(S)MN_a] - k_7 \cdot [(S)MN_a] + 0.5 \cdot k_8 \cdot [BA_a] \cdot [HCN_a] \quad /A4/$$

$$\frac{d[(R)MN_t]}{dt} = -k_7 \cdot [(R)MN_a] + 0.5 \cdot k_8 \cdot [BA_a] \cdot [HCN_a] \quad /A5/$$

$$\frac{d[(R)MN_t]}{dt} = -K_L a_{BA} \cdot ([BA_{eq}] - [BA_a]) + k_5 \cdot [E - BA] + k_6 \cdot [E] \cdot [BA_a] + k_7 \cdot [(S)MN_a] + k_8 \cdot [(R)MN_a] + k_8 [BA_a] \cdot [HCN_a] \quad /A6/$$

$$\frac{d[HCN_a]}{dt} = K_L a_{HCN} \cdot ([HCN_{eq}] - [HCN_a]) + k_3 \cdot [E - (S)MN] - k_4 \cdot [E - BA] \cdot [HCN_a] + k_7 \cdot [(S)MN_a] + k_7 [(R)MN_a] + k_8 \cdot [BA_a] \cdot [HCN_a] \quad /A7/$$

$$\frac{d[(S)MN_o]}{dt} = K_L a_{MN} \cdot ([(S)MN_{eq}] - [(S)MN_o]) \quad /A8/$$

$$\frac{d[(R)MN_o]}{dt} = K_L a_{MN} \cdot ([(R)MN_{eq}] - [(R)MN_o]) \quad /A9/$$

$$[BA_{eq}] = f([BA_o]) \quad /A10/ \qquad [HCN_{eq}] = f([HCN_o]) \quad /A11/$$

$$[(S)MN_{eq}] = f([(S)MN_t]) \quad /A12/ \qquad [(R)MN_{eq}] = f([(R)MN_t]) \quad /A13/$$

References

1. E. E. Conn, Cyanogenic glycosides. In: *The Biochemistry of Plants: A Comprehensive Treatise, Vol. 7: Secondary Plant Products*, P. K. Stumpf, E. E. Conn (Eds.), Academic Press, New York (1981) pp. 479–500.
2. F. Effenberger, *Angew. Chem. Int. Ed. Engl. 33* (1994) 1555–1564.
3. H. Griengl, A. Hickel, D. V. Johnson, C. Kratky, M. Schmidt, H. Schwab, *Chem. Commun. 20* (1997) 1933–1940.
4. H. Griengl, N. Klempier, P. Pöchlauer, M. Schmidt, N. Shi, A. A. Zabelinskaja-Mackova, *Tetrahedron, 54* (1998) 14477–14486.
5. D. V. Johnson, H. Griengl, *Chimica oggi, Sept./Oct.* (1997) 9–13.
6. D. V. Johnson, H. Griengl, *Adv. Biochem. Eng. Biotech. 63* (1999) 31–57.
7. C. G. Kruse: Chiral cyanohydrins-their manufacture and utility as chiral building blocks. In: *Chirality in Industry*, A. N. Collins, G. N. Sheldrake, J. Crosby (Eds.), John Wiley and Sons, New York (1992) pp. 279–299.
8. D. Selmar, F. J. P. Carvalho, E. E. Conn, *Anal. Biochem. 166* (1987) 208–211.
9. M. Bauer, R. Geyer, M. Boy, H. Griengl, W. Steiner, *J. Mol. Cat. B-Enzym. 5* (1998) 343–347.
10. A. Hickel, M. Graupner, D. Lehner, A. Hermetter, O. Glatter, H. Griengl, *Enzyme Microb. Technol. 21* (1997) 361–366.
11. E. Antonini, G. Carrea, P. Cremonesi, *Enzyme Microb. Technol. 3* (1981) 291–296.
12. M. D. Lilly, *J. Chem. Tech. Biotechnol. 32* (1982) 162–169.
13. M. D. Lilly, S. Harbron, T. Narendranathan, *Methods Enzymol. 136* (1987) 138–149.
14. H. M. van Sonsbeek, H. H. Beeftink, J. Tramper, *Enzyme Microb. Technol. 15* (1993) 722–729.
15. M. Bauer, H. Griengl, W. Steiner, *Biotechnol. Bioeng. 62* (1999) 20–29.
16. M. Bauer, H. Griengl, W. Steiner, *Enzyme Microb. Technol. 24* (1999) 514–522.
17. J. B. Lewis, *Chem. Eng. Sci. 3* (1954) 248–259.

18. J. M. Woodley: Two-liquid phase biocatalysis reactor design. In: *Biocatalysis*, D. A. Abramowicz (Ed.), Van Nostrand Reinhold, New York (1990) pp. 337–357.

19. J. M. Woodley, M. D. Lilly, *Chem. Eng. Sci. 45* (1990) 2391–2396.

20. J. M. Woodley, M. D. Lilly, *Progr. Biotechnol. 8* (1992) 147–154.

21. J. M. Woodley, A. J. Brazier, M. D. Lilly, *Biotechnol. Bioeng. 37* (1991) 133–140.

22. J. M. Woodley, P. J. Cunnah, M. D. Lilly, *Biocatalysis*, *5* (1991) 1–22.

23. A. S. Ghatorae, G. Bell, P. J. Halling, *Biotechnol. Bioeng. 13* (1994) 331–336.

24. A. S. Ghatorae, M. J. Guerra, G. Bell, P. J. Halling, *Biotechnol. Bioeng. 44* (1994) 1355–1361.

25. U. G. Wagner, M. Hasslacher, H. Griengl, H. Schwab, C. Kratky, *Structure*, *4* (1996) 811–822.

26. A. Hickel, C. J. Radke, H. W. Blanch, *J. Mol. Cat. B-Enz. 5* (1998) 349–354.

27. A. Moser: Prediction of enhancement of OTR in bioreactors. In: *Bioconversion and Biochemical Engineering, Vol. II*, T. K. Ghose (Ed.), Pramodh Kapur, New Delhi (1981) pp. 253–273.

28. A. Moser: *Bioprocess Technology, Kinetics and Reactors*, Springer, New York-Wien (1988).

29. R. A. Noulty, D. G. Leaist, *J. Chem. Eng. Data*, *32* (1987) 418–420.

Korištenje Lewisovih stanica u istraživanju enzimske kinetike (*S*)-hidroksinitril liaze u dvofaznom sustavu

Sažetak

(*S*)-hidroksinitril liaza iz *Hevea brasiliensis* (HbHnl) (EC 4.1.2.39) katalizira reverzibilnu sintezu kiralnih cianohidrina iz aldehida ili ketona i HCN. Enzimska sinteza (*S*)-mandelonitrila (MN) iz benzaldehida (BA) i HCN ispitivana je u dvofaznim sustavima pufera i organskih otapala (diizopropileter, metil-*t*-butileter) koristeći Lewisovu stanicu kako bi se istražilo međudjelovanje prijenosa mase i biokatalitičke reakcije. Brzina enzimske reakcije u vodenoj fazi, zasićenoj organskim otapalima, drastično je smanjena u usporedbi s reakcijom u čistom puferu zbog povišenja Michaelis-Mentenove konstante supstrata. Prijenos mase supstrata iz organske u vodenu fazu te prijenos mase produkta u suprotnom smjeru može se opisati teorijom dvostrukog filma. Stvaranje (*S*)-mandelonitrila u Lewisovoj stanici slijedi reakcijski model u vodenoj fazi, što znači da se enzimska reakcija provodi uglavnom u vodenoj fazi i u tankom filmu u blizini međufaze odnosno izravno na međufazi. Koristeći Hattin broj moglo se pokazati da je prijenos mase benzaldehida iz organske u vodenu fazu pojačan biokatalitičkom reakcijom (*S*)-hidroksinitril liaze iz *Hevea brasiliensis*.