Model based development of hydroxynitrile lyase catalyzed processes

Wouter Francois Willeman
Model based development
of hydroxynitrile lyase catalyzed processes

Wouter François Willeman

2002
Model based development
of hydroxynitrile lyase catalyzed processes

PROEFSCHRIFT

ter verkrijging van de graad van doctor
aan de Technische Universiteit Delft,
op gezag van de Rector Magnificus prof. dr. ir. J.T. Fokkema,
voorzitter van het College voor Promoties,
in het openbaar te verdedigen op dinsdag 9 april 2002 om 16:00 uur

door Wouter François WILLEMAN

scheikundig ingenieur
geboren te Alblasserdam
Dit proefschrift is goedgekeurd door de promotor:
Prof. dr. ir. J.J. Heijnen

Samenstelling promotiecommissie:

Rector Magnificus
Prof. dr. ir. J.J. Heijnen
Prof. dr. A. van der Gen
Dr. ir. A.J.J. Straathof
Prof. dr. C. Wandrey
Dr. ir. F. van Rantwijk
Prof. dr. F. Kapteijn
Dr. P. Pöchlauer

voorzitter
Technische Universiteit Delft, promotor
Universiteit Leiden
Technische Universiteit Delft
University of Bonn, Germany
Technische Universiteit Delft
Technische Universiteit Delft
DSM Fine Chemicals Linz, Austria

Reservelid:
Prof. dr. ir. M.C.M. van Loosdrecht
Technische Universiteit Delft

Dr. ir. A.J.J. Straathof heeft als begeleider in belangrijke mate bijgedragen aan de
totstandkoming van dit proefschrift.

The research reported in this thesis was performed at the Kluyver Laboratory for
Biotechnology, Delft University of Technology, The Netherlands and partly at DSM Fine
Chemicals Linz, Austria.

This work was carried out as part of the Innovation Oriented Research Program on
Catalysis (IOP Katalyse, IKA94026B) sponsored by the Ministry of Economic Affairs
from The Netherlands.
STELLINGEN
behorende bij het proefschrift
Model based development of hydroxynitrile lyase catalyzed processes
Wouter François Willeman

1. Een vrije verklaring van de afkorting PhD zou kunnen zijn “Professional help Desk”, voor een bijzonder stukje wetenschap.

2. De waarde van de evenwichtsconstante voor de synthese van één enantiomeer bedraagt de helft van de waarde voor het racemaat. 
   Hoofdstuk 2 van dit proefschrift

3. Er bestaat geen regel zonder uitzondering, behalve deze.

4. Wanneer een enzym zich bevindt in de waterfase van een water-organisch tweefasen-systeem en de waterfase wordt hergebruikt, zoals aangegeven in dit proefschrift, vervalt de noodzaak tot immobilisatie op een drager.

5. Alle modellen zijn fout maar sommige zijn uiterst bruikbaar.
   Dit proefschrift

6. Alle enzym-gekatalyseerde reacties worden vergezeld door niet-enzymatische reacties, echter de experimentele condities bepalen of we deze nevenreacties ook kunnen waarnemen.
   Dit proefschrift

7. Fijnchemicaliën zijn niet altijd zo fijn als de naam suggereert.

8. In hun zoektocht naar een enzym dat vooral actief is aan het grensvlak tussen een waterige en een organische fase hebben Hickel e.a. het optreden van stoftransport-limitatie in de waterfase over het hoofd gezien.

9. De introductie van zogenaamde “Euro-proof” portemonnaies suggereert dat het de gewoonste zaak van de wereld zal zijn om met €500 biljetten op zak te lopen.

10. De jonge doctor is niet altijd zo jong als men denkt.
STATEMENTS
As part of the thesis
Model based development of hydroxynitrile lyase catalyzed processes
Wouter François Willeman

1. A free translation of the abbreviation PhD could be “Professional help Desk”, for a special tiny part of science.

2. The value of the equilibrium constant for the synthesis of one enantiomer amounts to half the value of its racemate.
   Chapter 2, this thesis

3. There is no rule without exception, except this one.

4. When an enzyme resides in the aqueous phase of an aqueous-organic biphasic system and the aqueous phase is recycled, as indicated in this thesis, then there is no necessity for immobilization on a carrier.

5. All models are wrong but some are very useful.
   This thesis

6. All enzyme-catalyzed reactions are accompanied by non-enzymatic reactions. However, the experimental conditions will determine whether these side reactions can be observed.
   This thesis

7. Fine chemicals are not always as fine as their name supposes.

8. In their search for an enzyme acting at the aqueous-organic interface, Hickel et al. forgot to investigate the possibility of mass transfer limitation in the aqueous phase.

9. The introduction of so called “Euro-proof” wallets supposes that walking around with €500 notes in your wallet will be quite usual.

10. The young doctor is not always as young as people think.
Ter herinnering aan Opa Rietveld
# Contents

1 Introduction and outline 9

2 Estimation of kinetic parameters by progress curve analysis for the synthesis of (R)-mandelonitrile by *Prunus amygdalus* hydroxynitrile lyase 15

3 Development of a process model to describe the synthesis of (R)-mandelonitrile by *Prunus amygdalus* hydroxynitrile lyase in an aqueous-organic biphasic reactor 37

4 Comparison of a batch, fed batch and continuous operated stirred tank reactor for the enzymatic synthesis of (R)-mandelonitrile by using a process model 55

5 Reaction temperature optimization procedure for the synthesis of (R)-mandelonitrile by *Prunus amygdalus* hydroxynitrile lyase using a process model approach 73

6 Development of (R)-4-hydroxymandelonitrile synthesis in an aqueous-organic biphasic stirred tank batch reactor 89

Outlook 111

Summary 113

Samenvatting 117

Curriculum Vitae 121

Oral presentations and publications 123

Dankwoord 125
Introduction
and
Outline
Introduction

With regard to the recent worldwide industrial activity, the use of enantiopure cyanohydrins as building blocks for a large number of complex bioactive compounds has a great market potential. The versatility of cyanohydrins, due to the hydroxyl and nitrile functionality, for follow-up chemistry has been visualized in Figure 1.

Figure 1  *A world beyond cyanohydrins, used with permission [1].*

To enable the synthesis of the different compound classes from Figure 1, enantiopure cyanohydrins are required. Methods available for their synthesis are asymmetric catalysis by hydroxynitrile lyases, resolution by lipases or chemocatalytic methods. The chemocatalytic methods use chiral complexes controlling enantioselective hydrocyanation or silylcyanation, diastereoselective hydrocyanation or silylcyanation of chiral carbonyl compounds or the preparation from non-carbonyl compounds [2].
Comparing the obtained enantiomeric excesses for different substrates and methods, the hydroxynitrile lyases in general appear to be the best choice for most substrates.

Hydroxynitrile lyases (Hnls) are highly enantiospecific enzymes and different types were found in over 3000 plants species and even in insects [3]. In nature cyanogenic glycosides are decomposed into cyanohydrins by the glycosidase enzymes. Subsequently, Hnl catalyzes the decomposition of chiral cyanohydrins into hydrogen cyanide and an aldehyde or ketone. The function of HCN liberation can be twofold. The HCN is used for nitrogen storage and to protect young seedling tissues against grazing insects [3].

It is the reverse reaction towards chiral cyanohydrins which is of interest for the synthesis of enantiopure compounds and at which we will concentrate. The (S)-specific hydroxynitrile lyase from the rubber tree Hevea brasiliensis (HbHnl) is the first enzyme originating from plants which has been successfully used for the industrial production of fine chemicals. (S)-m-Phenoxybenzaldehyde cyanohydrin is produced on multi-ton scale by DSM [4]. The market economics required that the enzyme obtained in low amounts from the leaves of the rubber tree Hevea brasiliensis had to be cloned and overexpressed in Pichia pastoris [5].

Prunus amygdalus hydroxynitrile lyase (PaHnl) is abundant in almonds, can easily be obtained by extraction and is (R)-specific [6]. PaHnl has a molar weight of 72 kDa and is yellow colored due the presence of flavine adenine dinucleotide (FAD). This co-enzyme is essential for the stability and activity of PaHnl [7]. However, its role in the reaction is not clear. It was found that the PaHnl is more abundant in the sweet cultivated than in the bitter wild variety of almond [8]. The results described in this thesis were obtained by using PaHnl from sweet cultivated almonds.

The enzymatic synthesis of enantiopure cyanohydrins is hampered by a nonenzymatic (base catalyzed) reaction. The nonenzymatic reaction yields racemic cyanohydrin and therefore decreases the enantiomeric excess of the final product. To synthesize enantiopure cyanohydrins the nonenzymatic reaction has to be minimized. The Hnls have been tested for a large number of substrates in different systems of aqueous solvents with or without cosolvents, organic solvents and aqueous-organic biphasic systems [2,9,10]. Among the different systems aqueous-organic biphasic systems have been found to be particularly successful in reducing the nonenzymatic reaction. The organic phase serves as a buffer reservoir for the substrates and products whereas the conversion takes place in the aqueous phase. The organic phase lowers the corresponding concentrations in the aqueous phase and the enzyme is immobilized in the aqueous phase and may be reused. Figure 2, based on data of Niedermeyer [11], shows that the enzymatic reaction for benzaldehyde is favored at low substrate concentrations.

Until now the development of the synthesis of enantiopure cyanohydrins has been performed by means of empirical optimization, which is costly and time consuming. Empirical optimization of an aqueous-organic biphasic system requires a large number of experiments for every new product on every scale, from laboratory through pilot plant up to production scale. For a particular aqueous-organic biphasic system in a particular reactor process variables like temperature, pH, phase volume ratio, enzyme concentration,
substrate concentrations, stirrer speed and processing times can be varied experimentally resulting in a large amount of experiments. Scaling up by the empirical approach might result in expensive optimization experiments at large scale.

![Graph showing enzyme catalyzed vs. base catalyzed conversion of benzaldehyde](image)

*Figure 2* The calculated initial reaction rates for the Hnl and base catalyzed conversion of benzaldehyde.

Mathematical process models may facilitate process optimization, leading to considerable savings in experimental effort and enabling a straightforward scale-up. The process modeling approach is new for the enzymatic synthesis of enantiopure cyanohydrins which is accompanied by a nonenzymatic reaction, using aqueous-organic biphasic systems in stirred tank reactors. Two recent examples of a process modeling approach describing enzyme catalyzed reactions in aqueous-organic biphasic systems were studied in a Lewis cell type reactor: the synthesis of optically active epoxides by kinetic resolution [12] and the hydroxylation of phenol by tyrosinase [13].

This thesis describes the development of a general strategy towards the development and optimization of the industrial synthesis of enantiopure cyanohydrins, catalyzed by hydroxynitrile lyases, in aqueous-organic biphasic systems.

In 1964 the International Botanical Congress renamed the cultivated almond Prunus dulcis, until then known as *Prunus amygdalus*. The almonds you are eating, came a long way. Although 75% of the total almonds on the world market today originate from California (USA), the cultivated almond came a long way and originates from the deserts and lower mountain slopes of central and southwest Asia. The original wild almonds grew at small, thorny trees which produced hard-shelled and bitter nuts. At some point in prehistory, humans discovered that these trees sometimes produce sweet, edible kernels. The people of that time prized the sweet seeds as a staple food that had many uses and travelled well. As almonds travelled with humans, they took root wherever they fell. By 4000 B.C. almonds were in use in nearly every ancient civilization. King Tutankhamun took several handfuls of almonds to his grave in 1352 B.C., to nourish him on his journey into the afterlife... (http://www.bluediamond.com)
Outline

In December 1995, a joint project proposal of Leiden University and Delft University of Technology was approved for sponsorship by The Dutch Ministry of Economic Affairs as part of the Innovation Oriented Research Program on Catalysis (IOP-katalyse). The objective of the project was to build a bridge between science and industry enabling the application of the high potential synthesis strategy for enantiopure cyanohydrins within the fine chemical industry. The results of this project are described in this thesis. The project was supported by the generous gift of the enzyme Prunus amygdalus hydroxynitrile lyase by Solvay Pharmaceuticals, Weesp, The Netherlands.

The development of the synthesis of enantiopure cyanohydrins by using a process model approach was started by the development of a process model and the determination of its parameters for the model substrate benzaldehyde. After validation the process model was used for investigation and extended to the synthesis development for the substrate 4-hydroxybenzaldehyde. In Chapter 2-5 the research is focused on the conversion of benzaldehyde into (R)-mandelonitrile. The determination of the reaction kinetics is described in Chapter 2. In this chapter the available kinetic data are evaluated and improved by new methods. In Chapter 3, the process model is developed to describe the synthesis of (R)-mandelonitrile in a batch aqueous-organic biphasic system. The process model parameters are determined and the simulation results are validated by experimental results. Chapter 4 describes a model based method to determine the suitability of batch, fed batch or continuously operated stirred tank reactor for the synthesis of (R)-mandelonitrile. In Chapter 5 the synthesis of (R)-mandelonitrile is optimized by using the process model. The considered variables are enzyme concentration, conversion time, aqueous/organic phase volume ratio and reaction temperature. In Chapter 6 the developed process model and methods are successfully applied to a difficult substrate for PaHnl, in order to produce (R)-4-hydroxymandelonitrile.

The different chapters can be read independently. Since they were submitted separately for publication this resulted in some overlap between the chapters.
References

Estimation of kinetic parameters by progress curve analysis for the synthesis of (R)-mandelonitrile by *Prunus amygdalus* hydroxynitrile lyase

Abstract

Consistent sets of kinetic parameters were estimated for the synthesis of (R)-mandelonitrile, catalyzed by *Prunus amygdalus* hydroxynitrile lyase, at 5 and 25°C and pH 5.5 by progress curve analysis. The rate constants and equilibrium constants of the non-enzymatic reaction were determined separately to reduce the number of parameters to be estimated simultaneously. At a lower temperature the equilibrium is much more favorable and the formation of rac-mandelonitrile by the non-enzymatic reaction is suppressed. The estimated kinetic parameters were used to identify that the rate determining step in the catalytic cycle is the release of (R)-mandelonitrile from the ternary complex.

Introduction

Cyanohydrins are valuable building blocks for the synthesis of drugs and agrochemicals [1-3]. For their asymmetric synthesis from HCN and aldehydes or ketones enzymatic methods using hydroxynitrile lyases are widely used [1-7]. E.g. the hydroxynitrile lyase from *Prunus amygdalus*, *sativa* (*PaHnl*) has a broad substrate specificity and catalyzes the formation of the *(R)*-cyanohydrins with high enantiospecificity [8]. *PaHnl* can readily be obtained in pure form from almonds, which contain 4-5 g enzyme per kg, but it can also be used as a crude preparation [9,10]. The yellow glycoprotein *PaHnl* (Mandelonitrile lyase, E.C. 4.1.2.10) contains one flavine adenine nucleotide (FAD). This co-enzyme is essential for the stability and activity of *PaHnl* [11-13]. It does however not participate in the reaction. The molecular mass of *PaHnl* is 72 kDa [14,15].

To develop industrial production methods for *(R)*-cyanohydrins several strategies have been used to suppress the simultaneous non-enzymatic formation of racemic cyanohydrin [1-10,15-16]. Aqueous processes are optimized with respect to pH and temperature. Aqueous-organic biphasic processes are used to minimize the non-enzymatic reaction by decreasing the reactant concentration in the aqueous phase and therefore the non-enzymatic reaction. The use of only organic solvent is an extreme example of a two-phase process in which the amount of aqueous phase is just enough to retain activity of the enzyme and to saturate the organic solvent.

Until now optimization of the process has generally be carried out by the experimental approach which is costly and time consuming. Mathematical process models are very useful to achieve a faster and cheaper process optimization. To describe the reaction in a mathematical model a consistent description of the reaction kinetics is required. The formation of *(R)*-mandelonitrile from benzaldehyde and hydrogen cyanide was chosen as a model reaction (Figure 1). *PaHnl* is enantiospecific towards *(R)*-mandelonitrile [4,5,6,10]. In this study the available kinetic data [15,17] will be evaluated and improved by using new methods.

\[ \text{Benzaldehyde} + \text{HCN} \xleftrightarrow{\text{PaHnl}} \text{**(R)**-Mandelonitrile} \]

Figure 1. Reaction of benzaldehyde to *(R)*-mandelonitrile.
Theory

Kinetic model

The kinetic model, which describes the rate of formation of the two enantiomers of mandelonitrile, consists of two parts: a non-enzymatic and an enzymatic part.

The rate of the non-enzymatic (chemical) formation \( r_{p,chem}^R \) of rac-mandelonitrile is described by two equations, one for each enantiomer:

\[
r_{p,chem}^R = k_{chem,f}^R C_A C_B - k_{chem,r}^R C_p^R = k_{chem,f}^R \left( \frac{C_A C_B - \frac{C_p^R}{K_{eq}^R}}{K_{eq}^R} \right)
\]

(1)

and

\[
r_{p,chem}^S = k_{chem,f}^S C_A C_B - k_{chem,r}^S C_p^S = k_{chem,f}^S \left( \frac{C_A C_B - \frac{C_p^S}{K_{eq}^S}}{K_{eq}^S} \right)
\]

(2)

In this reaction hydrogen cyanide (B) is added to benzaldehyde (A) from which both enantiomers of mandelonitrile (\( p^R \) and \( p^S \)) are formed in equal amounts. The forward rate constant is equal for both enantiomers and the same applies to the reverse rate constant and the equilibrium constant:

\[
k_{chem,f}^R = k_{chem,f}^S = k_{chem,f}
\]

(3)

\[
k_{chem,r}^R = k_{chem,r}^S = k_{chem,r}
\]

(4)

\[
K_{eq}^R = K_{eq}^S = K_{eq} = \frac{k_{chem,f}^R}{k_{chem,r}^R} = \frac{k_{chem,f}^S}{k_{chem,r}^S} = \frac{k_{chem,f}}{k_{chem,r}}
\]

(5)

The macroscopic balance for rac-mandelonitrile (\( C_p = C_p^R + C_p^S \)) in a batch reactor with a non-enzymatic reaction only, can be found using eqs. (1) to (5):

\[
r_{p,chem}^R + r_{p,chem}^S = \frac{dC_p}{dt} = 2k_{chem,f} C_A C_B - k_{chem,r} C_p = 2k_{chem,f} \left( C_A C_B - \frac{C_p}{2 K_{eq}} \right)
\]

(6)

Usually this macroscopic balance is written as:

\[
\frac{dC_p}{dt} = k_{chem,f}^R C_A C_B - k_{chem,f}^S C_p = k_{chem,f}^{RS} \left( C_A C_B - \frac{C_p}{K_{eq}^{RS}} \right)
\]

(7)

Where \( K_{eq}^{RS} = \frac{k_{chem,f}^{RS} C_p}{k_{chem,r}^{RS} C_A C_B} \)

(8)
From eq. (6) and (7) it is clear that the equilibrium constant $K_{eq}^{RS}$ for formation of the racemate is twice the equilibrium constant $K_{eq}$ for the formation of one enantiomer. The same applies to the forward rate constant, but not to the reverse rate constant.

$$k_{chem,f}^R = k_{chem,f}^S = \frac{1}{2} k_{chem,f}^{RS}$$  \hspace{1cm} (9)

$$k_{chem,r}^R = k_{chem,r}^S = k_{chem,r}^{RS}$$  \hspace{1cm} (10)

$$K_{eq}^R = K_{eq}^S = \frac{1}{2} K_{eq}^{RS}$$  \hspace{1cm} (11)

When additionally an enzymatic reaction producing only the (R)-enantiomer takes place, the macroscopic balance becomes:

$$\frac{d(C_P^R + C_P^S)}{dt} = r_{P,enz}^R + r_{P,chem}^R + r_{P,chem}^S$$  \hspace{1cm} (12)

Synthesis and cleavage of (R)-mandelonitrile by ParHnl have been described by an ordered bi-uni model [17]. The kinetic mechanism of this model is shown in Figure 2. The rate equation for the formation of (R)-mandelonitrile according to the bi-uni model can be expressed as [18]:

$$r_{P,enz}^R = \frac{k_{cat} f}{K_{IA} K_{mB}} \left( \frac{C_A C_B - C_{P^A}}{K_{eq}^R} \right) \frac{C_E}{1 + \frac{C_A}{K_{IA}} + \frac{K_{mA}}{K_{mB}} + \frac{C_B}{K_{IA}} + \frac{C_{P^A}}{K_{mP}} + \frac{C_{P^B}}{K_{mP}}}$$  \hspace{1cm} (13)

With the two known Haldane equations:

$$K_{eq}^R = \frac{k_{cat} f}{k_{cat} K_{mB} K_{IA}^{1/2}} = \frac{k_{cat} f}{k_{cat} K_{mB} K_{IA}^{1/2}}$$  \hspace{1cm} (14)

Figure 2. Kinetic mechanism according to the ordered bi-uni model. A: benzaldehyde, B: HCN and P: (R)-mandelonitrile.
This bi-uni rate equation, eq. (13), contains 7 parameters \( k_{\text{cat}^p}, K_{mA}, K_{mB}, K_{mF}, K_{IA}, K_{IB}, \) and \( K^R_{eq} \). The two Haldane equations introduce 2 additional parameters \( k_{\text{cat}^p} \) and \( K_{ip} \) and therefore give no additional consistency constraints. However there is one additional, recently derived, consistency constraint, eq. (15), between the parameters which reduces the number of independent parameters to 6 [19].

\[
K_{mb} = K_{ip} \left[ 1 - \frac{k_{\text{cat}^p}}{k_{\text{cat}^l}} \left( \frac{K_{mA}}{K_{IA}} - 1 \right) \right]
\] (15)

The six microscopic rate constants that are defined in Figure 2 can be obtained from the six independent kinetic parameters, defined in eq. (13) to (15) as shown in Appendix I.

Non-enzymatic kinetic parameters and equilibrium constants

From literature a number of values are obtained for the forward and reverse reaction, shown in Table 1. Both a low pH and a low temperature seem favorable regarding the suppression of the non-enzymatic reaction.

Table 1 Literature values for the non-enzymatic rate constants of the formation and decomposition of rac-mandelonitrile.

<table>
<thead>
<tr>
<th>pH</th>
<th>T</th>
<th>( k_{\text{chem,f}}^{RS} ) [M(^{-1}).s(^{-1})]</th>
<th>( k_{\text{chem,r}}^{RS} ) [s(^{-1})]</th>
<th>source</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.75</td>
<td>20</td>
<td>242 (10^4)</td>
<td>0.856 (10^6)</td>
<td>[15]</td>
</tr>
<tr>
<td>3.75</td>
<td>20</td>
<td>218 (10^4)</td>
<td>-</td>
<td>[15]</td>
</tr>
<tr>
<td>3.75</td>
<td>30</td>
<td>-</td>
<td>4.3 (10^6)</td>
<td>[15]</td>
</tr>
<tr>
<td>3.75</td>
<td>40</td>
<td>-</td>
<td>13 (10^6)</td>
<td>[15]</td>
</tr>
<tr>
<td>3.75</td>
<td>50</td>
<td>-</td>
<td>32 (10^6)</td>
<td>[15]</td>
</tr>
<tr>
<td>5</td>
<td>25</td>
<td>9340 (10^6)</td>
<td>24.0 (10^6)</td>
<td>[20]</td>
</tr>
</tbody>
</table>

Literature values for the equilibrium constant \( K_{eq}^{RS}, K_{eq}^N \) and \( K_{eq}^S \) are shown in Table 2. They were determined in four different ways: from the ratio of forward and reverse non-enzymatic kinetic parameters [eq. (8)] or enzyme kinetic parameters [eq. (14)], by progress curve fitting or from the ratio of equilibrium concentrations [eq. (8)].
For the investigated reaction no pH effects are known in the considered pH-range which could influence the value of the equilibrium constant (hydrogen cyanide $pK_a = 9.21$). However, benzaldehyde may be oxidized to benzoic acid, which can give rise to errors. Still $K^R_{eq}$ ranges from 142 to 389 M$^{-1}$. Moreover, equilibrium constants for the enzymatic and non-enzymatic reaction should obey eq. (11), but it seems that occasionally the assumption has been made that they are the same, which is not the case.

<table>
<thead>
<tr>
<th>T [°C]</th>
<th>pH</th>
<th>buffer</th>
<th>$K_{eq}^{RS}$</th>
<th>$K_{eq}^{R}$</th>
<th>$K_{eq}^{S}$</th>
<th>Method</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>3.75</td>
<td>45 mM sodium citrate</td>
<td>142</td>
<td>227 ± 28</td>
<td>179</td>
<td>eq. (8)</td>
<td>[15]</td>
</tr>
<tr>
<td>25</td>
<td>2.55-7.5</td>
<td>1 M EDTA</td>
<td>192</td>
<td>389</td>
<td>211</td>
<td>Equilibration</td>
<td>[21]</td>
</tr>
<tr>
<td>25</td>
<td>3.75</td>
<td>25 mM sodium phosphate</td>
<td>179</td>
<td>192</td>
<td>211</td>
<td>eq. (8)</td>
<td>[22]</td>
</tr>
<tr>
<td>25</td>
<td>3.8-4.7</td>
<td>acetate</td>
<td>389</td>
<td>313</td>
<td>233*</td>
<td>Progress curve</td>
<td>[23,24]</td>
</tr>
<tr>
<td>25</td>
<td>5.0</td>
<td>20 mM glutamate</td>
<td>313</td>
<td>313</td>
<td>233*</td>
<td>eq. (14)</td>
<td>[23,24]</td>
</tr>
<tr>
<td>25</td>
<td>5.0</td>
<td>20 mM glutamate</td>
<td>238</td>
<td>238</td>
<td>233*</td>
<td>Equilibration</td>
<td>[17]</td>
</tr>
<tr>
<td>25</td>
<td>5.5</td>
<td>100 mM sodium citrate</td>
<td>238</td>
<td>238</td>
<td>233*</td>
<td>eq. (14)</td>
<td>[17]</td>
</tr>
<tr>
<td>25</td>
<td>5.5</td>
<td>100 mM sodium citrate</td>
<td>238</td>
<td>238</td>
<td>233*</td>
<td>Equilibration</td>
<td>[20]</td>
</tr>
</tbody>
</table>

* recalculated with eq. (14), using the kinetic parameters from Jorns [17]

Enzymatic kinetic parameters

Kinetic parameters for (R)-mandelonitrile formation by PaHnl at 25°C and pH 5.5, according to the ordered bi-uni model have been determined by Jorns [17] by initial rate experiments, see Table 3. For synthesis reactions 170 mM and for cleavage reactions 100 mM sodium citrate buffer was used as reaction medium.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>T [°C]</th>
<th>pH</th>
<th>$K_{eq}^R$</th>
<th>$K_{eq}^S$</th>
<th>$k_{cat}^f$</th>
<th>$k_{cat}^r$</th>
<th>$K_{mA}$</th>
<th>$K_{mB}$</th>
<th>$K_{mA}$</th>
<th>$K_{mB}$</th>
<th>$K_{mB}$</th>
<th>$K_{mB}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>PaHnl</td>
<td>25</td>
<td>5.5</td>
<td>233</td>
<td>1627</td>
<td>605</td>
<td>0.15</td>
<td>57</td>
<td>0.59</td>
<td>0.12</td>
<td>18</td>
<td>0.23</td>
<td></td>
</tr>
<tr>
<td>HbHnl</td>
<td>25</td>
<td>5</td>
<td>211</td>
<td>2111</td>
<td>131</td>
<td>4.51</td>
<td>295</td>
<td>2.93</td>
<td>0.76</td>
<td>72</td>
<td>4.21</td>
<td></td>
</tr>
</tbody>
</table>

Another kinetic parameter set for (R)-mandelonitrile formation by PaHnl was determined by initial rate experiments at 20°C and pH 3.75 by Kragl and Niedermeyer [15]. For both synthesis and cleavage reactions 45 mM sodium citrate was used.
In order to use kinetic parameter sets safely for process modeling they should be consistent and therefore meet the consistency constraints. The parameter set obtained by Jorns does fulfill all consistency constraints except one, which was discovered after her results were published [19]. Kragl and Niedermeyer used a simplified version of the established rate equation, eq. (13). Although this equation described their results perfectly, it was not suitable for describing reversible kinetics. Recalculations with the non-simplified equation, which describes reversible kinetics, did not change the parameter values significantly [25]. However also here the consistency constraints are not obeyed. Therefore the kinetic parameter sets cannot be used safely for process modeling with eq. (13).

Kinetic parameters can be determined by initial rate experiments or progress curve analysis. Kinetic parameter estimation by initial rate experiments can be performed graphically or numerically. The graphical methods (e.g. Lineweaver-Burk) require a set of experiments for each individual kinetic parameter. So far the consistency constraints, eqs. (14) and (15) have not been used systematically in this initial rate method. However, it is possible to use the consistency constraints in a numerical method for initial rate experiments. From the kinetic rate equation, eq. (13), a number of hyperbolic equations can be obtained which describe the reaction rate at different boundary conditions. The obtained set of equations, together with the consistency constraints can be used to fit the kinetic parameters against the measured initial rates.

Progress curve fitting is another method to obtain a consistent set of kinetic parameters. Two methods are available in progress curve fitting: by directly fitting the microscopic rate constants or by fitting the kinetic parameters together with the Haldane constraints. Bauer et al. [24] determined the kinetic parameters of the S-specific Hevea brasiliensis hydroxynitrile lyase (HbHnl) by fitting the microscopic rate constants to the rate equations of the individual steps of the reaction mechanism.

With progress curve analysis initial estimates of the kinetic parameters to be fitted must be given. If the initial estimates are poor, the optimization procedure can result in a local minimum. It is very difficult to chose good initial estimates for the microscopic rate constants. Besides, the full enzyme dynamics must be calculated which involves very long integration times due to the very stiff differential equations.

Our approach is different using the pseudo steady state solution of the enzyme kinetics, eq. (13) and the constraints between the kinetic parameters eqs. (14) and (15). This approach is much less time consuming and initial estimates are found more easily. Initial estimates of the correct order of magnitude are required for any non-linear regression method. By inspection of the progress curves it is relatively easy to find good initial estimates for the kinetic parameters. The described approach, also applicable to other kinetic mechanisms, can be used both for refining existing parameters (e.g. those of Jorns [17]) as well as for obtaining kinetic parameters de novo.

The independent kinetic parameters were fitted against the progress curves, using eqs. (12) to (15). To reduce the number of kinetic parameters that have to be fitted simultaneously, $K_{m}^{as}$ is measured separately by means of equilibration and the non-
enzymatic reaction is studied independently and taken into account during the fitting process. With this procedure only 5 enzymatic kinetic parameters are left which are fitted simultaneously. Subsequently, the microscopic rate constants are calculated from the kinetic parameters according to equations A1-A6, given in appendix I.

Method of approach

We aim to optimize processes and investigate novel process designs for the production of (R)-cyanohydrins catalyzed by the hydroxynitrile lyase from *Prunus amygdalus*, by using mathematical models. This approach requires the description of the process kinetics using consistent sets of kinetic parameters. A temperature of 5°C and a pH of 5.5 were chosen as interesting process conditions. Here the non-enzymatic reaction is sufficiently suppressed and the enzyme is stable [26]. At 5°C and pH 5.5 kinetic parameter sets were not available in literature. Besides the kinetic parameter sets from literature were not consistent. Therefore we decided to determine the kinetic parameters ourselves, taking the consistency constraints into account.

The same applied for the non-enzymatic rate constants, which are also not known at the mentioned conditions. With regard to the equilibrium constant, values from literature showed a large variation and occasionally no difference is made between the enzymatic and the non-enzymatic equilibrium constant. Therefore we also decided to measure the equilibrium constant ourselves.

Materials and Methods

Chemicals and enzymes

Chemically pure rac-mandelonitrile (98%) and enantiopure (99%, ee 99+%) (R)-mandelonitrile were a gift from Leiden University (Leiden, The Netherlands). rac-Mandelonitrile used for the *PaHnl* activity test was a gift from DSM-Chemie Linz (Linz, Austria). rac-Mandelonitrile (98%) pro synthesis was purchased from Merck and used for kinetic experiments at 25°C. Benzaldehyde was distilled under nitrogen before use. A buffered HCN-solution was obtained by dissolving NaCN, citric acid and NaOH in deaerated water in a closed system to prevent loss of HCN. NaCN was purchased from Aldrich. *PaHnl* enzyme powder was a gift from Solvay Pharmaceuticals (Weesp, The Netherlands) and contains 98% protein and about 65% *PaHnl* [16]. The specific activity of the powder was 185,000 IU per gram. The *HbHnl* solution, used for the equilibrium experiments, was made available by Boehringer Mannheim/Roche Diagnostics (Penzberg, Germany) and has a specific activity of 5,100 IU per ml [21].

Hydroxynitrile lyase activity test

The enzyme activity was determined by monitoring the cleavage of rac-mandelonitrile into benzaldehyde and HCN at 25°C. This activity test is comparable to tests used for *HbHnl* [21,24]. However, a different kind of buffer or slightly different volumes of the solutions were used. Benzaldehyde was measured spectrophotometrically at 280 nm, in a quartz cuvette, 1 cm light path. At this wavelength the absorption of
mandelonitrile can be neglected, which is not allowed at the absorption maximum of benzaldehyde at 248 nm. The assay was performed in 46 mM potassium citrate/phosphate buffer pH 5.0 as reaction medium. Three buffer solutions were prepared.
Buffer 1: 5 mM potassium citrate/phosphate buffer pH 5.0;
Buffer 2: 0.5 mM potassium phosphate buffer pH 6.5;
Buffer 3: 0.3 mM potassium citrate/phosphate buffer pH 3.5.

A solution of 60 mM rac-mandelonitrile in buffer 3 was prepared, which is stable for 4 h at 25°C. The enzyme was dissolved in buffer 2 at a concentration of about 400-1000 IU.l⁻¹. All solutions were stored at 25°C.

To 1.4 ml buffer 1 was added 0.2 ml enzyme solution or buffer 2 in case of the blank reaction. The reaction was started by adding 0.4 ml of the rac-mandelonitrile solution. The closed cuvette was mixed by turning it upside down twice and the absorption was monitored for at least 5 min.

The slope ΔA₂₈₀ was determined at the linear range of the curve and corrected for the blank. The change in absorbance, after deduction of the blank should be 0.06 to 0.140 absorption units per minute. By using the measured extinction coefficient, ε₂₈₀ = 1.405 mM⁻¹.cm⁻¹, the enzyme activity was calculated according to eq. (16).

\[
Activity = \frac{V_{\text{total}}}{\varepsilon_{280} \cdot I \cdot V_{\text{enzyme}}} \cdot \Delta A_{280} \quad \text{[IU.ml⁻¹enzyme]} \quad (16)
\]

One International Unit (IU) is defined as the amount of enzyme that cleaves one μmol (R)-mandelonitrile per minute at the conditions mentioned.

**Kinetic experiments**

Progress curves were recorded by measuring the absorption of benzaldehyde at 280 nm. The reactions were monitored in a 4 ml quartz cuvette, mounted in a temperature controlled cuvette holder. The cuvette was closed by a septum to avoid the release of HCN. To prevent reaction beforehand, the solutions were injected through the septum in the following order: buffer, enzyme, benzaldehyde and HCN for synthesis, and buffer, enzyme and mandelonitrile for cleavage reactions. To obtain a well mixed solution the cell was turned upside down twice. With the exception of the experiments at 25°C, in which case rac-mandelonitrile was used, (R)-mandelonitrile was used. Mandelonitrile in 5 mM sodium citrate buffer at pH 3.5 is stable for up to 4 h without significant decomposition. The used buffer concentrations were 100 mM sodium citrate at 5°C and 150 mM at 25°C. All buffers were deaerated (20 min vacuum sonification) and stored under nitrogen before use, to prevent oxydation of benzaldehyde.

**Equilibrium experiments**

Equilibrium experiments were performed in a similar way as the kinetic experiments. To obtain the equilibrium fast both HbHnI as PaHnI were added to the
reaction mixture at concentrations of about 100,000 IU/liter for each enzyme. For instance, at 5°C in this way a non-enzymatic equilibrium towards the racemate was reached within 15 min, where otherwise this would take over 1 day. The equilibrium constant was determined from both the forward and reverse reaction at concentrations similar as those used in the kinetic experiments.

Progress curve fitting

The kinetic parameters were estimated by fitting them against the progress curves by using Encora [27]. Encora is a program for estimating the values for independent kinetic parameters of single enzymes which act according to established kinetic models, here the bi-unii model. The equations (1), (2) and (12) to (15) and the stoichiometric relations were used simultaneously during the fitting procedure. From the measured progress curves, data files were created and loaded into Encora with the concentration of substrates, product enantiomers and enzyme as function of time. Internally the concentrations are recalculated to "relative concentrations" \( X \) using \( X = (C_P - C_{P,0})/C_{A,0} \) for forward reactions and \( X = (C_A - C_{A,0})/C_{P,0} \) for reverse reactions.

The fitting procedure is similar to that described by Rakels et al. [28]. Every progress curve is simulated by numerically integrating the kinetic equations (using a fourth order Runge-Kutta routine). By comparing the experimental value of \( X \) to the one calculated by the model, a residual sum of squared residuals (SSres) is obtained for each progress curve, which is divided by the number of measurements of that curve. The total of these SSres values of all curves is minimized, new estimates of the parameter set being generated by the Simplex-like algorithm of Nelder and Mead. The stop criterion is that SSres does not change anymore.

Results and Discussion

Equilibrium constant

The equilibrium constant, \( K_{eq}^{RS} \) was determined at different concentrations at 5 and 25°C by equilibration of the forward and reverse reaction. The used concentrations were in the same range as those of the kinetic experiments. The equilibration was accelerated using a mixture of R- and S-specific hydroxynitrile lyase, \( PaHnl \) and \( HbHnl \). Values found for the equilibrium constant, shown in Table 4, were in the range of values mentioned in literature, Table 2. For the enzyme catalyzed reaction the equilibrium constant is \( K_{eq} = \gamma K_{eq}^{RS} \), according to eqs. (1) to (11).

At lower temperatures the equilibrium is much more favorable (factor 3) for \((R)\)-mandelonitrile formation, resulting in a higher product yield or a reduction of the amount of HCN necessary to obtain the same conversion at a higher temperature. This is in accordance with data for other bi-unii reactions [29] and is most likely an entropic effect.
Table 4 Equilibrium constants and rate constants determined for the non-enzymatic synthesis of rac-mandelonitrile at pH 5.5 according to eq. (8).

<table>
<thead>
<tr>
<th>temperature [°C]</th>
<th>$k_{\text{chem,f}}^{RS}$ [M$^{-1}$s$^{-1}$]</th>
<th>$k_{\text{chem,r}}^{RS}$ [s$^{-1}$]</th>
<th>$K_{\text{eq}}^{RS}$ [M$^{-1}$]</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>$4400\ 10^{-6}$</td>
<td>$5.96\ 10^{-6}$</td>
<td>738</td>
</tr>
<tr>
<td>25</td>
<td>$28000\ 10^{-6}$</td>
<td>$105\ 10^{-6}$</td>
<td>266</td>
</tr>
</tbody>
</table>

Chemical kinetics

The kinetics of the non-enzymatic reaction to rac-mandelonitrile were determined by recording progress curves for the forward and reverse reaction at different initial concentrations, shown in Table 5. The progress curves were in agreement with eq. (7) when $K_{\text{eq}}^{RS}$ was fixed at the determined values. The obtained values for $k_{\text{chem,f}}^{RS}$ (Table 4) are higher than the values found in literature (Table 1). The difference is accounted for by a pH-effect. It is known that the non-enzymatic reaction is accelerated at a higher pH [15].

Table 5 Initial concentrations used for the determination of the non-enzymatical rate constant for formation of rac-mandelonitrile (A = benzaldehyde; B = HCN; P = racemic mandelonitrile).

<table>
<thead>
<tr>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td>$C_A$ [mM]</td>
<td>1.94</td>
<td>1.87</td>
<td>1.85</td>
<td>0.52</td>
<td>0.52</td>
<td>0.034*</td>
<td>0.031*</td>
<td>0.15*</td>
<td>0.015*</td>
</tr>
<tr>
<td>$C_B$ [mM]</td>
<td>50</td>
<td>200</td>
<td>10</td>
<td>50</td>
<td>10</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>$C_P$ [mM]</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2.54</td>
<td>2.03</td>
<td>12.7</td>
<td>1.05</td>
</tr>
</tbody>
</table>

* benzaldehyde was present as a contaminant of rac-mandelonitrile

Enzyme kinetics

Progress curves for the enzymatic reaction were recorded for both the forward and the reverse reaction. The progress curves are shown in Figure 3 to 6 and correspond to the experiments of Table 6. The concentrations of (R)- and (S)-mandelonitrile were calculated from the measured benzaldehyde concentrations as shown in appendix II. Using the aforementioned values of $k_{\text{chem,f}}^{R}$ and $K_{\text{eq}}^{R}$ and the given rate equations (1), (2) and (12) to (15), the 5 unknown independent parameters were fitted. Results are shown in Table 7.

Figure 3 shows that acceptable fits are obtained for the forward reaction at pH 5.5 and 25°C, but for the cleavage of rac-mandelonitrile at pH 5.5 and 25°C the rates are overestimated, shown in Figure 4. For these cleavage experiments the rac-mandelonitrile that was used may have contained an inhibiting contaminant. Comparing Table 3 and 7 shows that the estimated values for the kinetic parameters are close to the values of Jorns [17]. This demonstrates that the progress curve analysis gives satisfactory results.
Figure 3. Enzymatic conversion of benzaldehyde at 25°C, pH 5.5. Markers are experimental values, lines are the fitted curves. The amount of markers was reduced to improve the visibility. 0, 1; Δ, 2; □, 3; +, 4; ○, 5; the numbers correspond to the experiments in table 6.

Figure 4. Enzymatic formation of benzaldehyde at 25°C, pH 5.5. Markers are experimental values, lines are the fitted curves. The amount of markers was reduced to improve the visibility. 0, 6; Δ, 7; *, 8; +, 9; ○, 10; □, 11; the numbers correspond to the experiments in table 6.
We further concentrated on conditions where the non-enzymatic reaction is suppressed. At pH 5.5 and 5°C the reaction can be applied for synthetic purposes [26]. For these experiments we used chemically and enantiomerically pure (R)-mandelonitrile.

Table 6 Initial concentrations used for recording progress curves. (A = benzaldehyde; B = HCN; \( P^R \) = (R)-mandelonitrile; \( P = \) racemic mandelonitrile; \( E = \) PaHnl).

<table>
<thead>
<tr>
<th>( 25^\circ C, ) pH 5.5</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
</tr>
</thead>
<tbody>
<tr>
<td>( C_A ) [mM]</td>
<td>2.02</td>
<td>2.02</td>
<td>2.02</td>
<td>0.50</td>
<td>0.10</td>
<td>0.09*</td>
<td>0.04*</td>
<td>0.01*</td>
<td>1.06</td>
<td>0.04*</td>
<td>0.61</td>
</tr>
<tr>
<td>( C_B ) [mM]</td>
<td>201</td>
<td>50.4</td>
<td>10.1</td>
<td>50.4</td>
<td>50.4</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>49.6</td>
<td>4.96</td>
<td></td>
</tr>
<tr>
<td>( C_P ) [mM]</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>3.88</td>
<td>1.55</td>
<td>0.39</td>
<td>1.61</td>
<td>1.61</td>
<td>4.21</td>
<td></td>
</tr>
<tr>
<td>( C_E ) [U.l(^{-1})]</td>
<td>376</td>
<td>376</td>
<td>376</td>
<td>150</td>
<td>75</td>
<td>369</td>
<td>369</td>
<td>369</td>
<td>369</td>
<td>369</td>
<td></td>
</tr>
</tbody>
</table>

| \( 5^\circ C, \) pH 5.5 |  |  |  |  | 1 | 1 | 1 | 1 | 1 |
|-------------------------|---|---|---|---|---|---|---|---|---|---|---|---|
| \( C_A \) [mM]          | 1.96 | 1.88 | 2.04 | 0.52 | 0.09 | 0 | 0 | 0 | 1.69 | 0 | 0.83 |
| \( C_B \) [mM]          | 200 | 50.1 | 10.0 | 50.1 | 50.1 | 0 | 0 | 0 | 0 | 9.70 | 9.70 |
| \( C_P^R \) [mM]        | 0 | 0 | 0 | 0 | 0 | 2.02 | 0.81 | 0.20 | 0.81 | 2.03 | 2.03 |
| \( C_E \) [U.l\(^{-1}\)] | 905 | 905 | 905 | 453 | 181 | 3796 | 3796 | 759 | 759 | 759 |

* benzaldehyde was present as contaminant of rac-mandelonitrile

The fits for both the forward reaction (Figure 5) and reverse reaction at 5°C (Figure 6) were satisfactory. Most Michaelis and inhibition constants were not very dependent on the temperature, but some showed a large change, especially \( K_{mA} \). The catalytic constants (\( k_{cat}^f \) and \( k_{cat}^r \)) decreased 4-5 fold upon a decrease from 25 to 5°C.

At 5°C a smaller proportion of (S)-mandelonitrile is formed than at 25°C, according to simulations with the present parameter values. This shows that the non-enzymatic reaction is suppressed to a larger extent than the enzymatic reaction by decreasing the temperature, which confirms the observation of Wolfenden et al. [30].

Table 7 Estimated kinetic parameters for the enzymatic synthesis of (R)-mandelonitrile catalyzed by PaHnl.

<table>
<thead>
<tr>
<th>T ( [\circ C] )</th>
<th>pH</th>
<th>( K_{eq}^P ) [M(^{-1})]</th>
<th>( k_{cat}^f ) [s(^{-1})]</th>
<th>( k_{cat}^r ) [s(^{-1})]</th>
<th>( K_{mA} ) [mM]</th>
<th>( K_{mB} ) [mM]</th>
<th>( K_{mP} ) [mM]</th>
<th>( K_{IA} ) [mM]</th>
<th>( K_{IB} ) [mM]</th>
<th>( K_{IP} ) [mM]</th>
</tr>
</thead>
<tbody>
<tr>
<td>25</td>
<td>5.5</td>
<td>133</td>
<td>899*</td>
<td>653*</td>
<td>0.16</td>
<td>25</td>
<td>0.38</td>
<td>0.16</td>
<td>25</td>
<td>0.38</td>
</tr>
<tr>
<td>5</td>
<td>5.5</td>
<td>369</td>
<td>156*</td>
<td>215*</td>
<td>0.016</td>
<td>18</td>
<td>1.07</td>
<td>0.12</td>
<td>59</td>
<td>0.49</td>
</tr>
</tbody>
</table>

* Assuming the enzyme powder contained 65% PaHnl, with a molecular weight of 72 kDa.

b Calculated by using the consistency relations, eqs. (14) and (15).
Figure 5. Enzymatic conversion of benzaldehyde at 5°C, pH 5.5. Markers are experimental values, lines are the fitted curves. The amount of markers was reduced to improve the visibility. 0, 1; 2; *, 3; +, 4; o, 5; the numbers correspond to the experiments in table 6.

Figure 6. Enzymatic formation of benzaldehyde at 5°C, pH 5.5. Markers are experimental values, lines are the fitted curves. The amount of markers was reduced to improve the visibility. 0, 6; A, 7; *, 8; o, 9; o, 10; the numbers correspond to the experiments in table 6.
A parameter sensitivity analysis of the 5 estimated kinetic parameters was carried out by individually raising or lowering the values of the parameters by 10%. The influence of the changed parameter values on the SSres value was determined by simulation in Encora. According to these simulations the rate equation was found to be insensitive towards $K_{mA}$ and $K_{mP}$ values at 5°C. Subsequently the terms in the denominator of the rate equation (eq. (13)) were analyzed for their contribution to the rate at different concentrations. These concentrations ranged from 0.5 to 25 mM for benzaldehyde, 1-250 mM for HCN and 0-75 mM for (R)-mandelonitrile. Analysis showed that the influence of the third term in the denominator of eq. (13) was negligible at these concentrations. Hence, the following simplified rate equation can describe the experiments at 5°C and pH 5.5:

$$k_{p,enz}^{R} = \frac{k_{cat}^{f}}{K_{iA}K_{mB}} \left( \frac{C_{A}C_{B} - C_{p}^{k}}{K_{eq}^{R}} \right) \cdot C_{E}$$

(17)

The same analysis can be applied to 25°C and pH 5.5. It followed that no denominator term can be omitted in the rate equation at the considered concentrations.

From the kinetic parameters the microscopic rate constants, shown in Table 8, were calculated according to appendix I.

### Table 8 Microscopic rate constants at pH 5.5.

<table>
<thead>
<tr>
<th>$T$ [°C]</th>
<th>$k_{1}$ [mM$^{-1}$s$^{-1}$]</th>
<th>$k_{1}$ [s$^{-1}$]</th>
<th>$k_{2}$ [mM$^{-1}$s$^{-1}$]</th>
<th>$k_{2}$ [s$^{-1}$]</th>
<th>$k_{3}$ [mM$^{-1}$s$^{-1}$]</th>
<th>$k_{3}$ [s$^{-1}$]</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>9650</td>
<td>1149</td>
<td>24</td>
<td>266</td>
<td>155</td>
<td>319</td>
</tr>
<tr>
<td>25</td>
<td>5640</td>
<td>894</td>
<td>134</td>
<td>2429</td>
<td>899</td>
<td>2358</td>
</tr>
</tbody>
</table>

To determine the rate determining step of PahHnL according to the ordered bi-uni model the following method was used. The value of each pair of microscopic rate constants, e.g. $k_{1}$ and $k_{1}$, was raised or lowered by 25%. When only a single rate constant would be varied, the equilibrium constant would be changed, which is unrealistic. Then in a simulation the time was determined at which 50% conversion was obtained. This procedure was followed for the three pairs of microscopic rate constants and at different initial concentrations. Finally the percentage difference in time was calculated at which 50% conversion would be obtained, compared to the base case in which the original values for the microscopic rate constants were used. The results are shown in Table 9.
Table 9 Percentage difference in time necessary to obtain 50% conversion at different initial concentrations when the values of the microscopic constants of the individual steps were raised or lowered by 25%. Microscopic rate constants for HbHnl were from [17]. Differences less than 1% are reported as 0%.

<table>
<thead>
<tr>
<th>$C_{A0}$ [mM]</th>
<th>5°C, pH 5.5</th>
<th>25°C, pH 5.5</th>
<th>25°C, pH 5.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5</td>
<td>25</td>
<td>2.5</td>
<td>25</td>
</tr>
<tr>
<td>25</td>
<td>25</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>$C_{R0}$ [mM]</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>50</td>
<td>50</td>
<td>50</td>
</tr>
</tbody>
</table>

$\begin{array}{|c|c|c|c|}
\hline
k_{2}, k_{1} & +25\% & -2 & -2 & -2 \\
& -25\% & -4 & -4 & -4 \\
\hline
k_{3}, k_{3} & +25\% & -14 & -14 & -14 \\
& -25\% & -12 & -12 & -12 \\
\hline
\end{array}$

Based on the calculated kinetic parameters the rate determining step at 5°C and 25°C was identified as the conversion of the ternary complex into enzyme and (R)-mandelonitrile. This is in contrast to HbHnl. There, by using kinetic data from Bauer [24], shown in Table 3, the rate determining step was identified as the conversion of the enzyme-benzaldehyde-complex into the ternary complex. Note that the rate determining step in the kinetic mechanism can be dependent on the substrate and product concentrations. In general when one of the substrates is saturating the accompanying reaction step is not rate determining.

The inaccuracy of $K_{mA}$ only influences the calculated values of $k_1$ and $k_2$ significantly. As expected these non-rate-determining rate constants cannot be estimated with high accuracy. The inaccuracy of $K_{mp}$ influences the calculated values of $k_2$, $k_3$ and $k_4$. However simulations showed that raising and lowering of $K_{mp}$ by 50% did not change the time necessary to obtain 50% conversion. Changing the value of $K_{mp}$ did not influence the identification of the rate determining step.

Conclusions

The kinetic parameters for the enzymatic conversion of HCN and benzaldehyde into (R)-mandelonitrile, catalyzed by Prunus amygdalus hydroxynitrile lyase, have been estimated by using progress curve fitting. Consistent sets of kinetic parameters were obtained at pH 5.5, for 5°C and 25°C with a minimal experimental and computational effort. The number of parameters to be fitted was reduced by separate determination of the equilibrium constants and the non-enzymatic rate constants. It was found that the non-enzymatic equilibrium constant, $K_{eq}^{RS}$, amounts twice the value of the enzymatic equilibrium constant, $K_{eq}^R$ or $K_{eq}^S$.

At 5°C the rates slow down when compared to 25°C. However, the non-enzymatic formation of racemic mandelonitrile is suppressed to a larger extent than the enzymatic
formation of (R)-mandelonitrile, and the equilibrium is shifted towards the side of mandelonitrile. Therefore low temperatures are favorable for this reaction.

A new method to determine the rate determining step in the catalytic cycle was proposed. The rate determining step in the catalytic cycle of *Prunus amygdalus* hydroxynitrile lyase seems to be the conversion of the ternary complex into free enzyme and mandelonitrile. For *Hevea brasiliensis* hydroxynitrile lyase the rate determining step was found to be different. In this case the conversion of the enzyme-benzaldehyde-complex into the ternary complex was identified as the rate determining step.

**Symbols**

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
<th>Unit</th>
</tr>
</thead>
<tbody>
<tr>
<td>$A_{280}$</td>
<td>absorption at 280 nm</td>
<td>[-]</td>
</tr>
<tr>
<td>$C_j$</td>
<td>concentration</td>
<td>[mM]</td>
</tr>
<tr>
<td>$k_{cat}$</td>
<td>forward catalytic constant</td>
<td>[s$^{-1}$]</td>
</tr>
<tr>
<td>$k_{cat}^{-1}$</td>
<td>reverse catalytic constant</td>
<td>[s$^{-1}$]</td>
</tr>
<tr>
<td>$k_{chem,f}$</td>
<td>forward chemical reaction rate constant</td>
<td>[mM$^{-1}$s$^{-1}$]</td>
</tr>
<tr>
<td>$k_{chem,r}$</td>
<td>reverse chemical reaction rate constant</td>
<td>[s$^{-1}$]</td>
</tr>
<tr>
<td>$k_{1,3}$</td>
<td>forward microscopic rate constant</td>
<td>[mM$^{-1}$s$^{-1}$]</td>
</tr>
<tr>
<td>$k_{1,3}^{-1}$</td>
<td>reverse microscopic rate constant</td>
<td>[s$^{-1}$]</td>
</tr>
<tr>
<td>$K_{eq}$</td>
<td>equilibrium constant</td>
<td>[M$^{-1}$]</td>
</tr>
<tr>
<td>$K_{mj}$</td>
<td>Michaelis-Menten constant for compound j</td>
<td>[mM]</td>
</tr>
<tr>
<td>$K_{ij}$</td>
<td>inhibition constant for compound j</td>
<td>[mM]</td>
</tr>
<tr>
<td>$l$</td>
<td>light path cuvette</td>
<td>[dm]</td>
</tr>
<tr>
<td>$r_j$</td>
<td>reaction rate for compound j</td>
<td>[mM.s$^{-1}$]</td>
</tr>
<tr>
<td>$t$</td>
<td>time</td>
<td>[s]</td>
</tr>
<tr>
<td>$T$</td>
<td>temperature</td>
<td>[$^\circ$C]</td>
</tr>
<tr>
<td>$V$</td>
<td>volume</td>
<td>[m$^3$]</td>
</tr>
<tr>
<td>$\varepsilon_{280}$</td>
<td>extinction coefficient at 280 nm</td>
<td>[m$^2$.mol$^{-1}$]</td>
</tr>
</tbody>
</table>

**Compound j:**

- A: benzaldehyde
- B: hydrogen cyanide, HCN
- P: rac-mandelonitrile
- $P^R$: (R)-mandelonitrile
- $P^S$: (S)-mandelonitrile
- E: enzyme, PaHnl
Appendix I

Microscopic rate constants were calculated from the kinetic parameters by:

\[ k_1 = \frac{k_{cA}'}{K_{mA}} \]  \hspace{1cm} (A1)

\[ k_{-1} = k_{cA}' \frac{K_{IA}}{K_{mA}} \]  \hspace{1cm} (A2)

\[ k_2 = \frac{k_{-2} + k_3}{K_{mB}} \]  \hspace{1cm} (A3)

\[ k_{-2} = \frac{1}{\frac{1}{k_{cA}'} - \frac{1}{k_{-1}}} \]  \hspace{1cm} (A4)

\[ k_3 = k_{cA}' \]  \hspace{1cm} (A5)

\[ k_{-3} = \frac{k_{cA}'}{K_{lp}} \]  \hspace{1cm} (A6)

The rate equation for the ordered bi-uni model as a function of the microscopic rate constants is:

\[ r_{pA} = \frac{\left( \frac{k_1 k_3}{k_{-3}} C_A C_B - \frac{k_3 k_{-2}}{k_{-3} + k_{-2}} C_{pA} \right) C_B}{1 + \frac{k_1}{k_{-1}} C_A + \frac{k_1 k_2}{k_{-1} (k_{-1} + k_{-2})} C_B + \frac{k_3 k_1}{(k_{-1} + k_{-2}) k_{-1}} C_A C_B + \frac{k_3 (k_{-2} + k_{-1})}{k_{-1} (k_{-1} + k_{-2})} C_{pA} + \frac{k_3 k_2}{k_{-1} (k_{-1} + k_{-2})} C_B C_{pA}} \]
Appendix II

The concentration of (S)-mandelonitrile was calculated from the measured benzaldehyde concentrations according to the procedure below.

The macroscopic balance:

$$\frac{dC_{\mu}}{dt} = k_{\text{chem},f} \left( C_A C_B - \frac{C_{\mu}}{K_{eq}^S} \right)$$

is integrated with the boundary condition $C_{\mu} = C_{\mu}^0$ at $t = 0$:

$$C_{\mu} = C_{\mu}^0 \exp \left( -\frac{k_{\text{chem},f}^S}{K_{eq}^S} t \right) + K_{eq}^S C_A C_B \left[ 1 - \exp \left( -\frac{k_{\text{chem},f}^S}{K_{eq}^S} t \right) \right]$$

Each data point for HCN ($C_B$), follows from the measured benzaldehyde concentration $C_A$ and a stoichiometric balance. $C_{\mu}$ is calculated with the known $K_{eq}^S$ and $k_{\text{chem},f}^S$. This concentration is subtracted from the racemic product concentration to obtain the concentration of the (R)-enantiomer.
References


Development of a process model to describe the synthesis of \((R)\)-mandelonitrile by \textit{Prunus amygdalus} hydroxynitrile lyase in an aqueous-organic biphasic reactor

Abstract

A process model for the enzymatic synthesis of \((R)\)-cyanohydrins in an aqueous-organic biphasic stirred tank reactor has been developed. The conversion of benzaldehyde into \((R)\)-mandelonitrile, catalyzed by \textit{Prunus amygdalus} hydroxynitrile lyase at 5°C and pH 5.5 is chosen as a model system with methyl tert-butyl ether as the organic phase. The process model consists of a description of the reaction kinetics, mass transfer kinetics and the mass balances for both the aqueous and the organic phase. Values for the enzyme kinetic parameters, according to ordered bi-uni kinetics, the lumped mass transfer coefficient for benzaldehyde and the partition coefficients are determined separately. The process model is validated by using 11 experimental data sets of batch conversions in the aqueous-organic biphasic stirred tank reactor. In these 11 experiments different enzyme concentrations and phase volume ratios have been used. The model has been found to be valid with respect to both the conversion and the enantiomeric excess. To synthesize cyanohydrins with a high enantiomeric excess it is required to work at mass transfer limited conditions. The developed process model will be used to investigate other process concepts and other substrates.

Introduction

R-Hydroxynitrile lyase from almonds, *Prunus amygdalus sativa*, (PaHnl) catalyzes the addition of HCN to a wide range of aldehydes and ketones. The (R)-cyanohydrins that are formed are interesting chiral building blocks (Brussee and Van der Gen, 2000; Gregory, 1999; Griengl et al., 2000; Johnson and Griengl, 1999; Pöchlauer, 1998; Van Scharrenburg et al., 1993; Effenberger, 1994). PaHnl can readily be obtained in pure form from almonds, which contain 4-5 g enzyme per kg, but it can also be used as a crude preparation (Lin et al., 1999; Zandbergen et al., 1995). The yellow glycoprotein PaHnl (Mandelonitrile lyase, E.C. 4.1.2.10) contains one flavin adenine dinucleotide (FAD) molecule per molecule of protein. This co-enzyme is essential for the stability and activity of PaHnl. However, it does not appear to participate directly in the reaction (Bärwald and Jaenicke, 1978). The molecular mass of PaHnl is 72 kDa (Hickel et al., 1996).

In order to develop industrial production methods for (R)-cyanohydrins, several strategies have been used to suppress the simultaneous but undesired non-enzymatic, formation of racemic cyanohydrin (Niedermeyer, 1990; Loos et al., 1995). Aqueous processes have been optimized with respect to pH and temperature. Aqueous-organic biphasic processes have been developed to minimize the non-enzymatic reaction by decreasing the concentration of the reactants in the aqueous phase (Loos et al., 1995) and by making use of mass transfer and partitioning behavior of substrates and products between the aqueous and the organic phase (Gerrits et al., 2001). In general for aqueous-organic biphasic systems it is possible to reduce the amount of water to a level that is just enough to saturate the organic solvent and maintain the activity of the enzyme. Those systems consist of an organic phase without a bulk aqueous phase while the enzyme is often immobilized on a carrier (Wehtje, 1993).

Aqueous-organic biphasic processes using dissolved enzymes are widely applied in biocatalytic conversions (Cheetham, 1999; Crosby, 1992; Dahod and Empie, 1987; Griengl et al., 1998; Kosugi et al., 1990; Matsumae et al., 1999; Pöchlauer, 1998; Van den Tweel et al., 1987).

Until now, optimization of aqueous-organic biphasic systems for the synthesis of enantiopure cyanohydrins in a batch process have generally been carried out by an empirical approach (Griengl et al., 1998; Loos et al., 1995). The influence of all known parameters was investigated and these parameters were optimized one by one. Suitable process conditions are readily obtained by this experimental approach. Such data driven optimization, however, cannot be used efficiently to explore other process concepts, for example continuous or fed-batch processes. To develop new process concepts for (R)-cyanohydrin formation, a model driven approach is required.

The objective of this research is the development and the validation of a process model for the production of (R)-cyanohydrins in an aqueous-organic biphasic stirred tank reactor with an unknown interfacial area operated in batch mode. The formation of (R)-mandelonitrile from benzaldehyde and hydrogen cyanide, catalyzed by *Prunus amygdalus*
hydroxynitrile lyase is chosen as a model reaction (Figure 1). Methyl tert-butyl ether is selected as the organic solvent (Loos et al., 1995) and the reaction conditions will be 5°C and pH 5.5, at which the non-enzymatic reaction towards rac-mandelonitrile is largely suppressed.

\[
\begin{align*}
\text{Benzaldehyde} & \quad \xleftrightarrows{\text{PaHnl}} \quad \text{(R)-Mandelonitrile} \\
\text{C} & \quad \text{H} \quad + \quad \text{HCN} \\
\end{align*}
\]

Figure 1. Conversion of benzaldehyde into (R)-mandelonitrile.

**Modeling Aspects**

The process model is developed for an aqueous-organic biphasic stirred tank reactor operating in batch mode. The reactions, both enzymatic and non-enzymatic, are considered to take place in the bulk of the aqueous phase only. The compounds involved in the process model are benzaldehyde (A), hydrogen cyanide (B), (R)- and (S)-mandelonitrile (P^R and P^S) and the enzyme PaHnl (E).

**Balances**

The volumes of both the aqueous (\(V_{aq}\)) and the organic phase (\(V_{org}\)) are assumed to be constant during the conversion. The reaction is considered to only take place in the aqueous phase.

The mass balance for the aqueous phase (\(\text{j} = A, B, P^R \text{ or } P^S\)) is:
\[
V_{aq} \frac{dC_{aqj}}{dt} = r_j V_{aq} - \phi_j V_{aq} \quad [\text{mol.s}^{-1}] 
\]  
(1)

Where \(C_j\) is the molar concentration, \(r_j\) the reaction rate and \(\phi_j\) the mass transfer rate from the aqueous to the organic phase. The mass balance for the organic phase is:
\[
V_{org} \frac{dC_{orgj}}{dt} = \phi_j V_{aq} \quad [\text{mol.s}^{-1}] 
\]  
(2)

The initially present HCN is allowed to partitionate over the aqueous and the organic phase whereas the benzaldehyde initially is only present in the organic phase.

The stoichiometric balances are:
\[
- r_A = r_P^R + r_P^S 
\quad [\text{mol.l}_{aq}^{-1}.s^{-1}] 
\]  
(3)
\[
- r_A = - r_B 
\quad [\text{mol.l}_{aq}^{-1}.s^{-1}] 
\]  
(4)
Reaction kinetics

The enzymatic and non-enzymatic reaction rates both contribute to the overall reaction rate \( r_j \) in eq (1).

\[
r_j = r_{j, \text{enz}} + r_{j, \text{non-enz}} \quad \text{[mol.l}_{eq}^{-1}.s^{-1}] \tag{5}
\]

Synthesis and cleavage of \((R)\)-mandelonitrile by \(P_a\)Hnl are described by an ordered bi-uni model. The enzyme is considered to be enantiospecific towards the \((R)\)-enantiomer, \( r_{j, \text{enz}}^R = 0 \) (Willeman et al., 2000). The rate equation for the synthesis of \((R)\)-mandelonitrile according to the bi-uni model is:

\[
r_{j, \text{enz}}^R = \frac{k_{cat}^j}{K_{\text{IA}}K_{mB}} \left( \frac{C_{eq,A}C_{eq,B} - \frac{C_{eq,p^R}}{K_{eq}}}{1 + \frac{C_{eq,A}}{K_{\text{IA}}} + \frac{C_{eq,B}}{K_{mB}}} + \frac{C_{eq,A}C_{eq,B}}{K_{mB}} + \frac{C_{eq,B}C_{eq,p^R}}{K_{mp}} \right) \cdot C_{eq,E} \text{[mol.l}_{eq}^{-1}.s^{-1}] \tag{6}
\]

In eq. (6) \( K_{eq}^R \) is the equilibrium constant for the reaction towards \(P^R\), which value equals to half of the equilibrium constant \( K_{eq}^{RS} \), for reaction towards the racemate (Willeman et al., 2000).

The reversible non-enzymatic kinetics for product formation are described by eq. (7) and eq. (8). A clear distinction is made between the \((R)\)- and \((S)\)-product.

\[
r_{j, \text{non-enz}}^R = k_{\text{non-enz}}^R \left( C_A C_B - \frac{C_{p^R}}{K_{eq}} \right) \quad \text{[mol.l}_{eq}^{-1}.s^{-1}] \tag{7}
\]

\[
r_{j, \text{non-enz}}^S = k_{\text{non-enz}}^S \left( C_A C_B - \frac{C_{p^S}}{K_{eq}} \right) \quad \text{[mol.l}_{eq}^{-1}.s^{-1}] \tag{8}
\]

Although the enzymatic and non-enzymatic kinetic parameters are known for 100 mM sodium citrate aqueous buffer (Willeman et al., 2000), the saturation of the aqueous buffer by the organic solvent might effect these values considerably (Bauer, 1998).

Mass transfer kinetics

Mass transfer is described by:

\[
\phi_j = k_{L,j} \left( \frac{C_{eq,j}}{m_j} - C_{eq,j} \right) \quad \text{[mol.l}_{eq}^{-1}.s^{-1}] \tag{9}
\]

In eq. (9) \( a \) is the interfacial area per volume of aqueous phase and \( k_{L,j} \) is the lumped mass transfer coefficient defined by:

\[
k_{L,j} = \frac{K_{eq} K_{org} K_{L,j} m_j}{K_{org} m_j + K_{eq} K_{L,j}} \quad \text{[s}^{-1}] \tag{10}
\]

In eq. (10) \( K_{L,j}^{eq} \) and \( K_{L,j}^{org} \) are the mass transfer coefficients for the aqueous and the organic phase. The partition coefficient \( m_j \) is defined by the ratio of the equilibrium concentrations (organic over buffer).
The mass transfer rate \( \phi \) is based on the aqueous phase volume and therefore easily comparable to the reaction rates, since the reaction is considered to take place solely in the aqueous phase. If the size of aqueous droplets in the organic phase is assumed to be independent of the phase volume ratio, an increase in volume of aqueous phase is proportional to an increase in interfacial area. Therefore the mass transfer coefficient \( k_{l,a} \) is defined for the aqueous rather than the total volume. A constant value over a certain range of phase volume ratios is expected. A change in the value for \( k_{l,a} \) may indicate a change in droplet diameter or an inversion of the phase system from a dispersion of aqueous phase into a dispersion of organic phase.

The partition coefficients will be determined ourselves at the investigated experimental conditions.

Measurements of \( k_{l,a} \) can be performed by monitoring the rate of equilibration of compound \( j \) between the organic and aqueous phase. Preliminary studies showed that for realistic values of \( k_{l,a} \) this requires an extremely accurate and fast analysis. Alternatively, \( k_{l,a} \) can be determined in a reacting system, when the reaction rate is much faster than the mass transfer rate, leading to \( C_{eq,i} \approx 0 \). This enabled us to determine \( k_{l,a} \) independent of the reaction kinetics. By measuring the concentration in the organic phase in a reactive system the experimental time scale is lengthened.

Calculation of conversion and enantiomeric excess

In the experiments described in this work, both conversion and enantiomeric excess are based on analysis of the organic phase. Therefore the definitions for the extent of conversion and the enantiomeric excess for the simulations are:

\[
\xi = 1 - \frac{V_{org} C_{org,A}}{n_A^0} \quad [-] \quad (11)
\]

\[
e_{R} = \frac{C_{org,R^+} - C_{org,P}}{C_{org,R^+} + C_{org,P}} \quad [-] \quad (12)
\]

\( n_A^0 \) is the initial amount (mmol) of benzaldehyde present in the reactor.

Materials and Methods

Chemicals and enzyme

Chemically pure and enantiopure (99%, ee 99+%) \((R)\)-mandelonitrile was prepared as described before (Zandbergen et al., 1995). \( rac\)-Mandelonitrile used for the \( PaHnI \) activity test was a gift from DSM Fine Chemicals Austria (Linz, Austria). \( rac\)-Mandelonitrile pro synthesis used for the partition experiments was purchased from Merck. Benzaldehyde was distilled under nitrogen before use. A buffered HCN-solution was obtained by dissolving NaCN, citric acid and NaOH in deaerated water in a closed system to prevent loss of HCN. NaCN was purchased from Aldrich. Methyl \( tert\)-butyl ether was purchased from Aldrich, washed with demineralized water and distilled. \( PaHnI \) enzyme powder was a gift from Solvay Pharmaceuticals (Weesp, The Netherlands) and
contains 98% protein and about 65% PaHnl (Loos et al., 1995). The specific activity of
the enzyme powder was determined to be 185,000 IU per gram.

**Hydroxynitrile lyase activity test**

The enzyme activity was determined by monitoring the cleavage of rac-
mandelonitrile into benzaldehyde and HCN at 25.0°C (Bauer M, 1998; Willeman et al.,
2000). Benzaldehyde was measured spectrophotometrically at 280 nm, in a quartz
cuvette, 1 cm light path. At this wavelength the absorption of mandelonitrile can be
neglected, which is not allowed at the absorption maximum of benzaldehyde at 248 nm.
The assay was performed in 46 mM potassium citrate/phosphate buffer pH 5.0 as the
reaction medium.

**Kinetic experiments**

Progress curves were recorded by measuring the absorption of benzaldehyde at
280 nm (Bauer M, 1998; Willeman et al., 2000) in a 4 ml quartz cuvette, mounted in a
temperature controlled cuvette holder. The reactions were performed at 5.0°C and pH 5.5,
in 100 mM sodium citrate buffer saturated with methyl tert-butyl ether. Buffers were
deaerated (20 min vacuum sonification) and stored under nitrogen before use, to prevent
the oxidation of benzaldehyde.

The kinetic parameters were estimated by fitting them against the measured
progress curves by using Encora version 1.2 (Straathof 2001; Willeman et al., 2000).
Encora is a program designed for estimating the values for independent kinetic
parameters of single enzymes which act according to established kinetic models, in this
case the ordered bi-uni model.

**Enzyme stability**

The stability of PaHnl (260-310 mg.l⁻¹) with respect to deactivation by dissolved
methyl tert-butyl ether was determined in 100 mM sodium citrate buffer of pH 5.5,
saturated with methyl tert-butyl ether, at 25°C. The activity of the enzyme was
determined by the described hydroxynitrile lyase activity test at the start of the incubation
and after 24 h.

In addition, the stability with respect to interfacial deactivation was determined in
the aqueous-organic biphasic batch reactor at 5°C. Aqueous sodium citrate buffer (100
ml) with 367 or 1497 mg.l⁻¹ enzyme powder was stirred with 100 ml of methyl tert-butyl
ether for 3 days.

**Partition coefficients**

The partition coefficients of benzaldehyde and mandelonitrile were determined by
using HPLC-analysis (µbondapack phenyl column and a 16% acetonitrile aqueous phase
as eluent with 1 ml phosphorus acid per liter eluent) of both phases. 10 ml of 100 mM
sodium citrate buffer of pH 2.7 and 10 ml methyl tert-butyl ether were incubated and
mixed with 0.25-3 g of rac-mandelonitrile, which contained about 5% benzaldehyde, at 5°C for 3 h.

The partition coefficient of hydrogen cyanide was determined using GC-analysis of both phases (CPSIL 5 CB column 10 m x 0.53 mm). 15 ml of 100 mM aqueous citrate buffer of pH 5.5 with HCN concentrations ranging from 50-200 mM were incubated with 15 ml methyl tert-butyl ether and mixed for 1 h.

Mass transfer kinetics

Progress curves of batch conversions were recorded for the non-enzymatic synthesis of rac-mandelonitrile in the biphasic system at pH 8, 9 and 10 at 5°C in a stirred tank reactor of 225 ml. This reactor is described by Gerrits et al. (2001). First 60 ml of 100 mM sodium phosphate buffer were added to the reactor. Subsequently, a solution of 160 mmol HCN in 120 ml methyl tert-butyl ether was added. While stirring, the reaction was started by the addition of 30 mmol of benzaldehyde. The conversion of benzaldehyde was determined by sampling the organic phase and performing HPLC-analysis (Gerrits et al., 2001). Since the aqueous phase volume is small compared to the organic phase volume and most of the benzaldehyde and mandelonitrile resides in the organic phase, the amounts in the aqueous phase were considered negligible for calculation of the extent of conversion.

Enzymatic conversion in biphasic system

Progress curves were recorded for different phase volume ratios and enzyme concentrations in the same reactor as used for the mass transfer kinetics experiments. First the aqueous 100 mM citrate buffer containing the enzyme was added. Then a solution of HCN in methyl tert-butyl ether was added to the reactor. This solution was prepared from a 100 ml aqueous solution containing about 200 mmol NaCN that was brought to pH 5.5 by addition of citric acid and extracting three times with 40 ml of methyl tert-butyl ether. The HCN concentration is calculated by using the partition coefficient of HCN. After 10 min. of stirring, the batch conversion was started by adding the benzaldehyde in one portion in pure form. However, in experiments 7-11 the benzaldehyde was dissolved in 10 ml methyl tert-butyl ether and added over a period of 2 minutes to the reactor.

Sampling during the reaction was performed by turning off the stirrer and taking <2 µl samples from the organic phase. The conversion of benzaldehyde and the enantiomeric excess of mandelonitrile were determined immediately by HPLC (Gerrits et al., 2001).

Process simulation

The system of mathematical equations, the process model, was programmed within Matlab version 5.2 (The Mathworks), using the Simulink Block Library version 2.2.
Results and Discussion

Reaction kinetics

No decrease in the activity of PaHnl in buffer saturated with methyl tert-butyl ether at 25°C was observed over a period of 24 h, according to the standard assay. Moreover in a stirred biphasic mixture of buffer and methyl tert-butyl ether at 5°C no decrease in enzyme activity was observed over a period of 72 h. Since all other experiments were performed on a much shorter time scale it was concluded that the enzyme activity was constant throughout all experiments described here.

To estimate the enzyme kinetic parameters, eleven progress curves were recorded in buffer saturated with methyl tert-butyl ether at 5°C and pH 5.5 for both the forward and the reverse reaction. The progress curves are shown in Figure 2 and 3 and correspond to the experiments listed in Table 1. The concentration mandelonitrile was calculated from the online measured benzaldehyde concentrations. The concentrations (R)- and (S)-mandelonitrile were calculated according to a procedure described by Willeman et al. (2000). Calculations (not shown) show that presence of dissolved methyl tert-butyl ether slows the initial reaction rate down by 75%.

Table 1 Initial concentrations used for recording progress curves at pH 5.5 and 5°C. (A = benzaldehyde; B = HCN; P^R = (R)-mandelonitrile; E = PaHnl).

<table>
<thead>
<tr>
<th>exp. no.</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
</tr>
</thead>
<tbody>
<tr>
<td>C_{eqA}</td>
<td>[mM]</td>
<td>2.04</td>
<td>2.10</td>
<td>2.00</td>
<td>0.59</td>
<td>0.11</td>
<td>0.10</td>
<td>0.04</td>
<td>0.02</td>
<td>0.56</td>
<td>0.02</td>
</tr>
<tr>
<td>C_{eqB}</td>
<td>[mM]</td>
<td>101</td>
<td>50.5</td>
<td>10.1</td>
<td>50.5</td>
<td>10.1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>25.2</td>
<td>5.05</td>
</tr>
<tr>
<td>C_{eq,R}</td>
<td>[mM]</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2.30</td>
<td>0.92</td>
<td>0.23</td>
<td>0.92</td>
<td>0.92</td>
<td>2.30</td>
</tr>
<tr>
<td>C_{eq,E}</td>
<td>[U.l^{-1}]</td>
<td>1776</td>
<td>3571</td>
<td>7123</td>
<td>1776</td>
<td>1776</td>
<td>8418</td>
<td>8418</td>
<td>8418</td>
<td>4218</td>
<td>4218</td>
</tr>
</tbody>
</table>

Concerning the non-enzymatic reaction kinetics, progress curves for decomposition of rac-mandelonitrile at 25°C and pH 5.5 without enzyme showed no difference in the reaction rate between buffer and buffer saturated with methyl tert-butyl ether. The equilibrium constant was assumed not to change when a solvent saturated buffer is used instead of a pure aqueous buffer (Carrea, 1984). Therefore the values 2.2 \times 10^{-3} \text{ l eq.mol}^{-1}.\text{s}^{-1} for K_{chem,f}^R and 369 \text{ l eq.mol}^{-1} for K_{eq}^R were used, as determined at 5°C in the absence of methyl tert-butyl ether (Willeman et al., 2000) while fitting the independent enzyme kinetic parameters. Results are shown in Table 2, together with the results from experiments in absence of methyl tert-butyl ether.
Table 2 Estimated kinetic parameters for the enzymatic synthesis of \((R)\)-mandelonitrile catalyzed by \(PaHnl\) at pH 5.5 and 5°C. The values in buffer (Willeman et al., 2000) are given for comparison.

<table>
<thead>
<tr>
<th></th>
<th>(k_{cat}^f)</th>
<th>(k_{cat}^{f,b})</th>
<th>(K_{mA})</th>
<th>(K_{mB})</th>
<th>(K_{mp})</th>
<th>(K_{iA})</th>
<th>(K_{iB}^{b})</th>
<th>(K_{iP}^{b})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer</td>
<td>156*</td>
<td>215*</td>
<td>0.016</td>
<td>18</td>
<td>1.07</td>
<td>0.12</td>
<td>59</td>
<td>0.49</td>
</tr>
<tr>
<td>Buffer saturated with MTBE</td>
<td>176*</td>
<td>601*</td>
<td>3.84</td>
<td>27</td>
<td>129</td>
<td>3.85</td>
<td>27</td>
<td>128</td>
</tr>
</tbody>
</table>

* The enzyme powder contained 65% \(PaHnl\), with a molecular weight of 72 kDa.

b Calculated by using consistency relations [Willeman et al., 2000].

The values for \(k_{cat}^f\), \(k_{cat}^{f,b}\), \(K_{mB}\) and \(K_{iB}^{b}\) are of the same order of magnitude as in the absence of methyl tert-butyl ether. However, the values obtained for the kinetic parameters \(K_{mA}\), \(K_{mp}\), \(K_{iA}\) and \(K_{iP}^{b}\) in buffer saturated with methyl tert-butyl ether increased by a factor of 30 to 250, when compared with the values obtained for pure aqueous buffer. This might be caused by competitive inhibition by methyl tert-butyl ether, but as the methyl tert-butyl ether concentration was kept constant at near saturating conditions, the effect of this concentration was not investigated any further.

Figure 2. Enzymatic conversion of benzaldehyde at 5°C, pH 5.5. Markers are experimental values, lines are the fitted curves. The amount of markers was reduced to improve the visibility. \(\times\), \(1\); \(\circ\), \(2\); \(\Delta\), \(3\); \(\circ\), \(4\); \(\times\), \(5\); the numbers correspond to the experiments listed in table 1.
Figure 3. Enzymatic formation of benzaldehyde at 5°C, pH 5.5. Markers are experimental values, lines are the fitted curves. The amount of markers was reduced to improve the visibility. ◆, 6; ○, 7; Δ, 8; ○, 9; ×, 10; *, 11; the numbers correspond to the experiments listed in table 1.

Figure 2 shows that acceptable fits are obtained for the forward reaction at pH 5.5 and 5°C. On the other hand the cleavage rate of (R)-mandelonitrile is overestimated, as shown in Figure 3. Since we will focus on using PaHni for synthetic purposes, it is assumed that the kinetic parameters of Table 2 are sufficiently accurate to describe our experiments in the biphasic system.

Mass transfer kinetics

The partition coefficients for benzaldehyde is 43, for mandelonitrile 46 and for HCN 2.8 at 5°C. The mass transfer coefficient was determined in a biphasic system of buffer and methyl tert-butyl ether that was mass transfer limited due to a very fast non-enzymatic, base catalyzed, cyanohydrin formation. Progress curves were recorded for the non-enzymatic reaction at pH 8, 9 and 10 with 60 ml buffer and 120 ml of methyl tert-butyl ether, see Figure 4. The values for the non-enzymatic forward reaction rate constants at pH 8, 9 and 10 are 0.43, 2.6 and 15.7 l_mol⁻¹.s⁻¹ respectively, as determined by extrapolation of the values reported by Niedermeyer (1990).

Since no increase in conversion rate was observed, the system has to be mass transfer rate limited. Besides the mass transfer is not enhanced by the reaction. The observed mass transfer coefficient related to the aqueous phase volume corresponds to a value of 0.08 s⁻¹ for benzaldehyde using the described process model.
In these experiments HCN was present at a relatively high concentration in the aqueous phase due to its relatively low partition coefficient and the excess used. Therefore its mass transfer rate is assumed to be not limiting. Moreover its diffusion coefficient is expected to be larger than that of benzaldehyde and mandelonitrile, which results in a larger value for the mass transfer coefficient.

The value for $k_L a$ of mandelonitrile is assumed to be approximately equal to that of benzaldehyde, as these compounds are expected to have similar diffusion coefficients.

**Enzymatic conversion in biphasic system**

Enzymatic synthesis of ($R$)-mandelonitrile in the biphasic batch stirred tank reactor was performed at pH 5.5 and 5°C. Progress curves were determined for 11 experiments in which the phase volumes ranged from 10 to 105 ml of buffer and from 120 to 240 ml of methyl tert-butyl ether. In addition the enzyme concentration and initial amounts of reactants were varied, as shown in Table 3.
Table 3 Experimental conditions for the synthesis of (R)-mandelonitrile catalyzed by PaHnI in the considered aqueous organic biphasic system at pH 5.5 and 5°C.

<table>
<thead>
<tr>
<th>Exp. no.</th>
<th>V_{aq} [ml]</th>
<th>V_{org} [ml]</th>
<th>n_A [mmol]</th>
<th>n_B [mmol]</th>
<th>C_E [U.ml_{aq}^{-1}]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>105</td>
<td>120</td>
<td>62.7</td>
<td>180</td>
<td>185</td>
</tr>
<tr>
<td>2</td>
<td>20</td>
<td>120</td>
<td>46.9</td>
<td>206</td>
<td>925</td>
</tr>
<tr>
<td>3</td>
<td>21</td>
<td>240</td>
<td>59.2</td>
<td>410</td>
<td>176</td>
</tr>
<tr>
<td>4</td>
<td>11</td>
<td>240</td>
<td>60.3</td>
<td>410</td>
<td>333</td>
</tr>
<tr>
<td>5</td>
<td>10</td>
<td>120</td>
<td>61.3</td>
<td>176</td>
<td>89</td>
</tr>
<tr>
<td>6</td>
<td>15</td>
<td>120</td>
<td>65.8</td>
<td>207</td>
<td>23</td>
</tr>
<tr>
<td>7</td>
<td>11</td>
<td>130</td>
<td>53.7</td>
<td>173</td>
<td>37</td>
</tr>
<tr>
<td>8</td>
<td>11</td>
<td>130</td>
<td>54.7</td>
<td>173</td>
<td>19</td>
</tr>
<tr>
<td>9</td>
<td>11</td>
<td>130</td>
<td>56.8</td>
<td>176</td>
<td>185</td>
</tr>
<tr>
<td>10</td>
<td>11</td>
<td>130</td>
<td>59.4</td>
<td>176</td>
<td>555</td>
</tr>
<tr>
<td>11</td>
<td>20</td>
<td>130</td>
<td>54.7</td>
<td>173</td>
<td>185</td>
</tr>
</tbody>
</table>

The process model was validated by simulation of these experiments. The values of the reaction kinetic parameters, partition coefficients and mass transfer coefficient were fixed during the simulation. Benzaldehyde was assumed to be solely present in the organic phase at the start of the reaction.

The resulting simulated conversion progress curves are shown together with the experimental data in Figure 5 and 6. The choice for grouping the different data sets into mass transfer limited and non mass transfer limited is somewhat arbitrary.

![Conversion vs Time](image)

Figure 5. Enzymatic conversion of benzaldehyde in the mass transfer rate limited experiments at 5°C, pH 5.5 in the aqueous-organic biphasic system. Markers are experimental values; o, 1; □, 2; ◊, 3; ◊◊, 4; △, 5; △△, 6; ○, 7; □□, 8; □□□, 9; □□□□, 10; ◊◊◊, 11. The numbers correspond to the experiments in table 3. The lines represent the simulated experiments. Solid lines are the well fitting curves. Dashed lines are the less fitting curves; − − − −, 3; − − −, 4; − − − −, 10; − − − − − −, 11.
Figure 6. Enzymatic conversion of benzaldehyde for the experiments that are not mass transfer limited at 5°C, pH 5.5 in the aqueous-organic biphasic system. Markers are experimental values; •, 5; □, 6; △, 7; ▲, 8. The numbers correspond to the experiments in table 3. The lines represent the simulated experiments. Solid lines are the well fitting curves. Dashed lines are the less fitting curves; ---, 6; ——7.

Figure 7. Enantiomeric excess of mandelonitrile for the experiments that are not mass transfer limited at 5°C, pH 5.5 in the aqueous-organic biphasic system. Markers are experimental values; •, 5; □, 6; △, 7; ▲, 8. The numbers correspond to the experiments listed in table 3. The lines represent the simulated experiments. Solid lines are the well fitting curves. Dashed lines are the less well fitting curves; ---, 6; ——7.

In Figure 5 the results are shown of the experiments that are considered to be operated at mass transfer limitation. This results in an enantiomeric excess of over 99%, experimentally as well as simulated (data not shown).
In Figure 6 the results are shown of the other experiments which are initially not mass transfer limited, as a result of initially high concentrations of benzaldehyde in the aqueous phase. This results in a lower enantiomeric excess, as shown in Figure 7.

In the simulations the enzyme activity had to be halved to obtain reasonable correspondence with the experimental enantiomeric excess in Figure 7 and extent of conversion in Figure 6. As expected this does not influence the simulation results of the mass transfer limited experiments, neither with respect to conversion nor with respect to enantiomeric excess. A possible explanation for the reduced enzyme activity in the batch reactor is that during the kinetics experiments using aqueous buffer with dissolved methyl tert-butyl ether, the aqueous phase was not fully saturated. Although slight deviations from saturation could change the enzyme activity, it is unlikely that this could account for a 50% decrease of the enzyme activity. Better explanations for this decrease were not found, however.

Concerning the conversion, the simulation results agree with the experimental data. About one half of the simulated progress curves describes the experimental data well, however the other half gives a good qualitative description of the data but shows differences of up to 20%. We cannot account for these differences by our model. When comparing experiment 9 and 10, experiment 9 shows a larger conversion rate than 10, although less enzyme was used. The structure of our model will predict a lower conversion rate when less enzyme is used, irrespective of the values of the parameters in the model.

Some discrepancies between the experimental and simulated enantiomeric excess that are shown in Figure 7, however, can be easily explained. In experiments 5 and 6 the enantiomeric excess is increasing in time and finally approaching the simulated value. The process model simulates a drop in enantiomeric excess. This discrepancy is accounted for by the start-up procedure. In experiments 5 and 6 benzaldehyde was added in one portion to the biphasic liquid in the reactor. Before the benzaldehyde merged with the organic phase a three phase system may have been present. This may have resulted in high local concentrations of benzaldehyde in the aqueous phase favoring the non-enzymatic reaction and resulting in an initially low enantiomeric excess. To test this hypothesis in experiments 7 and 8 benzaldehyde was added dissolved in 10 ml methyl tert-butyl ether in the course of 2 min. As expected, in these experiments the initial enantiomeric excess is considerably higher. Since fast sampling of the phases is extremely difficult the initial drop that is seen in the simulated enantiomeric excess cannot be measured.

From the results in Figure 5 and 6 it can be concluded that the conversion rate is increased by increasing the mass transfer rate. The mass transfer rate is increased by increasing the aqueous phase volume and by increasing the enzyme concentration. From the results in Figure 7 it can be concluded, see also Gerrits et al. (2001), that the enantiomeric excess is increased by increasing the ratio between the enzymatic reaction rate and the mass transfer rate resulting in lower initial benzaldehyde concentrations in
the aqueous phase (results not shown). This ratio is increased by either increasing the enzyme concentration and/or decreasing the aqueous phase volume.

The results also indicate that there should exist an optimum aqueous phase volume fraction for the synthesis of cyanohydrins when using an aqueous-organic biphasic batch stirred tank reactor. This will depend on whether the enantiomeric excess or the reaction time is most important.

**Mass transfer versus interfacial enzyme activity**

An important question to be addressed for the efficient use of *PaHnl* is the actual site of the enzymatic reaction. Hickel et al. (1999) found that stirring of the organic phase had no effect on the conversion rate. It was concluded that mass transfer had no influence. Nevertheless, variation in interfacial area showed changes in the conversion rate. This was explained by activity of the enzyme at the aqueous-organic interface. Their research was performed in a Lewis cell type reactor and progress curves were determined by spectrophotometric measurement of the concentration of benzaldehyde in the organic phase.

When dealing with aqueous-organic biphasic systems with compounds that have high partition coefficients, mass transfer limitation will probably reside at the aqueous side of the interface. When the mass transfer coefficients at either side of the interface are in the same range \( K_{L}^{aq} \approx K_{O}^{aq} \), eq. (10) shows that \( k_L \approx K_{L}^{aq} \) for \( m \gg 1 \), and \( k_L \) is not improved when \( K_{L}^{aq} \) is improved due to stirring in the organic phase. \( k_L \) is improved when \( K_{L}^{aq} \) is improved due to mixing of the aqueous phase.

As the stirring rate in the aqueous phase was not varied, mass transfer limitation could not be detected by Hickel et al. (1999). The correlation they found between the magnitude of the interfacial area and the reaction rate was ascribed to activity of the enzyme at the interface. To account for the measured reaction rates the enzyme at the interface was considered hyperactive. However, we assume that the intrinsic activity of the enzyme at the interface equals the intrinsic activity in the bulk. Close to the interface the benzaldehyde concentration will be higher than in the bulk and the reaction rate will be higher. At the conditions of Hickel et al. (1999), where the aqueous phase was stagnant due to the absence of mixing in that phase, the benzaldehyde and reaction rate gradients may have been steeper than in our case, where the aqueous phase is mixed.

**Conclusions**

A process model for the synthesis of *(R)*-cyanohydrins in an aqueous-organic biphasic stirred tank reactor with an unknown interfacial area has been developed. The process model incorporates reaction kinetics, mass transfer kinetics and mass balances. Values for the enzyme kinetic parameters, according to ordered bi-uni kinetics, the lumped mass transfer coefficient for benzaldehyde and the partition coefficients have been determined separately.
The process model has been validated by using 11 experimental data sets for batch conversions in an aqueous-organic biphasic batch operated stirred tank reactor with varying volumes of both phases and varying enzyme concentrations. The correspondence between experiments and simulations was good with respect to both the conversion and the enantiomeric excess.

The process model assumes that the enzyme shows normal behavior at the interface and predicts that an increase in interfacial area increases the mass transfer rate and therefore the conversion rate.

The way of benzaldehyde addition to start the conversion has a large effect on the enantiomeric excess. Adding benzaldehyde slowly and dissolved in methyl tert-butyl ether results in a higher initial enantiomeric excess compared to the case where benzaldehyde was added pure and in one portion to the aqueous-organic biphasic mixture.

The process model confirms that to synthesize cyanohydrins with a high enantiomeric excess it is required to work under mass transfer rate limiting conditions. The developed process model can be used to investigate other process concepts and other substrates.

**Nomenclature**

- $C_{aq,i}$: concentration in aqueous phase \([\text{mol.l}_{aq}^{-1}]\)
- $C_{org,i}$: concentration in organic phase \([\text{mol.l}_o^{-1}]\)
- $C_{aq,E}$: enzyme concentration \([\text{g. l}_{aq}^{-1}]/[\text{U/ l}_{aq}^{-1}]\)
- $ee_j^R$: enantiomeric excess \([-]\)
- $k_{cat}^f$: forward catalytic constant \([\text{s}^{-1}]\)
- $k_{cat}^r$: reverse catalytic constant \([\text{s}^{-1}]\)
- $k_{chem}$: forward chemical reaction rate constant \([\text{l}_{aq}.\text{mol}^{-1}.\text{s}^{-1}]\)
- $k_{L,m}$: lumped mass transfer coefficient \([\text{s}^{-1}]\)
- $K_{eq}^R, K_{eq}^S$: enantiomeric equilibrium constant \([\text{l}_{aq}.\text{mol}^{-1}]\)
- $K_{eq}^R$: racemic equilibrium constant \([\text{l}_{aq}.\text{mol}^{-1}]\)
- $K_m$: Michaelis constant for compound \(j\) \([\text{mol.l}_{aq}^{-1}]\)
- $K_i$: inhibition constant for compound \(j\) \([\text{mol.l}_{aq}^{-1}]\)
- $m_j$: partition coefficient \([\text{l}_{aq}.\text{l}_o^{-1}]\)
- $n_j$: amount of compound \(j\) present in the reactor \([\text{mol}]\)
- $n_{0j}^0$: initial amount of compound \(j\) present in the reactor \([\text{mol}]\)
- $r_j$: reaction rate for compound \(j\) \([\text{mol.l}_{aq}^{-1}.\text{s}^{-1}]\)
- $t$: time \([\text{s}]\)
- $V_{aq}$: volume aqueous phase \([\text{l}_{aq}]\)
- $V_{org}$: volume organic phase \([\text{l}_o]\)
- $\phi_j$: mass transfer rate \([\text{mol.l}_{aq}^{-1}.\text{s}^{-1}]\)
- $\xi$: extent of conversion of aldehyde \([-]\)
Compound j:
A benzaldehyde
B hydrogen cyanide, HCN
PR (R)-mandelonitrile
PS (S)-mandelonitrile
E enzyme, PaHnl

References
Comparison of a batch, fed-batch and continuously operated stirred-tank reactor for the enzymatic synthesis of (R)-mandelonitrile by using a process model

Abstract

The suitability of a batch, fed-batch and continuously operated stirred-tank reactor for the enzymatic production of (R)-mandelonitrile in an aqueous-organic biphasic system was investigated by using a process model. The considered biphasic system is 10-50% (v/v) 100 mM sodium citrate buffer of pH 5.5 dispersed in methyl tert-butyl ether.

The constraints were that 750 moles of benzaldehyde per m³ should react towards (R)-mandelonitrile with an enantiomeric excess of 99% and a conversion of 98%.

A continuously operated stirred-tank reactor could not meet the constraints, but the production in a batch or fed-batch reactor was feasible. The choice for a batch or fed-batch reactor is dependent on the influence of the costs for reactor operation and for the enzyme on the product costs. The choice for operating at a small or large aqueous phase volume fraction is dependent on the costs and reusability of the enzyme.

The volumetric productivity is maximal when operating as batch. The enzymatic productivity and turnover are maximal when operating as fed-batch. In the fed-batch mode the enzymatic productivity increased by 24-37%, the turnover increased by 50-60% and the volumetric productivity decreased by 33-71% as compared to a batch reactor.

By enhancement of mass transfer both the volumetric and enzymatic productivity can be increased considerable while the turnover is only slightly decreased.

This chapter is published in Bioprocess and Biosystems Engineering (2001) 24:281-287.
Introduction

Cyanohydrins are valuable building blocks for the synthesis of drugs and agrochemicals [1-4]. For their asymmetric synthesis from hydrogen cyanide and aldehydes or ketones enzymatic methods using hydroxynitrile lyases are widely used [1-10]. In order to develop industrial production methods for \((R)\)-cyanohydrins, using \(R\)-hydroxynitrile lyase from almonds, the non-enzymatic formation of racemic cyanohydrin has to be suppressed.

Aqueous-organic biphasic processes have been developed to minimize the non-enzymatic reaction by decreasing the concentration of aldehyde in the aqueous phase and by making use of mass transfer and partitioning of substrates and products between the aqueous and the organic phase [11]. To synthesize cyanohydrins with a high enantiomeric excess it is required to work under mass transfer rate limiting conditions [12, 13].

Until now, the optimization of the aqueous-organic biphasic system was carried out for a batch process only. In order to explore other process concepts, for example fed-batch or continuous processes, we have developed a process model [13].

The objective of this research is to use this process model and to investigate by simulation the synthesis of \((R)\)-mandelonitrile in an aqueous-organic biphasic stirred-tank reactor operating in batch, fed-batch and continuous mode. Methyl tert-butyl ether is selected as the organic solvent [11] and the reaction conditions are 5°C and pH 5.5, at which the non-enzymatic reaction towards \(rac\)-mandelonitrile is largely suppressed.

The different reactor options will be compared to each other with respect to their volumetric productivity, enzymatic productivity and turnover.

The constraint to be met is that per m\(^3\) reactor, 98% of 750 moles of benzaldehyde are converted into \((R)\)-mandelonitrile with an enantiomeric excess of 99%. The amount of HCN which is available will be just enough to obtain an equilibrium conversion of 99% with respect to benzaldehyde. This amount will be calculated from the equilibrium constant, phase volumes and the partition coefficients.

The batch, fed-batch and continuously operated stirred-tank reactor will be studied over a range of aqueous phase volumes where the aqueous phase is expected to be disperse, i.e. up to a 50% aqueous phase volume fraction.

Theory

The process model is developed for an aqueous-organic biphasic stirred-tank reactor operating batch, fed-batch or continuous. The compounds involved in the process model are benzaldehyde (A), hydrogen cyanide (B), \((R)\)- and \((S)\)-mandelonitrile (P\(^R\) and P\(^S\)) and the enzyme \(PaHnl\) (E).
Balances

The volume of the aqueous phase is considered to be constant during the conversion. When operating continuously the aqueous phase is retained in the reactor. It is assumed that both reactions only take place in the aqueous phase [12, 13].

The mass balance for the aqueous phase \((j = A, B, P^k \text{ or } P^S)\) is:

\[
V_{\text{aq}} \frac{dC_{\text{aq,j}}}{dt} = r_j V_{\text{aq}} - \phi_j V_{\text{aq}} \quad [\text{mol.s}^{-1}] 
\]  

\(V_{\text{aq}}\) is the volume of the aqueous phase, \(C_{\text{aq,j}}\) the concentration in the aqueous phase, \(r_j\) the reaction rate and \(\phi_j\) the mass transfer rate from the aqueous to the organic phase.

The volume of the organic phase was considered to be constant as well during the reaction. For the fed-batch it was assumed that the feed was neat benzaldehyde and HCN and had a negligible influence on the reaction volume.

The mass balance for the organic phase of the (fed) batch operated stirred-tank reactor is:

\[
V_{\text{org}} \frac{dC_{\text{org,j}}}{dt} = \phi_j V_{\text{org}} + N_j^F \quad [\text{mol.s}^{-1}] 
\]  

\(V_{\text{org}}\) is the volume of the organic phase, \(C_{\text{org,j}}\) the concentration in the organic phase and \(N_j^F\) the feed rate of neat substrate, which is instantaneously mixed with the organic phase. When operating as batch \(N_j^F = 0\).

For a continuous operated stirred-tank reactor the balance is:

\[
V_{\text{org}} \frac{dC_{\text{org,j}}}{dt} = \phi_j V_{\text{org}} + F_{\text{org,j}}^{\text{conf}} \left(C_{\text{org,j}}^{\text{in}} - C_{\text{org,j}}\right) \quad [\text{mol.s}^{-1}] 
\]  

\(F_{\text{org,j}}^{\text{conf}}\) is the flow rate of the mobile organic phase and the entering substrate concentration is \(C_{\text{org,j}}^{\text{in}}\).

The initial amount of HCN is assumed to be partitioned between the aqueous and the organic phase whereas the benzaldehyde initially is present only in the organic phase.

The stoichiometric balances are:

\[-r_A = r_p^A + r_p^S \quad [\text{mol.m}^{-3}.s^{-1}] \]  

\[-r_A = -r_B \quad [\text{mol.m}^{-3}.s^{-1}] \]

Reaction kinetics

The enzymatic and non-enzymatic reaction rates both contribute to the overall reaction rate \(r_j\) in eq (1), as described before [14].

\[
r_j = r_{j,\text{enz}} + r_{j,\text{non-enz}} \quad [\text{mol.m}^{-3}.s^{-1}] 
\]

Synthesis and cleavage of \((R)\)-mandelonitrile by \(PaHnI\) are described by an ordered bi-uni model. The enzyme is considered to be enantiospecific towards the \((R)\)-
enantiomer, $r_{P,eu}^S = 0$ [14]. The rate equation for the synthesis of $(R)$-mandelonitrile according to the bi-uni model is:

$$r_{P,eu}^R = \frac{k_{cat}^R}{K_{IA}^R K_{mb}^R} \left( C_{aq,A} C_{aq,B} - \frac{C_{aq,R}^P}{K_{eq}^R} \right) \times C_{aq,E} [\text{mol.m}_aq^{-3}.s^{-1}]$$  (7)

$$1 + \frac{C_{aq,A}}{K_{IA}^R} + \frac{C_{aq,B}}{K_{mb}^R} + \frac{C_{aq,A} C_{aq,B}}{K_{IA}^R K_{mb}^R} + \frac{C_{aq,R}^P}{K_{eq}^R} + \frac{C_{aq,B}^P}{K_{mb}^P} + \frac{C_{aq,B}^P}{K_{mb}^P}$$

The reversible non-enzymatic kinetics for product formation are described by eq. (8) and eq. (9). A clear distinction is made between the $(R)$- and $(S)$-product.

$$r_{P,non-eu}^R = k_{non-eu}^R \left( C_A C_B - \frac{C_R^P}{K_{eq}^R} \right) [\text{mol.m}_aq^{-3}.s^{-1}]$$  (8)

$$r_{P,non-eu}^S = k_{non-eu}^S \left( C_A C_B - \frac{C_S^P}{K_{eq}^S} \right) [\text{mol.m}_aq^{-3}.s^{-1}]$$  (9)

The enzymatic and non-enzymatic kinetic parameters are given in Table 1 [14].

Table 1 Estimated kinetic parameters for the enzymatic synthesis of $(R)$-mandelonitrile catalyzed by PaHnl at 5°C and pH 5.5 [Willeman et al., 2000].

<table>
<thead>
<tr>
<th>$k_{cat}^R$</th>
<th>$k_{cat}^S$</th>
<th>$K_{mA}$</th>
<th>$K_{mB}$</th>
<th>$K_{mb}^R$</th>
<th>$K_{mb}^S$</th>
<th>$K_{IA}$</th>
<th>$K_{IB}$</th>
<th>$K_{IP}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>[s$^{-1}$]</td>
<td>[s$^{-1}$]</td>
<td>[mM]</td>
<td>[mM]</td>
<td>[mM]</td>
<td>[mM]</td>
<td>[mM]</td>
<td>[mM]</td>
<td>[mM]</td>
</tr>
<tr>
<td>176$^a$</td>
<td>601$^a$</td>
<td>3.84</td>
<td>27</td>
<td>129</td>
<td>3.85</td>
<td>27</td>
<td>128</td>
<td></td>
</tr>
</tbody>
</table>

$^a$ The enzyme powder contained 65% PaHnl, with a molecular weight of 72 kDa.

Mass transfer kinetics

Mass transfer is described by:

$$\phi_j = k_{L,j} a \left( \frac{C_{org,j}}{m_j} - C_{aq,j} \right) [\text{mol.m}_aq^{-3}.s^{-1}]$$  (10)

In eq. (10), $a$ is the interfacial area per volume of aqueous phase, $k_{L,j}$ the lumped mass transfer coefficient and $m_j$ the partition coefficient. The partition coefficients (org/aq) of benzaldehyde, HCN and mandelonitrile are 43, 2.8 and 46 [13].

The mass transfer rate is based on the aqueous phase volume and therefore easily comparable to the reaction rates. For simplicity, the value $k_{L,j} a = 0.05$ s$^{-1}$ is used for each simulation. This value for $k_{L,j} a$ is 60% of the value determined earlier for a laboratory reactor [13]. In a full scale reactor $k_{L,j} a$ should be known as a function of the aqueous phase volume fraction.

Initial amount of HCN

The amount of HCN added to the aqueous-organic biphasic system is restricted to the amount required for an equilibrium conversion of $\xi^{eq} = 99$ % of benzaldehyde. This is a rather arbitrary percentage, which guarantees that in each situation 98% conversion is
possible without using excessive amounts of HCN or using extreme long reaction times. For the batch situation this amount is calculated according to eq (11), derived according to appendix 1.

\[
n_B^0 = \frac{\xi^eq}{1 - \xi^eq} \frac{V_{tot}}{K_{eq}^{RS}} \left[ \frac{1 + \frac{V_{org}}{V_{tot}} (m_A - 1)}{1 + \frac{V_{org}}{V_{tot}} (m_B - 1)} \right] + \xi^eq n_A^0 \quad [\text{mol}] \quad (11)
\]

\[K_{eq}^{RS}\] is the equilibrium constant and \(n_A^0\) the amount of benzaldehyde to be converted. Depending on the aqueous phase volume fraction 975-1075 moles HCN were needed for 99% conversion of 750 moles benzaldehyde.

The initial concentrations of HCN were calculated according to:

\[
C_{B,sq}^0 = \frac{n_B^0}{V_{sq} + m_B V_{org}} \quad [\text{mol.m}^{-3}] \quad (12)
\]

\[
C_{B,org}^0 = \frac{n_B^0}{V_{org} + \frac{m_B}{V_{sq}}} \quad [\text{mol.m}^{-3}] \quad (13)
\]

The systems of mathematical equations (the process model) were programmed within Matlab version 5.3 (The Mathworks), using the Simulink Block Library version 3.

**Results**

The described models were used to determine the concentration of enzyme, \(C_{sq,E}\), that is just enough to meet the constraints of 98.00% conversion and 99.00% enantiomeric excess, resulting in the reactor time requirement (t_{conv}). This was performed for different aqueous phase volume fractions and for a batch, fed-batch and a continuous operating stirred-tank reactor. For a certain enzyme concentration at 98.00% conversion a certain enantiomeric excess value was found by simulation. Depending on this value, the enzyme concentration was iterated until at 98.00% conversion the required 99.00% enantiomeric excess was obtained. The corresponding reaction time is t_{conv}.

The stirred-tank reactors operating in batch, fed-batch or continuous mode were compared in terms of volumetric productivity (\(\eta_{volumetric}\)), enzymatic productivity (\(\eta_{enzymatic}\)) and turnover, which are defined by:

\[
\eta_{volumetric} = \frac{0.98 n_A^0}{V_{tot} t_{conv}} \quad [\text{mol.m}^{-3}.h^{-1}] \quad (14)
\]

\[
\eta_{enzymatic} = \frac{0.98 n_A^0}{t_{conv} V_{sq} C_{sq,E}} \quad [\text{mol.g}^{-1}.h^{-1}] \quad (15)
\]

\[
\text{turnover} = \frac{0.98 n_A^0}{V_{sq} C_{sq,E}} \quad [\text{mol.g}^{-1}] \quad (16)
\]
Batch stirred-tank reactor

Simulations of batch reactions at 20 and 50% aqueous phase volume fraction were compared. Figure 1 shows the extent of conversion as well as the aqueous phase concentration of benzaldehyde as a function of time. Figure 2 shows the enantiomeric excess curve.

Figure 1  Benzaldehyde conversion by a batch operated stirred-tank reactor and the aqueous benzaldehyde concentration as function of time for different aqueous phase volumes. The solid lines represent a 50 % and the dashed lines a 20 % aqueous phase volume fraction. The initial value of $C_{eq,A}$ is 0 mM, but the very steep increase in the first minute is hardly visible.

Figure 2  Benzaldehyde conversion by a batch operated stirred-tank reactor into (R)-mandelonitrile with the enantiomeric excess function of time for different aqueous phase volumes. The solid line represents the 50% and the dashed line the 20% aqueous phase volume fraction. The initial enantiomeric excess is 100%, but the very steep decrease in the first minute is hardly visible.
In both cases the enantiomeric excess initially drops from 100 to 98.3%, within the first minute of the reaction. This caused by the assumption that at the start of the reaction all benzaldehyde is present in the organic phase only. While benzaldehyde is being transferred to the aqueous phase the concentration of benzaldehyde in the aqueous phase is increasing. This results in the initial drop in enantiomeric excess. As the benzaldehyde concentration in the organic phase is decreasing in time the system becomes mass transfer limited in benzaldehyde resulting in the observed increase in enantiomeric excess. The enantiomeric excess is increasing until the reversibility of the reaction is becoming important and results in a decrease of the enantiomeric excess due to accumulation of the unwanted (S)-enantiomer. When the organic phase, which contains most of the product, would not be separated from the aqueous phase at the end of the reaction, the enantiomeric excess would continuously decrease until a racemate is obtained. The rate of the decrease in enantiomeric excess can be suppressed by lowering the aqueous phase volume.

In Figure 3 the ratio between the enzymatic and the non-enzymatic reaction rate is shown as function of time, which clearly explains the aforementioned behavior.

![Graph showing enzymatic and non-enzymatic reaction rates over time](image-url)

**Figure 3** Benzaldehyde conversion by a batch operated stirred-tank reactor into (R)-mandelonitrile with the ratio of the enzymatic over the non-enzymatic reaction rate as function of time for different aqueous phase volumes. The solid line represents the 50% and the dashed line the 20% aqueous phase volume fraction.

Lowering the aqueous phase volume decreases the total amount of mass transfer. This retards the synthesis of (R)-mandelonitrile more than the synthesis of unwanted (S)-mandelonitrile.
The results for the batch simulations for a range of aqueous phase volumes are shown in Figure 4 and 5. The required enzyme concentrations and conversion times are shown in Table 2. According to these results an increase in the aqueous phase volume increases both the volumetric and the enzymatic productivity. However, this is accompanied by a decrease in turnover, because more enzyme is used per unit of product. When comparing the cases of 10 and 50% aqueous phase volume fraction it appears that at 50% aqueous phase volume, the volumetric productivity is 10 times higher and the enzymatic productivity 6.4 times higher but the turnover is 7.5 times lower.

Table 2 Results of the simulations of the aqueous organic biphasic batch system at 5°C and pH 5.5 in a stirred-tank reactor.

<table>
<thead>
<tr>
<th>( V_{aq} ) [%]</th>
<th>( C_{aq,B} ) [g.L(^{-1})]</th>
<th>( t_{conv} ) [h]</th>
<th>( \eta_{volumetric} ) [mol.m(^{-3}).h(^{-1})]</th>
<th>( \eta_{enzymatic} ) [mol.g(^{-1}).h(^{-1})]</th>
<th>Turnover [mol.g(^{-1})]</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>1.50</td>
<td>18.9</td>
<td>38.9</td>
<td>0.052</td>
<td>4.90</td>
</tr>
<tr>
<td>20</td>
<td>1.60</td>
<td>8.3</td>
<td>88.6</td>
<td>0.111</td>
<td>2.30</td>
</tr>
<tr>
<td>30</td>
<td>1.75</td>
<td>4.7</td>
<td>155</td>
<td>0.177</td>
<td>1.40</td>
</tr>
<tr>
<td>40</td>
<td>1.95</td>
<td>3.0</td>
<td>246</td>
<td>0.252</td>
<td>0.94</td>
</tr>
<tr>
<td>50</td>
<td>2.25</td>
<td>2.0</td>
<td>374</td>
<td>0.332</td>
<td>0.65</td>
</tr>
</tbody>
</table>

The optimal aqueous phase volume for a batch operated stirred-tank reactor is dependent on both the reactor costs and the enzyme costs as part of the price per unit product.

The results described above were obtained for \( k_{L,a} = 0.05 \) s\(^{-1}\). When the value for \( k_{L,a} \) is doubled for a batch operated stirred-tank reactor the volumetric productivity is increased by 69-78%, the enzymatic productivity by 45-49% while the turnover is decreased by only 12-18%. Clearly, the efficiency of the process depends heavily on the mass transfer rate.
Figure 4  Volumetric productivity as function of the aqueous phase volume for 98% conversion of 750 moles of benzaldehyde into (R)-mandelonitrile with an enantiomeric excess of 99% in 1 m³ reactor volume. The solid line represents the batch reactor and the dashed lines the fed-batch reactor where the aqueous benzaldehyde concentration is controlled at 1 (-----) and 2.5 mM (---).

Figure 5  Enzymatic productivity as function of the aqueous phase volume for 98% conversion of 750 moles of benzaldehyde into (R)-mandelonitrile with an enantiomeric excess of 99% in 1 m³ reactor volume. The solid line represents the batch reactor and the dashed lines the fed-batch reactor where the aqueous benzaldehyde concentration is controlled at 1 (-----) and 2.5 mM (---).
Figure 6  
Turnover as function of the aqueous phase volume for 98% conversion of 750 moles of benzaldehyde into (R)-mandelonitrile with an enantiomeric excess of 99% in 1 m³ reactor volume. The solid line represents the batch reactor and the dashed lines the fed-batch reactor where the aqueous benzaldehyde concentration is controlled at 1 (-----) and 2.5 mM (———).

Fed-batch stirred-tank reactor

For the simulations of the stirred-tank reactor operating as a fed-batch, both substrates were fed as neat liquids and in equal molar amounts until 750 moles of benzaldehyde were added per m³. This fed-batch period was followed by a batch period where the benzaldehyde in the reactor, largely residing in the organic phase volume, was converted until the required conversion and enantiomeric excess were obtained.

The additional degree of freedom for the fed-batch compared to the batch stirred-tank reactor is the substrate feeding rate. Instead of investigating a large number of feeding profiles we decided to study the fed-batch reactor by using a controller, which kept the concentration of benzaldehyde in the aqueous phase at a fixed value. The optimal value of this setpoint had to be determined.

From the study of the batch reactor we learned that the initial high concentration of benzaldehyde in the aqueous phase resulted in an initial production of more (S)-product than wanted. This was partly compensated for by using a high enzyme concentration, which prevented (S)-product formation in a later stage.

We studied the influence of the controllable aqueous concentration of benzaldehyde at two extremes, 10 and 50% aqueous phase volume fraction.
Table 3 Results of the simulations for the aqueous organic biphasic fed-batch system at 5°C and pH 5.5 in a stirred-tank reactor with 10% aqueous phase volume fraction.

<table>
<thead>
<tr>
<th>C_{aq,A} [mM]</th>
<th>C_{aq,E} [g.l_{aq}^{-1}]</th>
<th>t_{conv} [h]</th>
<th>η_{volumetric} [mol.m^{-3}.h^{-1}]</th>
<th>η_{enzymatic} [mol.g_{E}^{-1}.h^{-1}]</th>
<th>turnover [mol.p.g_{E}^{-1}]</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>0.83</td>
<td>66.5</td>
<td>11.1</td>
<td>0.027</td>
<td>8.86</td>
</tr>
<tr>
<td>0.75</td>
<td>0.72</td>
<td>46.5</td>
<td>15.8</td>
<td>0.044</td>
<td>10.21</td>
</tr>
<tr>
<td>1</td>
<td>0.75</td>
<td>36.1</td>
<td>20.4</td>
<td>0.054</td>
<td>9.80</td>
</tr>
<tr>
<td>1.5</td>
<td>0.85</td>
<td>27.5</td>
<td>26.8</td>
<td>0.063</td>
<td>8.65</td>
</tr>
<tr>
<td>2</td>
<td>0.96</td>
<td>23.8</td>
<td>30.9</td>
<td>0.064</td>
<td>7.66</td>
</tr>
<tr>
<td>2.25</td>
<td>1.04</td>
<td>22.6</td>
<td>32.5</td>
<td>0.062</td>
<td>7.07</td>
</tr>
<tr>
<td>2.5</td>
<td>1.10</td>
<td>21.6</td>
<td>34.1</td>
<td>0.062</td>
<td>6.68</td>
</tr>
<tr>
<td>3</td>
<td>1.20</td>
<td>20.5</td>
<td>35.9</td>
<td>0.060</td>
<td>6.13</td>
</tr>
<tr>
<td>4</td>
<td>1.38</td>
<td>19.2</td>
<td>38.3</td>
<td>0.055</td>
<td>5.33</td>
</tr>
<tr>
<td>6</td>
<td>1.46</td>
<td>18.9</td>
<td>38.3</td>
<td>0.053</td>
<td>5.03</td>
</tr>
<tr>
<td>Batch</td>
<td>1.50</td>
<td>18.9</td>
<td>38.3</td>
<td>0.052</td>
<td>4.90</td>
</tr>
</tbody>
</table>

Table 4 Results of the simulations for the aqueous organic biphasic fed-batch system at 5°C and pH 5.5 in a stirred-tank reactor with 50% aqueous phase volume fraction.

<table>
<thead>
<tr>
<th>C_{aq,A} [mM]</th>
<th>C_{aq,E} [g.l_{aq}^{-1}]</th>
<th>t_{conv} [h]</th>
<th>η_{volumetric} [mol.m^{-3}.h^{-1}]</th>
<th>η_{enzymatic} [mol.g_{E}^{-1}.h^{-1}]</th>
<th>turnover [mol.p.g_{E}^{-1}]</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>1.95</td>
<td>10.1</td>
<td>73.0</td>
<td>0.075</td>
<td>0.75</td>
</tr>
<tr>
<td>0.75</td>
<td>1.10</td>
<td>8.7</td>
<td>85.0</td>
<td>0.154</td>
<td>1.34</td>
</tr>
<tr>
<td>1</td>
<td>0.90</td>
<td>6.8</td>
<td>108</td>
<td>0.240</td>
<td>1.63</td>
</tr>
<tr>
<td>2</td>
<td>1.00</td>
<td>3.8</td>
<td>194</td>
<td>0.388</td>
<td>1.47</td>
</tr>
<tr>
<td>2.5</td>
<td>1.10</td>
<td>2.9</td>
<td>250</td>
<td>0.455</td>
<td>1.34</td>
</tr>
<tr>
<td>3</td>
<td>1.25</td>
<td>2.9</td>
<td>249</td>
<td>0.399</td>
<td>1.18</td>
</tr>
<tr>
<td>4</td>
<td>1.5</td>
<td>2.6</td>
<td>282</td>
<td>0.376</td>
<td>0.98</td>
</tr>
<tr>
<td>6</td>
<td>1.9</td>
<td>2.4</td>
<td>305</td>
<td>0.321</td>
<td>0.77</td>
</tr>
<tr>
<td>10</td>
<td>2.18</td>
<td>1.9</td>
<td>383</td>
<td>0.351</td>
<td>0.67</td>
</tr>
<tr>
<td>25</td>
<td>2.25</td>
<td>2.0</td>
<td>374</td>
<td>0.332</td>
<td>0.65</td>
</tr>
<tr>
<td>Batch</td>
<td>2.25</td>
<td>2.0</td>
<td>374</td>
<td>0.332</td>
<td>0.65</td>
</tr>
</tbody>
</table>

From Table 3 and 4 it appears that when the setpoint for the benzaldehyde concentration is high the fed-batch system approaches the batch system and the volumetric productivity is at the maximum value. Regarding the enzymatic productivity and the turnover, for the 10% aqueous phase volume fraction optimal values are found at 2 and 0.75 mM and for the 50% aqueous phase volume fraction at 2.5 and 1 mM, respectively. In comparison to the batch reactor the enzymatic productivity's and turnovers increased, whereas the volumetric productivity's decreased.

This evaluation shows that the benzaldehyde setpoint should be chosen on basis of the relative costs of reactor time and enzyme.
Arbitrarily, the fed-batch operated stirred-tank reactor was calculated over the whole range of aqueous volumes at fixed aqueous concentrations of 1 and 2.5 mM benzaldehyde. The results of the simulations are shown together with the results for the batch systems in Figures 4-6. These figures show that the choice for batch or fed-batch again will be dependent on the cost determining factor: the reactor time or the enzyme.

When the volumetric productivity is cost determining a batch operated stirred-tank reactor at a large aqueous phase volume fraction of 50% appears to be the best choice. When the enzymatic productivity is cost determining a fed-batch operated stirred-tank reactor operating at a fixed aqueous benzaldehyde concentration of around 2.5 mM appears to be the best choice.

When the enzyme is a major cost factor, operating at the smaller aqueous phase volumes is more favorable. When the enzyme is stable during operation it is possible to reuse the aqueous phase volume. Then, the costs for the enzyme are reduced and it would be beneficial to operate at a larger aqueous phase volume fraction of 50%.

Continuous stirred-tank reactor

The study of an ideal continuous stirred-tank reactor in which the organic phase is continuous and the aqueous phase is retained in the reactor revealed that it is impossible to meet the requirements.

For the continuous reactor the residence time had to be calculated. The maximum obtainable conversion and enantiomeric excess are 97.2 and 97.9 % at a residence time of 66.7 h and 25 g of enzyme per liter at 25% aqueous phase volume fraction. A continuous stirred-tank reactor is therefore not suitable for (R)-mandelonitrile production. The reason for this is the high product concentration in the reactor combined with a high residence time, favoring the reversibility of the reaction and the accumulation of the unwanted (S)-enantiomer.

Plug flow reactors

For a continuous production method, plug flow reactor types are potentially more interesting in fulfilling the requirements. A plug flow reactor that has a similar \( k_{L}a \) value as the aforementioned stirred-tank reactor and in which both liquids have the same residence time naturally will show the same performance as a batch reactor, with respect to the productivity's and turnover, as defined here. However, it is difficult to have a high \( k_{L}a \) value and at the same time an ideal plug flow behavior. With a series of stirred tanks, ideal plug flow behavior might be approached.

Alternatively, the centrifugal reactor of Den Hollander et al. [15] can be used for a plug flow of organic liquid while the aqueous liquid is stationary and dispersed. Values for \( k_{L}a \) are in the range of 0.01 - 0.07 s\(^{-1}\), according to Van Buel et al. [16]. However, as these plug flow reactors require different equipment than the considered single stirred-tank reactor we will not try to evaluate these systems here.
Conclusions

Model calculations showed that it is not feasible to obtain both a conversion of 98.00% and an enantiomeric excess of 99.00% for \((R)\)-mandelonitrile when operating a stirred-tank reactor in continuous mode. However, the batch or fed-batch operated stirred-tank reactor are feasible. The choice between a batch and a fed-batch reactor is dependent on the costs of the reactor and the enzyme. The volumetric productivity is maximal for batch, resulting in low reactor costs. The enzymatic productivity and turnover are maximal for fed-batch, resulting in low enzyme costs. By fed-batch operation the enzymatic productivity is increased by 24-37%, the turnover is increased by 50-60% whereas the volumetric productivity is decreased by 33-71% as compared to a batch operating stirred-tank reactor.

When the enzyme is stable the aqueous phase may be reused favoring the use of larger aqueous phase volumes. By enhancement of mass transfer both the volumetric and enzymatic productivity can be increased considerably while the turnover is only slightly decreased.
List of symbols

$C_{aq,j}$  concentration in aqueous phase  [mol.m$_{aq}^{-3}$]
$C_{aq,E}$  enzyme concentration  [g. m$_{aq}$]
$C_{org,j}$  concentration in organic phase  [mol.m$_{org}^{-3}$]
$C_{in}$  concentration in entering organic flow  [mol.m$_{org}^{-3}$]
$ee$  enantiomeric excess of (R)-mandelonitrile  [%]
$F_{org,j}^{cont}$  flow rate continuous reactor  [m$_{org}^3$.s$^{-1}$]
$k_{cat}^{f}$  forward catalytic constant  [s$^{-1}$]
$k_{cat}^{r}$  reverse catalytic constant  [s$^{-1}$]
$k_{non-enz}$  forward chemical reaction rate constant  [m$_{aq}^3$.mol$^{-1}$.s$^{-1}$]
$k_{LiQa}$  lumped mass transfer coefficient  [s$^{-1}$]
$K_{eq}^{R}, K_{eq}^{S}$  enantiomeric equilibrium constant  [m$_{aq}^3$.mol$^{-1}$]
$K_{eq}^{RS}$  racemic equilibrium constant  [m$_{aq}^3$.mol$^{-1}$]
$K_{mij}$  Michaelis constant for compound j  [mol.m$_{aq}^{-3}$]
$K_{ij}$  inhibition constant for compound j  [mol.m$_{aq}^{-3}$]
$m_{j}$  partition coefficient  [m$_{aq}^3$.mol$_{org}^{-3}$]
$n_{j}$  total amount of compound j  [mol]
$n_{i}^{0}$  initial total amount of compound j  [mol]
$N_{j}^{f}$  feed rate of compound j  [mol.s$^{-1}$]
$r_{j}$  reaction rate for compound j  [mol.m$_{aq}^{-3}$.s$^{-1}$]
$t$  time  [s]
$t_{conv}$  time to obtain 98% conversion  [s]
$V_{aq}$  volume aqueous phase  [m$^3$]
$V_{org}$  volume organic phase  [m$^3$]
$V_{tot}$  total reaction volume  [m$^3$]
$\eta_{volumetric}$  volumetric productivity  [mol$_{p}$.m$^3$.h$^{-1}$]
$\eta_{enzymatic}$  enzymatic productivity  [mol$_{p}$.g$_{e}$h$^{-1}$]
$\phi_{j}$  mass transfer rate  [mol.m$_{aq}^{-3}$.s$^{-1}$]
$\xi, \xi^{eq}$  extent of conversion of aldehyde  [%]

Compound j:
A  benzaldehyde
B  hydrogen cyanide, HCN
$P^{R}$  (R)-mandelonitrile
$P^{S}$  (S)-mandelonitrile
E  enzyme, R-hydroxynitrile lyase

68
Appendix

The amount of HCN required for an equilibrium conversion of $\xi^\text{eq} = 0.99$ was calculated using the mass balance of the product

$$n_p = V_{\text{org}} C_{\text{org},p} + V_{\text{aq}} C_{\text{aq},p}$$

By substitution of the partition coefficient $m_p$

$$m_p = \frac{C_{\text{org},p}}{C_{\text{aq},p}}$$

one obtains

$$C_{\text{aq},p} = \frac{n_p}{V_{\text{tot}} \left[ 1 + \frac{V_{\text{org}}}{V_{\text{tot}}} (m_p - 1) \right]}$$

Similar relations for the aqueous concentrations of A and B can be found and substituted in the equation for the equilibrium constant

$$K_{eq}^{RS} = \frac{C_{\text{eq},p}}{C_{\text{aq},A} C_{\text{aq},B}} = \frac{n_p}{V_{\text{tot}} \left[ 1 + \frac{V_{\text{org}}}{V_{\text{tot}}} (m_p - 1) \right]}$$

From this equation the amount of B is calculated which is present in the aqueous-organic biphasic system at the equilibrium conversion $\xi^\text{eq} = 0.99$.

$$n_B^{eq} = \frac{\xi^\text{eq}}{1 - \xi^\text{eq}} V_{\text{tot}} \left[ 1 + \frac{V_{\text{org}}}{V_{\text{tot}}} (m_A - 1) \right] \frac{1}{K_{eq}^{RS} \left[ 1 + \frac{V_{\text{org}}}{V_{\text{tot}}} (m_p - 1) \right]}$$

An additional amount of B, $\xi^\text{eq} n_A^0$, is needed for the conversion of A. The total amount of B to be added to the aqueous-organic biphasic system is:

$$n_B^0 = \xi^\text{eq} n_A^0 + \frac{\xi^\text{eq}}{1 - \xi^\text{eq}} V_{\text{tot}} \left[ 1 + \frac{V_{\text{org}}}{V_{\text{tot}}} (m_A - 1) \right] \frac{1}{K_{eq}^{RS} \left[ 1 + \frac{V_{\text{org}}}{V_{\text{tot}}} (m_p - 1) \right]}$$
References


Reaction temperature optimization procedure for the synthesis of (R)-mandelonitrile by *Prunus amygdalus* hydroxynitrile lyase using a process model approach

**Abstract**

The reaction temperature was optimized for the enzymatic synthesis of (R)-mandelonitrile in a biphasic system of aqueous buffer and methyl tert-butyl ether by including temperature effects in an existing process model. (R)-Mandelonitrile was synthesized by addition of hydrogen cyanide to benzaldehyde using *Prunus amygdalus* hydroxynitrile lyase as the catalyst. The reaction was investigated within a temperature range of 5 to 25°C and at pH 5.5.

At low temperatures the nonenzymatic reaction is reduced to a larger extent than the enzymatic reaction. The mass transfer rate is reduced to a smaller extent. Mass transfer limitation is required for a high enantiomeric excess and determines the conversion rate. Therefore the volumetric productivity decreases at lower temperatures. The equilibrium constant is considerably higher at low temperatures resulting in a higher extent of conversion, or a lower hydrogen cyanide requirement. Both the volumetric productivity and the required enzyme concentration increase by increasing the reaction temperature and aqueous-phase volume while meeting the required conversion degree and enantiomeric excess.

For the conversion of 750 moles benzaldehyde per m$^3$ into (R)-mandelonitrile with an enantiomeric excess of 99.00% and an 98.00% extent of conversion in benzaldehyde, an economic optimum was calculated. A reaction temperature of 15°C and an aqueous-phase volume of 50% containing 1.5 kg enzyme could lead to a volumetric productivity of 130 kg (R)-mandelonitrile m$^3$.h$^{-1}$.

Introduction

Cyanohydrins are valuable building blocks for the synthesis of drugs and agrochemicals. For their asymmetric synthesis from HCN and aldehydes or ketones enzymatic methods using hydroxynitrile lyases are widely used [1-7]. E.g., the hydroxynitrile lyase from Prunus amygdalus, sativa (PaHnl) has a broad substrate specificity and catalyzes the formation of the (R)-cyanohydrins with a high enantiospecificity [8].

The enzymatic formation of (R)-cyanohydrin is accompanied by a nonenzymatic formation of unwanted racemic product. Research on cyanohydrin production has focused on the prevention of the nonenzymatic reaction in order to obtain the required enantiomeric excess of the final product.

One of the most successful strategies to reduce the nonenzymatic reaction is the use of aqueous-organic biphasic systems, consisting of an organic phase that serves as a reservoir of substrates and product and an aqueous phase in which the enzymatic and the nonenzymatic reaction take place. The reason for this success is the occurrence of mass transfer limitation for the aldehyde to the aqueous phase [9,10]. This results in aldehyde concentrations in the aqueous phase that are lower than those resulting from partitioning. At these low concentrations the nonenzymatic reaction hardly occurs and the enzymatic reaction is favored resulting in a high enantiomeric excess.

Optimization of the aqueous-organic biphasic process can be performed by empirical methods, which are costly and time consuming. Mathematical process models may facilitate process optimization, leading to considerable savings in experimental effort and enabling a straight-forward scale-up. A process model for aqueous-organic biphasic systems has been developed and validated for the synthesis of (R)-mandelonitrile [10] and it was used for the optimization of the aqueous phase volume fraction [11].

Here we will demonstrate that the process model can be extended and used for temperature optimization as well. Model-based optimization of temperature for enzymatic reactions is rarely carried out. A well known published example is the model-based temperature optimization of glucose isomerase by Roels and Van Tilburg [12]. This concerns a continuous plug flow reactor with immobilized enzyme. The influence of temperature on the reaction equilibrium, enzyme activity and enzyme stability was determined, and subsequently an optimum in productivity was determined.

Concerning the synthesis of enantiopure cyanohydrins in aqueous-organic biphasic systems, the optimization of temperature is complicated by the introduction of the organic phase, interfacial mass transfer and the competition between the enzymatic and nonenzymatic reactions.

In earlier work the choice for a reaction temperature for synthesis of enantiopure cyanohydrins in aqueous-organic biphasic systems was based on experimental experience [9] or on systematic data driven optimization [13].
Method of approach

The influence of the reaction temperature will be investigated for the synthesis of (R)-mandelonitrile in an aqueous-organic biphasic system within a temperature range of 5 to 25°C in a batch stirred tank reactor using a process model. Working at a lower temperature might result in ice formation while a higher temperature might give problems with the stability of cyanohydrins and the safety when working with hydrogen cyanide and methyl tert-butyl ether. The dispersed aqueous-phase volume will be studied up to 50% of the total volume. It is expected that phase inversion will occur above 50% dispersed phase volume. Besides, an increase in the aqueous-phase volume decreases the amount of aldehyde that can be accommodated due to a reduced organic-phase volume.

A batch instead of a fed-batch operated stirred tank reactor is investigated since a fed-batch reactor will introduce the feeding profile as an additional degree of freedom and would complicate the discussion. This does not rule out that a fed-batch operated reactor might be an attractive option [11].

The process model will be based on reaction kinetics and mass transfer kinetics with parameters that are interpolated from experimental values at 5 and 25°C. Although such a model is rather crude, it is suitable for demonstrating our method of temperature optimization.

Theory

The process model for a stirred tank reactor operating in batch mode has been described before in detail [10,11]. The reactions, both enzymatic and nonenzymatic, are assumed to take place in the bulk of the aqueous phase only. The compounds involved in the process model are benzaldehyde (A), hydrogen cyanide (B), (R)- and (S)-mandelonitrile (P^R and P^S) and the enzyme PaHnl (E).

Balances

The mass balances are:

\[ V_{aq} \frac{dC_{aq,j}}{dt} = r_j V_{aq} - \phi_j V_{aq} \quad [\text{mol.s}^{-1}] \quad (1) \]

\[ V_{org} \frac{dC_{org,j}}{dt} = \phi_j V_{aq} \quad [\text{mol.s}^{-1}] \quad (2) \]

\( V \) is the phase volume, \( C_j \) the concentration of \( j \) (A, B, P^R or P^S), \( r_j \) its reaction rate and \( \phi_j \) its mass transfer rate from the aqueous to the organic phase defined per aqueous-phase volume. The subscripts aq and org refer to the phases.

Following usual experimental procedures to execute the batch conversions [9] the initial amount of HCN is partitioned between the aqueous and the organic phase whereas the aldehyde initially is only present in the organic phase. HCN is assumed to be continuously at equilibrium between both phases due to its relatively high concentration, low partition coefficient and the excess used [10].

75
Reaction kinetics

The enzymatic and nonenzymatic reaction rates both contribute to the overall reaction rate $r_j$ in eq (1). The enzyme is enantiospecific towards the $(R)$-enantiomer, $r_{P,enz}^S = 0$. The rate equation for the synthesis of $(R)$-mandelonitrile according to the bi-uni model is:

$$r_{P,enz}^R = \frac{k_{f,enz}^{R}}{K_{iA} K_{mB}} \left( \frac{C_{sq,A} C_{sq,B} - C_{sq,p^A}}{K_{eq}^R} \right) \cdot C_{eq,E} \ [\text{mol.l eq}^{-1}.\text{s}^{-1}]$$

In eq. (3) $K_{eq}^R$ is the equilibrium constant for the reaction towards $P^R$. Its value equals to half of $K_{eq}^{RS}$, the equilibrium constant for reaction towards the racemate [14].

The reversible nonenzymatic kinetics for product formation are described by eqs. (4) and (5). A distinction is made between the $(R)$- and $(S)$-product, but values for the $R$- and $S$-constants are identical.

$$r_{P,non-enz}^R = k_{non-enz}^R \left( C_{sq,A} C_{sq,B} - C_{sq,p^A} \right) \ [\text{mol.l eq}^{-1}.\text{s}^{-1}]$$  \hspace{1cm} (4)

$$r_{P,non-enz}^S = k_{non-enz}^S \left( C_{sq,A} C_{sq,B} - C_{sq,p^S} \right) \ [\text{mol.l eq}^{-1}.\text{s}^{-1}]$$  \hspace{1cm} (5)

Mass transfer kinetics

Mass transfer is described by [10]:

$$\phi_j = k_{i,j} \alpha \left( \frac{C_{org,j}}{m_j} - C_{aq,j} \right) \ [\text{mol.l eq}^{-1}.\text{s}^{-1}]$$

(6)

In eq. (6) $\alpha$ is the interfacial area per volume of aqueous phase, $k_{i,j}$ the lumped mass transfer coefficient and $m_j$ the partition coefficient, which is defined by the ratio of the equilibrium concentrations (organic over aqueous buffer).

The mass transfer rate $\phi_j$ is based on the aqueous-phase volume and therefore easily comparable to the reaction rates. If the size of aqueous droplets in the organic phase is assumed to be independent of the phase volume ratio, an increase in volume of aqueous phase is proportional to an increase in interfacial area and $\alpha$ is not dependent on the phase volumes [10].

Temperature dependent phenomena

The phenomena that are assumed to be temperature dependent are the reaction kinetics, reaction equilibria, partition equilibria and the mass transfer kinetics. The enzyme is assumed to be stable during the experiments, so temperature effects on enzyme inactivation are not taken into account. The parameters considered to be temperature dependent are the catalytic constant of the enzyme $k_{f,enz}^R$, chemical rate constant $k_{non-enz}^R$. 

76
(= \kappa_{\text{non-enz}}^S), reaction equilibrium constant \( K_{eq}^R (= K_{eq}^S) \), partition coefficients \( m_j \) and the mass transfer parameter \( k_l \alpha \).

The temperature dependence of both the enzymatic and the nonenzymatic kinetics may be described by the Arrhenius equation. For the enzyme catalyzed reaction this concerns the catalytic constant \( k_{cat}' \). For simplicity, the other kinetic constants in eq. (3) are assumed to be temperature independent. The justification for this simplification is that temperature effects that originate from microscopic rate constants may cancel to a large extent in \( K_m \) and \( K_i \) values, which are ratios of these rate constants [14]. For the nonenzymatic reaction the temperature dependence concerns \( k_{\text{non-enz}}^R \).

\[
k_{cat}' = k_{cat,ref}' \exp \left[ \frac{E_{act}^{enz}}{RT_{ref}} \right] \exp \left[ -\frac{E_{act}^{enz}}{RT} \right] \quad \text{[mol.g\text{-}1.s\text{-}1]} \tag{7}
\]

\[
k_{\text{non-enz}}^R = k_{\text{non-enz,ref}}^R \exp \left[ \frac{E_{act}^{\text{non-enz}}}{RT_{ref}} \right] \exp \left[ -\frac{E_{act}^{\text{non-enz}}}{RT} \right] \quad \text{[l.mol\text{-}1.s\text{-}1]} \tag{8}
\]

In eqs. (7) and (8) \( E_{act}^{enz} \) and \( E_{act}^{\text{non-enz}} \) are the activation energies for the enzymatic and nonenzymatic reaction, respectively, and \( R \) is the gas constant.

The temperature dependence of the equilibrium constant \( K_{eq}^R \) is derived from the standard enthalpy of reaction \( (\Delta_r H^0) \) assuming that the standard enthalpy and entropy of reaction are not temperature dependent within the studied temperature range.

\[
K_{eq}^R = K_{eq,ref}^R \exp \left[ -\frac{\Delta_r H^0}{RT_{ref}} \right] \exp \left[ \frac{\Delta_r H^0}{RT} \right] \quad \text{[l.mol\text{-}1]} \tag{9}
\]

With the usual assumption that the standard molar enthalpy change of transfer \( (\Delta_r H^0) \) is not temperature dependent in the range studied the temperature dependence of the partition coefficient \( m_j \) is given by [15]

\[
\frac{\partial \ln m_j}{\partial T} = \frac{\Delta_r H^0}{RT^2} \quad \text{[K\text{-}1]} \tag{10}
\]

The main influence of temperature on mass transfer kinetics is via the diffusion coefficient. For oxygen diffusion, as a rule of thumb, an increase of \( k_l \) by 2.5% for each °C has been proposed [16]. Over a limited temperature range, as in our case, the description of the temperature dependence for mass transfer might be approached by:

\[
k_l \alpha = (k_l \alpha)_{ref} \left( \frac{T}{T_{ref}} \right)^a \quad \text{[s\text{-}1]} \tag{11}
\]

The simulated extent of conversion and the enantiomeric excess are based on both phases.

Initial amount of HCN

The amount of HCN to be added to the aqueous-organic biphasic system was calculated for an equilibrium conversion of \( \xi_{eq} = 99\% \) of benzaldehyde according to eq. (12) [11].
\[ n_B^0 = \frac{\xi^{eq} \cdot V_{tot}}{1 - \xi^{eq} \cdot \frac{K_{eq}}{K_{eq}^{ref}}} \left[ 1 + \frac{V_{org}}{V_{tot}} (m_A - 1) \right] \left[ 1 + \frac{V_{org}}{V_{tot}} (m_B - 1) \right] + \xi^{eq} \cdot n_A^0 \]  \quad \text{[mol]} \quad (12)

\[ n_A^0 \] is the amount of benzaldehyde to be converted. The initial concentrations of HCN were calculated according to:

\[ C_{B,\text{aq}}^0 = \frac{n_B^0}{V_{\text{aq}} + m_B \cdot V_{\text{org}}} \quad \text{[mol.m}_{\text{aq}}^{-3}] \quad (13) \]

\[ C_{B,\text{org}}^0 = \frac{n_B^0}{V_{\text{org}} + \frac{V_{\text{aq}}}{m_B}} \quad \text{[mol.m}_{\text{org}}^{-3}] \quad (14) \]

Materials and Methods

Chemicals and enzymes

\textit{rac-Mandelonitrile (98\%)} pro synthesis was purchased from Merck and used to determine the partition coefficients of mandelonitrile and benzaldehyde. Benzaldehyde was purchased from Aldrich and distilled under nitrogen before use. NaCN was purchased from Aldrich. To determine the partition coefficient of HCN, a buffered HCN-solution was prepared by dissolving NaCN, citric acid and NaOH in deaerated water in a closed system to prevent loss of HCN. Solutions of HCN in methyl \textit{tert}-butyl ether were obtained by extraction of an aqueous HCN solution. Methyl \textit{tert}-butyl ether was purchased from Aldrich. \textit{PaHnl} enzyme powder was a gift from Solvay Pharmaceuticals (Weesp, The Netherlands) and contains 98\% protein and about 65\% \textit{PaHnl} [13]. The specific activity of the powder was 185,000 IU per gram.

Partition coefficients

The partition coefficients of benzaldehyde and mandelonitrile were determined by using HPLC-analysis (\textmu bonadpack phenyl column and a 16\% acetonitrile aqueous phase as eluent with 1 ml phosphoric acid per liter eluent) of both phases. 10 ml of 100 mM sodium citrate buffer of pH 2.7 and 10 ml methyl \textit{tert}-butyl ether were incubated and mixed with 0.25-3 g of \textit{rac}-mandelonitrile, which contained about 1\% benzaldehyde, at 25°C for 2 h.

The partition coefficient of hydrogen cyanide was determined using GC-analysis of both phases (CPSIL 5 CB column 10 m x 0.53 mm). 15 ml of 100 mM aqueous citrate buffer of pH 5.5 with HCN concentrations ranging from 50-200 mM were incubated with 15 ml methyl \textit{tert}-butyl ether at 25°C and mixed for 1 h.
Mass transfer kinetics

A progress curve for a batch conversion was recorded for the nonenzymatic synthesis of rac-mandelonitrile at pH 9 and 25°C in the 225 ml reactor as described by Gerrits et al. [9]. The value of $k_{\text{l,a}}$ was determined using 50 ml aqueous and 120 ml organic phase volume. A 73 ml solution of 100 mmol HCN in methyl tert-butyl ether was prepared according to Gerrits et al. [9].

First 50 ml buffer, 50 mM sodium phosphate pH 9, were added to the reactor. Subsequently the 73 ml organic phase with HCN were added. The liquid induced impeller was placed 1 cm above the bottom of the reactor. While stirring at 500 min$^{-1}$, 3.9 g (37 mmol) benzaldehyde, dissolved in methyl tert-butyl ether and diluted to 50 ml was added to the reactor within 1 min. Samples were taken from the organic phase after stopping the stirrer at distinct intervals and analyzed by HPLC, as described for determination of the partition coefficients.

The $k_{\text{l,a}}$ value was determined according to Willeman et al. [10] by fitting $k_{\text{l,a}}$ to the experimental data using $k_{\text{non-enz}}^R = 1.3 \text{ l.mol}^{-1}.\text{s}^{-1}$ in the process model.

Results and Discussion

Reaction kinetics and equilibrium constant

The enzyme kinetic parameters are known for buffer saturated with methyl tert-butyl ether at pH 5.5 and 5°C and shown in Table 1. The equilibrium constant and the reaction rate constants for the nonenzymatic reaction are known for aqueous buffer of pH 5.5 at 5 and 25°C and shown in Table 2.

The values of the parameters describing the temperature dependence for the equilibrium constant, enzymatic kinetics and nonenzymatic kinetics were calculated by interpolation between the values known at 5 and 25°C from Table 1 and 2 using eqs. (8)-(10). The activation energy for the enzyme kinetics in buffer and buffer saturated with methyl tert-butyl ether were assumed to be equal. The calculated values for the parameters determining the temperature dependence are shown in Table 3.

From the relative values of the kinetic parameters, shown in Figure 1, it appears that the enzymatic reaction is less temperature sensitive than the nonenzymatic reaction. At fixed substrate concentrations, at low temperatures the nonenzymatic reaction rate will be reduced more than the enzymatic reaction rate. This confirms the observations of Wolfenden et al. [17].
Table 1
Enzyme kinetic parameters for the synthesis of mandelonitrile at pH 5.5 and 5°C in aqueous buffer saturated with methyl tert-butyl ether [10]

<table>
<thead>
<tr>
<th></th>
<th>( k_{\text{cat}} )</th>
<th>( K_{\text{mA}} )</th>
<th>( K_{\text{mB}} )</th>
<th>( K_{\text{mP}} )</th>
<th>( K_{\text{IA}} )</th>
<th>( K_{\text{IB}} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>([\text{mol.g}^{-1}.\text{s}^{-1}])</td>
<td>([\text{mM}])</td>
<td>([\text{mM}])</td>
<td>([\text{mM}])</td>
<td>([\text{mM}])</td>
<td>([\text{mM}])</td>
<td>([\text{mM}])</td>
</tr>
<tr>
<td>1.59 (10^3)</td>
<td>3.84</td>
<td>27</td>
<td>129</td>
<td>3.85</td>
<td>27</td>
<td></td>
</tr>
</tbody>
</table>

Figure 1. Temperature dependency of parameters for the conversion of benzaldehyde at pH 5.5. The parameters have been normalized through division by their value at 5°C.

Table 2
Equilibrium constants and rate constants for enzymatic and nonenzymatic synthesis of mandelonitrile at pH 5.5 in aqueous buffer [12]

<table>
<thead>
<tr>
<th>( T )</th>
<th>( K_{\text{eq}}^R )</th>
<th>( k_{\text{cat}}^f )</th>
<th>( k_{\text{non-enz}}^R )</th>
</tr>
</thead>
<tbody>
<tr>
<td>[°C]</td>
<td>[l.mol(^{-1})]</td>
<td>[mol.g(^{-1}).s(^{-1})]</td>
<td>[l.mol(^{-1}).s(^{-1})]</td>
</tr>
<tr>
<td>5</td>
<td>369</td>
<td>1.41 (10^3)</td>
<td>2.2 (10^3)</td>
</tr>
<tr>
<td>25</td>
<td>133</td>
<td>7.21 (10^3)</td>
<td>14 (10^3)</td>
</tr>
</tbody>
</table>

Table 3
Values for the parameters describing the temperature dependence of the reaction and mass transfer kinetics for the synthesis of mandelonitrile.

<table>
<thead>
<tr>
<th>( pH )</th>
<th>( \Delta H^0 )</th>
<th>( E_{\text{act}}^{\text{non-enz}} )</th>
<th>( E_{\text{act}}^{\text{enz}} )</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>[kJ.mol(^{-1})]</td>
<td>[kJ.mol(^{-1})]</td>
<td>[kJ.mol(^{-1})]</td>
</tr>
<tr>
<td>5.5</td>
<td>35.2</td>
<td>66.0</td>
<td>62.5</td>
</tr>
</tbody>
</table>
Mass transfer kinetics and partition equilibria

Values for the partition coefficients for benzaldehyde, HCN and mandelonitrile determined at 25°C together with the values earlier determined at 5°C are shown in Table 4. The partition coefficients appear to be constant in the considered temperature range which is not uncommon [15]. Arbitrarily the values determined at 5°C will be used within the process model.

The value for $k_l \alpha$ determined at 25°C amounts 0.21 s$^{-1}$. Using the already known value of 0.08 s$^{-1}$ at 5°C [10], a value of 13.90 for $\alpha$ in eq. (13) is calculated. The large effect of temperature on $k_l \alpha$, 150% increase upon a temperature increase of 20°C, appears to be an effect on the diffusion coefficient and the interfacial area through the viscosity. According to calculations, the influence of temperature on the diffusion coefficient through the Stokes-Einstein relation would account for about 50% increase of the diffusion coefficient (and hence on $k_l$). An increase of the interfacial area, based on the dispersed aqueous phase, due to a smaller droplet size could account for the remaining effect of temperature on $k_l \alpha$. As shown in Figure 1, the temperature dependence of $k_l \alpha$ is a factor 2-3 lower than that of the reaction kinetics.

<table>
<thead>
<tr>
<th>$T$ [$\degree$C]</th>
<th>$k_l \alpha$ [s$^{-1}$]</th>
<th>$m_A$ [l$<em>{aq}$/l$</em>{org}$$^{-1}$]</th>
<th>$m_B$ [l$<em>{aq}$/l$</em>{org}$$^{-1}$]</th>
<th>$m_P$ [l$<em>{aq}$/l$</em>{org}$$^{-1}$]</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>0.08</td>
<td>43</td>
<td>2.8</td>
<td>46</td>
</tr>
<tr>
<td>25</td>
<td>0.21</td>
<td>47</td>
<td>2.5</td>
<td>44</td>
</tr>
</tbody>
</table>

**Table 4**

Mass transfer and partition coefficients at pH 5.5 (A = benzaldehyde; B = HCN; P = mandelonitrile). Values at 5°C were obtained from Willeman et al. [10].

Temperature and aqueous phase volume optimization

The production of (R)-mandelonitrile with a required enantiomeric excess of 99.00% and a 98.00% extent of conversion in benzaldehyde was considered. We assumed that 750 moles of benzaldehyde had to be converted in a 1 m$^3$ reactor. The required amount of HCN was calculated and based on an equilibrium conversion of 99%. Since values of all required parameters are known in the range of 5-25°C, optimization can be performed.

The process model was used to determine the required concentration of enzyme, $C_{eq,E}$, that is just enough to meet the constraints of 98.00% conversion and 99.00% enantiomeric excess. This was performed for a temperature range of 5-25°C and an aqueous phase volume fraction range of 10-50%, where the aqueous phase was considered to be dispersed, for a batch stirred tank reactor. For an arbitrary enzyme concentration, at 98.00% conversion a certain enantiomeric excess value was found by simulation. Depending on this value, the enzyme concentration was iteratively increased or decreased until at 98.00% conversion the required 99.00% enantiomeric excess was obtained. The corresponding reaction time is $t_{conv}$, from which the volumetric productivity is calculated. The results of these simulations are shown in Figures 2 and 3.
Figure 2. Required enzyme concentration for the conversion of 750 moles of benzaldehyde per m$^3$ with 98.00 % conversion towards (R)-mandelonitrile with 99.00 % enantiomeric excess at different temperatures for different aqueous phase volume fractions at pH 5.5. α, 10%; 0, 20%; Δ, 30%; o, 40%; x, 50% aqueous phase volume fraction. The markers are for identification only and do not imply experiments.

Figure 3. Required conversion time matching the enzyme concentration for the conversion of 750 moles of benzaldehyde per m$^3$ with 98.00 % conversion towards (R)-mandelonitrile with 99.00 % enantiomeric excess at different temperatures for different aqueous phase volume fractions at pH 5.5. α, 10%; 0, 20%; Δ, 30%; o, 40%; x, 50% aqueous phase volume fraction. The markers are for identification only and do not imply experiments.
The volumetric productivity, the enzyme concentration and moreover the total amount of enzyme required decrease when operating at low temperatures and low aqueous-phase volumes. For the optimization of the reaction temperature and the aqueous-phase volume, prices are required for both the enzyme and the time the reactor is used for the reaction step. The prices given here are estimated values, serving just the purpose of illustrating the method of optimization. A price of € 100 per kg PaHml enzyme was used (compare Sheldon [18]) and € 100 per h of reactor use, based on a 1 m³ stirred-tank reactor, including labor costs. The effect of substrate costs does not have to be taken into account since a fixed amount of aldehyde is converted and the amount of HCN showed little variation. It was assumed that the enzyme was used only once.

There is an optimum situation, see Figure 4. The optimum is € 2.3 per kg of product obtained at a reaction temperature of 15°C and an aqueous phase volume fraction of 50% with an enzyme concentration of 1.51 g.l⁻¹. For the conversion of 750 mol benzaldehyde at these conditions 1150 moles HCN are required, a 50% excess. The corresponding volumetric productivity amounts 130 kg (R)-mandelxonitrile m⁻³.h⁻¹.

Earlier an experiment has been performed at conditions relatively close to these optimum conditions but at 5°C [9,10]. This is one of the experiments that has been used to validate the process model. According to Figure 4 the difference between 5 and 15°C at 50% aqueous-phase volume is relatively small. So the present analysis did not reveal operating conditions that were clearly better than those previously studied. For other cyanohydrins, much less conditions have been studied experimentally and optimum conditions may not have been found yet.

At production scale, the optimum conditions might be different, since mass transfer properties can be different and too short reaction times are not practical. Besides, prices for enzyme and reactor use are likely to change and are dependent on the kind of enzyme, the used amounts, the kind of reactor and the reactor times.

Decreasing enzyme costs, relative to the reactor costs, e.g. by reusing the enzyme, results in an increase of the optimum temperature and the aqueous phase volume and therefore the volumetric productivity. Decreasing reactor costs, relative to the enzyme costs, result in a decrease of the optimum temperature and the aqueous phase volume fraction.
Figure 4. Production costs per kg (R)-mandelonitrile using estimated costs of 100 € per kg enzyme and 100 € per h reactor use at different temperatures for different aqueous phase volume fractions at pH 5.5. σ, 10%; 0, 20%; Δ, 30%; ◊, 40%; x, 50% aqueous phase volume fraction. The markers are for identification only and do not imply experiments.

Figure 5. The extent of conversion, quotient of enantiomeric rates and the enantiomeric excess for the conversion of 750 moles benzaldehyde per 1 m³ into (R)-mandelonitrile at 15°C (solid lines) and 25°C (dashed lines), pH 5.5., 50% aqueous phase and 1.51 gE.L⁻¹. 
**Quotient of enantiomeric reaction rates**

The enantiomeric excess of the \((R)\)-mandelonitrile at the end of a conversion is the result of the competing enzymatic and nonenzymatic reactions. The rates of both reactions change and differ during the conversion. At a particular time the reaction should be stopped and the product should be harvested. Waiting longer for a higher conversion degree will decrease the enantiomeric excess due to the reversibility of the reaction. The desired \((R)\)-mandelonitrile racemizes via benzaldehyde into undesired \((S)\)-mandelonitrile, which accumulates in the system. The rate at which this racemization takes place is dependent on the reaction conditions.

To calculate the time at which the reaction should be stopped a new quantity \(Q_p^R\) is introduced, the quotient of enantiomeric reaction rates, which is the enantiomeric excess of newly formed product at a certain time or degree of conversion.

\[
Q_p^R = \frac{r_{p^+} - r_{p^-}}{r_{p^+} + r_{p^-}} \quad [-]
\]

(15)

\(r_{p^+}\) and \(r_{p^-}\) are the enantiomeric reaction rates for \((R)\) and \((S)\)-mandelonitrile, respectively. As the conversion proceeds \(r_{p^+} - r_{p^-}\) decreases and results in a sharp decrease of the quotient value \(Q_p^R\) at the required conversion, see Figure 5. The reaction should be stopped before \(Q_p^R\) becomes zero \(r_{p^+} = r_{p^-}\) and racemic mandelonitrile is produced. Beyond this time \(Q_p^R\) becomes negative which means that the \((R)\)-mandelonitrile starts racemizing.

Although it appears that the reaction is at equilibrium, regarding the extent of conversion, it is not. The enantiomeric excess is steadily decreasing until a racemate is obtained and its rate of decrease is dependent on the experimental conditions. The relatively small differences in enantiomeric excess, especially at the high values usually required, are difficult to measure. Therefore it is impossible to determine the ultimate time to stop the reaction by measurement of the enantiomeric excess.

At 25°C the racemization of \((R)\)-mandelonitrile takes place earlier and proceeds faster than at 15°C, see Figure 5. To obtain an enantiomeric excess of 99% at a 98% conversion of benzaldehyde at 25°C the enzyme concentration has to be increased from 1.5 to 4.2 g l\(^{-1}\). At 25°C the reaction should be stopped much earlier and stopping at the right time is more critical due to the faster racemization.
Conclusions

A model based procedure has been developed for the optimization of the reaction temperature for the enzymatic conversion of benzaldehyde and HCN into (R)-mandelonitrile, catalyzed by Prunus amygdalus hydroxynitrile lyase, in a biphasic system of aqueous buffer and methyl tert-butyl ether.

At low temperatures the nonenzymatic reaction is reduced to a larger extent than the enzymatic reaction. The volumetric productivity increases with an increasing reaction temperature. When prices are ascribed to the costs of enzyme and reactor use the process can be optimized.

For the conversion of 750 moles benzaldehyde per m$^3$ into (R)-mandelonitrile with an enantiomeric excess of 99.00% and an 98.00% extent of conversion in benzaldehyde an economic optimum was determined. The economic optimum was found at a temperature of 15°C and an aqueous phase volume fraction of 50% using 1.51 g enzyme per liter aqueous phase and results in a volumetric productivity of 130 kg (R)-mandelonitrile m$^3$.h$^{-1}$.

This temperature optimization procedure is expected to be very useful in finding appropriate reaction conditions for the synthesis of other hydroxynitriles.
Symbols

\( a \)  
specific area  
\( [m^2 \cdot m^{-3}] \)

\( C_{acq,i} \)  
concentration in aqueous phase  
\( [\text{mol} \cdot m^{-3}] \)

\( C_{aq,E} \)  
enzyme concentration  
\( [\text{g} \cdot m_{aq}^{-3}] \)

\( C_{org,i} \)  
concentration in organic phase  
\( [\text{mol} \cdot m_{org}^{-3}] \)

\( ee \)  
enantiomeric excess of \((R)\)-mandelonitrile  
\( [%] \)

\( E_{\text{act}}^{\text{env}}, E_{\text{act}}^{\text{non-env}} \)  
activation energy  
\( [\text{kJ} \cdot \text{mol}^{-1}] \)

\( k_{\text{cat}} \)  
forward catalytic constant  
\( [\text{mol} \cdot g_{E}^{-1} \cdot \text{s}^{-1}] \)

\( k_{\text{non-env}} \)  
forward chemical reaction rate constant  
\( [\text{m} \cdot \text{s}^{-1}] \)

\( k_{i,j} \)  
mass transfer coefficient  
\( [\text{m} \cdot \text{s}^{-1}] \)

\( K_{eq}^{R}, K_{eq}^{S} \)  
enantiomeric equilibrium constant  
\( [\text{mol}^{-1}] \)

\( K_{eq}^{R E} \)  
racemic equilibrium constant  
\( [\text{mol} \cdot \text{mol}^{-1}] \)

\( K_{mj} \)  
Michaelis constant for compound j  
\( [\text{mol} \cdot \text{mol}^{-1}] \)

\( K_{ij} \)  
inhibition constant for compound j  
\( [\text{mol} \cdot \text{mol}^{-1}] \)

\( m_{j} \)  
partition coefficient  
\( [\text{mol} \cdot \text{mol}^{-1}] \)

\( n_{j}^{0} \)  
initial total amount of compound j  
\( [\text{mol}] \)

\( Q_{P}^{R} \)  
quotient of enantiomeric rates  
\( [%] \)

\( r_{j} \)  
reaction rate for compound j  
\( [\text{mol} \cdot \text{mol}^{-1} \cdot \text{s}^{-1}] \)

\( R \)  
gas constant  
\( [\text{J} \cdot \text{mol}^{-1} \cdot \text{K}^{-1}] \)

\( t \)  
time  
\( [\text{s}] \)

\( t_{\text{conv}} \)  
time to obtain 98% conversion  
\( [\text{s}] \)

\( T_{\text{ref}} \)  
reference temperature  
\( [\text{°C}] \)

\( V_{aq} \)  
volume aqueous phase  
\( [\text{m}^3] \)

\( V_{org} \)  
volume organic phase  
\( [\text{m}^3] \)

\( V_{\text{tot}} \)  
total reaction volume  
\( [\text{m}^3] \)

\( \alpha \)  
temperature coefficient  
[\%]

\( \Delta_{r}H_{0} \)  
reaction enthalpy  
\( [\text{kJ} \cdot \text{mol}^{-1}] \)

\( \Delta_{f}H_{0} \)  
transfer enthalpy  
\( [\text{kJ} \cdot \text{mol}^{-1}] \)

\( \phi_{j} \)  
mass transfer rate  
\( [\text{mol} \cdot \text{mol}^{-1} \cdot \text{s}^{-1}] \)

\( \xi \)  
extent of conversion of aldehyde  
\( [%] \)
References


Development of (R)-4-hydroxymandelonitrile synthesis in an aqueous-organic biphasic stirred tank batch reactor

Abstract

A hydroxynitrile lyase-catalyzed reaction that has been hardly explored before, was optimized in a way that it is suitable for the rapid and efficient development of a full-scale production process.

The conversion of 4-hydroxybenzaldehyde into (R)-4-hydroxymandelonitrile, catalyzed by Prunus amygdalus hydroxynitrile lyase, was carried out in a biphasic system of aqueous buffer (pH 5.5) and methyl tert-butyl ether and was described by using a process model. The process model consists of a description of the reaction kinetics, mass transfer kinetics and the mass balances for both the aqueous and the organic phase. Values were determined for the equilibrium constant, the enzyme kinetic parameters, the lumped mass transfer coefficient for benzaldehyde and the partition coefficients.

By using estimated prices of enzyme and reactor use, the optimum aqueous phase volume fraction and required enzyme concentration were calculated at a temperature of 20°C for a batch operated stirred tank reactor. According to the process model it was possible to convert 90% of the 4-hydroxybenzaldehyde into (R)-4-hydroxymandelonitrile with 95% enantiomeric excess. The price optimum for this reaction was found at an aqueous phase volume of 17% of the total volume. The required enzyme concentration to meet the targets was 28.6 g/l aqueous phase.

At the predicted optimum the synthesis was experimentally performed and the results were in accordance with the simulation regarding the extent of conversion and the enantiomeric excess.

This chapter is accepted for publication in Biotechnol Bioeng.
Introduction

R-Hydroxynitrile lyase from almonds, *Prunus amygdalus sativa*, (*PaHnl*) catalyzes the addition of HCN to a wide range of aldehydes and ketones. The (*R*)-cyanohydrins that are formed are interesting chiral building blocks (Brussee and Van der Gen, 2000; Effenberger et al., 2000; Gregory, 1999; Griengl et al., 2000; Johnson and Griengl, 1999; Pöchlauer, 1998; Van Scharrenburg et al., 1993; Effenberger, 1994).

In this paper we focus on the *PaHnl*-catalyzed synthesis of (*R*)-4-hydroxymandelonitrile by addition of HCN to 4-hydroxybenzaldehyde, Figure 1.

![Conversion of 4-hydroxybenzaldehyde into (*R*)-4-hydroxymandelonitrile.](image)

This cyanohydrin is interesting as precursor for the synthesis of (*R*)-4-hydroxymandelic acid which is a key intermediate for [*R*-(R*,S*)]-N,N-dimethyl-2-[[5,6,7,8-tetrahydro-7-[[2-hydroxy-2-(4-hydroxyphenyl)ethyl]amino]-2-naphthalenyl]oxy]-acetamide, Figure 2, which has potential for treating colic or facilitating stone discharge in urolithiasis patients (Hiroaki et al., 1999). Using almond meal containing 2% buffer pH 5.4 to maintain the enzyme activity, (*R*)-4-hydroxymandelonitrile has previously been synthesized with an isolated yield of 64% and an enantiomeric excess of 96%. The reaction took 68 h and was carried out at 5°C in diisopropyl ether (Kiljunen and Kanerva, 1996). Using purified *PaHnl* in a biphasic system of aqueous buffer pH 5.5 and methyl tert-butyl ether, (*R*)-4-hydroxymandelonitrile has previously been synthesized within 96 h at 5°C with a yield of 90% and an enantiomeric excess of 91% (Gerrits et al., 2001).

For other Hnl-catalyzed reactions, aqueous-organic biphasic liquid processes have been developed (Loos et al., 1995). The nonenzymatic reaction can be minimized by decreasing the concentration of the reactants in the aqueous phase by making use of mass transfer limitation and in addition to the partitioning behavior of substrates and products between the aqueous and the organic phase (Gerrits et al., 2001).

![Pharmaceutical with potential for treating colic or facilitating stone discharge in urolithiasis patients. The synthesis requires (*R*)-hydroxymandelic acid as key intermediate.](image)
Until now, optimization of aqueous-organic biphasic systems for the synthesis of enantiopure cyanohydrins in a batch process has generally been carried out by an empirical approach (Griengl et al., 1998; Loos et al., 1995). The influence of all known parameters was investigated and these parameters were optimized one by one. Suitable process conditions are readily obtained by this empirical approach. However, experiments for such a data driven optimization have to be repeated if other substrates are used. Also, due to the requirement of mass transfer limitation, it is difficult to scale-up processes obtained by data driven experiments, therefore necessitating additional and expensive optimization experiments at full-scale.

The model driven approach (Willeman et al., 2002B) that was used earlier for the optimization of the (R)-mandelonitrile production should easily be extendable to (R)-cyanoxyhydrins that have hardly been examined. The model driven approach enables a fast and inexpensive optimization and scale-up for full-scale systems.

The objective of this research is the development of the (R)-4-hydroxymandelonitrile synthesis in a biphasic system of aqueous buffer at pH 5.5 and methyl tert-butyl ether in a stirred tank reactor by using the model driven approach in order to limit the number of full-scale experiments to a minimum. The influence of temperature and aqueous phase volume fraction on the enzyme concentration and volumetric productivity will be studied.

**Modeling Aspects**

The process model described in this work is limited to a stirred tank reactor operating in batch or fed batch mode. The reactions, both enzymatic and nonenzymatic, are considered to take place in the bulk of the aqueous phase only. The compounds involved in the process model are 4-hydroxybenzaldehyde (A), hydrogen cyanide (B), (R)- and (S)-4-hydroxymandelonitrile (P<sup>R</sup> and P<sup>S</sup>) and the enzyme PdHmt (E).

**Balances**

The volume of the aqueous phase is considered to be constant during the conversion. The mass balance used for the aqueous phase (j = A, B, P<sup>R</sup> or P<sup>S</sup>) is:

\[
V_{aq} \frac{dC_{aq,j}}{dt} = r_j V_{aq} - \phi_j V_{aq}
\]  

\[\text{[mol.s}^{-1}]\] (1)

\(V_{aq}\) is the volume of the aqueous phase, \(C_{aq,j}\) the concentration in the aqueous phase, \(r_j\) the reaction rate and \(\phi_j\) the mass transfer rate from the aqueous to the organic phase.

The volume of the organic phase was considered to be constant as well during the reaction. For the fed batch it was assumed that the feed was neat benzaldehyde and HCN which had a negligible influence on the reaction volume.
The mass balance used for the organic phase of the (fed) batch operated stirred tank reactor is:

\[
V_{\text{org}} \frac{dC_{\text{orgj}}}{dt} = \phi_j V_{\text{eq}} + N_j' \quad [\text{mol.s}^{-1}]
\]  

(2)

\(V_{\text{org}}\) is the volume of the organic phase, \(C_{\text{orgj}}\) the concentration in the organic phase and \(N_j'\) is the feed rate of neat substrate, which is instantaneously mixed with the organic phase. When operating as batch \(N_j' = 0\). The initial amount of HCN is assumed to be partitioned between the aqueous and the organic phase whereas the aldehyde initially is only present in the organic phase.

**Reaction kinetics**

The enzymatic and nonenzymatic reaction rates both contribute to the overall reaction rate \(r_j\) in eq (1).

\[
r_j = r_{j, \text{enz}} + r_{j, \text{non-enz}} \quad [\text{mol.l}^{-1} \text{s}^{-1}]
\]

(3)

Synthesis and cleavage of \((R)\)-4-hydroxymandelonitrile by \(P\alpha\text{HInl}\) are described by an ordered bi-uni model. The enzyme is considered to be enantiospecific towards the \((R)\)-enantiomer, \(r_{p, \text{enz}}^R = 0\). The rate equation for the synthesis of \((R)\)-4-hydroxymandelonitrile according to the bi-uni model is:

\[
r_{p, \text{enz}}^R = \frac{k_{\text{cat}}}{K_{\text{eq}}} \cdot \frac{C_{\text{eqA}} C_{p, B} - C_{\text{eq}} p^2}{1 + \frac{C_{\text{eqA}}}{K_A} + \frac{C_{\text{eqB}}}{K_B} + \frac{C_{\text{eq}} p^2}{K_{\text{eq}}} + \frac{C_{\text{eqB}}}{K_{\text{eq}}} + \frac{C_{\text{eq}} p^2}{K_{\text{eq}}}} \cdot C_{\text{eqB}} \quad [\text{mol.l}^{-1} \text{s}^{-1}]\]

(4)

In eq. (4) \(K_{\text{eq}}^R\) is the equilibrium constant for the reaction towards \(P^R\). Its value equals to half of the equilibrium constant \(K_{\text{eq}}^{R^S}\), for reaction towards the racemate (Willeman et al., 2000).

The reversible nonenzymatic kinetics for product formation are described by eq. (5) and eq. (6). A distinction is made between the \((R)\)- and \((S)\)-product, but values for the \(R\)- and \(S\)-constants are identical.

\[
r_{p, \text{non-enz}}^R = k_{\text{non-enz}}^R \left( C_A C_B - \frac{C_{p, B}^R}{K_{\text{eq}}} \right) \quad [\text{mol.l}^{-1} \text{s}^{-1}]
\]

(5)

\[
r_{p, \text{non-enz}}^S = k_{\text{non-enz}}^S \left( C_A C_B - \frac{C_{p, B}^S}{K_{\text{eq}}} \right) \quad [\text{mol.l}^{-1} \text{s}^{-1}]
\]

(6)
Mass transfer kinetics

Mass transfer is described by:

\[ \phi_j = k_{L,j}a \left( \frac{C_{\text{org,j}}}{m_j} - C_{\text{aq,j}} \right) \]  
\[ \text{[mol.l^{-1}.s^{-1}]} \] \hspace{1cm} (7)

In eq. (7) \( a \) is the interfacial area per volume of aqueous phase, \( k_{L,j} \) the lumped mass transfer coefficient and \( m_j \) the partition coefficient, which is defined by the ratio of the equilibrium concentrations (organic over aqueous buffer).

The mass transfer rate \( \phi_j \) is based on the aqueous phase volume and therefore easily comparable to the reaction rates, since the reaction is considered to take place solely in the aqueous phase. If the size of aqueous droplets in the organic phase is assumed to be independent of the phase volume ratio, an increase in volume of aqueous phase is proportional to an increase in interfacial area and \( a \) is not dependent on the phase volumes.

The partition coefficient of hydrogen cyanide at 20°C amounts 2.6 (Gerrits et al., 2001). The partition coefficients of 4-hydroxybenzaldehyde and 4-hydroxymandelonitrile have not been reported. The value of \( k_{L,j}a \) can be determined in a reacting system where the reaction rate is much faster than the mass transfer rate, leading to \( C_{\text{aq,j}} \approx 0 \) (Willemen et al., 2002A). This enables the determination of \( k_{L,j}a \) independent of the reaction kinetics.

Temperature dependent parameters

The phenomena that are considered temperature dependent are the reaction kinetics, both enzymatic and nonenzymatic, and the mass transfer kinetics. The description of Willemen et al. (2002B) is used. The temperature dependence for the enzymatic and the nonenzymatic kinetics are described by Arrhenius relations for \( k_{\text{cat}}^f \) and \( k_{\text{non-enz}}^R \), respectively.

\[ k_{\text{cat}}^f = k_{\text{cat,ref}}^f \exp \left[ \frac{E_{\text{act,cat}}}{R} \left( \frac{1}{T_{\text{ref}}} - \frac{1}{T} \right) \right] \]  
\[ \text{[mol.gE^{-1}.s^{-1}]} \] \hspace{1cm} (8)

\[ k_{\text{non-enz}}^R = k_{\text{non-enz,ref}}^R \exp \left[ \frac{E_{\text{act,non-enz}}}{R} \left( \frac{1}{T_{\text{ref}}} - \frac{1}{T} \right) \right] \]  
\[ \text{[l.mol^{-1}.s^{-1}]} \] \hspace{1cm} (9)

In eq. (8) and (9) \( E_{\text{act,cat}} \) and \( E_{\text{act,non-enz}} \) are the activation energies and \( R \) is the gas constant.
The temperature dependence of the equilibrium constant $K_{eq}^R$ is derived from the standard enthalpy of reaction ($\Delta H^0$) assuming that the enthalpy and entropy of reaction are not very temperature dependent within the studied temperature range.

\[
K_{eq}^R = K_{eq,T_{ref}}^R \exp \left[ -\frac{\Delta_r H^0}{R} \left( \frac{1}{T_{ref}} - \frac{1}{T} \right) \right] \quad [\text{mol}^{-1}] 
\]  

(10)

The influence on mass transfer kinetics was described according to Willeman et al. (2002B).

\[
k_{L,a} = k_{L,T_{ref}} \left( \frac{T}{T_{ref}} \right)^{\alpha} \quad [\text{s}^{-1}] 
\]  

(11)

Initial amount of HCN

We assume that the amount of HCN to be added to the aqueous-organic biphasic system can be restricted to the amount required for an equilibrium conversion of $\xi_{eq} = 95\%$ of 4-hydroxybenzaldehyde. This percentage guarantees that in each situation at least 90\% conversion is possible without using excessive amounts of HCN or using extreme long reaction times. This amount is calculated according to eq (12) (Willeman et al., 2001).

\[
n_b^0 = \frac{\xi_{eq}}{1 - \xi_{eq}} \frac{V_{tot}}{K_{eq}^{R^5}} \left[ 1 + \frac{V_{org}}{V_{tot}} (m_A - 1) \right] \left[ 1 + \frac{V_{org}}{V_{tot}} (m_B - 1) \right] \quad [\text{mol}] 
\]  

(12)

$n_A^0$ is the amount of 4-hydroxybenzaldehyde to be converted. The initial concentrations of HCN were calculated according to:

\[
C_{B,eq}^0 = \frac{n_b^0}{V_{eq} + m_B V_{org}} \quad [\text{mol.m}^{-3}] 
\]  

(13)

\[
C_{B,org}^0 = \frac{n_b^0}{V_{org} + m_B V_{eq}} \quad [\text{mol.m}^{-3}] 
\]  

(14)

Calculation of conversion and enantiomeric excess

In the experiments described in this work, both conversion and enantiomeric excess are based on analysis of the organic phase only. Therefore the definitions for the extent of conversion and the enantiomeric excess for the simulations are:

\[
\xi = 1 - \frac{V_{org} C_{org,A}}{n_A^0} \quad [-] 
\]  

(15)

\[
e e_{R}^R = \frac{C_{org,R^R} - C_{org,R^L}}{C_{org,R^R} + C_{org,R^L}} \quad [-] 
\]  

(16)
Materials and Methods

Chemicals and enzymes

Chemically pure (99.7%) 4-hydroxybenzaldehyde was obtained from Chuo Chemical Co. (Tokyo, Japan). HCN was prepared by distillation of an acidified NaCN solution. NaCN was purchased from Fluka. Methyl tert-butyl ether was purchased from J.T. Baker. PaHnl enzyme powder was a generous gift from Solvay Pharmaceuticals (Weesp, The Netherlands) and contains 98% protein and about 65% PaHnl (Loos et al., 1995). The specific activity of the enzyme powder is 185,000 U per gram (Willeman et al., 2000). The PaHnl solution, used for the preparative synthesis of (R)-4-hydroxybenzaldehyde, was obtained from Roche Diagnostics (Penzberg, Germany) and had a specific activity of 1,600 U per ml. The *Hevea brasiliensis* Hnl (HbHnl) solution, used for the equilibrium experiments, was also obtained from Roche Diagnostics and had a specific activity of 6,500 U per ml. All mentioned activities are based on using racemic mandelonitrile as substrate. Both racemic and enantiopure 4-hydroxymandelonitrile are synthesized ourselves.

The double walled reactor, H/D 1.35, was obtained from Schmizo (Switzerland). Its effective volume was 200 ml, of which 150 ml was used, and 4 baffles were placed at equal distance from each other. A 6-blade turbine stirrer, was used and placed about 1 cm above the bottom of the reactor. During all experiments described here the stirring frequency was 950 min⁻¹.

Preparative synthesis of (R)-4-hydroxymandelonitrile

(R)-4-Hydroxymandelonitrile had to be synthesized for the enzymatic kinetic experiments. 75 ml methyl tert-butyl ether, 30 ml PaHnl solution adjusted to pH 5.5 and 7.5 ml HCN (192 mmol) were added to the reactor vessel. The reactor was closed and stirred at 3°C, favoring a high enantiomeric excess. 5.0 g (41 mmol) 4-hydroxybenzaldehyde were dissolved in 45 ml methyl tert-butyl ether and added to the reactor by using a dropping funnel within 5 minutes. After 2 h 25% of the aldehyde was converted and 30 ml PaHnl solution at pH 5.5 were added to the reactor. After 3 h 55% of the aldehyde was converted and after 7 h 50 ml methyl tert-butyl ether were added and the reaction mixture was cooled down to 1°C. After 20 h 80% of the aldehyde was converted into (R)-4-hydroxymandelonitrile, >99% ee, and the reaction was stopped by drying and filtration of the organic phase. A trace of citric acid was added for stabilization and after evaporation of the methyl tert-butyl ether at 30°C at reduced pressure, 4.2 g product, containing about 20% aldehyde, was obtained. It was found that the aldehyde dissolves better in dichloromethane than the 4-hydroxymandelonitrile. The remaining 4-hydroxybenzaldehyde was removed by extracting the solid product three times with 25 ml of dichloromethane. Less than 0.1% aldehyde remained.
Preparative synthesis of rac-4-hydroxymandelonitrile

 rac-4-Hydroxymandelonitrile had to be synthesized for the partition, equilibrium and nonenzymatic kinetic experiments. 3.7 g (30 mmol) 4-hydroxybenzaldehyde were dissolved in 40 ml methyl tert-butyl ether and 500 mg weakly basic ion exchanger Amberlyst A-21 were added and stirred. Then, 4 ml (2.8 g; 102 mmol) HCN were added. The reaction vessel was closed and stirred at room temperature. After 90 min 95% of the aldehyde was converted. After filtration some citric acid was added for stabilization and by evaporation of the methyl tert-butyl ether at 30°C at reduced pressure 4.1 g product was obtained. The remaining 4-hydroxybenzaldehyde was removed by extracting the solid product with 25 ml dichloromethane. 2.9 g of rac-4-hydroxymandelonitrile was obtained with less than 0.2% aldehyde.

Kinetic experiments

Reaction kinetics, both enzymatic as nonenzymatic, were measured at 20.0°C and pH 5.5, in 100 mM sodium citrate buffer saturated with methyl tert-butyl ether. Reaction mixtures of 25 ml were prepared from 4-hydroxybenzaldehyde, HCN, rac- and (R)-4-hydroxymandelonitrile, enzyme solutions and buffers. Depending on the direction of the reaction it was started either by addition of the 4-hydroxybenzaldehyde or the 4-hydroxymandelonitrile solution. Within seconds the reaction mixtures were drawn into a UV-cuvette for recording the progress curves. The 1 mm quartz flow through cuvette, mounted in a temperature controlled cuvette holder, had a calculated volume of 0.5 ml, including tubing.

Progress curves were recorded by measuring the absorption of 4-hydroxybenzaldehyde at 290 (absorption maximum) or 315 nm. The extinction coefficient for 4-hydroxybenzaldehyde in 100 mM sodium citrate buffer pH 5.5 amounts 1437 and 166.6 m²mol⁻¹ at 290 and 315 nm, respectively. At either wavelength the extinction coefficient of 4-hydroxybenzaldehyde is over 100 times the absorption coefficient of 4-hydroxymandelonitrile, so the influence of the latter compound is negligible.

The kinetic parameters were estimated by fitting them against the measured progress curves by using Encora version 1.2 (Straathof 2001; Willeman et al., 2000). Encora is a program designed for estimating the values for independent kinetic parameters of single enzymes which act according to established kinetic models, in this case the ordered bi-uni model.

Equilibrium experiments

Equilibrium experiments were performed in a similar way as the kinetic experiments. To obtain a fast equilibrium HbHnl as well as PaHnl were added to the reaction mixture at concentrations of about 1,000,000 U/liter for each enzyme. The equilibrium constant was determined from both the forward and reverse reaction at concentrations similar to those in the kinetic experiments.
Partition coefficients

The partition coefficient of 4-hydroxybenzaldehyde was determined by using UV-spectroscopy. 15 ml of 100 mM sodium citrate buffer of pH 5.5 and 15 ml methyl tert-butyl ether were incubated and mixed with 10-200 mg of 4-hydroxybenzaldehyde, at 20°C for 4 h. Samples from the aqueous phase were measured by UV-spectroscopy. By using a mass balance the concentrations in the organic phase were determined, which was relatively accurate since the compound predominately resides in the organic phase.

The partition coefficient of rac-4-hydroxymandelonitrile was determined by using HPLC-analysis (reversed phase Nucleosil-100 column and a 16% acetonitrile aqueous phase as eluent with 1 ml phosphoric acid per liter eluent) of both phases. 15 ml of 100 mM sodium citrate buffer of pH 3.25 and 15 ml methyl tert-butyl ether were incubated and mixed with 50-250 mg of rac-4-hydroxymandelonitrile at 20°C for 1 h.

Mass transfer kinetics

A progress curve of a batch conversion was recorded for the nonenzymatic, base catalyzed synthesis of rac-4-hydroxymandelonitrile in the biphasic system at pH 9 and 20°C in the 200 ml reactor. The value of $k_{1\alpha}$ was determined using 25 ml aqueous and 125 ml organic phase volume. To obtain 25 ml aqueous phase volume 30 ml aqueous buffer was required to compensate for the solubility of water in methyl tert-butyl ether. This solubility was about 2% at 20°C and increased to about 4% by addition of the HCN. Apparently, HCN improves the polarity of methyl tert-butyl ether.

First 66 ml of methyl tert-butyl ether were added to the reactor. Subsequently, 30 ml of 50 mM sodium phosphate buffer of pH 9 was added. While stirring, 9 ml (6.21 g) HCN were added and at the moment that the reaction mixture was at 20.0°C the reaction was started by the addition of 4 g 4-hydroxybenzaldehyde dissolved in 50 ml methyl tert-butyl ether. The extent of conversion of 4-hydroxybenzaldehyde was determined by sampling the organic phase and performing GC-analysis after derivatization.

The value of $k_{1\alpha}$ was determined according to Willeman et al. (2002A) by fitting $k_{1\alpha}$ to the experimental data using $k_{\text{non-enz}}^R = 3.8 \text{ l}\cdot\text{mol}^{-1}\cdot\text{s}^{-1}$ in the process model.

Enzymatic conversion in biphasic system

The progress curve for the conversion of 8 g 4-hydroxybenzaldehyde, which has a solubility of about 100 g/l methyl tert-butyl ether at 20°C, was recorded for the estimated optimum aqueous and organic phase volumes of 25 and 125 ml and an enzyme concentration of 28.6 g/l aqueous phase in the same reactor as used for the mass transfer kinetics experiments. To compensate for the solubility of water in the organic phase 5 ml additional aqueous buffer were added, as in the mass transfer experiment.

First 12.5 ml methyl tert-butyl ether were added. Then the solution of 8 g 4-hydroxybenzaldehyde dissolved in 100 ml methyl tert-butyl ether was added to the reactor. While stirring, 12.4 ml (8.71 g) of HCN were added and the system was allowed
to equilibrate at 20.0°C. The reaction was started by the addition of 30 ml aqueous buffer of pH 5.5 with 0.756 g dissolved PaHnl.

Sampling during the reaction was performed by turning off the stirrer and taking a 1 ml sample from the organic phase. The conversion of 4-hydroxybenzaldehyde and the enantiomeric excess of (R)-4-hydroxymandelonitrile were determined after derivatization by GC.

Analysis

Unless mentioned otherwise the samples were analyzed by GC after derivatization. The samples were derivatized by dissolving 150 µl organic phase in 1 ml dichloromethane, adding 60 µl acetyl chloride and after 1 minute 68 µl pyridine. The derivatized samples were analyzed on a CP-Chirasil-DEX CB Column.

Process simulation

The system of mathematical equations was programmed within Matlab version 5.3 (The Mathworks), using the Simulink Block Library version 3.

Results and Discussion

Equilibrium constant

The equilibrium constant of the synthesis reaction was determined using different concentrations by equilibration of the forward and reverse reaction and a mixture of R- and S-specific hydroxynitrile lyase, PaHnl and HbHnl. At 20°C \( K_{eq}^{RS} \) amounts 39 M\(^{-1}\), so \( K_{eq}^{R} \) amounts 19.5 M\(^{-1}\). At 5 and 25°C \( K_{eq}^{R} \) amounts 35 and 17 M\(^{-1}\), respectively, which is considerably less than for the reaction with benzaldehyde (\( K_{eq}^{R} = 133 \) M\(^{-1}\) at 25°C, Willeman et al., 2000). The reason for this is the stabilizing effect of the 4-hydroxy group on benzaldehyde.

Chemical kinetics

The kinetics of the nonenzymatic reaction to rac-4-OH-mandelonitrile were determined by recording progress curves for the forward and reverse reaction at different initial concentrations, shown in Table 1. The value obtained for \( k_{ree^{-}}^{R} \) at 20°C amounts 1.25 \( 10^{-3} \) l.mol\(^{-1}\).s\(^{-1}\), which is small as compared to the values obtained for benzaldehyde at 5 and 25°C, \( k_{ree^{-}}^{R} = 2.2 \ 10^{-3} \) and 14 \( 10^{-3} \) l.mol\(^{-1}\).s\(^{-1}\), respectively (Willeman et al., 2000). Since the influence of the background reaction appeared to be relatively small, 20°C was chosen as present reaction temperature, whereas 5°C had been used for benzaldehyde.
Table 1 Initial concentrations used for the determination of the nonenzymatic rate constant for formation of rac-4-hydroxymandelonitrile at 20°C and pH 5.5 (A = 4-hydroxybenzaldehyde; B = HCN; P = racemic 4-hydroxymandelonitrile).

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
</tr>
</thead>
<tbody>
<tr>
<td>C_A [mM]</td>
<td>4.00</td>
<td>5.65</td>
<td>6.00</td>
<td>11.31</td>
<td>15.3</td>
<td>0.01*</td>
<td>0.01*</td>
<td>0.02*</td>
<td>0.08*</td>
</tr>
<tr>
<td>C_B [mM]</td>
<td>203</td>
<td>204</td>
<td>190</td>
<td>204</td>
<td>204</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>C_P [mM]</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.98</td>
<td>1.96</td>
<td>3.93</td>
<td>16.8</td>
<td></td>
</tr>
</tbody>
</table>

*4-hydroxybenzaldehyde was present as a contaminant of rac-4-hydroxymandelonitrile

Table 2 Initial concentrations used for recording progress curves at 20°C and pH 5.5. (A = 4-hydroxybenzaldehyde; B = HCN; P^R = (R)-4-hydroxymandelonitrile; E = PaHai).

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
</tr>
</thead>
<tbody>
<tr>
<td>C_A [mM]</td>
<td>0.13</td>
<td>0.51</td>
<td>1.00</td>
<td>1.00</td>
<td>0.51</td>
<td>0.51</td>
<td>5.11</td>
</tr>
<tr>
<td>C_B [mM]</td>
<td>204</td>
<td>102</td>
<td>51</td>
<td>203</td>
<td>204</td>
<td>51</td>
<td>0</td>
</tr>
<tr>
<td>C_P^R [mM]</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.50</td>
<td>2.01</td>
<td>2.01</td>
</tr>
<tr>
<td>C_E [U.ml^-1]</td>
<td>198</td>
<td>198</td>
<td>148</td>
<td>148</td>
<td>198</td>
<td>379</td>
<td>396</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
<th>13</th>
<th>14</th>
</tr>
</thead>
<tbody>
<tr>
<td>C_A [mM]</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>C_B [mM]</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>102</td>
</tr>
<tr>
<td>C_P^R [mM]</td>
<td>0.25</td>
<td>1.00</td>
<td>2.01</td>
<td>8.40</td>
<td>16.8</td>
<td>2.01</td>
<td>2.01</td>
</tr>
<tr>
<td>C_E [U.ml^-1]</td>
<td>379</td>
<td>725</td>
<td>759</td>
<td>1156</td>
<td>1156</td>
<td>379</td>
<td>2.05</td>
</tr>
</tbody>
</table>

Enzyme kinetics

To estimate the enzyme kinetic parameters, 14 progress curves were recorded in buffer saturated with methyl tert-butyl ether at 20°C and pH 5.5 for both the forward and the reverse reaction. The progress curves are shown in Figure 3 and 4 and correspond to the experiments listed in Table 2. The concentration 4-hydroxymandelonitrile was calculated from the online measured 4-hydroxybenzaldehyde concentrations. The concentrations (R)- and (S)-4-hydroxymandelonitrile were calculated according to a procedure described by Willeman et al. (2000). Using the aforementioned values of k_{non-enz} and K_{eq}^R, the 5 unknown independent parameters were fitted. No convergence was obtained unless K_{mb} was fixed at a high value. Arbitrarily a value of 5000 mM was used. Results are shown in Table 3.

Figure 3 and 4 show that satisfying fits are obtained for the forward and reverse reaction at pH 5.5 and 20°C. For the cleavage of rac-4-hydroxymandelonitrile at pH 5.5 and 20°C the rates are a little overestimated, especially for the higher concentrations in Figure 4.

Just like the nonenzymatic reaction, the enzyme catalyzed reaction towards 4-hydroxymandelonitrile appeared to be much slower at 20°C compared to the reaction towards mandelonitrile at 5°C. The present value of k_{cat} for 4-hydroxybenzaldehyde amounts 67 at 20°C compared to 176 s^{-1} for benzaldehyde at 5°C (Willeman et al., 2002A). The initial rates of the enzymatic and nonenzymatic reaction for the conversion of benzaldehyde at 5°C were calculated for 5 mM benzaldehyde and 500 mM HCN and extrapolated to 20°C. Comparison of these values with the initial reaction rates for 4-hydroxybenzaldehyde revealed that the initial enzymatic reaction rate for benzaldehyde is
200 times faster than for 4-hydroxybenzaldehyde, whereas the initial nonenzymatic
reaction rate is only 5 times faster.

Table 3 Estimated kinetic parameters for the enzymatic synthesis of (R)-4-hydroxymandelonitrile catalyzed
by PahHnl in buffer saturated with methyl tert-butyl ether at pH 5.5 and 20°C (A = 4-hydroxybenzaldehyde;
B = HCN; P = (R)-4-hydroxymandelonitrile).

<table>
<thead>
<tr>
<th>$k_{cat}^f$</th>
<th>$k_{cat}^b$</th>
<th>$K_{mA}$</th>
<th>$K_{mB}$</th>
<th>$K_{mP}$</th>
<th>$K_{IA}$</th>
<th>$K_{IB}$</th>
<th>$K_{IP}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>[s$^{-1}$]</td>
<td>[s$^{-1}$]</td>
<td>[mM]</td>
<td>[mM]</td>
<td>[mM]</td>
<td>[mM]</td>
<td>[mM]</td>
<td>[mM]</td>
</tr>
<tr>
<td>67$^a$</td>
<td>0.9$^a$</td>
<td>15.8</td>
<td>5000</td>
<td>22.5</td>
<td>16.9</td>
<td>5400</td>
<td>22.4</td>
</tr>
</tbody>
</table>

$^a$ The enzyme powder contained 65% PahHnl, with a molecular weight of 72 kDa.
$^b$ Calculated by using consistency relations [Willeman et al., 2000].

Mass transfer kinetics

The partition coefficients determined at 20°C are 21 for 4-hydroxybenzaldehyde
and 14 for 4-hydroxymandelonitrile. The partition coefficients for benzaldehyde and
mandelonitrile at 5°C are 43 and 46 respectively (Willeman et al., 2002A). These higher
values can be attributed to the absence of a hydrophilic hydroxyl group. The mass transfer
coefficient was determined in a biphasic system of buffer and methyl tert-butyl ether that
was mass transfer limited due to a very fast nonenzymatic, base catalyzed, cyanohydrin
formation. A progress curve was recorded for the nonenzymatic reaction at pH 9 with 25
ml buffer and 125 ml of methyl tert-butyl ether, see Figure 5.

The experiment was repeated at pH 8 and 10. Since no increase (pH 10) or
decrease (pH 8) in conversion rate was observed (results not shown here) the system has
to be mass transfer rate limited and the mass transfer is not enhanced by the reaction rate.
The calculated value of $k_{cat}$, related to the aqueous phase volume, corresponded to 0.12 s$^{-1}$
for 4-hydroxybenzaldehyde using the described process model. Experiments at higher
and lower aqueous phase volume fractions indicated lower $k_{cat}$ values. One accurate
measurement was performed at 15 ml buffer and 135 ml of methyl tert-butyl ether, see
Figure 5, resulting in a calculated value for $k_{cat}$ of 0.09 s$^{-1}$. Thus the assumption that the
droplet diameter of the dispersed aqueous buffer, and hence $k_{cat}$, is constant within a
certain range of aqueous phase volumes is a crude approximation. Instead of trying to
find a relation between $k_{cat}$ and the aqueous phase volume fraction, we chose to use $k_{cat}$ =
0.12 s$^{-1}$ in the subsequent optimization, thus overestimating $k_{cat}$ for most conditions, and
to perform a sensitivity analysis afterwards.

In the experiments HCN was present at high concentrations in the aqueous phase
due to its relatively low partition coefficient and the excess used. Therefore, its mass
transfer rate is assumed to be not limiting. Moreover, its diffusion coefficient is expected
to be larger than that of benzaldehyde and mandelonitrile, which results in a larger value
for the mass transfer coefficient.

The value for $k_{cat}$ of 4-hydroxymandelonitrile is assumed to be equal to that of 4-
hydroxybenzaldehyde, as these compounds are expected to have approximately similar
diffusion coefficients.
Figure 3. Enzymatic conversion of 4-hydroxybenzaldehyde at 20°C, pH 5.5. Markers are experimental values, lines are the fitted curves. The amount of markers was reduced to improve the visibility. 0, 1; ○, 2; Δ, 3; x, 4; ○, 5; the numbers correspond to the experiments in table 2.

Figure 4. Enzymatic formation of 4-hydroxybenzaldehyde at 20°C, pH 5.5. Markers are experimental values, lines are the fitted curves. The amount of markers was reduced to improve the visibility. ●, 6; x, 7; Δ, 8; ○, 9; +, 10; −, 11; 0, 12; ○, 13; Δ, 14; the numbers correspond to the experiments in table 2. The fitted curves for experiment 10, 11 and 13 are dashed.
Figure 5. Nonenzymatic conversion of 4-hydroxybenzaldehyde in the experimental determination of the mass transfer coefficient at pH 9 and 20°C in the aqueous-organic biphasic system. Markers are the experimental values, lines are the fitted curves. □, 25 ml buffer and 125 ml methyl tert-butyl ether; Δ, 15 ml buffer and 135 ml methyl tert-butyl ether.

Process mode

The process model was developed and studied earlier for an aqueous-organic biphasic stirred tank reactor operating in batch, fed batch or continuous mode (Willeman et al., 2001). From that study a stirred tank reactor operating in continuous mode appeared to show no cost advantage.

For the conversion of benzaldehyde into (R)-mandelonitrile, it was found that the amount of enzyme could be reduced by operating in fed batch mode (Willeman et al., 2001). However, preliminary simulations for the conversion of 4-hydroxybenzaldehyde revealed that operating the stirred tank reactor in fed batch mode did not result in a decrease of enzyme consumption (results not shown). A batch operated stirred tank reactor appeared to be the best choice for the conversion of 4-hydroxybenzaldehyde into (R)-4-hydroxymandelonitrile.

Optimization of aqueous volume fraction and enzyme concentration

Since all values of the required process parameters have been determined, optimization of the synthesis of (R)-4-hydroxymandelonitrile in a batch operated stirred tank reactor can be performed by using the process model and the values for the process parameters.

Preliminary simulations revealed that at pH 5.5 and 20°C it is practically impossible to obtain both a high conversion degree and a high enantiomeric excess. Although the enzyme used is very active and pure, 185,000 U/g, an enantiomeric excess of over 99% would require more enzyme than the reactor can accommodate. Furthermore,
the relatively low equilibrium constant indicates that to obtain a high conversion degree a large excess of HCN is required.

In comparison to benzaldehyde, (Willeman et al., 2002B), the targets were therefore adjusted. Simulations revealed that a conversion of 90% of 8 g 4-hydroxybenzaldehyde in 150 ml reactor volume in combination with an enantiomeric excess of 95% is possible with regard to the required amount of enzyme and HCN. The required amount of HCN was calculated by eq. (12) and based on an equilibrium conversion degree of 95% 4-hydroxybenzaldehyde. This enables us to obtain a 90% conversion within a reasonable time.

The described model was used to determine the required concentration of enzyme, $C_{\text{aq,E}}$, that is just enough to meet the constraints of 90.00% conversion and 95.00% enantiomeric excess. This was performed for different aqueous phase volume fractions, at which the aqueous phase was considered to be dispersed, for a batch operating stirred tank reactor. For an arbitrary enzyme concentration, at 90.00% conversion a certain enantiomeric excess value was found by simulation. Depending on this value, the enzyme concentration was iteratively increased or decreased until at 90.00% conversion the required 95.00% enantiomeric excess was obtained. The corresponding reaction time is $t_{\text{conv}}$. The results of these simulations are shown in Table 4.

The concentration and moreover the amount of enzyme decrease when operating at lower aqueous phase volume fractions, whereas the time to obtain a conversion of 90.00% in 4-hydroxybenzaldehyde and a 95.00% enantiomeric excess for 4-hydroxymandelonitrile increases.

To obtain the optimum aqueous phase volume fraction and enzyme concentration, prices are estimated for the enzyme and for the time the reactor is used for the conversion. A price of € 100 per kg PaHn1 enzyme was used (compare Sheldon, 1993) and € 100 per h of reactor use, based on a 1 m$^3$ stirred reactor, including labor costs. The effect of substrate costs does not have to be taken into account as a fixed amount of aldehyde is converted and the amount of HCN showed little variation. Using the enzyme and reactor costs an optimum for the aqueous phase volume fraction and the enzyme concentration can be found at which the process should be operated. The enzyme was considered to be used only once. There is a clear cost optimum around € 13.5 per kg of product at 17% aqueous volume fraction and 28.6 g enzyme per liter, see Table 4.

According to the process model, this optimum at which 90.00% of 4-hydroxybenzaldehyde is converted to 4-hydroxymandelonitrile with an enantiomeric excess of 95.00% is obtained after 3 h using 25 ml aqueous phase and 125 ml organic phase. For the conversion of 8 g 4-hydroxybenzaldehyde 8.6 g HCN is required, a 5-fold molar excess.
Table 4 Results of the simulations of the aqueous organic biphasic batch system at 20°C and pH 5.5 in a batch operated stirred tank reactor, when considering enzyme costs of 100 €.kg⁻¹ and reactor usage costs of 100 €.h⁻¹.

<table>
<thead>
<tr>
<th>$V_{aq}$ [ml]</th>
<th>$V_{org}$ [ml]</th>
<th>$C_{E}^{tw}$ [g.l⁻¹]</th>
<th>$t_{conv}$ [min]</th>
<th>Reaction price [€.kg_product⁻¹]</th>
</tr>
</thead>
<tbody>
<tr>
<td>75</td>
<td>75</td>
<td>36.6</td>
<td>36.8</td>
<td>32.3</td>
</tr>
<tr>
<td>65</td>
<td>85</td>
<td>34.4</td>
<td>48.5</td>
<td>26.8</td>
</tr>
<tr>
<td>55</td>
<td>95</td>
<td>32.5</td>
<td>64.5</td>
<td>22.2</td>
</tr>
<tr>
<td>45</td>
<td>105</td>
<td>31.0</td>
<td>87.7</td>
<td>18.4</td>
</tr>
<tr>
<td>35</td>
<td>115</td>
<td>29.7</td>
<td>124.1</td>
<td>15.4</td>
</tr>
<tr>
<td>25</td>
<td>125</td>
<td>28.6</td>
<td>189.6</td>
<td>13.5</td>
</tr>
<tr>
<td>15</td>
<td>135</td>
<td>27.6</td>
<td>343.1</td>
<td>14.5</td>
</tr>
<tr>
<td>5</td>
<td>145</td>
<td>26.8</td>
<td>1110</td>
<td>33.1</td>
</tr>
</tbody>
</table>

However, it should be noticed that at production scale, e.g. 1 m³, this picture can be different, since mass transfer properties can be different. Besides, prices for enzyme and reactor use are likely to change and are scale dependent. Figure 6 shows the effect of these prices for the conversion when the prices for enzyme and reactor usage are halved and doubled. The optimum phase volume ratio remains around 20% aqueous phase volume, independent of costs for enzyme or reactor use. It also remains around 20% when it would have been taken into account that at other aqueous phase volume fractions the value of $k_{L}a$ may be lower than the value of 0.12 s⁻¹ that we used in the simulations.

At the estimated optimum an enzymatic experimental conversion was performed.

![Figure 6. Influence of the costs for the enzyme PaaHnl and the reactor use on the costs for the reaction step as function of the percentage of the aqueous phase volume. The full lines represent the cases in which the reactor costs are 100 € per h and where the enzyme costs ranges from 50-200 € per kg. The dashed lines represent the cases in which the enzyme costs are 100 € per kg and the reactor use costs range from 50-200 € per h.](image-url)
Figure 7. Enzymatic conversion of 8 g 4-hydroxybenzaldehyde with 8.7 g HCN at 20°C, pH 5.5 in the aqueous-organic biphasic system with 0.756 g PAAm dissolved in 25 ml aqueous phase and 125 ml organic phase. Markers are experimental values; 0, conversion (ξ); 0, enantiomeric excess. The lines represent the simulated experiments. The dashed lines represent the simulations for which the value of k_{L,a} was increased and decreased by 25%.

Experimental conversion in biphasic system

The enzymatic synthesis of (R)-4-hydroxymandelonitrile in the biphasic batch stirred tank reactor was performed at the aforementioned optimum conditions. After 3 h, the experimental extent of conversion for 4-hydroxybenzaldehyde was 85% and the ee of (R)-4-hydroxymandelonitrile was 97%. The experimental results confirm the results obtained by the process model, as shown in Figure 7. The extent of conversion is 5% overestimated whereas the enantiomeric excess is 2% underestimated.

The sensitivity of the system on the value of k_{L,a} was determined by simulating a 25% increase and decrease of the k_{L,a} value and found to have a negligible influence on the extent of conversion, shown in Figure 7 by the dashed lines, and no influence on the enantiomeric excess. An increase in volumetric productivity can only be accomplished by a simultaneous increase in mass transfer capacity and amount of enzyme.

Reaction temperature

Earlier work by Willeman et al. (2002B) revealed that at a low temperature the enzyme is used more efficiently whereas at a high temperature the reactor is used more efficiently. The procedure developed for optimization of the reaction temperature for the production of (R)-mandelonitrile is applied to (R)-4-hydroxymandelonitrile subsequently.

The values of the parameters describing the temperature dependence of the reaction kinetics were calculated from literature data. Niedermeyer (1990) determined the kinetics of the nonenzymatic conversion of benzaldehyde and 4-hydroxybenzaldehyde as a function of temperature at pH 3.75 instead of our value pH 5.5. The difference between
the activation energies for the conversion of 4-hydroxybenzaldehyde and benzaldehyde amounts 1.7 kJ.mol⁻¹. Using this value and the earlier determined activation energy for the nonenzymatic conversion of benzaldehyde at pH 5.5 (Willeman et al., 2002B) the activation energy for the nonenzymatic conversion of 4-hydroxybenzaldehyde at pH 5.5 is calculated. For the enzymatic conversion of 4-hydroxybenzaldehyde at pH 5.5, it is assumed that the difference between the activation energy of the nonenzymatic and the enzymatic conversion of benzaldehyde at pH 5.5 applies. This difference amounts 3.5 kJ.mol⁻¹. The calculated activation energies for the conversion of 4-hydroxybenzaldehyde at pH 5.5 are shown together with the earlier reported values in Table 5.

The enthalpy of reaction \( \Delta H^\circ \) is calculated from eq. (10) by using the values for the equilibrium constants at 5, 20 and 25°C, given in Table 6. The calculated enthalpy \( \Delta H^\circ \) amounts 28.6 kJ.mol⁻¹. The value for the constant \( \alpha \) by which the \( k_2a \) is dependent on the reaction temperature according to eq. (11) was considered to amount 13.9 as determined earlier for benzaldehyde (Willeman et al., 2002B).

The influence of temperature on the product costs is shown in Figure 8. The temperature of 20°C used for the earlier determined optimum of 17% aqueous volume phase fraction appears to be very close to the temperature optimum. Therefore it is unlikely that by changing the temperature the extent of conversion and the enantiomeric excess can be increased simultaneously above 90 and 95%, respectively, within the present constraints.

Table 5 Activation energies for the conversion of aldehydes into their corresponding cyanohydrins at pH 5.5 and 3.75.

<table>
<thead>
<tr>
<th></th>
<th>pH 5.5</th>
<th>pH 3.75</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( E_{\text{act}}^{\text{non-enz}} )</td>
<td>( E_{\text{act}}^{\text{enz}} )</td>
</tr>
<tr>
<td></td>
<td>[kJ.mol⁻¹]</td>
<td>[kJ.mol⁻¹]</td>
</tr>
<tr>
<td>4-hydroxybenzaldehyde</td>
<td>67.7ᵃ</td>
<td>64.2ᵃ</td>
</tr>
<tr>
<td>benzaldehyde</td>
<td>66.0ᵇ</td>
<td>62.5ᵇ</td>
</tr>
</tbody>
</table>

ᵃ This work;ᵇWilleman et al (2002B);ᶜNiedermeyer (1990)

Table 6 Equilibrium constants for the enzymatic addition of HCN to benzaldehyde and 4-hydroxybenzaldehyde towards their corresponding cyanohydrins at pH 5.5.

<table>
<thead>
<tr>
<th>( T ) [°C]</th>
<th>( K_{eq}^R ) [L.mol⁻¹]</th>
<th>( K_{eq}^R ) [L.mol⁻¹]</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>35</td>
<td>369</td>
</tr>
<tr>
<td>20</td>
<td>20</td>
<td>-</td>
</tr>
<tr>
<td>25</td>
<td>15</td>
<td>133</td>
</tr>
</tbody>
</table>

ᵃ Conversion towards (R)-4-hydroxymandelonitrile
ᵇ Conversion towards (R)-mandelonitrile from Willeman et al. (2000)
Figure 8. Production costs per kg (R)-4-hydroxymandelonitrile using estimated costs of 100 € per kg enzyme and 100 € per h reactor use as function of the reaction temperature and at different aqueous phase volume fractions at pH 5.5. Δ, 10%; ○, 17%; ●, 30%; ×, 50% aqueous phase volume fraction.

Conclusions

A methodology to develop the synthesis of (R)-cyanohydrins in aqueous-organic biphasic stirred tank reactors by using a process model has been developed. (R)-4-Hydroxymandelonitrile was synthesized by the addition of HCN to 4-hydroxybenzaldehyde, catalyzed by *Prunus amygdalus* hydroxynitrile lyase, *PaHnl*. Methyl tert-butyl ether was used as the organic solvent and the reaction conditions were 20°C and pH 5.5.

The optimization was directed towards the conversion of 4-hydroxybenzaldehyde with 90.00% conversion into (R)-4-hydroxymandelonitrile with an enantiomeric excess of 95.00%. Using cost prices of € 100 per kg *PaHnl* enzyme and € 100 per h reactor a cost optimum of € 13.5 per kg (R)-4-hydroxymandelonitrile is obtained. A batch operated stirred tank reactor operating at 17% (v/v) of the total reaction mixture volume being aqueous phase is required using a 5-fold molar excess of HCN, a *PaHnl* enzyme concentration of 28.6 g/l buffer yielding a volumetric productivity of 18.5 kg (R)-4-hydroxymandelonitrile m⁻³.h⁻¹. A temperature of 20°C appears to be very close to the temperature optimum.

The calculated optimum conditions were experimentally applied in a laboratory stirred tank reactor. The correspondence between experiments and simulations was good with respect to the enantiomeric excess and satisfying with respect to the extent of conversion.

It is not possible to conclude that the reaction conditions are the true optimum, without studying a very large range of other conditions. However, this study shows that once the *kₐlα* value of a full-scale system is known, only some small-scale experiments are required to find suitable conditions for a full-scale conversion, which may result in large savings on experimental expenses.
Nomenclature

\( a \) interfacial area per volume aqueous phase \([\text{m}^2 \cdot \text{m}^3_{\text{eq}}]\)

\( C_{\text{aq},j} \) concentration in aqueous phase \([\text{mol} \cdot \text{l}_{\text{eq}}^{-1}]\)

\( C_{\text{org},j} \) concentration in organic phase \([\text{mol} \cdot \text{l}_0^{-1}]\)

\( C_{\text{eq},E} \) enzyme concentration \([\text{g} \cdot \text{l}_{\text{eq}}^{-1}]\) or \([\text{U} \cdot \text{l}_{\text{eq}}^{-1}]\)

\( e e^R_j \) enantiomeric excess [-]

\( k_{\text{cat}}^\prime \) forward catalytic constant \([\text{s}^{-1}]\)

\( k_{\text{cat}}^r \) reverse catalytic constant \([\text{s}^{-1}]\)

\( k_{\text{non-enz}} \) forward nonenzymatic reaction rate constant \([\text{l}_{\text{eq}} \cdot \text{mol}^{-1} \cdot \text{s}^{-1}]\)

\( k_{l,j} \) mass transfer coefficient \([\text{s}^{-1}]\)

\( K_{\text{eq}}^R, K_{\text{eq}}^S \) enantiomeric equilibrium constant \([\text{l}_{\text{eq}} \cdot \text{mol}^{-1}]\)

\( K_{\text{eq}}^{RS} \) racemic equilibrium constant \([\text{l}_{\text{eq}} \cdot \text{mol}^{-1}]\)

\( K_{mj} \) Michaelis constant for compound \( j \) \([\text{mol} \cdot \text{l}_{\text{eq}}^{-1}]\)

\( K_{ij} \) inhibition constant for compound \( j \) \([\text{mol} \cdot \text{l}_{\text{eq}}^{-1}]\)

\( m_j \) partition coefficient \([\text{l}_{\text{eq}} \cdot \text{l}_0^{-1}]\)

\( n_j \) amount of compound \( j \) present in the reactor \([\text{mol}]\)

\( n_j^0 \) initial amount of compound \( j \) present in the reactor \([\text{mol}]\)

\( N_j^\prime \) feed rate of substrate \([\text{mol} \cdot \text{s}^{-1}]\)

\( r_j \) reaction rate for compound \( j \) \([\text{mol} \cdot \text{l}_{\text{eq}}^{-1} \cdot \text{s}^{-1}]\)

\( t \) time \([\text{s}]\)

\( V_{\text{aq}} \) volume aqueous phase \([\text{l}_{\text{eq}}]\)

\( V_{\text{org}} \) volume organic phase \([\text{l}_0]\)

\( \dot{q} \) mass transfer rate \([\text{mol} \cdot \text{l}_{\text{eq}}^{-1} \cdot \text{s}^{-1}]\)

\( \xi \) extent of conversion of aldehyde [-]

Compound \( j \):

A benzaldehyde

B hydrogen cyanide, HCN

\( P^R \) \((R)-\text{mandelonitrile}\)

\( P^S \) \((S)-\text{mandelonitrile}\)

References


Outlook

Recently, expanding interest from the fine chemical industry can be noticed for the synthesis of enantiopure cyanohydrins, indicating their market potential and the need for the development of Hnl catalyzed processes. A process model based tool has been developed enabling the rapid application from laboratory into production of the high potential synthesis strategy for enantiopure cyanohydrins within the fine chemical industry. Process models can be used for the optimization of the reactor type, process conditions, process economics and for scaling up.

The process model that has been developed was successfully applied to the conversion of 4-hydroxybenzaldehyde into its corresponding cyanohydrin by Prunus amygdalus hydroxynitrile lyase. The process model can be readily extended for optimization of the conversion of other substrates with a specified degree of conversion into their corresponding cyanohydrins with a specified enantiomeric excess. The optimization involves temperature, phase volumes, substrate concentrations, enzyme concentration, reactor type, conversion time and costs for enzyme and reactor use.

The research described in this thesis was limited to the use of purified Prunus amygdalus hydroxynitrile lyase in the system of methyl tert-butyl ether as organic phase and an aqueous phase pH 5.5 in a stirred tank reactor. Interesting aspects for future research may be the optimization of the type of organic solvent and the pH. Furthermore, it would be useful to incorporate correlations for mass transfer. Regarding the enzyme, it would be interesting to investigate the possibility to recycle the enzyme, to use crude enzyme preparations and to use other hydroxynitrile lyases. From a technical point of view the study of process integration, handling of substrates and downstream processing could lead to alternative process options. Nevertheless, the basic features of the process model as presented in this thesis will probably remain unaltered.

Compared to traditional optimization methods, where all these variables are experimentally varied, the process model based optimization should lead to considerable savings on the experimental expenses and process development time, minimization of scale-up risks ensuring a short time to the market and a strong long term market position.
Summary
Development of hydroxynitrile lyase catalyzed processes

The enzyme hydroxynitrile lyase from almonds, *Prunus amygdalus* hydroxynitrile lyase (*PaHnl*), catalyzes the addition of hydrogen cyanide to aldehydes or ketones towards enantiopure cyanohydrins. The synthesis of enantiopure cyanohydrins is a strategic route towards an extended range of more complex chiral compounds to be used as pharmaceuticals or agrochemicals, e.g.. The objective of the research described in this thesis was to develop tools to enhance the introduction of this promising synthesis route into an industrial process.

The enzymatic synthesis of enantiopure cyanohydrins is hampered by a nonenzymatic aspecific reaction yielding racemic cyanohydrins and therefore lowering the enantiomeric excess. This nonenzymatic reaction should be minimized compared to the enzymatic reaction to obtain cyanohydrins with a high enantiomeric excess.

Several methods have been used more or less successfully to minimize the nonenzymatic reaction compared to the enzymatic reaction, e.g. adjustment of pH and/or temperature, use of low substrate concentrations, large amounts of enzyme and the use of immobilized enzymes in organic solvents. The aqueous solubility of the more complex aldehydes and ketones is usually low, which limits the amount of substrate or product present in the reactor for systems using an aqueous phase only.

In aqueous-organic biphasic systems low substrate concentrations are combined with the possibility to accommodate large amounts of substrate. Aqueous-organic biphasic systems have been found to be successful for the synthesis of enantiopure cyanohydrins on laboratory scale and are considered as promising for application at industrial scale. The enhancement of their application requires process development and methods to scale up and optimize.

Optimization of aqueous-organic biphasic processes can be carried out by empirical methods, which are costly and time consuming. Mathematical process models may facilitate process optimization, leading to considerable savings in experimental effort.

In this thesis the development of a process model for aqueous-organic systems is described, experimentally validated and used for process optimization purposes. The process model consists of a description of the reaction kinetics, thermodynamics, mass transfer kinetics and a set of mass balances for a stirred tank reactor (batch, fed batch or continuous).

The available reaction kinetic data from literature have been validated and improved by using new methods. Consistent sets of kinetic parameters were estimated for the synthesis of *(R)*-mandelonitrile at 5 and 25°C by progress curve analysis. At a lower temperature the reaction equilibrium is much more favorable and the formation of racemic mandelonitrile is suppressed. The estimated kinetic parameters were used to identify that the rate determining step in the catalytic cycle is the release of *(R)*-mandelonitrile from the ternary complex.
The conversion of benzaldehyde into \((R)\)-mandelonitrile in an aqueous-organic biphasic system at 5°C and pH 5.5 is chosen as a model system with methyl tert-butyl ether as the organic phase. The model parameters: reaction kinetic parameters, partition coefficients and the lumped mass transfer coefficient were experimentally determined. The process model was validated by using 11 experimental datasets of batch conversions with different enzyme concentrations and phase volume ratios in a stirred tank reactor. The model was found to be valid with respect to both the extent of conversion and the enantiomeric excess. It was found that for synthesizing cyanohydrins with a high enantiomeric excess working at mass transfer limiting conditions is required.

The suitability of a batch, fed batch and continuous operated stirred tank reactor for the conversion of 750 moles benzaldehyde per m\(^3\) reactor into \((R)\)-mandelonitrile was investigated by using the process model. The constraints were that 98% of the benzaldehyde should react towards \((R)\)-mandelonitrile with an enantiomeric excess of 99%. A continuous operated stirred tank reactor could not meet the constraints, but the production in a batch or fed batch reactor was feasible. The choice for a batch or fed batch reactor is dependent on the influence of the costs for reactor operation and for the enzyme on the product costs. The enzymatic productivity and turnover are maximal when operating as fed batch whereas the volumetric productivity is maximal when operating as batch. By enhancement of mass transfer the volumetric and enzymatic productivity are increased considerable while the amount of product per kg enzyme is slightly decreased.

The reaction temperature was optimized for the synthesis of \((R)\)-mandelonitrile in the considered aqueous-organic biphasic system. At low temperatures the nonenzymatic reaction is reduced to a larger extent than the enzymatic reaction resulting in lower required enzyme concentrations. The mass transfer rate is reduced to a smaller extent. Mass transfer limitation is required for a high enantiomeric excess and determines the conversion rate. Therefore the volumetric productivity decreases at lower temperatures. The equilibrium constant is considerably higher at low temperatures resulting in a higher extent of conversion, or a lower hydrogen cyanide requirement. Both the volumetric productivity and the required enzyme concentration decrease by decreasing the reaction temperature and aqueous-phase volume while meeting the required conversion degree and enantiomeric excess. When prices are ascribed to the enzyme and reactor use, the reaction temperature can be optimized using the process model.

The model driven optimization approach for \((R)\)-mandelonitrile was extended to the development of the synthesis of the 4-hydroxy derivative. According to the process model it was possible to convert 90% of the 4-hydroxybenzaldehyde into \((R)\)-4-hydroxymandelonitrile with 95% enantiomeric excess. At the predicted optimum the synthesis was experimentally performed and the results were in accordance to the simulation regarding both the extent of conversion and the enantiomeric excess.

Compared to traditional optimization methods, where all variables are experimentally varied, the process model based optimization should lead to considerable savings on the experimental expenses and process development time and minimization of scale-up risks, ensuring a short time to the market and a strong long term market position.
Samenvatting
Ontwikkeling van hydroxynitril-lyase gekatalyseerde processen

Amandelen (Prunus amygdalus) bestaan voor 0,5% uit het enzym Prunus amygdalus hydroxynitril-lyase (PaHnl). PaHnl katalyseert de synthese van chirale cyaanhidrinen door de enantiocese specifieke koppeling van blauwzuur (HCN) aan aldehyde en ketonen. Chirale cyaanhidrinen zijn de grondstoffen voor een uitgebreid scala aan andere chirale verbindingen met een uiteindelijke toepassing als bijvoorbeeld pharmaceutica of landbouwchimicaleiën. Het doel van het in dit proefschrift beschreven onderzoek bestond uit het ontwikkelen van methoden ten einde de introductie van deze veelbelovende synthese van cyaanhidrinen in de fijnchemische industrie te bevorderen.

De toepassing van de enzymatische synthese van enantiomere-zuivere cyaanhidrinen wordt belemmerd door het optreden van een specifieke niet-enzymatische reactie waardoor racemisch cyaanhidrine wordt gevormd dat tot een ongewenste verlaging van de enantiomere overmaat van het cyaanhidrine leidt. Voor de synthese van cyaanhidrinen met een hoge enantiomere zuiverheid moet de niet-enzymatische reactiesnelheid tot een minimum gereduceerd worden ten opzichte van de enzymatische reactiesnelheid.

Verschillende methoden zijn toegepast, de ene met meer succes dan de andere, om de niet-enzymatische ten opzichte van de enzymatische reactiesnelheid te reduceren. Voorbeelden hiervan zijn: het werken bij een andere pH en/of temperatuur, het gebruik van lage aldehydeconcentraties en/of hoge enzymconcentraties en het gebruik van geïmobiliseerde enzymen in organische oplosmiddelen. De oplosbaarheid van complexere aldehyde en ketonen in water is vaak laag. Dit beperkt de hoeveelheid substraat en produkt in de reactor bij het gebruik van uitsluitend een waterige fase.

In waterige-organische twee-fasen systemen kunnen lage aldehydeconcentraties worden gecombineerd met grote hoeveelheden substraat en produkt. Waterige-organische twee-fasen systemen zijn succesvol gebleken in de synthese van enantiomerezuivere cyaanhidrinen op laboratoriumschaal en kunnen als veelbelovend worden beschouwd voor toepassing op industriële schaal. Teneinde de toepassing van deze veelbelovende laboratoriumsynthese binnen de fijnchemische industrie te bevorderen, zijn methoden ten behoeve van opschaling en optimalisatie benodigd.

Empirische methoden kunnen gebruikt worden maar zijn op industriële schaal duur en kosten veel tijd. Procesmodellen vereenvoudigen de opschaling en optimalisatie van processen en kunnen leiden tot aanzienlijke besparingen op de experimentele inspanningen. In dit proefschrift wordt de ontwikkeling van een procesmodel voor waterige-organische twee-fasen systemen beschreven, experimenteel getest en gebruikt voor de uiteindelijke optimalisatie. Het procesmodel beschrijft de reactiekinetiek en stoftransportkinetiek voor een waterig-organisch twee-fasen systeem in een geroerde tankreactor.

De vanuit de literatuur afkomstige reactiekinetiek-data zijn gecontroleerd en verbeterd door gebruik te maken van nieuwe technieken. Een consistent verzameling waarden voor de kinetische parameters in water bij pH 5,5 is bepaald voor de synthese van (R)-amandelzuurnitril bij 5 en 25°C met behulp van de analyse van progressiecurves. Bij lagere temperaturen is de evenwichtsligging van cyaanhidrine-vorming veel gunstiger en wordt de niet-enzymatische reactie gereduceerd ten opzichte van de enzymatische reactie. De waarden van de kinetische parameters zijn onder meer gebruikt ter bepaling.
van de snelheidsbepalende stap in het reactiemechanisme van het enzym: het loslaten van (R)-amandelzuurnitril uit het enzym-produkt complex.

De omzetting van benzaldehyde in (R)-amandelzuurnitril bij 5°C en pH 5,5 met methyl-tert-butylether als organische fase is gekozen als modellsysteem voor het testen van het eerder genoemde procesmodel. Ter beschrijving van het systeem zijn de waarden voor de reactiekinetiekparameters, partitiecoëfficiënten en de stofoverdrachtscoëfficiënt experimenteel bepaald. Het procesmodel is getest met behulp van 11 experimentele datasets. De datasets betroffen batch-omzettingen in een geroerde tankreactor bij verschillende enzymconcentraties en fase-volume verhoudingen. De experimentele en gecommueleerde datasets kwamen goed met elkaar overeen wat betreft de conversie en de enantiomere overmaat. Voor de synthese van enantiomeerzuivere cyaanhydrinen bleken stoftransport-limiterende condities een vereiste.

Een batch, fed-batch en continu bedreven geroerde tankreactor zijn met elkaar vergeleken met behulp van het procesmodel voor de omzetting van 750 mol benzaldehyde per m³ reactor tot (R)-amandelzuurnitril. De randvoorwaarden waren dat 98% van het benzaldehyde omgezet moest worden in (R)-amandelzuurnitril met een enantiomere overmaat van 99%. Een continu bedreven geroerde tankreactor kon niet voldoen aan de gestelde randvoorwaarden. De batch en fed-batch bedreven reactor zijn beide een optie. De keuze voor een batch of fed-batch bedreven reactor is afhankelijk van de invloed van de reactiekosten, bepaald door enzym- en reatorgebruik, op de kosten per eenheid produkt. De enzymatische produktiviteit en turnover zijn maximaal voor een fed batch reactor terwijl de volumetrische produktiviteit maximaal is voor een batch reactor. Het verhogen van de stofoverdracht veroorzaakt een sterke toename van zowel de volumetrische als de enzymatische produktiviteit terwijl de hoeveelheid produkt per kg gebruikt enzym nauwelijks afneemt.

De reactietemperatuur is geoptimaliseerd voor de synthese van (R)-amandelzuurnitril in het waterige-organische twee-fasen systeem. Bij lage temperaturen wordt de niet-enzymatische reactie sterker gereduceerd dan de enzymatische reactie waardoor het gebruik van lagere enzymconcentraties mogelijk is. Lage temperaturen zijn voordelig vanwege de betere evenwichts ligging. De volumetrische produktiviteit en de benodigde enzymconcentratie dalen bij een afnemende reactietemperatuur en/of het volume van de waterfase. Door een aannames te doen voor de kosten voor het enzym- en reatorgebruik werd een optimale reactietemperatuur bepaald met behulp van het procesmodel.

De ontwikkelde optimalisatie methode is uitgebreid naar het ontwikkelen van de synthese van het 4-hydroxyderivaat van (R)-amandelzuurnitril. Met het procesmodel was het mogelijk de procescondities te voorspellen voor de omzetting van 90% van het 4-hydroxybenzaldehyde in (R)-4-hydroxyamandelzuurnitril met een enantiomere overmaat van 95%. Het voorspelde optimum is experimenteel gevalideerd en de resultaten waren in overeenstemming met de simulatie voor zowel de conversie als de enantiomere overmaat.

Ten opzichte van traditionele optimalisatie-methoden, waar alle variabelen experimenteel worden gevarieerd, leidt een procesoptimalisatie gebaseerd op procesmodellen tot grote besparingen op de experimentele uitgaven en de ontwikkelingstijd van een proces en worden de opschalingsrisico's tot een minimum beperkt. Dit resulteert in een snelle marktintroductie en een langdurige sterke marktpositie.
Curriculum vitae


In 1989 werd gestart met de opleiding Chemische Technologie aan de toenmalige Hogere Technische School Dordrecht. Stages werden doorlopen bij DSM Research te Geleen en Analytical Controls te Rotterdam waar ook de afstudeeropdracht is uitgevoerd op het gebied van reversibele alkaan/alkeen scheiding.

In 1993 werd gestart met de opleiding Scheikundige Technologie aan de Technische Universiteit Delft waar een bioprocestechnologische wereld voor hem open ging. In 1996 werd de studie afgesloten op het Kluyverlaboratorium voor Biotechnologie middels het onderzoeken van de stofoverdracht in vallende-film reactoren in aanwezigheid van biomass. Het onderzoek maakte deel uit van een nieuw proces voor de productie van elementair zwavel vanuit rookgassen uit kolengestookte electriciteitscentrales en werd uitgevoerd onder directe begeleiding van Prof. dr. ir. Mark van Loosdrecht en Prof. dr. ir. Sef Heijnen.

In 1997 begon hij aan een onderzoeksperiode als promovendus op het Kluyverlaboratorium voor Biotechnologie bij de faculteit Toegepaste Natuurwetenschappen van de Technische Universiteit Delft en werkte aan de ontwikkeling van door het enzym hydroxynitril lyase gekatalyseerde processen onder begeleiding van Dr. ir. Adrie Straathof en zijn promotor Prof. dr. ir. Sef Heijnen. Als onderdeel van het onderzoek werkte hij 5 maanden bij DSM Fine Chemicals Linz, Oostenrijk in de groep van Dr. Peter Pöchlauer aan het toepassen van de reeds ontwikkelde methoden op een substraat met industriële relevantie.

Tijdens het promotieonderzoek werkte hij op het grensvlak van ontwikkeling en productie van fijnchemisch middels biokatalyse. Hij ontwikkelde op procesmodellen gebaseerde technieken voor het snel vertalen van een op laboratoriumschaal succesvolle synthese strategie voor enantiomeerzuivere cyaanhydrinen en de implementatie ervan op industriële schaal.

Sinds 1 november 2001 is hij werkzaam bij Centocor BV te Leiden, onderdeel van Johnson&Johnson.
Oral Presentations
2nd-7th September 2001, Darmstadt, Germany
5th International Symposium on Biocatalysis and Biotransformations
Oral presentation: "Development of hydroxynitrile lyase catalyzed processes"

20th April 2001, Wageningen, The Netherlands
Netherlands Biotechnology Society working meeting. Applied Biocatalysis and Plant Biotechnology
Oral presentation: "Can almonds be used in fine chemicals production?"

10th-13th September 2000, Copenhagen, Danmark
3rd European Symposium on Biochemical Engineering Science
Oral presentation: "Development of enantioselective processes in biphasic liquids"

Publications


Dankwoord

Als allerlaatste maar zeker niet het minste wil ik graag diegenen bedanken die mij op één of andere manier ondersteund hebben gedurende mijn periode als promovendus op het Kluyverlaboratorium voor Biotechnologie.

Adrie en Sef, gedurende vier jaar kon ik een beroep op jullie doen. Ik dankjulliewel voor het in mij gestelde vertrouwen. Jullie waren onmisbaar en betere begeleiders kon ik mij niet wensen. Woorden schieten tekort! Ulf, ook jij bedankt voor je bijdrage, jouw engels is van zeer goede kwaliteit.

Het onderzoek in Delft maakte deel uit van een gezamenlijk onderzoek met de Universiteit Leiden, afdeling Toegepaste Organische Chemie. Mijn start als onderzoeker bestond uit een stage bij onze onderzoeksverkoper. Tijdens deze periode ben ik met veel enthousiasme ingewerkt door Pieter Jan Gerrits (of moest er toch een streepje tussen?). De stage leerde mij dat werken met blauwzuur een delicate bezigheid is die de nodige veiligheidsmaatregelen vereist. Met veel plezier denk ik terug aan onze samenwerking, die echter lang niet altijd gemakkelijk was. Verder bedank ik ook zijn begeleiders Hans Brussee en professor Arne van der Gen voor hun inbreng in onze vruchtbare samenwerking. Dankzij Hans en zijn contacten binnen Solvay Pharmaceuticals kon een tekort aan enzym binnen no-time weer worden aangevuld.

Gedurende de gehele onderzoeksperiode bleek het lastig te zijn studenten te werven voor een research-practicum of afstudeeropdracht. De naam blauwzuur alleen al bleek voldoende om studenten tot ver buiten Delft af te schrikken met uitzondering van de volgendeelden en heldinnen: Samantha van den Bergh, Thorsten Friedrich, Danielle Homeman en Martin Smit. Het door jullie gefabriceerde FabrieksVoorOntwerp is een mooie bron van ideeën geweest. Bas Derissen en Chantal Mathijssen vormden de basis van het procesmodel. Dankjulliewel!

Ik wil graag het Ministerie van Economische Zaken (via Senter) bedanken voor de financiële ondersteuning van dit onderzoek dat deel uitmaakte van het programma IOP Katalyse. Daarnaast wil ik graag het IOP Katalyse bedanken voor de meestal vlekkeloze IOP begeleidingscommissiedagen te Ede, het mogelijk maken van een buitenlandse stage en het geven van een zeer gerichte effectieve presentatiecursus. Deze cursus alsmede de halfjaarlijkse oefeningen tijdens de BC-dagen heeft het presenteren van mijn onderzoeksresultaten gemaakt van een verschrikking tot een happening. Alle leden van de begeleidingscommissie dank ik hartelijk voor hun inbreng en interesse. Met name Guus van Scharrenburg van Solvay Pharmaceuticals voor de zeer gulle gift van 2 maal 10 gram PaHnI van superkwaliteit en Birgit Schulze als uiterst stabiele factor in de commissie en tevens voorzitter. De halfjaarlijkse beoordelingsrapporten vormden bijna altijd (post was een keer verwisseld door het IOP) een mooie pluim op het werk. Op deze plaats wil ik de secretaris van het IOP Katalyse, Merlijn van Rijswijk, bedanken. Jouw inbreng en visie waren voor mij soms van onschatbare waarde en brachten mij weer op nieuwe ideeën. Volgende week weer een balletje slaan, Rotjeknor of Delft?
Doordat DSM deel uitmaakte van de IOP-begeleidingscommissie was een buitenlandse stage in het makka van enantiomerezuivere cyaanhydrines snel geregeld. Gedurende 4 bijzonder zomerse maanden heb ik kunnen werken aan het toepassen van mijn onderzoeksresultaten in een industriële omgeving, de afdeling Forschung & Entwicklung van DSM Fine Chemicals Linz in Oostenrijk. Gedurende deze tijd ben ik verliefd geworden op Oostenrijk, op zijn adembenemende bergen maar bovenal op het leven in Mullviertel en zijn Mostbauern. Ik dacht dat ik me wel zou redden met zo'n 6 jaar duitse les achter de rug. Mullviertel Mundart is echter heel iets anders. Het werken in een perfect team van chemici en technici is mij bijzonder goed bevallen. Zo goed dat ik de hele maand december weer ben teruggegaan voor enkele afrondende experimenten om pas op Silvesterabend weer terug te keren naar Nederland. Rudi, ik blijf erbij dat Kirchschlag gedeeltelijk een zwarte piste was (voor mij althans).

Een Dankjulliewel voor Irma Wirth, Herbert Mayrhofer, Martin Emsenhuber, Peter Pöchlauer, Wolfgang Skranc maar bovenal SuperRudi Neuhofer. Woorden schieten wederom tekort! (ook voor de in de wijnkelder gegeten en door zijn vader gevangen en gerookte wilde forel, in combinatie met veel Sturm na afloop van de Wachau-marathon, september 2001). Ik betreurt het nog altijd dat een vervolgonderzoek moest stuk lopen op: "immer das Geld".

Via Professor Franz Effenberger is medio 2001 contact gelegd met Daniel Decker en Bettina Kirschbaum van Clariant (Frankfurt am Main, Duitsland). Gedurende een succesvol congres Biotrans2001 in Darmstadt is gesproken over de toepassing van mijn onderzoeksresultaten binnen Clariant. Ondanks een wederzijdse intentie tot samenwerking mocht ook ditmaal de toepassing van de resultaten niet doorgaan. Zullen de resultaten van 4 jaar onderzoek dan toch op die bekende boekenplank terecht komen?

Een doorzetter wint echter vaak! Na terugkomst uit Oostenrijk heb ik voor mijzelf besloten dat er na 4 jaar promotieonderzoek een concept proefschrift met goedkeuring moest liggen. Met nog een half jaar en 2 manuscripten te gaan heb ik meer dan 2 handjes laten wapperen. De inzet en betrokkenheid van Max Zomerdiijk, Angela Pröll, Cor Ras en Rudi Neuhofer waren hierbij onmisbaar. Dankjulliewel!

Pfirti!