New process concepts for the enzymatic synthesis of amoxicillin from penicillin G
Stellingen

behorende bij het proefschrift
"New process concepts for the enzymatic synthesis of amoxicillin from penicillin G"

1. In de geïntegreerde aanpak voor de biotransformatie van penicilline G naar ampicilline hebben Rindfleisch et al. beide enzymreacties sequentieel uitgevoerd om simultane synthese van penicilline G en ampicilline onder de gebruikte condities te voorkomen. Hierbij is over het hoofd gezien dat bij het simultaan uitvoeren van beide reacties de ampicilline-synthesereactie de evenwichtsligging van de penicilline-hydrolyse naar de produktkant kan verschuiven, waardoor een verdere procesintegratie bereikt kan worden.
   

2. Door het kiezen van optimale reactiecondities kunnen hoge opbrengsten worden behaald in een suspensie-naar-suspensie batch-produktie van amoxicilline zonder het product te hoeven kristalliseren m.b.v. bipolaire membranen in een extra bypass-loop.
   
   Hoofdstuk 4 van dit proefschrift

   
   Hoofdstuk 4 van dit proefschrift

4. De invloed van ionsterkte op enzymgekatalyseerde reacties is in vele studies een onderbelichte variabele.

5. Het gebruik van gepubliceerde data van andere onderzoeksgroepen voor eigen onderzoek vereist een goed inzicht in de nauwkeurigheid van de gebruikte meet- en analyse-methodes.

6. Om verwarring binnen verschillende onderzoeksgebieden te voorkomen moet er in literatuur een duidelijk onderscheid worden gemaakt tussen suspensie-naar-suspensie omzettingen en vast-naar-vast omzettingen.

7. Voor een objectieve beoordeling van manuscripten ter publikatie zouden de manuscripten anoniem moeten worden aangeboden aan de beoordelaars en zouden bovendien de namen van de beoordelaars bij de artikelen moeten worden gepubliceerd.

8. Behalve de bedreiging die het toepassen van genetische modificatie vormt voor de biodiversiteit is het ook een laatste redmiddel van de biodiversiteit.
   
   Bionieuws 16, 2000

9. Acceptatie van genetisch gmodificeerde produkten door consumenten zal alleen plaatsvinden bij een direct waarneembaar voordeel voor de consumenten.

10. Het toedienen van anti-depressiva bij varkens ter verbetering van hun welzijn getuigt niet van een diervriendelijke produktiemethode.
    
   Bionieuws 15, 2000

11. Het hebben van een internationale (werk)omgeving is een uitstekende manier om je talenkennis te vergroten, dit gaat echter ten koste van je moedertaal.

Marjon Diender
Delft, januari 2001
English translation

1. In the integrated approach for the biotransformation of penicillin G to ampicillin, Rindfleisch et al. performed both enzymatic reactions sequentially to prevent simultaneous synthesis of penicillin G and ampicillin under the conditions they used. In this way, they have not seen the opportunity for further process integration, which is provided by the synthesis reaction of ampicillin to shift the equilibrium of the penicillin G hydrolysis to the product side when both reactions would be performed simultaneously.

2. By choosing optimal reaction conditions high yields can be achieved in the suspension-to-suspension batch production of amoxicillin without having to crystallise the product with bipolar membranes in a bypass-loop.
   Chapter 4 of this thesis

3. According to Spieß (2000), the solubility of APA should not be exceeded, because precipitated APA limits the nucleophile concentration in the liquid phase. By assuming this Spieß misses one of the large advantages of suspension-to-suspension conversion, which is that during almost the complete conversion the substrates are present in the liquid phase at the maximal concentration (solubility).
   Chapter 4 of this thesis

4. In most studies, the influence of ionic strength on enzyme-catalysed reactions is a neglected variable.

5. The use of published data of other research groups for your own research requires good insight in the accuracy of the used experimental and analysis methods.

6. To avoid confusion within different research areas, solid-to-solid and suspension-to-suspension conversions should be distinguished.

7. For an objective refereeing of manuscripts that are submitted for publication, the manuscripts should be offered to the reviewers anonymously. In addition, the names of the reviewers should be published with the articles.

8. Apart from the threat genetic modification is to biodiversity, it is biodiversity's last remedy as well.
   Bionieuws 16, 2000

9. Acceptance of genetically modified products by the public will only take place when a direct advantage for the consumer is noticeable.

10. Administration of anti-depressives to pigs to improve their well-being is not an animal-friendly production method.

11. An international (working) environment enhances your knowledge of other languages, however this is at the cost of your native language.
New process concepts for the enzymatic synthesis of amoxicillin from penicillin G

Marjon Diender

Print: Offsetdrukkerij Ridderprint B.V., Ridderkerk
New process concepts for the enzymatic synthesis of amoxicillin from penicillin G

PROEFSCHRIFT

ter verkrijging van de graad van doctor
aan de Technische Universiteit Delft,
op gezag van de Rector Magnificus prof. K.F. Wakker,
voorzitter van het College voor Promoties,
in het openbaar te verdedigen
op dinsdag 20 maart 2001 om 16.00 uur

door Marjon Brigitte DIENDER

ingenieur in de landbouw- en milieuwetenschappen
geboren te Zwolle
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 Bruggink
Prof. dr P.J. Halling
Prof. dr D.B. Janssen
Prof. dr R.A. Sheldon
Dr ir A.J.J. Straathof
Dr T. van der Does

voorzitter
Technische Universiteit Delft, promotor
Katholieke Universiteit Nijmegen
University of Strathclyde, UK
Rijksuniversiteit Groningen
Technische Universiteit Delft
Technische Universiteit Delft
DSM Anti-infectives

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


Dit werk werd financieel ondersteund door DSM en het Ministerie van Economische Zaken (via Senter).

ISBN 90-9014318-1
# Table of contents

## Chapter 1  Introduction

1.1 General introduction 2
1.2 Production of semisynthetic antibiotics 3
1.3 Enzymatic production of Amox 4
1.4 Shortcut routes from pen G to Amox 6
1.5 Scope and outline of this thesis 7
1.6 References 9

## Chapter 2  Feasibility of the thermodynamically-controlled synthesis of amoxicillin

2.0 Abstract 11
2.1 Introduction 12
2.2 Materials and methods 13
  2.2.1 Materials 13
  2.2.2 Solubility measurements 13
  2.2.3 Reaction equilibrium measurements in water 13
  2.2.4 Analysis 14
2.3 Results and discussion 14
  2.3.1 Reaction equilibria in water as a function of pH 14
  2.3.2 Suspension-to-suspension process 16
2.4 Conclusions 18
2.5 Acknowledgements 18
2.6 References 19

## Chapter 3  Predicting enzyme catalysed reaction equilibria in cosolvent-water mixtures as a function of pH and cosolvent composition

3.0 Abstract 21
3.1 Introduction 22
3.2 Materials and methods 23
Table of contents

3.2.1 Materials 23
3.2.2 Titration of ionizable groups 23
3.3 Results and discussion 24
  3.3.1 Dissociation and reaction equilibria in water 24
  3.3.2 The equilibrium in cosolvent-water mixtures 26
  3.3.3 Effect of organic solvents on apparent pKₐ 27
  3.3.4 Choice of reference reaction 29
  3.3.5 Effect of organic solvents on synthetic yield of Pen G 30
  3.3.6 Prediction of Amox synthesis equilibrium 33
3.4 Conclusions 34
3.5 Notation 35
3.6 References 36

Chapter 4  Course of pH during the formation of amoxicillin by a suspension-to-suspension reaction

4.0 Abstract 39
4.1 Introduction 40
4.2 Theory 41
  4.2.1 Initial pH 42
  4.2.2 pH during reaction 43
4.3 Materials and methods 45
  4.3.1 Materials 45
  4.3.2 Enzyme activity 46
  4.3.3 Synthesis reaction in water 46
  4.3.4 Analysis 46
  4.3.5 Simulations 46
4.4 Results and discussion 46
  4.4.1 Suspension-to-suspension reactions 46
  4.4.2 Simulations 49
4.5 Conclusions 52
4.6 Notation 52
4.7 References 53
Chapter 5  Equilibrium modelling of extractive enzymatic hydrolysis of penicillin G with concomitant 6-aminopenicillanic acid crystallisation

5.0 Abstract 55
5.1 Introduction 56
5.2 Theory 57
  5.2.1 One-phase reaction equilibrium 57
  5.2.2 Partitioning 58
  5.2.3 Two-phase reaction equilibria 59
  5.2.4 Three-phase reaction equilibria 61
5.3 Materials and methods 62
  5.3.1 Materials 62
  5.3.2 Enzymatic reaction in 1:1 (w/w) water/butyl acetate two-phase system 62
  5.3.3 Analysis 63
5.4 Results and discussion 63
  5.4.1 Hydrolysis of Pen G 63
  5.4.2 Partitioning 65
  5.4.3 Modelling of hydrolysis of Pen G 68
5.5 Conclusions 72
5.6 Acknowledgements 72
5.7 Notation 72
5.8 References 73

Chapter 6  Modelling the influence of ionic strength on α-amino acid ester hydrolase in the salt-free production of amoxicillin

6.0 Abstract 77
6.1 Introduction 78
6.2 Theory 81
  6.2.1 Calculation initial inorganic salt concentration 82
  6.2.2 Calculation added amount of HPGM 83
6.3 Materials and methods 85
  6.3.1 Enzyme 85
  6.3.2 Enzyme activity 85
Table of contents

6.3.3 Materials 85
6.3.4 Enzymatic reactions 85
6.3.5 Analysis 86
6.3.6 Simulations 86
6.4 Results and discussion 86
6.4.1 Enzymatic reactions 86
6.4.2 Simulations 91
6.5 Conclusions 93
6.6 Acknowledgements 94
6.7 Notation 94
6.8 References 95

Chapter 7 Feasibility of a one-pot shortcut route from penicillin G to amoxicillin in anhydrous organic solvent

7.0 Abstract 97
7.1 Introduction 98
7.2 Theory 100
7.2.1 Reaction equilibria in water 100
7.2.2 Partitioning 102
7.2.3 Reaction equilibria in anhydrous organic solvent 103
7.2.4 Crystallisation 103
7.3 Materials and methods 105
7.3.1 Materials 105
7.3.2 Solubility measurements 105
7.3.3 Enzyme activity 105
7.3.4 Enzymatic reaction 105
7.3.5 Analysis 106
7.3.6 Calculations 106
7.4 Results and discussion 106
7.4.1 Enzyme activity 106
7.4.2 Reference reaction equilibrium constants 107
7.4.3 Reference solubilities 108
7.4.4 Reference partition coefficients 109
7.4.5 Predictions 109
7.5 Conclusions 112
7.6 Acknowledgements 113
7.7 Notation 113
7.8 References 114

Summary 117

Samenvatting 121

Curriculum vitae 126

Dankwoord 127
Introduction
and
Outline of this thesis
1.1 General introduction

The antibiotic penicillin G (Pen G) is the most common raw material for semisynthetic β-lactam antibiotics. Key-intermediate for β-lactam antibiotics is 6-aminopenicillanic acid (APA), the β-lactam nucleus, which has a worldwide annual production volume of approximately 10,000 tons. Pen G is produced fermentatively by adding phenylacetic acid (PAA) to a crude fermentation broth. Pen G is converted, either chemically or enzymatically, to APA and PAA. By coupling chemically or enzymatically different side chains to APA, a wide range of semisynthetic penicillins with different specificities and stabilities can be obtained. Examples of bulk semisynthetic antibiotics are amoxicillin (Amox) and ampicillin (Ampi) (Van de Sandt and De Vroom, 2000; Bruggink, 1996). These antibiotics have market sales worth of 2200 (Amox) and 2000 (Ampi) million $ per year as bulk-formulated drug (Sheldon, 1994). Figure 1 shows a general overview of the route for synthesis of Amox.

![Diagram of fermentation process from Pen G to Amox](image)

**Figure 1** General production route of the penicillin-derived antibiotic Amox.
1.2 Production of semisynthetic antibiotics

Traditionally, the conversion of Pen G into semisynthetic antibiotics was performed chemically. In these chemical production methods, much waste is produced, i.e. 10-100 kg of waste per kg of product. These classical processes make use of halogenated organic solvents and need energy-consuming sub-zero temperatures (DSM magazine, 1997; Van de Sandt and De Vroom, 2000). The biocatalytic processes, however, are run in water at ambient or slightly elevated or decreased temperatures and use only a titrant as auxiliary chemical. The amount of waste using the biocatalytic processes compared to the chemical processes is reduced by a factor of 5 for the hydrolysis reaction of Pen G to APA and a factor of 3 for the coupling reaction of APA and side chain (Van de Sandt and De Vroom, 2000).

![Diagram](image)

**Figure 2** Schematic overview of conventional enzymatic process of Pen G purification (adapted from Hersbach et al., 1984) and biotransformation to Amox.

In the present downstream processing of Pen G, Pen G is extracted from the fermentation broth with an organic solvent like butyl acetate or amyl acetate.
Subsequently, Pen G is either back-extracted to an aqueous phase and then crystallised, or crystallised directly from the organic phase by adding solid potassium acetate or sodium acetate. The Pen G crystals are used for the production of APA by enzymatic hydrolysis. APA is recovered by crystallisation, dissolved again and may be coupled enzymatically with an activated side chain to form a semisynthetic antibiotic. A schematic overview of an enzymatic process of Pen G purification and biotransformation to Amox is given in Figure 2. As can be seen in this overview, this enzymatic process comprises many process steps. The next section focuses on the enzymatic coupling reaction of the β-lactam nucleus and the side chain. The succeeding section discusses a potentially more efficient antibiotic production process resulting from a combination of several process steps (shortcuts).

1.3 Enzymatic production of Amox

The enzymatic coupling reaction between the β-lactam nucleus and the side chain can be performed using two distinctive strategies:

1. *thermodynamically-controlled synthesis* in which a direct condensation of the nucleus and a non-activated side chain, such as hydroxyphenylglycine (HPG) takes place (see Figure 3a). This strategy can only be applied successfully if conditions for a favourable equilibrium position can be found. The enzyme does

\[
\begin{align*}
\text{APA} + \text{HPG} & \rightleftharpoons \text{Amox} + \text{H}_2\text{O} \\
\text{APA} + \text{HPGM} + \text{H}_2\text{O} & \xrightarrow{\text{I}} \text{Amox} + \text{MeOH} + \text{H}_2\text{O} \\
\text{APA} + \text{HPG} + \text{MeOH} & \xrightarrow{\text{II}} \text{HPG} + \text{APA} + \text{MeOH} \\
\end{align*}
\]

*Figure 3 Enzymatic production of Amox via a. thermodynamically-controlled synthesis, b. kinetically-controlled synthesis.*
not influence the position of the equilibrium, but only the rate at which the equilibrium is established (Kasche, 1986). To shift unfavourable reaction equilibria towards synthesis, moderate to high concentrations of water-miscible organic solvents may be added (Homandberg et al., 1978; Fernandez-Lafuente et al., 1991).

2. **kinetically-controlled synthesis** in which the nucleus is coupled to an activated side chain, such as hydroxyphenylglycine methyl ester (HPGM) or hydroxyphenylglycine amide (HPGA) (reaction I, Figure 3b). Using this strategy, high yields on nucleus are achievable, but part of the activated side chain hydrolyses (reaction II, Figure 3b) and is not coupled to the nucleus, so recycling and reactivation of side chain are necessary or extra waste is produced. Also part of the produced antibiotic may hydrolyse (reaction III, Figure 3b). An important parameter -the so-called synthesis/hydrolysis ratio (i.e. the molar ratio of produced antibiotic and hydrolysed side chain)- is influenced by reaction conditions and biocatalyst (enzyme) properties and formulation (Kasche, 1986; Bruggink et al., 1998).

Because of the low water solubility of semisynthetic antibiotics like Amox and Ampi, the antibiotic may crystallise during the synthesis reaction. This crystallisation can be very favourable for the thermodynamically-controlled synthesis when the equilibrium concentration of the antibiotic exceeds its solubility. Then the equilibrium is shifted towards the product side and higher yields will be achieved. The crystallisation of the antibiotic during the reaction is also favourable for the kinetically-controlled synthesis as this phenomenon avoids the unwanted product

![Figure 4](image)

**Figure 4**  *Schematic overview of suspension-to-suspension reaction.*
hydrolysis by the enzyme due to much lower dissolved concentrations. When hydrolytic side reactions of substrate or product (reactions II and III, Figure 3b) are to be reversed or suppressed, the so-called suspension-to-suspension reactions are especially advantageous. In these reactions the substrates and the products are mainly present as undissolved particles, while the enzymatic reactions take place in the aqueous phase (see Figure 4). In this type of reactions, the product to reactor volume ratio is much higher than in the conventional systems in which all reactants are completely dissolved (Halling et al., 1995).

1.4 Shortcut routes from Pen G to Amox

As described earlier, a reduction in the number of process steps in the production of semisynthetic antibiotics by combining several steps could lead to more efficient production processes, while producing less waste. A possibility for a shortcut route is to perform the hydrolysis reaction of Pen G salt and the synthesis reaction of Amox simultaneously. In this way, the equilibrium of the hydrolysis reaction will be shifted towards the product side as the intermediate APA is consumed in the synthesis reaction. Both reactions can be performed using the same enzyme, penicillin acylase. By performing the reactions simultaneously, APA formed in the hydrolysis reaction can be used directly for the synthesis reaction without having to purify it in between.

![Diagram showing process concept of Pen G purification and simultaneous enzymatic Pen G hydrolysis and Amox synthesis.]

Figure 5  Schematic overview of a process concept of Pen G purification and simultaneous enzymatic Pen G hydrolysis and Amox synthesis.
the reactions. This would reduce 1. the number of process units 2. losses of APA during downstream processing and 3. salt formation as the number of pH shifts (for enzymatic steps and crystallisation) can be decreased (see Figure 5).

An even more advanced shortcut route would be to start with Pen G acid dissolved in the organic solvent directly after its extraction from the fermentation broth. The hydrolysis reaction of Pen G and the kinetically-controlled synthesis reaction of Amox could both take place in the organic solvent or in organic solvent/water mixtures. This shortcut route might reduce the salt production and the number of process steps even more than the previous shortcut, because in this case also Pen G does not have to be purified prior to the enzymatic reactions (see Figure 6).

**Figure 6** Schematic overview of a process concept of partial Pen G purification and subsequent Pen G biotransformation to Amox in aqueous/organic solvent two-phase system.

### 1.5 Scope and outline of this thesis

In 1996, a large cluster project was formed between DSM, Gist-brocades (now part of DSM), five university groups and the Ministry of Economical Affairs (through Senter). The aim of this cluster project was to develop biocatalytic, cleaner and more efficient production processes for semisynthetic antibiotics. The particular aim of the work described in this thesis, which was part of the cluster project, was to develop new enzymatic process concepts for the production of semisynthetic penicillins, with the emphasis on the enzymatic coupling reaction of APA and a side chain. This thesis comprises two parts: the first part (chapters 2-4) focuses on the enzymatic coupling reaction of APA and a side chain, while the second part focuses on possibilities for
shortcut routes (a two-step shortcut route in chapter 5 and 6, and a one-pot shortcut route in chapter 7).

A thermodynamical approach is required to evaluate the earlier-mentioned systems to produce Amox. In this thermodynamical approach reaction equilibria, dissociation constants, solubilities and partition behaviour of the reactants play a role. The different process concepts for the production of Amox were described using the thermodynamical approach and tested experimentally.

**Coupling reaction**

The thermodynamically-controlled synthesis of Amox from APA and HPG is described in Chapter 2. In this chapter, the feasibility of suspension-to-suspension production of Amox in aqueous systems is studied. Chapter 3 describes the influence of water-miscible organic solvents on thermodynamic reaction equilibria. A model is presented for the prediction of thermodynamic equilibria in cosolvent-water mixtures. As a case study, the thermo-dynamically-controlled synthesis of Amox in water and water/cosolvent systems is shown. In Chapter 4, the kinetically-controlled synthesis of Amox from APA and HPGM is studied. A quantitative model is presented for predicting the pH and concentrations of the reactants during suspension-to-suspension reactions. As no inorganic salts are added to control the pH during the reaction, a salt-free production of Amox is achieved.

**Shortcut routes**

After fermentation, Pen G is extracted with an organic solvent. This organic solvent containing Pen G acid can be added directly to an enzymatic reactor and hydrolysed by extractive catalysis. Chapter 5 describes a model for the equilibrium conversion of Pen G in organic solvent/water two-phase mixtures as a function of initial concentrations and phase volume ratio. The organic phase after Pen G hydrolysis contains mainly extracted PAA, while the remaining aqueous phase contains mostly APA. This aqueous phase can then be used for the synthesis of Amox upon addition of activated side chain and a biocatalyst. Chapter 6 describes a quantitative model for the prediction of the influence of the ionic strength and the presence of components such as Pen G and PAA on the synthesis reaction of Amox by the enzyme α-amino acid ester hydrolase. In the last chapter, Chapter 7, the feasibility of a one-pot shortcut route from Pen G to Amox in anhydrous organic solvent is studied.
1.6 References


Feasibility of the thermodynamically-controlled synthesis of amoxicillin

Abstract
The enzymatic, thermodynamically controlled synthesis of amoxicillin in aqueous solution was measured in order to study the feasibility of a 'suspension-to-suspension' conversion. In aqueous solution, however, the synthetic yield of amoxicillin remains lower than the amoxicillin solubility. Therefore, a 'suspension-to-suspension' synthesis of amoxicillin in aqueous solution is not feasible. Synthetic yields in enzymatic condensation reactions can often be improved by adding organic solvents in monophasic systems. Addition of cosolvents is calculated to improve the apparent equilibrium constant of amoxicillin synthesis considerably, but probably not the synthetic yield, due to solubility restrictions of the reactants.

This chapter was published in J Mol Catal B: Enz (1998) 5:249-25
2.1 Introduction

Amoxicillin is one of the major β-lactam antibiotics, with sales of $2200 million as bulk formulated drug in 1994 (Sheldon, 1996). Traditionally, semisynthetic β-lactam antibiotics are produced by chemical modification of the basic penicillin structure. However, enzymatic procedures may offer important advantages over the conventional chemical transformations. For example, it can be performed in a one-step reaction with high yield and high specificity, and under mild reaction conditions (Kasche, 1986; Fernandez-Lafuente et al., 1996; Choi et al., 1981; Kasche et al., 1986).

The most frequently applied strategy for enzymatic synthesis is a kinetically controlled reaction, in which an activated acyl donor is coupled to an antibiotic nucleus. High yields on nucleus are achievable, but part of the activated acyl donor hydrolyses and is not coupled to the nucleus, so recycling and reactivation of the acyl donor is necessary, leading to costly extra process steps. In addition, hydrolysis of the product may occur (Fernandez-Lafuente et al., 1996). A simpler strategy for enzymatic synthesis would be a direct condensation of antibiotic nuclei and unactivated acyl donors in a thermodynamically controlled reaction, which avoids these recycles and reactivation. This strategy can only be applied successfully if conditions for a favorable equilibrium position can be found. The enzyme does not influence the position of the equilibrium, but only the rate at which the equilibrium is established. In water, however, hydrolysis of the antibiotic may be favored to synthesis, because of unfavourable thermodynamics. To shift the reaction equilibrium towards synthesis one may add moderate to high concentrations of water miscible organic solvents (Kim and Lee, 1996; Tewari et al., 1995) and/or increase the substrate concentration up to the solubility level or even higher, which gives a suspension of substrate. In favourable cases the concentration of the product that is formed exceeds its solubility. Then a suspension of solid substrates can be converted into a suspension of solid products. This situation, to which we refer as the 'suspension-to-suspension' process, has been achieved for several enzymatic reactions (Kasche, 1986; Halling et al., 1995).

The purpose of this chapter is to describe the thermodynamics of the synthesis of amoxicillin in order to study the feasibility of the 'suspension-to-suspension' concept. The thermodynamic condition for a 'suspension-to-suspension' conversion to occur is that the ratio of dissolved concentrations (i.e. the apparent equilibrium constant, $K_{app}$) is larger than the ratio of solubilities ($R_S$):
\[ K_{\text{app}} = \frac{c_{\text{Amox}}}{c_{\text{APA}} c_{\text{HPG}}} > \frac{S_{\text{Amox}}}{S_{\text{APA}} S_{\text{HPG}}} = R_s \]  

(1)

where \( c_i \) is the actual concentration of species \( i \) and \( S_i \) its solubility.

### 2.2 Materials and methods

#### 2.2.1 Materials

Immobilized penicillin G acylase (EC 3.5.1.11) from \( E. \) coli, 6-aminopenicillanic acid (6-APA, 98.5% pure) and amoxicillin trihydrate (Amox, 98.9% pure) were donated by Gist-brocades (Delft, The Netherlands). D-(\( \rho \)-) hydroxyphenylglycine (HPG) was a kind gift of DSM (Geleen, The Netherlands).

#### 2.2.2 Solubility measurements

Solubility measurements of Amox, 6-APA and HPG were performed as described by Gude et al. (1996).

#### 2.2.3 Reaction equilibrium measurements in water

Equilibrium measurements of the synthesis and hydrolysis of amoxicillin were performed by approaching equilibrium from both directions of reaction at pH 5.0, 5.6 and 6.0 using a pH-stat (Impulsomat 614, Dosimat 665, and pH meter 691 of Metrohm). For each experiment a solution was prepared in 0.1 mol\textsuperscript{-1} potassium phosphate buffer (pH 5.65) with an excess of APA and HPG to obtain saturation, and 0 mol Amox\textsuperscript{-1} (hydrolysis experiments), or 0.6\textsuperscript{-3} mol Amox\textsuperscript{-1} (synthesis experiments at pH 5.6 and 6) or 2.3\textsuperscript{-3} mol Amox\textsuperscript{-1} (synthesis experiments at pH 5.0). Equilibration and filtration were performed as described for the solubility experiments. The pH was adjusted using 1.0 mol\textsuperscript{-1} aqueous NaOH or HCl.

Reactions were performed in a thermostated reaction vessel at 25\( ^\circ \)C by addition of 5.0 ml of 0.11 g\textsuperscript{-1} enzyme stock solution (in 0.1 mol\textsuperscript{-1} potassium phosphate buffer, pH 5.65). Generally, reactions were carried out for approximately 20 to 44 h.

For calculation of \( K_{\text{app}} \) the ratios of concentrations were averaged for the flat region of the Amox curve in the synthesis experiments and for the corresponding region in the hydrolysis experiments.
2.2.4 Analysis

APA, HPG and Amox were identified and analyzed by HPLC using a Waters System with a Zorbax SB-C_{18} 4.6×75 mm 3.5 μm column, thermostated at 33 °C, and a Waters 996 PDA detector at 230 nm, a Waters 910 Wisp 10 μl injector, and using a Waters 590 pump with a flow of 1 ml·min^{-1}. Prior to analysis, the samples were diluted with eluens. The eluens was composed of 8 mmol·l^{-1} tetrabutylammonium bromide, 10 mmol·l^{-1} Na₂HPO₄ and 15% (v/v) acetonitril. It was brought to pH 6.60 with H₃PO₄.

2.3 Results and discussion

2.3.1 Reaction equilibria in water as a function of pH

Precise determination of the equilibrium constant for the synthesis of Amox from APA and HPG is difficult because of chemical degradation of these compounds during equilibration and because of the low equilibrium concentration of Amox. We carried out the reactions in duplicate or triplicate, starting from compositions close to either side of the equilibrium. In order to minimize chemical degradation the amounts of enzyme were maximized. Yet, degradation was not completely excluded during these experiments and equilibrium was not reached.

![Figure 1](image_url)  
**Figure 1** Equilibrium measurement of amoxicillin at pH 6.0 and 25 °C. Equilibrium was achieved from both directions of reaction. Concentrations of APA and HPG are not displayed.
For the Amox concentration profile this was hardly visible in some examples, and a reasonable correspondence between the concentration in hydrolysis and synthesis experiments was obtained (Figure 1), but for APA the degradation amounted up to 15% (data not shown). Still, in this dynamic state the equilibrium constant could be determined from the ratio of concentrations, because the pseudo-first order rate constants for the enzymatic reaction were much higher than for the chemical degradation according to blanks and literature data (Tsuji et al., 1978).

![Graph showing equilibrium constants for Amoxicillin synthesis and hydrolysis](image)

**Figure 2** Apparent equilibrium constants for the synthesis of amoxicillin in water and a 50% (w/w) DMF mixture as a function of pH at 25°C. Experimental values for synthesis (●) and hydrolysis (■), and predicted values of $K_{app,aq}$ (—) and $K_{app,org}$×10² in 50% (w/w) DMF mixture (—†—†). $K_{app}=\text{[Amox]}/\text{[APA][HPG]}$. Calculated ratio of solubilities $R_{S,aq}$ (…………), $R_{S,org}$ (— — — —). Experimental value of $R_{S,org}$ (◇).

As shown in Figure 2, there is a reasonable correspondence between equilibria achieved from either synthesis or hydrolysis experiments, except for pH 5.1. Nevertheless, results from all experiments were used for further calculations. The apparent equilibrium constant can be modeled as a function of the dissociation of the reactants and the pH-independent equilibrium constant of a reference reaction ($K_{ref}$) (Svedas et al., 1980). We have used a reference reaction for the synthesis of amoxicillin, which involves anionic APA, cationic HPG and zwitterionic Amox:
APA\(^{-}\) + HPG\(^{+}\) ⇌ Amox\(^{+}\) + H\(_{2}\)O

Using the dissociation constants of the reactants (Table I), the experimental values of \(K_{\text{app}}\) (see Figure 2) the average value of \(K_{\text{ref}}\) is 623 l·mol\(^{-1}\).

**Table 1**  
Effect of DMF on the apparent \(pK_a\) values of APA, HPG and Amox at 25°C.  
Experimental results were measured by Diender et al. (1998).

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Compound</th>
<th>(pK_{a,\text{app}})</th>
<th>water</th>
<th>50% (w/w) DMF</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>APA</td>
<td>(pK_{a,\text{APA1}})</td>
<td>2.5</td>
<td>3.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(pK_{a,\text{APA2}})</td>
<td>4.9</td>
<td>4.8</td>
</tr>
<tr>
<td></td>
<td>HPG</td>
<td>(pK_{a,\text{HPG1}})</td>
<td>2.2</td>
<td>3.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(pK_{a,\text{HPG2}})</td>
<td>9.2</td>
<td>9.2</td>
</tr>
<tr>
<td></td>
<td>Amox</td>
<td>(pK_{a,\text{Amox1}})</td>
<td>2.9</td>
<td>4.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(pK_{a,\text{Amox2}})</td>
<td>7.4</td>
<td>7.5</td>
</tr>
</tbody>
</table>

Using this value of \(K_{\text{ref}}\), \(K_{\text{app}}\) is calculated as a function of pH (see Figure 2, model line)(Diender et al., 1998). The measured values support the model, because experimental \(K_{\text{app}}\) values decrease with increasing pH according to the prediction. However, the \(K_{\text{app}}\) values observed in this work are an order of magnitude smaller than the values for ampicillin hydrolysis reported by Svedas et al. (1980) and Blinkovsky and Markaryan (1993). They gave values of \(K_{\text{app}}\) of 158 mol\(^{-1}\)·l at pH=4.5, 78 mol\(^{-1}\)·l at pH 5.0 (Svedas et al., 1980) and 5.5 mol\(^{-1}\)·l at pH 5.5 (Blinkovsky and Markaryan, 1993), all at 25°C. Ampicillin is very similar in structure to amoxicillin. We had expected the apparent equilibrium constant of amoxicillin and ampicillin to be approximately equal. The low value of \(K_{\text{app}}\) observed for amoxicillin has important implications for the feasibility of the 'suspension-to-suspension' reaction, as will be shown below.

**2.3.2 Suspension-to-suspension process**

In order to study the feasibility of a 'suspension-to-suspension' conversion of amoxicillin in water the solubilities in water of the reactants and product were
Thermodynamically controlled synthesis of Amox
determined as a function of pH. Results of these solubility measurements are represented in Figure 3. A study of the feasibility of the ‘suspension-to-suspension’ conversion of amoxicillin based on the equilibrium constant reported for ampicillin (Blinkovskv and Markaryan, 1993) and the solubilities of Fig. 3 may suggest that ‘suspension-to-suspension’ conversion could be successful. However, our experimental equilibrium concentrations of amoxicillin in water (e.g. Figure 1) are an order of magnitude lower than its solubility (Figure 3), so the ‘suspension-to-suspension’ conversion should not occur. Also, preliminary results by others indeed showed no ‘suspension-to-suspension’ conversion in water (L.M. van Langen and R. Sheldon, personal communication). This conclusion seems valid in the whole pH range from 4 to 8, because the calculated value of $R_S$ is always larger than $K_{app}$ (Fig. 2). Schroën et al. (1999) describe similar results for cephalexin.

![Graph showing solubility of Amox, APA, and HPG as a function of pH.](image)

**Figure 3** Experimental solubilities of Amox (▲), APA (■) and HPG (●) in water as a function of pH at 25 °C. The full curves are fits according to the model of Gude et al. (1996).

As mentioned in the Section 2.1, one may add moderate to high concentrations of water-miscible organic solvents to shift the reaction equilibrium towards synthesis. The addition of organic cosolvents lowers the water activity, and stabilizes the neutral forms of dissociating groups of the substrates (mainly carboxylic acid groups). To predict enzymatic reaction equilibria in monophasic water-organic solvent mixtures we recently developed a model that uses reaction equilibria in water and apparent $pK_a$ values of the reactants in different water-cosolvent mixtures as input parameters (Diender et al., 1998). This model confirms the high yields that have been found for
thermodynamically controlled synthesis of penicillin G from APA and phenylacetic acid in water-dimethylformamide (DMF) mixtures, and can also be used to predict the synthetic yield of amoxicillin in this water-cosolvent mixture.

In Table I the apparent dissociation constants of the amoxicillin related reactants in water and a 50\% (w/w) DMF mixture are presented, which we used to calculate $K_{\text{app.org}}$ using the model (Svedas et al., 1980) and $K_{\text{ref}} = 623$ 1 mol$^{-1}$. It is calculated that $K_{\text{app}}$ increases nearly a factor 100 in 50\% (w/w) DMF mixture compared to water (Figure 2). However, this does not mean that 'suspension-to-suspension' conversion will occur, because the solubilities of the substrates and the product will also change in the water-organic solvent mixtures. In a mixture with DMF, the solubilities of Amox, APA and HPG are much lower than in water, overall leading to an increase of $R_S$ in equation (1), that counteracts the increase of $K_{\text{app}}$. Thus a 'suspension-to-suspension' conversion of amoxicillin is expected not to be feasible in a water-cosolvent mixture, but this can be tested only if an enzyme is available that is and remains sufficiently active under such conditions.

2.4 Conclusions

In aqueous solution, the equilibrium constant of the enzymatic synthesis of amoxicillin from APA and HPG is much lower than was expected from extrapolated literature data on ampicillin synthesis. Therefore, a 'suspension-to-suspension' conversion in water is not feasible. The presence of an organic cosolvent, e.g. 50\% (w/w) DMF mixture is calculated to improve the equilibrium constant considerably by a factor of nearly 100. However, it is expected that, due to solubility restrictions in the DMF-water mixture, also here the 'suspension-to-suspension' concept is not feasible.

2.5 Acknowledgements

We are very grateful to H.H.J. Meuwissen for the solubility measurements.
2.6 References


Predicting enzyme catalysed reaction equilibria in cosolvent-water mixtures as a function of pH and solvent composition

Abstract
Synthetic yields in enzymatic condensation reactions can often be improved by using organic solvents in monophasic systems. However, the addition of these cosolvents may result in loss of stability of the enzyme. Therefore, knowing that stabilization of the enzyme is necessary and often laborious, it would be useful to predict the potential improvement of the reaction equilibrium in the presence of the solvents before modifying the enzyme.

This paper describes a model for predicting reaction equilibria in cosolvent-water mixtures by simply measuring the equilibrium in water, and the apparent pKₐ of all reactants in those mixtures. In the model a reference reaction must be chosen. This choice of reference reaction is crucial for the outcome of the model. The reference reaction should be chosen such that the same functional groups are present in the same ionic state both in the reactants as in the products. The model works quite well for the model system studied, i.e. synthesis of penicillin G from 6-aminopenicillanic acid and phenylacetic acid.

*This chapter was published in Biocatal Biotrans (1998) 16:275-289*
3.1 Introduction

Enzymatic syntheses are often limited by thermodynamic equilibria. For example, direct condensation of antibiotic nuclei and unactivated acyl donors by a thermodynamically controlled reaction in water may lead to negligible yields because hydrolysis of the antibiotic is favored over synthesis. To shift the equilibrium towards synthesis, the pH may be changed or moderate to high concentrations of organic cosolvents may be added. However, especially at high concentrations of water-miscible solvents (cosolvents) no enzyme activity may remain. In order to experimentally study the equilibrium at these conditions the enzyme needs to be modified, which often is laborious and time-consuming. (Homandberg et al., 1978; Fernandez-Lafuente et al., 1991a, b, 1996; Kasche et al., 1986; Ivanov et al., 1997) Before making efforts to modify the enzyme, it would be useful to predict the thermodynamic equilibrium, i.e. to predict the possible synthetic yield in those organic solvent/water mixtures as a function of pH. The purpose of this paper is to present a quantitative model for cosolvent-water mixtures to this end. This model is an extension of the work of Carpenter (1960), Homandberg et al. (1978) and Kasche (1986). Carpenter (1960) introduced a conjecture that peptide bond synthesis equilibria are independent of substituent effects if the equilibrium constant for the synthesis is defined in terms of the non-ionized forms of the reactants. Homandberg et al. (1978) extended the assumption of Carpenter to the effect of organic solvents and predicted reaction equilibrium positions for the synthesis of uncharged peptides in water and cosolvent-water mixtures. Kasche (1986) also described the synthesis of various condensation products in water and cosolvent-water mixtures. However, none of the previously mentioned models dealt with the problems originating from the use

\[
\begin{align*}
R^0 & \rightleftharpoons \frac{K_{a,A}}{} \quad R^- \\
\quad & \Downarrow K_{a,D} \\
R^+ & \rightleftharpoons \frac{K_{a,C}}{} \quad R^{++}
\end{align*}
\]

**Figure 1** Dissociation scheme of amphoteric reactant R.
of uncharged reactants or products in the model when one or more of the reactants is an amphoteric molecule. Amphoteric molecules often exist as zwitterions instead of uncharged molecules. Therefore, microscopic dissociation constants that are not easily accessible have to be used. This is demonstrated for the dissociation of an amphoteric reactant R in Figure 1. \( K_a \) are the microscopic dissociation constants. The microscopic dissociation constants of the uncharged molecule (\( K_{a,A} \) and \( K_{a,D} \)) can be calculated from the macroscopic dissociation constants (i.e. the experimentally determined dissociation constants) of the zwitterion as described by Greenstein and Winitz (1961). This calculation method, however, can cause errors in the prediction of the synthesis of condensation products, because the assumptions that have to be made in this calculation may not be valid. In the case of synthesis reactions involving zwitterions, it can be easier to choose a chemical reference reaction involving charged reactants instead of uncharged molecules, avoiding the problems arising by using microscopic dissociation constants.

In this paper we present an extended model to predict the synthetic yield in organic cosolvent/water mixtures as a function of pH and solvent composition. Also, we will describe the influence of the choice of reference reaction on the outcome of the model. For the validation of our model, the synthesis of penicillin G from phenylacetic acid and 6-aminopenicillinic acid will be used. Also, the prediction and validation of synthetic yield of amoxicillin will be discussed.

### 3.2 Materials and methods

#### 3.2.1 Materials

Penicillin G (Pen G, 99.0% pure), 6-aminopenicillanic acid (APA, 98.5% pure), and amoxicillin trihydrate (Amox, 98.9% pure) were donated by Gist-brocades (Delft, The Netherlands). D-(p-)-hydroxyphenylglycine (HPG, 99.0% pure) was a kind gift of DSM (Geleen, The Netherlands). Phenylacetic acid (PAA, 99.0% pure) was from Fluka Chemie AG, Buchs. Organic solvents were analytical grade.

#### 3.2.2 Titration of ionizable groups

Stock solutions of APA (0.02 M) and PAA or HPG (0.04 M) were used to prepare 50% (w/w) cosolvent-water mixtures. The pH of the stock solutions was previously adjusted to around 9.0 with 1.0 M aqueous KOH. The apparent \( pK_a \) values of the reacting species in the 50% (w/w) cosolvent-water mixtures were determined at
25°C by potentiometric titration at concentrations of 0.01-0.02 M with 0.25 M aqueous HCl as titrant. Titration curves were recorded by a Metrohm 665 Dosimat motor burette, a Metrohm 614 Impulsomat control unit and a Metrohm 691 pH-meter with on-line data acquisition and a stirred, thermostatted glass titration vessel. A simple model was set up for the relation between pH and added volume of titrant. This model (composed of a mass balance, a charge balance and the dissociation equilibrium) was fitted to the experimental data by non-linear regression, which resulted in an apparent dissociation constant.

Generally, experiments were carried out within 15-25 minutes, therefore it was assumed that no degradation of the compounds occurred during the experiments.

3.3 Results and discussion

3.3.1 Dissociation and reaction equilibrium in water

The penicillin acylase-catalyzed synthesis of Pen G from APA and PAA was chosen as a model system (Figure 2).

![Reaction Equation]

**Figure 2** Enzymatic synthesis of Pen G from PAA and APA.

This is an equilibrium reaction of which the equilibrium position can be influenced by shifting the pH and by adding cosolvents (Fernandez-Lafuente et al. 1991a; Tewari and Goldberg, 1988). To account for the different ionic species of the components involved, a chemical reference reaction should be chosen, e.g.

\[ \text{APA}^- + \text{PAA}^0 \rightleftharpoons \text{PenG}^- + \text{H}_2\text{O} \]  (1)
Because we are considering a system dilute in Pen G, APA and PAA molarity (c) can be used instead of molalities, and asymmetric activity coefficients (γ*) instead of symmetric activity coefficients (γ) for these compounds. But for water, mole fraction and symmetric activity coefficient have to be used. Therefore the equilibrium constant of the reference reaction is:

\[ K_{ref} = \frac{c_{PenG^{-}} \cdot x_{w}}{c_{PAA^{0}} \cdot c_{APA^{-}}} \cdot \Gamma \]  

(2)

where

\[ \Gamma = \frac{\gamma_{PenG}^{*} \cdot \gamma_{w}}{\gamma_{PAA^{0}}^{*} \cdot \gamma_{APA^{-}}^{*}} \]

In dilute aqueous solution, all activity coefficients are unity and the mole fraction of water is 1. When the equilibrium constant is measured at one pH, one can calculate \( K_{ref} \) and predict the equilibrium position at all other values of pH. The apparent equilibrium constant (\( K_{app} \)) is defined as:

\[ K_{app} = \frac{(c_{PenG^{0}} + c_{PenG^{-}})}{(c_{APA^{+}} + c_{APA^{+}} + c_{APA^{-}} + c_{PAA^{0}}) \cdot (c_{PAA^{0}} + c_{PAA^{-}})} \]

\[ = \frac{c_{PenG^{-}} \cdot F_{PAA^{0}} \cdot F_{APA^{-}}}{c_{APA^{-}} \cdot c_{PAA^{0}} \cdot F_{PenG^{-}}} \]  

(3)

where \( F_{PAA^{0}} \), \( F_{APA^{-}} \) and \( F_{PenG^{-}} \) are the fractions of uncharged PAA and anionic species of APA or Pen G. The fractions \( F_{PAA^{0}} \), \( F_{APA^{-}} \) and \( F_{PenG^{-}} \) are calculated from

\[ F_{PAA^{0}} = \frac{1}{1 + \frac{K_{a,PAA,app}}{c_{H^{+}}}} \]  

(4)

\[ F_{APA^{-}} = \frac{1}{1 + \frac{c_{H^{+}}}{K_{a,APA2,app}} + \frac{(c_{H^{+}})^{2}}{K_{a,APA1,app} \cdot K_{a,APA2,app}}} \]  

(5)
\[ F_{\text{PenG}} = \frac{1}{1 + \frac{c_{\text{H}^+}}{K_{a,\text{PenG,app}}}} \]  

where \(K_{a,\text{PAA,app}}, K_{a,\text{APA1,app}}, K_{a,\text{APA2,app}}, \) and \(K_{a,\text{PenG,app}}\) are the apparent dissociation constants of PAA, APA and Pen G, respectively.

### 3.3.2 The equilibrium in cosolvent-water mixtures

For an aqueous solvent (superscript aq) and an aqueous-organic solvent mixture (superscript org) the thermodynamic equilibrium constant is the same:

\[ K_{eq} = \frac{a_{\text{PenG}}^{eq} \cdot a_{w}^{eq}}{a_{\text{PAA}}^{eq} \cdot a_{\text{APA}}^{eq}} = \frac{a_{\text{org}}^{eq} \cdot a_{w}^{eq}}{a_{\text{PAA}}^{eq} \cdot a_{\text{APA}}^{eq}} \]

Using 1 molal concentration in water as the standard state for the dissolved compounds this becomes

\[ \Gamma_{\text{PenG}}^{eq} \cdot \frac{m_{\text{PAA}}^{eq} \cdot x_{\text{APA}}^{eq}}{m_{\text{PAA}}^{eq} \cdot m_{\text{APA}}^{eq}} = \Gamma_{\text{org}}^{eq} \cdot \frac{m_{\text{org}}^{eq} \cdot x_{\text{org}}^{eq}}{m_{\text{org}}^{eq} \cdot m_{\text{org}}^{eq}} \]

As indicated before, in water one can assume that \(x_{w}^{eq}=1\) and \(\Gamma_{w}^{eq}=1\). Also, because we are considering a system dilute in PAA, APA and Pen G, molalities are almost equal to molarities in water, but a density correction has to be included for the solvent mixture.

\[ \frac{c_{\text{PenG}}^{eq} \cdot \rho_{\text{org}}^{eq}}{c_{\text{PAA}}^{eq} \cdot c_{\text{APA}}^{eq}} = x_{w}^{eq} \cdot \Gamma_{\text{org}}^{eq} \cdot \frac{c_{\text{org}}^{eq} \cdot \rho_{\text{org}}^{eq}}{c_{\text{PAA}}^{eq} \cdot c_{\text{APA}}^{eq}} \]

where \(\rho_{\text{org}}^{eq}\) is the density in kg solvent mixture per liter solvent mixture. Suppose one wants to know

\[ K_{\text{org,app}}^{eq} = \frac{c_{\text{org}}^{eq} \cdot \rho_{\text{org}}^{eq}}{c_{\text{PAA}}^{eq} \cdot c_{\text{APA}}^{eq}} = \frac{c_{\text{org}}^{eq} \cdot \rho_{\text{org}}^{eq}}{c_{\text{PAA}}^{eq} \cdot c_{\text{APA}}^{eq}} \cdot \frac{F_{\text{org}}^{eq} \cdot F_{\text{org}}^{eq}}{F_{\text{org}}^{eq} \cdot F_{\text{org}}^{eq}} \]

Now one can derive using equations 9 and 10:
\[
K_{\text{org}}^{\text{app}} = \frac{c_{\text{aq}}^{\text{PenG}}}{c_{\text{aq}}^{\text{PAA}^0} \cdot c_{\text{aq}}^{\text{APA}^-}} \cdot 1 \cdot \rho_{\text{aq}}^{\text{org}} \cdot \frac{F_{\text{org}}^{\text{PAA}^0}}{F_{\text{org}}^{\text{APA}^-}} \cdot \frac{F_{\text{org}}^{\text{PenG}^-}}{F_{\text{org}}^{\text{PenG}^-}}
\]

\[
= \frac{k_{\text{app}}^{\text{aq}}}{x_{w}^{\text{org}}} \cdot 1 \cdot \rho_{\text{org}}^{\text{aq}} \cdot \frac{F_{\text{org}}^{\text{PAA}^0}}{F_{\text{org}}^{\text{APA}^-}} \cdot \frac{F_{\text{org}}^{\text{PenG}^-}}{F_{\text{org}}^{\text{PenG}^-}} \quad (11)
\]

In this paper we want to avoid measurement or prediction of \(pF^{\text{org}}\) values, because the methods for this are still at the development stage. One can choose a reference reaction for which \(F^{\text{org}}=1\). As will be shown later, the reference reaction described in equation 1 is such a suitable reaction. Then \(K_{\text{org}}^{\text{app}}\) can be calculated from quantities that can be determined easily:

\[
K_{\text{app}}^{\text{org}} = \frac{k_{\text{app}}^{\text{aq}}}{x_{w}^{\text{org}}} \cdot 1 \cdot \rho_{\text{org}}^{\text{aq}} \cdot \frac{F_{\text{org}}^{\text{PAA}^0}}{F_{\text{org}}^{\text{APA}^-}} \cdot \frac{F_{\text{org}}^{\text{PenG}^-}}{F_{\text{org}}^{\text{PenG}^-}} \quad (12)
\]

3.3.3 Effect of organic cosolvents on apparent p\(K_a\) value

Dissociation constants cannot be predicted properly for mixed solvents where a minimum of data on the compounds involved is available in the literature (Li et al., 1994), but they can be measured easily by titration. Apparent p\(K_a\) values (i.e. the logarithms of the observed dissociation constants) measured in water and cosolvent-water mixtures are listed in Table I. The pH-meter was calibrated in water at pH 4 and 7 at 25°C. No corrections of pH were made in the presence of organic solvent, and p\(K_a\) values were calculated as p\(K_{a,\text{app}}\). Measured apparent dissociation constants can be used in equations 4-6 to calculate the fractions of the components which are used in the reference reaction. The apparent p\(K_a\) value of Pen G in pure water was not measured, but taken from Tewari and Goldberg (1988). Because of the great similarity with the APA molecule, p\(K_{a,\text{PenG,app}}\) was assumed to be the same as p\(K_{a,\text{APA1,app}}\) in the different cosolvent-water mixtures. Note the effect of organic cosolvents on p\(K_{a,\text{app}}\) of the carboxylic acid groups, while the p\(K_{a}\) of the amine groups
Table I  Apparent $pK_a$ values, densities and water mole fractions in different solvent mixtures.

<table>
<thead>
<tr>
<th>Solvent</th>
<th>p$K_{a,APA}$</th>
<th>p$K_{a,PAA}$</th>
<th>p$K_{a,PenG}$</th>
<th>p$K_{a,HPG1}$</th>
<th>p$K_{a,HPG2}$</th>
<th>p$K_{a,Amox1}$</th>
<th>p$K_{a,Amox2}$</th>
<th>$x_w$</th>
<th>$\rho_{org}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pure H$_2$O</td>
<td>2.5</td>
<td>4.9</td>
<td>4.3</td>
<td>2.5$^a$</td>
<td>2.2</td>
<td>9.2</td>
<td>2.9</td>
<td>7.4</td>
<td>1</td>
</tr>
<tr>
<td>50% (w/w) glycerol</td>
<td>2.5</td>
<td>4.8</td>
<td>4.5</td>
<td>2.5</td>
<td>2.2</td>
<td>8.8</td>
<td>N.D.</td>
<td>7.3</td>
<td>0.832</td>
</tr>
<tr>
<td>50% (w/w) 1,4-butanediol</td>
<td>3.3</td>
<td>4.8</td>
<td>5.2</td>
<td>3.3</td>
<td>2.8</td>
<td>9.2</td>
<td>N.D.</td>
<td>7.2</td>
<td>0.834</td>
</tr>
<tr>
<td>50% (w/w) N,N-dimethylformamide</td>
<td>3.9</td>
<td>4.8</td>
<td>6.0</td>
<td>3.9</td>
<td>3.6</td>
<td>9.2</td>
<td>4.5</td>
<td>7.5</td>
<td>0.802</td>
</tr>
</tbody>
</table>

$^a$ Tewari and Goldberg (1988); These authors also mentioned $pK_{a,PenG}=9.0$ for Pen G and $pK_{a,APA}=9.9$ for APA, but these protonations do not seem to be relevant for the present paper.

N.D. Not determined
is little affected. These results agree with results published by Homandberg et al. (1978) and Fernandez-Lafuente et al. (1991a). The ΔpKₐ values (pKₐ,app in cosolvent minus pKₐ in water) obtained in the same cosolvents and at the same concentration were very similar to these studies.

Although the pKₐ of the carboxylic acid group of APA in pure water is far outside the pH range of 5-7.5 (in which penicillin G synthesis was measured by Fernandez-Lafuente et al. (1991a)), the apparent pKₐ of this group (pKₐ,APA1) was measured in cosolvent-water mixtures, because it might come close to this pH range. This turned out to be the case. Again, the pKₐ,app of the carboxylic acid group of Pen G was assumed to be similar to the pKₐ,app of the carboxylic acid group of APA.

As mentioned in the introduction it is possible to extrapolate the microscopic dissociation constants of zwitterions from the experimentally determined macroscopic dissociation constants using the method described by Greenstein and Winitz (1961). For this extrapolation the assumption is made that the effect of an uncharged -COOH group is identical to that of a -COOCH₃ or -COOC₂H₅ group in influencing the dissociation of a neighboring group. However, when the distance between the groups increases, the effect diminishes. As this effect is not known for APA one might assume the effect to be zero, because the amino group in APA is a relatively large distance from the carboxylic acid group. Consequently, Kₐ,A = Kₐ,C = Kₐ,APA₁,app and Kₐ,B = Kₐ,D = Kₐ,APA₂,app, which implies that the fraction of neutral APA is close to zero at any pH value.

3.3.4 Choice of reference reaction

By adding organic cosolvents (i.e. lowering the dielectric constant of the mixture) the electrostatic contribution to the Gibbs energy change of the reaction can be influenced. The presence of organic cosolvents has an effect on the activity coefficients of the reactants. By choosing the reference reaction such that the same functional groups are present in the same ionic state both in the reactants and in the products, this effect of the cosolvents on the activity coefficient cancels and Γ²org=1. Thus in the model system, Pen G has a carboxylic acid group, which can either be dissociated or undissociated. The same carboxylic acid group is present in APA, so for the assumption that Γ²org is unity, the same state of dissociation of this group has to be chosen in the reference reaction for APA as well as for Pen G. The carboxylic acid group of PAA is involved in the amide bond in Pen G. Thus to compensate in the Γ²org term for the absence of a charge in the amide bond in Pen G, the carboxylic acid

29
group in PAA has to be undissociated in the reference reaction. If we consider the
ionic species in the model system, a maximum of sixteen different reference reactions
are possible (4 ionic species for APA, 2 for PAA and 2 for Pen G; 4x2x2=16). Therefor e, the correct choice of reference reaction is of great importance for the
outcome of the model. This will be shown in the following cases involving three
different reference reactions:

I. \[ \text{APA}^0 + \text{PhAc}^0 \rightleftharpoons \text{PenG}^0 + \text{H}_2\text{O} \]

II. \[ \text{APA}^- + \text{PhAc}^0 \rightleftharpoons \text{PenG}^- + \text{H}_2\text{O} \] (13)

III. \[ \text{APA}^++ \text{PhAc}^- \rightleftharpoons \text{PenG}^0 + \text{H}_2\text{O} \]

For reference reaction II, the assumption of $\Gamma^{\text{org}}$ being unity will be valid and apparent
dissociation constants can be measured easily. For reference reaction I $\Gamma^{\text{org}}$ is also
unity, however, in this case some additional assumptions have to be made to calculate
the microscopic dissociation constants, which could possibly lead to deviations
between results predicted by the model and experimental results. In reference reaction
III, however, there are charges on the substrates which are absent in the product.

When equation 12 (adjusted for the ionic species in reference reaction III) is used to
predict the equilibrium constant of Pen G in water-solvent mixtures, the predictions
are expected to be poor. Also, for all of the other possible reference reactions that do
not meet the criterion that the same functional groups in the same ionic state should be
present both in the reactants as in the product, predictions are expected to be poor.
Therefore, reference reaction II was expected to give the best results for this model
system.

3.3.5 Effect of organic solvents on synthetic yield of Pen G

Using the measured $pK_{a,\text{app}}$ values of the reactants, $K_{\text{app}}^{\text{eq}}$ (Tewari and
Goldberg, 1988), the densities of the cosolvent-water mixtures and the mole fraction
of water (Table I), the apparent equilibrium constant ($K_{\text{app}}^{\text{org}}$) in the water-solvent
mixtures can be calculated using equation 12. The synthetic yield of Pen G can then
be calculated as a function of pH and solvent composition for the three different
reference reactions (equation 13). The influence of the reference reaction on the
predicted yield is illustrated in Figure 3, in which the synthetic yield of Pen G in a
50% (w/w) DMF/water mixture is predicted with the three different reference reactions.

Clearly, reference reaction II gives the best predictions. For reference reaction III the prediction is poor as expected, probably because the assumption that $I_{\text{org}} = 1$ does not hold. For reference reaction I, predictions are also poor, probably because the microscopic dissociation constants that have been extrapolated from the macroscopic dissociation constants are in error. The best choice of reference reaction is therefore a reference reaction which contains the same functional groups in the same ionic state both in the reactants as in the products. A reference reaction containing only uncharged species is a logical reference reaction. However, if one of the reactants is a zwitterion, it is better to use a reference reaction containing species for which the apparent dissociation constants can be measured easily. An example is reference reaction II in equation 13. This reference reaction is therefore used for the further predictions of the synthetic yield of Pen G in the other solvent-water mixtures.

![Graph showing yield vs pH for reactions I, II, and III.](image)

**Figure 3** Experimental yields (markers) (Fernandez-Lafuente et al., 1991a) and predicted yields (lines) of Pen G synthesis in a 50% (w/w) DMF/water mixture for three different reference reactions described in equation 13. Initial concentrations: 0.02 M APA, 0.02 M PAA.

Figure 4 shows the dramatic effect of the presence of organic cosolvents on the synthetic yield of Pen G at different pH values as measured by Fernandez-
Figure 4 Experimental yields (markers) (Fernandez-Lafuente et al., 1991a) and predicted yields (lines) of Pen G synthesis in different cosolvent/water mixtures. Initial concentrations: 0.02 M APA, 0.02 M PAA.

Lafuente et al. (1991a). The synthetic yield increases upon addition of organic solvents, and this effect becomes larger as the pH of the mixture increases. The model gives an acceptable prediction of the yield which will be achievable in the different cosolvent-water mixtures with a cosolvent content of over 50% (w/w). This means that the model can be used to select a suitable organic solvent and an optimal pH for the synthetic reaction. The reason for deviations between model and experiments are, in addition to the simplifying assumptions in the model, the limited experimental accuracy that can be obtained in equilibrium studies with these unstable compounds. For example, the pKₐ of the amine group of APA has been found by different researchers to range from 4.6 (Svedas et al., 1980) to 5.4 (Tewari and Goldberg, 1988), probably because APA degrades easily under the experimental conditions. This, however, can have some consequences for results of the model. If we use e.g. a value of 5.4 for the pKₐ of the amine group of APA in the model the prediction of the synthetic yield is better than in Figure 3, but for a value of 4.6 the prediction is worse.

The $K_{app}^{aq}$ value used in the model was taken from Tewari and Goldberg (1988). They calculated this value from experimental data by correcting for the ionic strength. However, in the studies of Fernandez-Lafuente et al. (1991a) data were not corrected for ionic strength. This also can cause deviations between the model and experiments. Equation 12 predicts that $K_{app}^{aq}$ becomes infinity if $x_{w}$ approaches zero. This seems
to be an overestimation of the synthetic yield, but experimental data that confirm or contradict the model prediction at a cosolvent content of over 50% (w/w) are missing.

3.3.6 Prediction of Amox synthesis equilibrium

As explained in the introduction, it is possible to predict the synthetic yield of a reaction simply by measuring the equilibrium in water and the apparent $pK_a$ values of the reactants in the cosolvent-water mixtures. As an example, the model will be used to predict the apparent reaction equilibrium constant for the synthesis of Amox in water and in a 50% (w/w) DMF mixture. Amox is a $\beta$-lactam antibiotic, which is formed by the coupling of APA and HPG. We have chosen the following reference reaction (with the same functional charged groups in both reactants as in the product):

$$\text{APA}^- + \text{HPG}^+ \rightleftharpoons \text{Amox}^{\text{+}} + \text{H}_2\text{O}$$ (14)

With the results of equilibrium measurements of Amox in water (Diender et al., 1998) and the apparent $pK_a$ values of Amox, APA and HPG (see Table I) the apparent equilibrium constant for the synthesis of amox in cosolvent-water mixtures can be predicted. Because Fernandez-Lafuente et al. (1991a) achieved the highest yields for Pen G synthesis in a 50% (w/w) DMF/water mixture, this solvent mixture was selected to predict the equilibrium of amox synthesis. Results of this prediction are shown in Figure 5.

Table I shows the measured apparent $pK_a$ values of HPG and Amox in water and 50% (w/w) DMF/water mixture that were used for the calculations. As can be concluded from these results, the equilibrium of Amox in water is heavily in favor of hydrolysis. However, when synthesis is performed in a 50% (w/w) DMF/water mixture, $K_{app}$ is calculated to increase nearly a factor 100 in the DMF/water mixture compared to water. To check this prediction, an enzyme preparation that would be active and stable in the DMF/water mixture would have to be available. However, it should be noticed that the actual equilibrium concentrations of Amox that might be reached in the DMF/water mixture probably are lower than in water because the solubilities of the reactants in the DMF/water mixture are much lower than in water (Diender et al., 1998).
Figure 5  Apparent equilibrium constants for the synthesis of amox in water and a 50% (w/w) DMF mixture as a function of pH at 25°C. Experimental results for $K_{\text{app,aq}}$ (■) were measured by Diender et al. (1998). Lines are predicted values for $K_{\text{app,aq}}$ and $K_{\text{app,org}}$.

3.4 Conclusions

A quantitative model has been presented for the equilibrium predictions of enzymatic synthesis reactions in cosolvent/water mixtures. The model is based on the reaction equilibrium in water, a properly chosen reference reaction and measured apparent $pK_a$ values of all substrates and products in water and in the cosolvent-water mixtures. For the validation of the model, the synthesis of Pen G was used. The model predicted synthetic yields of Pen G quite reasonably. However, the choice of reference reaction is of crucial importance for the outcome of the model. The reference reaction should be chosen such that the same functional groups are present in the same ionic state both in the reactants as in the products. A reference reaction which involves only uncharged species of the reactants can cause problems when one or more of the reactants or products is an amphoteric molecule. In that case microscopic dissociation constants must be used, which can not be measured, but must be extrapolated from the macroscopic dissociation constants. This extrapolation can be difficult and give poor results in predicting the synthetic yield of a reaction. It is better then to use a reference reaction containing species, for which the apparent dissociation constants can be measured easily.
3.5 Notation

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Definition</th>
<th>Units</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amox</td>
<td>amoxicillin</td>
<td></td>
</tr>
<tr>
<td>APA</td>
<td>6-aminopenicillanic acid</td>
<td></td>
</tr>
<tr>
<td>HPG</td>
<td>D-(p-)hydroxyphenylglycine</td>
<td></td>
</tr>
<tr>
<td>Pen G</td>
<td>penicillin G</td>
<td></td>
</tr>
<tr>
<td>PAA</td>
<td>phenylacetic acid</td>
<td></td>
</tr>
<tr>
<td>a</td>
<td>activity</td>
<td>(-)</td>
</tr>
<tr>
<td>c</td>
<td>concentration</td>
<td>(M)</td>
</tr>
<tr>
<td>F</td>
<td>fraction</td>
<td>(-)</td>
</tr>
<tr>
<td>K</td>
<td>reaction equilibrium constant</td>
<td>(M⁻¹)</td>
</tr>
<tr>
<td>Kₐ</td>
<td>dissociation constant</td>
<td>(M)</td>
</tr>
<tr>
<td>Kₚₑᶠ</td>
<td>equilibrium constant of the reference reaction</td>
<td>(-)</td>
</tr>
<tr>
<td>m</td>
<td>molality</td>
<td>(mol·kg⁻¹solution)</td>
</tr>
<tr>
<td>x</td>
<td>mole fraction</td>
<td>(-)</td>
</tr>
</tbody>
</table>

**Greek**

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Definition</th>
<th>Units</th>
</tr>
</thead>
<tbody>
<tr>
<td>γ</td>
<td>symmetric activity coefficient</td>
<td>(-)</td>
</tr>
<tr>
<td>γₘₚ</td>
<td>asymmetric activity coefficient</td>
<td>(-)</td>
</tr>
<tr>
<td>ρ</td>
<td>density</td>
<td>(kg solution·l⁻¹solution)</td>
</tr>
<tr>
<td>Γ</td>
<td>defined in equation 8</td>
<td>(-)</td>
</tr>
</tbody>
</table>

**Sub- or superscript**

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>app</td>
<td>apparent value</td>
</tr>
<tr>
<td>aq</td>
<td>of aqueous solvent</td>
</tr>
<tr>
<td>i</td>
<td>species i</td>
</tr>
<tr>
<td>org</td>
<td>of organic solvent/water mixtures</td>
</tr>
<tr>
<td>w</td>
<td>of the compound water</td>
</tr>
<tr>
<td>0</td>
<td>for uncharged compound</td>
</tr>
<tr>
<td>+</td>
<td>for cationic compound</td>
</tr>
<tr>
<td>-</td>
<td>for anionic compound</td>
</tr>
<tr>
<td>+−</td>
<td>for zwitterionic compound</td>
</tr>
</tbody>
</table>
3.6 References


Course of pH during the formation of amoxicillin by a suspension-to-suspension reaction

Abstract
Amoxicillin can be produced in an enzymatic suspension-to-suspension reaction in which the substrate(s) and product(s) are mainly present as solid particles, while the reaction takes place in the liquid phase. During these suspension-to-suspension reactions different subprocesses take place, such as dissolution/crystallization of substrates and products, enzymatic synthesis of the product(s) and undesired enzymatic hydrolysis of substrates and/or products. All these subprocesses are influenced by pH and also influence the pH because the reactants are weak electrolytes. This paper describes a quantitative model for predicting pH and concentrations of reactants during suspension-to-suspension reactions. The model is based on mass and charge balances, pH-dependent solubilities of the reactants, and enzyme kinetics. For the validation of this model, the kinetically controlled synthesis of amoxicillin from 6-aminopenicillanic acid and D-(p)-hydroxyphenylglycine methyl ester was studied. The pH and the dissolved concentrations took a very different course at different initial substrate amounts. This was described quite reasonably by the model. Therefore, the model can be used as a tool to optimize suspension-to-suspension reactions of weak electrolytes.

This chapter was published in Enzyme Microb Technol (2000) 27:576-582
4.1 Introduction

There is an increasing interest in enzymatic conversions for the production of biologically active compounds. Research has mainly been focused on enzymatic reactions in aqueous or organic solutions and on monophasic or biphasic mixtures thereof. Recently, another type of enzymatic reactions, involving solid substrate and/or product, is getting more attention. In the so-called suspension-to-suspension (or solid-to-solid, or solid phase) reactions the substrate(s) and product(s) are mainly present as undissolved particles, whereas the enzymatic reaction takes place in the liquid phase. The product to reactor volume ratio is much higher than in the conventional systems. This type of processes can also lead to yields similar to those in conventional enzymatic reactions. Suspension-to-suspension conversions are especially advantageous when hydrolytic reactions are to be reversed or suppressed (Erbeldinger et al., 1998; Halling et al., 1995; Straathof et al., in press; Lee and Kim, 1998; Kasche, 1986; Kasche and Galunsky, 1994).

During a suspension-to-suspension process different subprocesses take place, e.g. dissolution and crystallization of the substrate(s) and product(s), enzymatic synthesis of the product(s), and enzymatic hydrolysis of the substrate(s) and product(s). The enzymatic reaction will be influenced by pH and, for reactants that show acid-base dissociation in the pH range of interest, solubility and hence dissolution and crystallization will be influenced. Then the subprocesses themselves will produce or consume protons and hence influence the pH. A thorough understanding of these different subprocesses is necessary for optimizing the suspension-to-suspension process. Wandrey and Flaschel (1979) calculated the pH in homogeneous aqueous solutions as a function of conversion for the hydrolysis of N-acetyl-L-methionine. Later, Flaschel et al. (1992), Nakanishi and Matsuno (1986) and Nakanishi et al. (1986) predicted the pH profile in a water organic-solvent two-phase system. Different groups have investigated the kinetics of a suspension-to-suspension reaction (Lee and Kim, 1998; Erbeldinger et al., 1999). However, a quantitative analysis of a suspension-to-suspension reaction without pH control has not been performed yet. Therefore, it is not clear to which extent pH control is required in industrial processes.

In this article, we present a model, which can predict the pH and concentrations of the reactants during the kinetically controlled synthesis of the semisynthetic antibiotic amoxicillin without pH control. The kinetically controlled synthesis of amoxicillin (Amox) from D-p-hydroxyphenylglycine methyl ester (HPGM) and 6-aminopenicillanic acid (APA) was chosen as a model system. The enzyme penicillin
acylase catalyzes the synthesis (reaction I), by coupling HPGM and APA, but also the undesired substrate hydrolysis (of HPGM, reaction II) and product hydrolysis (of Amox, reaction III). Both side reactions lead to hydroxyphenylglycine (HPG).

I  Synthesis:          \[ \text{APA} + \text{HPGM} \rightarrow \text{Amox} + \text{MeOH} \]

II Substrate hydrolysis: \[ \text{HPGM} + \text{H}_2\text{O} \rightarrow \text{HPG} + \text{MeOH} \]

III Product hydrolysis: \[ \text{Amox} + \text{H}_2\text{O} \rightarrow \text{HPG} + \text{APA} \]

In a batch reactor, both substrates may initially be mostly undissolved, whereas most of the amoxicillin will crystallize during its production (also, the byproduct HPG might crystallize during reaction) (Bruggink et al., 1998; Clausen and Dekker, 1996). We will describe this by a quantitative model that is based on mass and charge balances, pH-dependent solubilities of the substrates and products and finally enzyme kinetics. This model should give an insight in the subprocesses taking place, for example, the extent of undesired substrate or product hydrolysis, and can be used to optimize process conditions.

4.2 Theory

The reactants in the model system are all weak electrolytes: they are present in solution as cationic, zwitterionic or anionic species depending on the pH. In the pH range that we studied the following dissociation reactions are important:

\[ \text{APA}^+ \leftrightarrow \text{APA}^- + \text{H}^+ \]
\[ \text{HPGM}^+ \leftrightarrow \text{HPGM}^0 + \text{H}^+ \]
\[ \text{Amox}^+ \leftrightarrow \text{Amox}^- + \text{H}^+ \]
\[ \text{HPG}^{++} \leftrightarrow \text{HPG}^- + \text{H}^+ \]

The dissociation constants \((K_a)\) of these reactions are given in Table 1. The concentration of each ionic species of a compound can be calculated as a function of the total dissolved concentration of the compound \((c)\) and the fraction of the ionic species
(F). As an example, the calculation of concentration and the fraction of the anionic form of APA are given in equations 1 and 2.

\[ c_{\text{APA}^-} = F_{\text{APA}^-} \cdot c_{\text{APA}} \]  

(1)

in which

\[ F_{\text{APA}^-} = \frac{c_{\text{APA}^-}}{c_{\text{APA}^-} + c_{\text{APA}^{2-}}} = \frac{1}{1 + \frac{c_{\text{H}^+}}{K_{a,\text{APA}}}} \]  

(2)

For the other compounds the calculations of the concentrations and the fractions of the ionic forms are analogous to equations 1 and 2.

**Table 1**  **Dissociation constants and reference solubilities of the reactants of the model system.**

<table>
<thead>
<tr>
<th>compound</th>
<th>pK_a</th>
<th>( S_{\text{ref}} ) (mol·L(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>APA(^a)</td>
<td>4.9</td>
<td>0.008</td>
</tr>
<tr>
<td>HPGM(^b)</td>
<td>8.3</td>
<td>0.0013</td>
</tr>
<tr>
<td>Amox(^a)</td>
<td>7.4</td>
<td>0.0091</td>
</tr>
<tr>
<td>HPG(^b)</td>
<td>9.2</td>
<td>0.17</td>
</tr>
</tbody>
</table>

\(^a\) Values are from Diender et al. (1998)

\(^b\) Value was determined (data not shown) similar as described by Diender et al.\(^b\). Solubilities were measured in the pH range of 6.5 to 7.5 (13 measurements) and \( S_{\text{ref}} \) was determined by fitting using the least squares method.

**4.2.1 Initial pH**

The initial pH in our suspension-to-suspension reaction can be derived from a charge balance (equation 3).

\[ c_{\text{HPGM}^-} = c_{\text{APA}^-} \]  

(3)
(In the range of concentrations used by us, the concentrations of OH\textsuperscript{-} and H\textsuperscript{+} can be neglected). If the substrates are completely dissolved, the concentrations are determined by the initial amounts. If the substrates are only partly dissolved, the concentrations are determined by the solubilities of the compounds. The solubilities of all reactants are influenced by pH, because APA, HPGM, Amox and HPG are weak electrolytes. For simplicity one may assume that the overall solubility is completely determined by the solubility of the neutral (HPGM) or zwitterionic species (APA, Amox and HPG)(Tsuji et al., 1978). Hence, the pH-dependence of the solubility can be described by equations 2 and 4, in which the overall solubility ($S_i$ of compound i) is calculated as a function of the reference solubility ($S_r$ of the neutral form or $S_{z+}$ of the zwitterionic form). Values of these reference solubilities are presented in Table 1.

Substitution of concentrations (equation 1) or the solubilities (equations 4a and 4b) in the charge balance (equation 3) leads to a second-order equation in proton concentration, from which the initial pH can be calculated.

\[
S_{\text{APA}} = \frac{S_{\text{APA}^-}}{F_{\text{APA}^-}} \tag{4a}
\]

\[
S_{\text{HPGM}} = \frac{S_{\text{HPGM}^0}}{F_{\text{HPGM}^0}} \tag{4b}
\]

\[
S_{\text{HPG}} = \frac{S_{\text{HPG}^-}}{F_{\text{HPG}^-}} \tag{4c}
\]

\[
S_{\text{Amox}} = \frac{S_{\text{Amox}^-}}{F_{\text{Amox}^-}} \tag{4d}
\]

4.2.2 pH during the reaction

To predict the pH during the reaction the aforementioned theory still applies. However, the charge balance contains four components now, i.e. APA, HPGM, Amox and HPG, which will give an equation fourth-order in proton concentration. To determine whether the concentrations of the components are below or above their solubilities, the concentrations should not be compared to the initial amounts, but to the
actual amounts. The actual amounts can be calculated using molar balances. The macroscopic balances for Amox and HPG in a batch reactor are:

\[
\frac{\mathrm{dn}_{\text{Amox}}}{\mathrm{dr}} = (r_{\text{synth}} - r_{\text{prodhydr}}) \cdot V
\]  
(5a)

\[
\frac{\mathrm{dn}_{\text{HPG}}}{\mathrm{dr}} = (r_{\text{subshyd}} + r_{\text{prodhydr}}) \cdot V
\]  
(5b)

in which \( n \) is the total amount (dissolved and solid) of moles of a compound present in the reactor, \( r \) is the rate of synthesis or hydrolysis and \( V \) is the reactor volume. Penicillin G acylase catalyzes three reactions, i.e. product synthesis (reaction I), substrate hydrolysis (reaction II) and product hydrolysis (reaction III). For these reactions the following simplified (mass action based) enzymatic rate equations can be formulated:

\[
r_{\text{synth}} = k_{\text{synth}} \cdot c_{\text{HPGM}} \cdot c_{\text{APA}} \cdot c_{\text{enzyme}}
\]  
(6a)

\[
r_{\text{subshyd}} = k_{\text{subshyd}} \cdot c_{\text{HPGM}} \cdot c_{\text{enzyme}}
\]  
(6b)

\[
r_{\text{prodhydr}} = k_{\text{prodhydr}} \cdot c_{\text{Amox}} \cdot c_{\text{enzyme}}
\]  
(6c)

in which \( k \) is the rate constant. The stoichiometric balances for APA and HPGM are:

\[
n_{\text{APA}} = n_{\text{APA,0}} - n_{\text{Amox}}
\]  
(7a)

\[
n_{\text{HPGM}} = n_{\text{HPGM,0}} - n_{\text{Amox}} - n_{\text{HPG}}
\]  
(7b)

in which \( n_0 \) denotes the initial amount of moles of a compound which was added. For simplicity we assume that the dissolution and crystallization are at equilibrium, i.e. these subprocesses are much faster than the enzymatic reaction. Furthermore, we assume that all enzymatic reactions are pH-dependent to the same degree (\( k_{\text{synth}}, k_{\text{subshyd}} \) and \( k_{\text{prodhydr}} \) are the same function of pH). If this pH-dependence is not known, the course of the concentrations and pH cannot be predicted as a function of time, but it can be predicted as a function of the extent of conversion (i.e. as a function of the ratio
between concentrations). This requires only two kinetic parameters, $k_{\text{synth}}/k_{\text{subshydr}}$ and $k_{\text{synth}}/k_{\text{prodshydr}}$, which are both ratios between the enzymatic rate constants. The ratio between $k_{\text{synth}}$ and $k_{\text{subshydr}}$ can be determined as follows. At the start of an experiment product hydrolysis does not (yet) occur, so combination of equations 5 and 6 leads to:

\[
\frac{dn_{\text{Amox}}}{dt} = \frac{k_{\text{synth}} \cdot c_{\text{APA},0} \cdot c_{\text{HPGM},0} \cdot c_{\text{enzyme}}}{k_{\text{subshydr}} \cdot c_{\text{HPGM},0} \cdot c_{\text{enzyme}}} \tag{8}
\]

Also, product crystallization will not yet occur, hence

\[
\frac{dn_{\text{Amox}}}{dt} = \frac{dc_{\text{Amox}}}{dt} = \frac{dc_{\text{HPG}}}{dc_{\text{HPG}}} \tag{9}
\]

Combination of equations 8 and 9 leads to:

\[
\frac{k_{\text{synth}}}{k_{\text{subshydr}}} = \frac{c_{\text{Amox}}}{c_{\text{HPG}}} \cdot \frac{1}{c_{\text{APA},0}} \tag{10}
\]

If the ratio between $k_{\text{synth}}$ and $k_{\text{subshydr}}$ is known, the ratio between $k_{\text{synth}}$ and $k_{\text{prodshydr}}$ can be determined by fitting the progress curve of a reaction.

### 4.3 Materials and methods

#### 4.3.1 Materials

Immobilized penicillin G acylase (EC 3.5.1.11) from *E. coli* (Van der Does et al., 1998), and 6-aminopenicillanic acid (APA, 98.5% pure) were from DSM-Gist (Delft, The Netherlands). D-$p$-Hydroxyphenylglycine methyl ester (HPGM, 99.0% pure) was from DSM Research (Geleen, The Netherlands).
4.3.2 Enzyme activity

To determine enzyme activity, a known amount of enzyme was added to a stirred 10 ml suspension of APA (63 g/l) and HPGM (65 g/l) at 25 °C. After 1 and 1.5 h a sample was drawn and the Amox concentration was determined by HPLC. 1 Unit of penicillin G acylase is defined as the amount of enzyme which produces 1 g Amox per h under these conditions.

4.3.3 Synthesis reaction in water

Synthesis reactions were performed by adding APA and HPGM to water in a continuously stirred, thermojacketed vessel at 25 °C. After reaching a constant pH 100 mU of immobilized enzyme were added. During the synthesis experiments, the pH was monitored and samples were taken. To stop the enzymatic reaction in the samples, the samples were filtered (0.2-µm Nylaflo® nylon membrane filters, Gelman Sciences) and diluted with water. The filtrate was analyzed.

4.3.4 Analysis

APA, Amox, HPGM and HPG were identified and analyzed by HPLC using a Waters System with a Zorbax SB-C18 4.6×75 mm 3.5-µm column, thermostated at 33 °C, and a Waters 996 PDA UV-detector at 230 nm, a Waters 910 Wisp 10-µl injector, and using a Waters 590 pump with a flow of 1 ml·min⁻¹. The eluent was composed of 5% (v/v) MeOH in water and contained 70 mM sodium phosphate buffer of pH 3.

4.3.5 Simulations

Simulations were performed with the program Psi (Boza, Pijnacker, The Netherlands), which calculated the proton concentration by iteration at every integration time step.

4.4 Results and discussion

4.4.1 Suspension-to-suspension reactions

To validate the model three reactions at different conditions (different initial amounts of APA and HPGM) have been tested. Results from the experiments are given in Figure 1 (0.1 M APA and 0.1 M HPGM), Figure 2 (0.2 M APA and 0.2 M HPGM) and Figure 3 (0.2 M APA and 0.4 M HPGM).
Figure 1  Concentrations of dissolved reactants and pH as a function of the extent of HPGM conversion. Initial conditions: 0.1 M APA and 0.1 M HPGM. Experimental values of APA (♦), HPGM (■), Amox (▲), HPG (×), and pH (∗), lines represent the simulated concentrations.

Figure 2  Concentrations of dissolved reactants and pH as a function of the extent of HPGM conversion. Initial conditions: 0.2 M APA and 0.2 M HPGM. For other legends see Figure 1.
We have not given the results as a function of time, because we cannot predict the time course with our model (see Theory section). The results can best be given as a function of the extent of conversion of HPGM. The experimental extent of conversion is not known (except for the initial situation) because we only measured the dissolved concentrations of the reactants (not the total amounts). Therefore, we have shown these concentrations as a function of the extent of conversion of HPGM according to the model, using experimental and theoretical pH to place the experimental data (of \( t > 0 \)) at the proper extent of conversion.

![Graph showing concentrations of dissolved reactants and pH as a function of conversion](image)

**Figure 3** *Concentrations of dissolved reactants and pH as a function of the extent of HPGM conversion. Initial conditions: 0.2 M APA and 0.4 M HPGM. For other legends see Figure 1.*

In all three experiments, the dissolved concentrations of APA and HPGM decrease, while Amox and HPG are produced. Every experiment shows a very different course of pH during the reaction. Figure 1 shows a sharp linear decrease of the pH in the initial stage, while later the pH decrease declines. The experiment of Figure 2 shows a constant pH during the initial stage of the reaction. Then the pH starts to decrease gradually, and this decrease becomes more significant towards the end of the experiment. In the last experiment (Figure 3) the pH gradually increases from 6.1 to 6.7 and then decreases sharply again.
4.4.2 Simulations

Using the results from the experiment starting with 0.1 M APA and 0.1 M HPGM (Figure 1) and equation 10, the ratio between \( k_{\text{synth}} \) and \( k_{\text{subshdyr}} \) was found to have a value of 21.4. The predicted concentrations and pH visually gave the best results to match the experimental values when \( k_{\text{synth}}/k_{\text{proshdyr}} \) was also 21.4. These relations between the enzymatic rate constants were used to predict the reaction course in the other two experiments (Figures 2 and 3).

As can be seen in Figures 2 and 3, the model predicts some concentrations quite well, whereas for others only the trends are predicted. This indicates that the model structure is correct. There could be several reasons for the deviations of the model from the experimental data. Firstly, in the model all enzymatic reactions are assumed to be similarly pH-dependent for simplicity of the model. However, this may be an oversimplification as not only enzyme dissociation is influenced by pH, but also substrate dissociation. Secondly, in a suspension-to-suspension conversion the dissolved concentrations are high, so the ionic strength may vary up to 0.4 mol/l. This could affect the enzyme kinetics, and also influence the dissociation constants. Dale and White (1983) showed that ribonuclease lost more than 50% activity upon increasing ionic strength from 0.02 to 0.2 mol/l. Fiol et al. (1998) and Alonso et al. (1998) found that the dissociation constant of the carboxylate group of glycine changed 0.3 pH units upon increasing the ionic strength from 0.10 to 0.95 mol/l. A change in dissociation constants would influence the solubility and accordingly the concentrations of the reactants dissolved in the liquid phase. This effect could explain the slight deviations between the experimental and theoretical values of the initial pH.

In the model changes in solubility are supposed to be instantaneous. However, in the course of the reaction there could be a rate limitation in the crystallisation or the dissolution of the crystals, so the actual concentration in the liquid phase may be higher or lower than calculated. This is shown by the concentration of Amox in Figures 1-3. Amox is initially produced to a certain concentration, but later the dissolved concentration decreases again and remains constant for the rest of the experiment. We assume that there is a lag period in the crystallisation of Amox, during which Amox is present in supersaturation.

As mentioned in the introduction an insight in the subprocesses taking place during suspension-to-suspension conversions could help to understand and optimize these conversions. This will be shown by the experiment starting with 0.2 M APA and 0.4 M HPGM (Figure 3). In this experiment, the pH is increasing gradually from the start and then suddenly decreasing sharply. Analyzing only the overall concentrations,
we would not be able to explain the pH profile. However, when the total amounts of the substrates are split into solid and dissolved amounts by performing a simulation with the model (Figure 4, for data of the substrates and 5, for data of the products), the causes of the pH changes become clear.

\[
\begin{align*}
\text{Figure 4} & \quad \text{Dissolved concentrations and total amounts per reactor volume of APA and HPGM and pH calculated as a function of HPGM conversion. Start conditions 0.2 M APA and 0.4 M HPGM.}
\end{align*}
\]

In the initial stage of the experiment, the pH is constant. In this part both APA and HPGM are saturating the liquid phase. In the crystals APA and HPGM are present as APA\(^+\) and HPGM\(^0\) respectively. When the predominant species are shown the initial enzymatic reactions become:

\[
\begin{align*}
\text{synthesis:} & \quad \text{HPGM}^+ + \text{APA}^- \longrightarrow \text{Amox}^{+\cdot} + \text{MeOH} \\
\text{substrate hydrolysis:} & \quad \text{HPGM}^+ + \text{H}_2\text{O} \longrightarrow \text{HPG}^+ + \text{MeOH} + \text{H}^+
\end{align*}
\]

During the dissolution of HPGM protons are consumed (in the liquid phase HPGM is present as cation), which are mostly provided by the dissolution of APA (in the liquid phase APA is predominantly present in the anionic form) while the rest is provided by HPGM hydrolysis. Hence the pH is constant. At the end of this stage, the pH starts to increase, because \(n_{\text{APA}}/V_{eq}\) drops below its solubility and becomes equal to \(c_{\text{APA}}\). There
is not sufficient proton production anymore, as the solid phase of APA is depleted, but the proton consumption continues as HPGM is still dissolving. Gradually product hydrolysis becomes more important. Like substrate hydrolysis, this leads to the production of protons. When the ratio between synthesis and hydrolysis decreases during the reaction, the pH increase declines. At the end of the second stage, the total amount of HPGM has decreased down to its solubility (n_{HPGM}/V_{aq} becomes equal to c_{HPGM}). At that moment, the pH starts to decrease sharply. In this third stage of the experiment almost no HPGM is used for Amox synthesis and most is hydrolysed to HPG and MeOH, causing proton production.

![Figure 5](image) Dissolved concentrations and total amounts per reactor volume of Amox and HPG and pH calculated as a function of HPGM conversion. Start conditions 0.2 M APA and 0.4 M HPGM.

As this experiment shows, the model can explain pH shifts during the suspension-to-suspension reaction. The discontinuities in pH changes during suspension-to-suspension reactions are caused by the depletion of the solid phase of one of the substrates. Similar discontinuities will be found when a product starts to crystallize, if the predominant dissolved product species is an anion or cation. The model can be used to find the optimal conditions to produce Amox. For example, when the enzyme stability or activity is low in a certain pH range the model can predict whether or when the pH will be in that range and pH control is necessary. In this way no unnecessary buffers, acids or bases are used for pH control, which can simplify downstream processing. The model can also predict when to stop the reaction to achieve the highest yield of product.
4.5 Conclusions

A quantitative model has been presented for the prediction of the course of pH and concentrations in enzymatic suspension-to-suspension conversions. The model is based on enzymatic conversion rates, pH-dependent solubilities, stoichiometric, macroscopic and charge balances. For the validation of this model, the kinetically controlled synthesis of Amox was used. The model predicted the pH profile and the trends in the concentrations quite reasonably. It explained the profiles that are observed on the basis of subprocesses taking place during the suspension-to-suspension reactions, such as the unwanted hydrolysis of HPGM and Amox. The model can be used as a tool for optimizing an enzymatic suspension-to-suspension reaction, when dissociating reactants are involved.

4.6 Notation

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Definition</th>
<th>Unit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amox</td>
<td>amoxicillin</td>
<td></td>
</tr>
<tr>
<td>APA</td>
<td>6-aminopenicillanic acid</td>
<td></td>
</tr>
<tr>
<td>HPG</td>
<td>D-(p-)hydroxyphenylglycine</td>
<td></td>
</tr>
<tr>
<td>HPGM</td>
<td>D-(p-)hydroxyphenylglycine methyl ester</td>
<td></td>
</tr>
<tr>
<td>MeOH</td>
<td>methanol</td>
<td></td>
</tr>
<tr>
<td>$c_i$</td>
<td>dissolved concentration of species i</td>
<td>(mol·l$^{-1}$)</td>
</tr>
<tr>
<td>$F$</td>
<td>fraction</td>
<td>(-)</td>
</tr>
<tr>
<td>$k$</td>
<td>rate constant</td>
<td>(mol$^{-1}$·l·s$^{-1}$)</td>
</tr>
<tr>
<td>$K_a$</td>
<td>dissociation constant</td>
<td>(mol$^{-1}$)</td>
</tr>
<tr>
<td>$n_i$</td>
<td>total amount of moles of species i per reactor volume</td>
<td>(mol·l$^{-1}$)</td>
</tr>
<tr>
<td>$r$</td>
<td>synthesis or hydrolysis rate</td>
<td>(mol·s$^{-1}$)</td>
</tr>
<tr>
<td>$S$</td>
<td>solubility</td>
<td>(mol·l$^{-1}$)</td>
</tr>
<tr>
<td>$t$</td>
<td>time</td>
<td>(s)</td>
</tr>
<tr>
<td>$V$</td>
<td>volume</td>
<td>(l)</td>
</tr>
</tbody>
</table>

Sub- or superscript

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>synth</td>
<td>synthesis</td>
</tr>
<tr>
<td>subhydr</td>
<td>substrate hydrolysis</td>
</tr>
<tr>
<td>prodhydr</td>
<td>product hydrolysis</td>
</tr>
<tr>
<td>52</td>
<td></td>
</tr>
</tbody>
</table>
4.7 References


Kasche V. 1986. Mechanism and yields in enzyme catalysed equilibrium and kinetically controlled synthesis of \( \beta \)-lactam antibiotics, peptides and other condensation products. Enzyme Microb Technol 8:4-16.


Straathof AJJ, Litjens MJJ, Heijnen JJ. Enzymatic transformations in suspensions. Meth Biotechnol, accepted for publication.


Equilibrium modelling of extractive enzymatic hydrolysis of penicillin G with concomitant 6-aminopenicillanic acid crystallisation

Abstract
In the present downstream processing of penicillin G, penicillin G is extracted from the fermentation broth with an organic solvent and purified as a potassium salt via a number of back-extraction and crystallisation steps. After purification, penicillin G is hydrolysed to 6-aminopenicillanic acid, a precursor for many semisynthetic β-lactam antibiotics. We are studying a reduction in the number of pH shifts involved and hence a reduction in the salt production. To this end, the organic penicillin G extract is directly to be added to an aqueous immobilised enzyme suspension reactor and hydrolysed by extractive catalysis. This paper describes a model for predicting the equilibrium conversion of penicillin G in water-organic two-phase batch systems with either potassium or hydroxyphenylglycine methyl ester as counter ion. The model incorporates the partitioning behaviour of the reactants, the enzymatic reaction equilibrium and the crystallisation of 6-aminopenicillanic acid. The model predicted the conversion of Pen G quite reasonably for different initial values of pH, penicillin G concentration and phase volume ratio. This conversion can exceed 90% if crystallisation of 6-aminopenicillanic acid occurs. The model can be used as a tool for optimising the enzymatic hydrolysis in water/organic solvent two-phase systems.

This chapter was submitted for publication.
5.1 Introduction

Enzymatic extractive catalysis, i.e. the coupling an enzymatic reaction and an extraction of the product(s) in a biphasic system has been applied and described by many researchers (e.g. Martinek et al., 1981\textsuperscript{ab}; Andersson et al., 1984; Eggens et al., 1989; Woodley and Lilly, 1990; Janssen et al., 1995; Hernandez-Justiz et al., 1998). One major advantage of extractive catalysis is that the equilibrium of the reaction can be shifted towards the product side by extracting the product from an aqueous to an organic phase. The organic phase can also be a reservoir for substrates with a low solubility in water. Substrate or product inhibition can be avoided if the inhibitor prefers the organic phase to the aqueous phase.

The antibiotic penicillin G (Pen G) is the most common raw material of semisynthetic β-lactam antibiotics. After fermentation at pH 6.2-6.8 (Hersbach et al., 1984), Pen G is extracted from the fermentation broth at pH 2.0-2.5 by organic solvents like butyl acetate or amyl acetate. Subsequently, Pen G is either back-extracted to an aqueous phase at pH 6.8-8.0 and then crystallised, or crystallised directly from the organic phase by adding solid potassium or sodium acetate. The Pen G crystals are used for the production of 6-aminopenicillanic acid (APA) by enzymatic hydrolysis at pH 7-8. APA is recovered at its isoelectric point (Mwangi, 1994), dissolved again and can be coupled enzymatically to a side chain base to form a semisynthetic antibiotic (Diender et al., 2000). In each process step salts are formed as side products due to the pH shifts (Reschke and Schugelr, 1984\textsuperscript{b}). A reduction of the salt formation would be achieved if the extracted Pen G in the organic phase could directly be added to the enzymatic reactor and could be hydrolysed efficiently in a two-phase system by extractive catalysis. In such a case, Pen G, initially only present in the organic phase, partitions between both phases depending on the pH and is hydrolysed in the aqueous phase. The side chain that is formed by hydrolysis, phenylacetic acid (PAA), also partitions between the aqueous and organic phase, while the second hydrolysis product, APA, having a zwitterionic nature, stays in the aqueous phase. By extracting PAA, the equilibrium position is shifted towards the product side and good yields can be achieved. This was shown by Barensehee et al. (1992) and Rindfleisch et al. (1997). They investigated several possibilities for integrated processes for the production and biotransformation of Pen G and in this way reduced the number of process steps. Further improvement of the Pen G conversion in those biphasic systems can be achieved by choosing the process conditions such that the equilibrium concentration of APA exceeds its solubility,
causing APA to crystallise. This crystallisation of APA could improve the yield considerably.

To adjust the pH from 2.0-2.5 of the extraction liquid to the pH at which the enzymatic hydrolysis is performed a base (e.g. ammonia or potassium hydroxide) should be added. To avoid salt production because of this pH shift, the side chain base that will be coupled to APA in a successive reaction (Diender et al., 2000) can be added instead. For example, hydroxyphenyl glycine methyl ester (HPGM), the side chain for amoxicillin formation, which would be added to APA after hydrolysis of Pen G, may also be added before hydrolysis.

To improve the existing processes in this manner, we are studying the enzymatic hydrolysis of Pen G in water/butyl acetate two-phase systems. The aim of this paper is to describe the equilibrium position of Pen G hydrolysis in water/butyl acetate two-phase batch systems as a function of the pH, cation presence, initial Pen G concentration and organic/aqueous phase volume ratio. Therefore, the partitioning behaviour of the reactants will be described as a function of these three parameters, and combined with a solubility model (for APA crystallisation) and a reaction equilibrium model. The feasibility of Pen G hydrolysis in two-phase batch systems can be studied by optimisation of the reaction conditions using the overall model.

5.2 Theory

5.2.1 One-phase reaction equilibrium

The equilibrium of Pen G hydrolysis in an aqueous system can be described by the apparent equilibrium constant \( K_{\text{eq,app}}^{sq} \) and the concentrations of the reactants (equation 1).

\[
K_{\text{eq,app}}^{sq} = \frac{c_{\text{PAA}}^{sq} \cdot c_{\text{APA}}^{sq}}{c_{\text{PenG}}^{sq}}
\]  \hspace{1cm} (1)

This equilibrium is a function of pH. The apparent equilibrium constant is a function of a reference reaction constant and the fractions of the reactants at a certain pH. The following reference reaction is chosen (Diender et al., 1998):

\[\text{PenG}^- + \text{H}_2\text{O} \rightleftharpoons \text{APA}^- + \text{PAA}^- + \text{H}^+\]
The apparent reaction constant is then as follows

\[ K_{eq,app}^{\infty} = K_{ref} \cdot \frac{F_{PenG^-}}{c_{H^+} \cdot F_{PAA^-} \cdot F_{APA^-}} \]  

in which \( K_{ref} \) is the pH-independent reference reaction constant and \( F_{PenG^-} \), \( F_{PAA^-} \), and \( F_{APA^-} \) are the fractions of the anionic species of Pen G, PAA and APA, respectively.

The fractions \( F_{PenG^-} \), \( F_{PAA^-} \) and \( F_{APA^-} \) are calculated from

\[ F_{PenG^-} = \frac{1}{1 + \frac{c_{H^+}}{K_{a,PenG}}} \]  

\[ F_{PAA^-} = \frac{1}{1 + \frac{c_{H^+}}{K_{a,PAA}}} \]  

\[ F_{APA^-} = \frac{1}{1 + \frac{c_{H^+}}{K_{a,APA}^1} + \frac{(c_{H^+})^2}{K_{a,APA}^2 \cdot K_{a,APA}^1 \cdot K_{a,APA}^2}} \]  

in which \( c_i \) is the concentration and \( K_{a,i} \) is the dissociation constant of component \( i \) (Tewari and Goldberg, 1988).

5.2.2 Partitioning

In a two-phase organic/aqueous system, components will partition between both phases. For weak electrolytes, mainly the uncharged species will partition to the organic phase. The main dissociation and partition equilibria are shown in Figure 1. The (overall) apparent partition coefficient of a monovalent acid \( i \) is defined as

\[ K_{p,i}^{app} = \frac{c_i^{org}}{c_i^{aq}} = \frac{c_i^{org}}{c_i^{aq} + c_i^{aq}} \]
The aqueous concentrations in the denominator can be calculated with equations similar to equations 3 to 5 and the measured aqueous concentrations (e.g. $c_{i,a}^{aq} = F_i \cdot c_{i}^{aq}$). Hence, the partitioning behaviour depends on the pH. This dependence of the apparent partition coefficient $K_{p,i}^{app}$ on the pH can be described as follows:

$$ K_{p,i}^{app} = K_{p,i}^{ref} \cdot F_i $$  \hspace{1cm} (7)

in which $K_{p,i}^{ref}$ is the intrinsic (pH-independent) partition coefficient, and $F_i$ is the fraction of the uncharged species of component $i$, which can be calculated with equations similar to equations 3 to 5.

From the initial amounts of the components, pH, mass balances and the organic/aqueous phase volume ratio the partition behaviour of the components can be predicted.

![Diagram](image)

**Figure 1** Schematic overview of dissociation and partition equilibria in aqueous/organic two-phase systems.

5.2.3 Two-phase reaction equilibria

When the enzymatic hydrolysis of Pen G is performed in a two-phase organic solvent/water system, the enzymatic reaction is assumed to take place only in the aqueous phase. As the hydrolysis of Pen G proceeds, the reactants will continuously establish a partitioning equilibrium between the organic and aqueous phase until a
final enzymatic and partitioning equilibrium has been established. For each compound a mass balance can be formulated over the organic and aqueous phase (equation 8)

\[ n_i = (c_i^{\text{aq}} + c_i^{\text{org}}) \cdot V^{\text{aq}} + c_i^{\text{org}} \cdot V^{\text{org}} \]  \hspace{1cm} (8) 

in which \( n \) is the total amount of moles and \( V \) is the volume of the aqueous (aq) or organic (org) phase. We assume that volume changes during the extractive reaction will be negligible. The stoichiometric balances for the enzymatic reaction are as follows:

\[ n_{\text{PenG}} = n_{\text{PenG}^\circ} - n_{\text{PAA}} \]  \hspace{1cm} (9) 

\[ n_{\text{APA}} = n_{\text{PAA}} \]  \hspace{1cm} (10) 

Combination of the partition coefficients (equation 6), mass balances (equation 8), and stoichiometric balances (equation 9 and 10) will give for each compound the concentration in the aqueous phase as a function of the concentration PAA in the aqueous phase, the partition coefficients and the volumes of both phases.

\[ c_{\text{PenG}}^{\text{aq}} = \frac{n_{\text{PenG}^\circ} - c_{\text{PAA}}^{\text{aq}} \cdot (V^{\text{aq}} + V^{\text{org}} \cdot K_{p,\text{PAA}}^{\text{app}})}{(V^{\text{aq}} + V^{\text{org}} \cdot K_{p,\text{PenG}}^{\text{app}})} \]  \hspace{1cm} (11) 

\[ c_{\text{APA}}^{\text{aq}} = \frac{c_{\text{PAA}}^{\text{aq}} \cdot (V^{\text{aq}} + V^{\text{org}} \cdot K_{p,\text{PAA}}^{\text{app}})}{(V^{\text{aq}} + V^{\text{org}} \cdot K_{p,\text{APA}}^{\text{app}})} \]  \hspace{1cm} (12) 

Substituting these aqueous concentrations in equation 1 and combination with equation 2 will give the aqueous concentration of PAA as a function of pH from which all concentrations in the organic and aqueous phase can be calculated. However, in biphasic systems it is more convenient to express the reaction equilibrium constant in terms of overall (biphasic) concentrations (Martinek and Semenov, 1981*). These biphasic concentrations are given by
\[ c_{i}^{bi} = c_{i}^{aq} \cdot \frac{1 + \alpha \cdot K_{\text{app}}^{\mu,\text{P,APA}}}{1 + \alpha} \] (13)

in which \( \alpha \) is the phase volume ratio (\( \alpha = V_{\text{org}}^{v} / V_{\text{aq}}^{v} \)). The apparent biphasic reaction equilibrium is then expressed as

\[ K_{\text{eq,app}}^{\text{bi}} = \frac{c_{\text{PAA}}^{\text{bi}} \cdot c_{\text{APA}}^{\text{bi}}}{c_{\text{PenG}}^{\text{bi}}} = K_{\text{eq,app}}^{\text{aq}} \cdot \frac{(1 + \alpha \cdot K_{\text{app}}^{\mu,\text{P,APA}}) \cdot (1 + \alpha \cdot K_{\text{app}}^{\mu,\text{APA}})}{(1 + \alpha) \cdot (1 + \alpha \cdot K_{\text{app}}^{\mu,\text{PenG}})} \] (14)

Using the dissociation constants of the reactants (Table I), a value of 9.06·10^{-8} \text{ mol·l}^{-1} for \( K_{\text{ref}} \) (Tewari and Goldberg, 1988) and the experimental phase volume ratio, the value of \( K_{\text{eq,app}}^{\text{bi}} \) can be calculated as a function of pH.

5.2.4 Three-phase reaction equilibria

If the equilibrium concentration of APA in the aqueous phase is higher than its solubility, APA may crystallise. This crystallisation phenomenon is beneficial for the yield as the enzymatic hydrolysis reaction is shifted towards the product side. The solubility of APA can be described as a function of pH with the following equation (Diender et al., in press):

\[ S_{\text{APA}} = \frac{S_{\text{APA}^{-}}}{F_{\text{APA}^{-}}} \] (15)

in which \( S_{\text{APA}^{-}} \) is the solubility of the zwitterionic species of APA (0.008 \text{ mol·l}^{-1}).

For the calculation of the apparent biphasic equilibrium constant, the solubility of APA (\( S_{\text{APA}} \)) must be used in equations 1 and 14 instead of \( c_{\text{APA}}^{\text{aq}} \), which simplifies these equations.
5.3 Materials and methods

5.3.1 Materials

Penicillin G acylase (EC 3.5.1.11) from E. coli was an aqueous suspension of cross-linked enzyme crystals (SynthaCLEC-PA, Altus Biologics Inc., Cambridge, MA, USA). Penicillin G potassium salt (Pen G-K salt, 99.0% pure), D-\(p\)-hydroxyphenylglycine methyl ester (HPGM, 99.0% pure), and 6-aminopenicillanic acid (APA, 98.5% pure) were kind gifts of DSM (Geleen, The Netherlands). Butyl acetate (BuAc) was obtained from Aldrich (99.7% pure, HPLC-grade) and phenylacetic acid (PAA, 99% pure) from Fluka.

5.3.2 Enzymatic reaction in 1:1 (w/w) water/butyl acetate two-phase system

A stock solution of Pen G in acidic form was prepared by dissolving Pen G-K salt in 20 g MilliQ water and 30 g BuAc at room temperature. While stirring this two-phase system, the pH was adjusted to a final pH of 2.6 by adding 0.3 mol\(\cdot\)l\(^{-1}\) HCl. At this low pH, most of the Pen G is present in the BuAc phase. This BuAc phase was used as stock solution of Pen G in the acidic form. A fresh stock solution was prepared for each experiment and was used immediately after preparation.

Equilibrium measurements of the hydrolysis of Pen G in the presence of different amounts of potassium ions or HPGM were performed to achieve different initial pH values. For the experiments involving potassium ions, Pen G was added to 1:1 (w/w) water BuAc mixture either as Pen G-K salt or in the acidic form in BuAc. By changing the ratio of the amount of Pen G added as K-salt and the acidic form of Pen G in BuAc, the initial pH of the biphasic mixture was adjusted. For the experiments involving HPGM, no Pen G-K salt was used but the initial pH was adjusted by adding different amounts of solid HPGM to the stock solution of Pen G in the acidic form. Initial Pen G concentrations varied between 38 and 82 mmol Pen G \(\cdot\) l\(^{-1}\) biphasic mixture (see Table II). Both phases were saturated with the other phase before mixing of the phases. (Densities were determined with Density meter DMA 48 (AP Paar, Austria): \(\rho\) water phase 1.007 g\(\cdot\)cm\(^{-3}\), \(\rho\) BuAc phase 0.880 g\(\cdot\)cm\(^{-3}\)).

Prior to adding the enzyme suspension to the reaction mixture, the original penicillin acylase suspension was centrifuged for 2 min at 13,000 rpm (MSE MicroCentaur) and washed twice with 1 ml of MilliQ water. After the washing step, the centrifuged penicillin acylase crystals were resuspended in a known amount of MilliQ water.
Reactions were performed in a 60 ml stirred, thermojacketed reaction vessel at 25 °C by addition of penicillin acylase suspension (containing 0.34-0.84 g centrifuged penicillin acylase crystals) to a 1:1 (w/w) water BuAc two-phase system containing Pen G (total volume of approximately 40 ml). During the experiment the pH of the two-phase mixture was monitored continuously (glass pH electrode, Metrohm, Switzerland), and 3 ml samples of the mixture were taken and filtered (0.2-μm Nylaflo® nylon membrane filters, Gelman Sciences, USA) to stop the enzymatic reaction. This also removed any solid APA formed. The liquid phases were allowed to separate and were analysed by HPLC. Generally, reactions were carried out for approximately 23 to 26 h. For calculation of $K_{eq,app}^{bi}$ the ratios of biphasic concentrations were averaged for the flat region of the curve in the experiments.

5.3.3 Analysis

Pen G, APA, PAA, HPGM and HPG were identified and analysed by HPLC using a Waters system with a Microbondapak-phenyl column, 3.9×300 mm thermostated at 33 °C (for the experiments using Pen G-K salt) or a PLATINUM EPS-C$_{18}$ column (particle size 5 μm, 10 nm pore size) 4.6×250 mm at room temperature (for Pen G/HPGM combinations). A Waters 2690 Alliance separation module was used with a flow of 1 ml·min$^{-1}$ and 10 μl injection volume. Detection was performed by a Waters 996 PDA detector at 220 nm. The eluens was composed of 0.64 g·l$^{-1}$ KH$_2$PO$_4$ and 28% (v/v) acetonitrile, which was brought to pH 2.75 with H$_3$PO$_4$.

5.4 Results and discussion

5.4.1 Hydrolysis of Pen G

Figure 2 shows an example of the course of pH and the concentrations of the reactants in both phases during a hydrolysis experiment. During some of the hydrolysis experiments (especially the experiments at low pH) chemical degradation of Pen G and APA occurred. The amounts of enzyme were maximized in order to minimize the chemical degradation in the experiments at low pH. In the experiment shown in Figure 2, the pH is decreasing from 7.0 at the start of the experiment to 5.4 at equilibrium. At this pH range the predominant enzymatic reaction is

$$\text{PenG}^+ + \text{H}_2\text{O} \rightleftharpoons \text{APA}^- + \text{PAA}^- + \text{H}^+$$
Protons are produced as Pen G is hydrolysed. This is only partly compensated by extraction of PAA to the organic phase, and a steep decrease of pH occurs at the start of the experiment. The enzymatic reaction is finally reaching its thermodynamic equilibrium causing the pH to become constant. As shown by the results, equilibrium was reached during the time of the experiment and $K_{eq,app}^b$ could be determined from the ratio of concentrations, assuming that the enzyme was still active at low pH and not denatured.

![Figure 2](image) **Figure 2** Concentration profile of reactants and pH profile during hydrolysis of Pen G in a 1:1 (w/w) water/butyl acetate two-phase batch system at 25 °C (experiment 1, Table II). Symbols: closed markers, concentrations in aqueous phase; open markers, concentrations in organic phase; ▽: Pen G; △: APA; □: PAA; solid line, no marker: pH.

In some experiments at low pH (experiments 5, 6, 8, 9 and 10, see Table II) APA crystallised during the experiment. This was observed by the appearance of crystals during the experiment. Also, the aqueous APA concentrations showed initially a maximum, decreased again and became constant at the solubility concentration. Apparently, at those pH values supersaturation of APA occurs. We did not find any literature about the occurrence of crystallisation of APA during Pen G hydrolysis.

For all experiments, mass balances were set up for the nucleus (Pen G/APA), and side chains (Pen G/PAA and HPGM/HPG, respectively). Because only dissolved aqueous concentrations were measured, the mass balances did not close for the
nucleus in the experiments in which APA crystallisation took place. In some of the other experiments, the mass balances did not close completely, probably due to chemical degradation of Pen G and APA. Reschke and Schügerl (1984) investigated the degradation of Pen G in different media. They reported a first-order degradation rate constant of $9.52 \times 10^{-3}$ h$^{-1}$ in water saturated with BuAc at pH 5.0 (degradation of Pen G in pure BuAc was found to be much slower). For Pen G and APA this rate constant was used to calculate aqueous concentrations corrected for chemical degradation. The mass balances corrected for degradation gave acceptable results (approximately 5%).

Penicillin acylase is able to hydrolyse HPGM into HPG and methanol. However, in experiments 9 and 10 only small amounts (less than 0.8 mmol·l$^{-1}$) of HPG were found in the aqueous phase and nothing in the organic phase. Apparently this hydrolysis reaction is not of importance.

5.4.2 Partitioning

During the Pen G hydrolysis experiments samples were taken from both phases in the course of the reaction. It was assumed that the mass transfer between phases was much faster than the enzymatic reactions, and that a dynamic partitioning equilibrium was established. Measured concentrations were used to determine the apparent partition coefficients of the components. The influence of the cation on the partitioning behaviour was tested by using either potassium or protonated HPGM as counter ion.

<table>
<thead>
<tr>
<th>Compound</th>
<th>$pK_a$</th>
<th>$S_{\text{ref}}$</th>
<th>$K_{p,i}^{\text{ref}}$ in presence of $K^+$</th>
<th>$K_{p,i}^{\text{ref}}$ in presence of HPGM$^+$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pen G</td>
<td>2.5$^a$</td>
<td>$\infty$</td>
<td>69</td>
<td>47</td>
</tr>
<tr>
<td>APA</td>
<td>2.5 / 4.9$^a$</td>
<td>0.008$^b$</td>
<td>$10^3$</td>
<td>$10^3$</td>
</tr>
<tr>
<td>PAA</td>
<td>4.3$^a$</td>
<td>$\infty$</td>
<td>29</td>
<td>26</td>
</tr>
<tr>
<td>HPGM</td>
<td>8.3$^b$</td>
<td>$\infty$</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>HPG</td>
<td>2.2 / 9.2$^a$</td>
<td>$\infty$</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

$^a$ Values are from Diender et al. (1998)
$^b$ Values are from Diender et al. (2000)
As the partitioning of APA, HPGM and HPG (the hydrolysis product of HPGM) in butyl acetate was very low in all experiments ($K_{p,APA}^{\text{app}}$ in the order of $10^{-3}$, partitioning of HPGM and HPG was negligible) results are not shown. For Pen G and PAA, the experimental concentrations in the organic phase from all samples from all experiments are shown as a function of the concentration of uncharged Pen G and PAA in the aqueous phase in Figures 3 and 4. The fractions of uncharged species were calculated with experimental pH values and the dissociation constants shown in table I.

A linear regression line was fitted through all experimental data. The slope of the regression line is equal to the reference partition coefficient, $K_{p,i}^{\text{ref}}$ (see Table I). The presence of a cation (potassium or HPGM\(^+\)) in the reaction medium has a slight effect on the slope of the regression line, i.e. the reference partition coefficient of Pen G and PAA. In the presence of HPGM, this reference partition coefficient is lower than the one in the presence of potassium as counter ion. It is possible that a complex is formed between a potassium ion and the anionic form of Pen G. This ion pair complex can also partition into the organic phase. However, an intercept with the Y-axis would be observed in Figures 3 and 4, as there would be still be Pen G in the

![Graph](image)

**Figure 3** Measured concentration of Pen G in organic phase as a function of the aqueous concentration of uncharged Pen G in a 1:1 (w/w) water/butyl acetate two-phase batch system at 25 °C. • experimental data with potassium as counter ion; ○ experimental data with HPGM\(^+\) as counter ion.
organic phase when \( e_{\text{PenG}}^{aq} \) is zero. This is not observed. Reschke and Schügerl (1984) found that coextraction of buffer and chloride ions did not have a large influence on penicillin G extraction. Another explanation for the different partition behaviour in the presence of potassium or HPGM was not found.

![Graph](image)

**Figure 4** Measured concentration of PAA in organic phase as a function of the aqueous concentration of uncharged PAA in a 1:1 (w/v) water/butyl acetate two-phase batch system at 25 °C. ● experimental data with potassium as counter ion; ● experimental data with HPGM as counter ion.

The value of 47 for the reference partition coefficient of Pen G in the presence of HPGM agrees fairly well with the data given in literature (\( K_{\text{p, PenG}}^{\text{ref}} = 48 \) at room temperature (Reschke and Schügerl, 1984) and \( K_{\text{p, PenG}}^{\text{ref}} = 54 \) or 63 (depending on the analysis method) at \( T = 0^\circ\text{C} \) (Whitmore et al., 1946). Reschke and Schügerl (1984) also found a value of 25 for the reference partition coefficient of PAA, which is comparable to what we found (\( K_{\text{p, PAA}}^{\text{ref}} = 26 \) in the presence of HPGM). The data for the partitioning of Pen G and PAA in the presence of potassium (\( K_{\text{p, PenG}}^{\text{ref}} = 69 \), and \( K_{\text{p, PAA}}^{\text{ref}} = 29 \)), are higher than these data, but still in the same range. The differences may be caused by the use of phosphate and citrate buffers to set the pH, which could have influenced the partitioning behaviour in the literature experiments. In our experiments, we did not add any buffers to set the pH, but the composition of the mixtures was different for each sample, as the enzymatic reaction took place. The
presence of the other components like Pen G, APA and PAA could also influence the partitioning behaviour of Pen G and PAA. Bento et al. (1962) reported that the partitioning of Pen G is influenced by the initial concentration of Pen G. They found that at initial aqueous concentrations of Pen G sodium salt between 0 and 90 mM (i.e. $c^{bi} = 45$ mM) the apparent partition coefficient increased only slightly from 50-80 in 1:1 (w/w) aqueous/ BuAc two-phase systems at pH 2 and 20°C. However, at concentrations in the range of 90-350 mM the apparent partition coefficient increased steeply to 160. This was ascribed to association of Pen G molecules. Association of Pen G molecules (and possible also PAA) could explain the scatter in our data points: the composition in the two-phase system is different for each sample as the enzymatic hydrolysis reaction takes place. At the start of each experiment the initial biphasic Pen G concentration is in the range of or above the concentrations Bento and coworkers found at which the partitioning behaviour changes. During the reaction, the concentration of Pen G decreases and the apparent partition coefficient becomes less dependent on the concentration.

5.4.3 Modelling of hydrolysis of Pen G

As described in the Theory section the apparent biphasic equilibrium constant can be modeled as a function of the dissociation of the reactants (i.e. pH), the phase volume ratio and the pH-independent equilibrium constant of the reference reaction (combination of equations 2, 13 and 14). Because in the hydrolysis experiments different initial biphasic Pen G concentrations and slightly different phase volume ratios were used (see Table II), it is not possible to represent all experimental and theoretical values of $K^{bi}_{eq,app}$ as a function of pH in a single graph. Therefore, we have represented the theoretical $K^{bi}_{eq,app}$ in a parity plot against the experimental value in Figure 5. As shown by the results, the model predicts the apparent biphasic equilibrium constant reasonably.

Table II presents the measured and predicted yield (based on PAA) of each experiment. In general, the measured yields are a bit lower than the predicted yields. An explanation for this could be that the solubility and $pK_a$'s used in the calculations (Table I) have been measured in the absence of organic solvent and at a different ionic strength than in the present experiments. Another explanation could be that
Figure 5  Parity plot of predicted values versus experimental values of $K_{\text{eq,app}}^{bi}$ for the hydrolysis of Pen G in a 1:1 (w/w) water/butyl acetate two-phase batch system at 25 °C. ● data of PenG / potassium experiments; ▲ data of Pen G/HPGM experiments.

Table II  Initial and equilibrium conditions of Pen G hydrolysis experiments in two-phase water/butyl acetate batch system at 25 °C.

<table>
<thead>
<tr>
<th>Exp.</th>
<th>Counter ion</th>
<th>$c_{\text{counter ion}}^{bi}$ (mM)</th>
<th>$\alpha$</th>
<th>$c_{\text{PenG}}^{bi}$ (mM)</th>
<th>pH initial</th>
<th>pH equilibrium</th>
<th>yield exper. (%)</th>
<th>yield pred. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>K⁺</td>
<td>43</td>
<td>1</td>
<td>41</td>
<td>7.0</td>
<td>5.4</td>
<td>64</td>
<td>71</td>
</tr>
<tr>
<td>2</td>
<td>K⁺</td>
<td>46</td>
<td>1.1</td>
<td>44</td>
<td>7.4</td>
<td>5.5</td>
<td>69</td>
<td>72</td>
</tr>
<tr>
<td>3</td>
<td>K⁺</td>
<td>37</td>
<td>1.1</td>
<td>39</td>
<td>5.4</td>
<td>5.2</td>
<td>80</td>
<td>80</td>
</tr>
<tr>
<td>4</td>
<td>K⁺</td>
<td>36</td>
<td>1.2</td>
<td>38</td>
<td>5.2</td>
<td>5.2</td>
<td>79</td>
<td>81</td>
</tr>
<tr>
<td>5</td>
<td>K⁺</td>
<td>19</td>
<td>1.2</td>
<td>42</td>
<td>4.4</td>
<td>4.5</td>
<td>86</td>
<td>92</td>
</tr>
<tr>
<td>6</td>
<td>K⁺</td>
<td>0</td>
<td>1.1</td>
<td>41</td>
<td>2.9</td>
<td>3.0</td>
<td>94</td>
<td>96</td>
</tr>
<tr>
<td>7</td>
<td>K⁺</td>
<td>73</td>
<td>1.2</td>
<td>67</td>
<td>7.0</td>
<td>5.5</td>
<td>69</td>
<td>73</td>
</tr>
<tr>
<td>8</td>
<td>K⁺</td>
<td>36</td>
<td>1.2</td>
<td>82</td>
<td>4.4</td>
<td>4.6</td>
<td>86</td>
<td>91</td>
</tr>
<tr>
<td>9</td>
<td>HPGM⁺</td>
<td>22</td>
<td>1.2</td>
<td>54</td>
<td>4.0</td>
<td>4.4</td>
<td>93</td>
<td>93</td>
</tr>
<tr>
<td>10</td>
<td>HPGM⁺</td>
<td>12</td>
<td>1.2</td>
<td>44</td>
<td>4.0</td>
<td>4.0</td>
<td>88</td>
<td>95</td>
</tr>
</tbody>
</table>
equilibrium was not reached during the experiment. This could be due to degradation of Pen G or APA or due to the crystallisation of APA. In experiment 10, for example, the measured aqueous concentration of APA at which we assumed equilibrium was still higher than the solubility at that pH. This indicates that equilibrium was not reached yet. However, the model predicts trends in the yield as a function of pH and the initial Pen G concentration quite well. Figure 6 shows the predicted yields of 0.1 mol·l⁻¹ Pen G/HPGM-salt in purely aqueous systems and in 1:1 or 1:10 (w/w) water/butyl acetate biphasic systems with and without crystallisation as a function of pH.

![Figure 6](image)

**Figure 6** Calculated yields as a function of pH for the enzymatic hydrolysis of 0.1 mol·l⁻¹ mixture Pen G/HPGM in different systems at 25 °C. ——— no APA crystallisation; —— with APA crystallisation; □ pure aqueous system; ○ 1:1 (w/w) water/butyl acetate two-phase systems; Δ 1:10 (w/w) water/butyl acetate two-phase systems.

In purely aqueous systems the yield is strongly dependent on pH, as reported before (e.g. Tewari and Goldberg, 1988). At pH 8 almost 99% conversion of 0.1 mol·l⁻¹ Pen G can be achieved, while at pH 4.5 only 33% of Pen G is converted. Due to entropic effects, higher yields can be achieved in more dilute solutions (Martinek and Semenov, 1981). At pH 8 in water/BuAc biphasic mixtures partitioning is negligible. Then the maximal yield achievable is lower than in aqueous systems (at similar initial amounts of Pen G per liquid volume). This is caused by the relatively high aqueous
concentrations of PAA and APA in the biphasic systems, due to the smaller volume of the aqueous phase. Below pH 6, the effect of partitioning is becoming visible. The produced PAA is extracted into the organic phase, shifting the equilibrium to the product side. Higher yields are achieved in the two-phase systems than in pure aqueous systems in that pH range. At lower pH values (depending on the initial aqueous Pen G concentration), APA crystallisation is taking place, which improves the yield considerably for all systems. For example, at pH 5, yield is calculated to increase from 40 to 90% by APA crystallisation in a 1:10 (w/w) water/BuAc system. The pH at which APA starts to crystallise is higher as the volume ratio of organic to aqueous phase is increasing. This is due to the high concentration of APA in the small remaining volume of aqueous phase. Figure 7 shows the influence of the initial Pen G concentration on the yield in a 1:1 (w/w) water/BuAc biphasic system.

![Graph showing yield vs pH](image)

**Figure 7** Calculated yields as a function of pH for the enzymatic hydrolysis Pen G/HPGM salt for different initial Pen G concentrations in 1:1 (w/w) water/BuAc at 25 °C. 

- - - no APA crystallisation; - - - - with APA crystallisation; □ 0.1 mol Pen G·L⁻¹ mixture; ○ 0.5 mol Pen G·L⁻¹ mixture; Δ 1.0 mol Pen G·L⁻¹ mixture.

This figure shows clearly that in more dilute solutions higher yields can be achieved, due to the entropic effects as mentioned before. However, due to the crystallisation of APA high conversions of Pen G can be achieved even at high initial concentrations. This favourable situation allows the further development of processes for the conversion of Pen G into semisynthetic antibiotics with reduced salt formation as mentioned in the Introduction.
for the conversion of Pen G into semisynthetic antibiotics with reduced salt formation as mentioned in the Introduction.

Although the combination of extractive enzymatic reaction and product crystallisation may seem to be exotic, Dahod and Empie (1986) reported the application of this combination in the enzymatic production process of L-phenylalanine.

### 5.5 Conclusions

By performing the penicillin G hydrolysis in a water/organic solvent two-phase batch system, the product PAA can be extracted into the organic phase at low pH. Also, at lower pH, crystallisation of APA can occur. Both phenomena shift the equilibrium to the product side and consequently high yields can be obtained (>90% for 50 mM Pen G). A quantitative model has been presented for the prediction of the hydrolysis equilibrium as a function of pH, initial Pen G concentration and phase volume ratio. The model includes the reaction equilibrium, the partitioning behavior of all reactants and the solubility equilibrium of APA. For the validation of this model, Pen G was hydrolysed at various conditions. The model predicted the conversion of Pen G in different batch systems satisfactory.

### 5.6 Acknowledgements

The section Organic Chemistry and Catalysis from Delft University of Technology are kindly acknowledged for providing penicillin acylase.

### 5.7 Notation

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>APA</td>
<td>6-aminopenicillanic acid</td>
</tr>
<tr>
<td>BuAc</td>
<td>butyl acetate</td>
</tr>
<tr>
<td>HPG</td>
<td>D-(p-)-hydroxyphenylglycine</td>
</tr>
<tr>
<td>HPGM</td>
<td>D-(p-)-hydroxyphenylglycine methyl ester</td>
</tr>
<tr>
<td>PAA</td>
<td>phenylacetic acid</td>
</tr>
</tbody>
</table>
Pen G  penicillin G

\( \alpha \)  phase volume ratio \((V_{\text{org}}/V_{\text{aq}})\)  (-)

\( c_i \)  dissolved concentration of species \( i \)  \((\text{mol}\cdot\text{l}^{-1})\)

\( F \)  fraction  (-)

\( K_a \)  dissociation constant  \((\text{mol}\cdot\text{l}^{-1})\)

\( K_{eq} \)  enzymatic equilibrium constant  \((\text{mol}\cdot\text{l}^{-1})\)

\( K_p \)  partition coefficient  (-)

\( n_i \)  total amount of moles of component \( i \)  \((\text{mol})\)

\( S \)  solubility  \((\text{mol}\cdot\text{l}^{-1})\)

\( V \)  volume  \((\text{l})\)

Sub- or superscript

app  apparent

aq  aqueous

bi  biphasic

org  organic

ref  reference

5.8 References


Modelling the influence of ionic strength on α-amino acid ester hydrolase in the salt-free production of amoxicillin

Abstract
Amoxicillin can be produced by enzymatically coupling 6-aminopenicillanic acid with hydroxyphenylglycine methyl ester, for example. In the reaction mixture, other molecules than the substrates and product can be present, e.g. inorganic salts resulting from pH adjustment or contaminants like penicillin G or phenylacetic acid. This paper describes the influence of the presence of these components and of the ionic strength on the synthesis of amoxicillin by the enzyme α-amino acid ester hydrolase (AEH) from Acetobacter pasteurianus ATCC 9325. The influence of phenylacetic acid and penicillin G was similar and correlated with the ionic strength, whereas the influence of some inorganic salts on the performance of AEH was more scattered. A quantitative model was based on enzyme kinetics (described as a function of the ionic strength), pH-dependent solubilities and stoichiometric, mass and charge balances. The model predicted the concentrations during the course of the experiments in the absence and presence of phenylacetic acid and penicillin G reasonably well. As decreasing the ionic strength in the reactor will increase the yield of amoxicillin, the synthesis reaction of amoxicillin might be done preferably in absence of phenylacetic acid and penicillin G, by adjusting the initial pH with hydroxyphenylglycine methyl ester instead of an inorganic base and by feeding hydroxyphenylglycine methyl ester during the reaction.

This chapter was submitted for publication.
6.1 Introduction

The antibiotic penicillin G (Pen G) is the raw material of many semisynthetic antibiotics. After fermentation, Pen G is extracted from the fermentation broth by organic solvents like butyl acetate or amyl acetate. Subsequently, Pen G is either back-extracted to an aqueous phase and then crystallised, or crystallised directly from the organic phase by adding solid potassium acetate or sodium acetate. The Pen G crystals are used for the production of 6-aminopenicillanic acid (APA) by enzymatic hydrolysis. APA is recovered by crystallisation, dissolved again and can be coupled enzymatically with an activated side chain to form a semisynthetic antibiotic. E.g., this is hydroxy-phenylglycine methyl ester (HPGM) or hydroxyphenylglycine amide (HPGA) for amox-icillin (Amox), and phenylglycine methyl ester or phenylglycine amide for ampicillin (Hersbach et al., 1984; Barendschee et al., 1992; Rindfleisch et al., 1997). A schematic overview of such a process is given in Figure 1.

Figure 1 Schematic overview of process concept of Pen G purification and subsequent biotransformation to Amox, using penicillin acylase as the only enzyme (adapted from Hersbach et al., 1984).
Both enzymatic reactions (Figure 2, reactions I and II) can be catalysed by the same enzyme, penicillin acylase (Cole, 1969; Svedas et al., 1980; Bruggink et al., 1998). A shortcut route with the two enzymatic reactions occurring simultaneously or subsequently could be very interesting for the following reasons: 1. the number of unit operations is reduced; 2. APA formed in the hydrolysis reaction does not need to be crystallised in between the enzymatic reactions, which decreases the losses of APA during downstream processing; 3. a reduction in the salt production may be achieved as the number of pH shifts (for the enzymatic reactions and crystallisation steps) can be decreased (Diender et al., submitted); 4. if both reactions are performed simultaneously, APA formed in the hydrolysis reaction is consumed in the synthesis reaction and in this way the equilibrium of the hydrolysis reaction is shifted towards the product side (i.e. a higher conversion is reached). However, so far this shortcut route has not been possible as one of the products from the Pen G hydrolysis reaction, phenylacetic acid (PAA), essentially inhibits the catalytic activity of penicillin acylase for the Amox synthesis (reaction II) (Svedas et al., 1980).

\[
\text{Pen G + H}_2\text{O} \xrightleftharpoons{I} \text{APA + PAA}
\]

\[
\text{APA + HPGM + H}_2\text{O} \xrightarrow{II} \text{Amox + MeOH + H}_2\text{O}
\]

\[
\text{HPG + MeOH} \xrightarrow{III} \text{HPG + APA}
\]

\[
\text{HPG + MeOH} \xrightarrow{IV} \text{HPG + APA}
\]

**Figure 2** Schematic overview of enzymatic reactions.

The aim of this paper is to study the possibilities for the shortcut route using another enzyme for the second reaction. This enzyme is α-amino acid ester hydrolase (AEH) from *Acetobacter pasteurianus* ATCC 9325. This enzyme is known to be able to catalyse the synthesis reaction in the presence of PAA (Nara et al., 1977). However, an important parameter, the so-called synthesis/hydrolysis ratio (S/H, the molar ratio of produced Amox and HPG, the hydrolysed side-chain donor), is negatively influenced by the presence of PAA (Figure 2, reactions II and III) (unpublished results). The extent of this influence is not known. This means that the shortcut route
using AEH requires removal of PAA to an unknown level. The removal does not have to be absolute, as is required for any route using penicillin acylase for the Amox synthesis.

Previous studies (Diender et al., submitted) have shown that performing the hydrolysis of Pen G in water/butyl acetate two-phase mixtures using penicillin acylase is an adequate method to achieve low levels of PAA in the aqueous phase and high conversions by extracting PAA into the organic phase. This extractive reaction can be carried out starting from an acidic Pen G solution in butyl acetate that originates from the extraction after the fermentation of Pen G (Figure 3). The pH that is required for the extractive reaction can be set by adding HPGM as a base. The remaining aqueous phase contains APA and HPGM (less than 1 equivalent, depending on the reaction conditions). This can be used for the synthesis reaction of Amox by addition of AEH and an additional amount of HPGM, because more than 1 equivalent of HPGM is required for synthesis. This aqueous phase may also contain some unextracted PAA and unhydrolysed Pen G.

![Figure 3](image_url)  
**Figure 3** Schematic overview of a process concept of Pen G purification and subsequent biotransformation to Amox using penicillin acylase and AEH as enzymes (shortcut route).

A quantitative model that describes the enzymatic kinetically-controlled synthesis of Amox in a batch reactor has been presented by Diender et al. (2000). The model is based on mass and charge balances, solubilities of the reactants and the kinetics of the penicillin acylase-catalysed synthesis, in the absence of Pen G and PAA. In this paper the kinetics of Amox synthesis catalysed by AEH from *Acetobacter pasteurianus* ATCC 9325 will be decribed. Kato et al. (1980) and Ryu and Ryu (1987) have studied AEH from *X. citri*, which closely resembles AEH from *A. pasteurianus* ATCC 9325 with respect to molecular mass, the ratio of specific
enzyme activities for cephalixin synthesis and hydrolysis and the pH optima for these reactions. They found that AEH is influenced by the ionic strength of the reaction mixture. Higher synthetic yields could be obtained by decreasing the ionic strength.

Therefore, we have studied the influence of the presence of different additives, such as PAA, Pen G, and some inorganic salts on the performance of AEH. The influence of the ionic strength (built up by all substrates, products and the additives) on AEH was studied as well. We have implemented the influence of the additives on AEH as an ionic strength effect in the model of Diender et al. (2000).

6.2 Theory

The reactants in this system are all weak electrolytes: they are present in solution as cationic, zwitterionic or anionic species depending on the pH. Also some of the additives as for example PAA or Pen G, can show acid/base dissociation behaviour. In the pH range that we studied the following dissociation reactions are important:

\[
\begin{align*}
\text{APA}^+ & \rightleftharpoons \text{APA}^- + H^+ \\
\text{HPGM}^+ & \rightleftharpoons \text{HPGM}^0 + H^+ \\
\text{Amox}^+ & \rightleftharpoons \text{Amox}^- + H^+ \\
\text{HPG}^+ & \rightleftharpoons \text{HPG}^- + H^+ \\
\text{PAAH} & \rightleftharpoons \text{PAA}^- + H^+ \\
\text{Pen GH} & \rightleftharpoons \text{Pen G}^- + H^+
\end{align*}
\]

The dissociation constants \((K_a)\) of these reactions are given in Table I. The concentration of each ionic species of a compound \(i\) can be calculated as a function of the total dissolved concentration of the compound \((c_i)\) and the fraction of the ionic species \((F_i)\). As an example, the calculation of the concentration and the fraction of the anionic form of APA are given in equation 1 and 2.

\[
c_{\text{APA}^-} = F_{\text{APA}^-} \cdot c_{\text{APA}} \tag{1}
\]

in which
\[ F_{\text{APA}^-} = \frac{c_{\text{APA}^-}}{c_{\text{APA}^-} + c_{\text{APAd}^-}} = \frac{1}{1 + \frac{c_{\text{H}^+}}{K_{\alpha,\text{APA}}}} \]  

(2)

For the other compounds the calculations of the concentrations and the fractions are analogous to equations 1 and 2.

Table I  Dissociation constants and reference solubilities of the reactants of the model system.

<table>
<thead>
<tr>
<th>compound</th>
<th>( pK_a )</th>
<th>( S_{\text{ref}} ) (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>APA(^a)</td>
<td>4.9</td>
<td>0.008</td>
</tr>
<tr>
<td>HPGM(^b)</td>
<td>8.3</td>
<td>0.0013</td>
</tr>
<tr>
<td>Amox(^a)</td>
<td>7.4</td>
<td>0.0091</td>
</tr>
<tr>
<td>HPG(^a)</td>
<td>9.2</td>
<td>0.17</td>
</tr>
<tr>
<td>PAA(^a)</td>
<td>4.6</td>
<td>n.r.</td>
</tr>
<tr>
<td>Pen G(^a)</td>
<td>2.6</td>
<td>n.r.</td>
</tr>
</tbody>
</table>

\(^a\) Values are from Diender et al., submitted.

\(^b\) Values are from Diender et al., 2000.

6.2.1 Calculation initial inorganic salt concentration

The synthesis reaction can be performed at a fixed pH by adding extra acid or base to the initial amounts of substrates, or at a changing pH when no extra acid or base is added. When performed at a fixed pH, the pH can be kept constant by adding inorganic acid/base or by adding HPGM. In the latter case, Amox will be produced in a salt-free manner. In all cases the initial pH is determined only by the initial concentration of the reactants and additives. As the pH is fixed at 6.2 in this study, the initial amount of the inorganic acid or base which is necessary to adjust the pH to 6.2 can be derived from a charge balance based on the concentrations APA\(^-\), HPGM\(^+\), the additives PAA\(^-\) and/or PenG\(^-\) and/or inorganic acid or base (equation 3).

\[ c_{\text{inorganic cations}} - c_{\text{inorganic anions}} = c_{\text{APA}^-} + c_{\text{PAA}^-} + c_{\text{PenG}^-} - c_{\text{HPGM}^+} \]

(3)
In the range of concentrations used by us, the concentrations of OH\(^{-}\) and H\(^{+}\) can be neglected in equation 3. PAA and Pen G are supposed to be present only in small dissolved quantities. APA and HPGM can be initially present in the reactor completely or partly dissolved. If the substrates are only partly dissolved, the concentrations are determined by the solubilities of the compounds. The solubilities of all reactants are influenced by pH, because APA, HPGM, Amox and HPG are weak electrolytes. As explained by Diender et al. (2000), the overall solubility of APA can be calculated as a function of a reference solubility (\(S_{\text{APA,ref}}\)) and pH using equation 4. For the other compounds analogous equations can be used.

\[
S_{\text{APA}} = \frac{S_{\text{APA,ref}}}{F_{\text{APA}^{+}}}
\]  

(4)

Values of these reference solubilities are presented in Table I. Substitution of concentrations (equation 1) in the charge balance (equation 3) taking into account the solubilities (equation 4) leads to a higher-order equation in proton concentration, from which the initial pH can be calculated by iteration (compare Diender et al., 2000).

### 6.2.2 Calculation added amount of HPGM

The pH during the reaction will be kept at the initial value by titration with solid HPGM. Because the added amount of HPGM to keep the pH constant is not measured, it must be calculated. The amount of moles of HPGM (\(n_{\text{HPGM,ad}}\)), which has to be added can be calculated using a stoichiometric balance (equation 5).

\[
n_{\text{HPGM,ad}} = n_{\text{HPG}} + n_{\text{Amox}} + n_{\text{HPGM}} - n_{\text{HPGM,0}}
\]  

(5)

in which \(n\) is the total amount (dissolved and solid) of moles of a compound present in the reactor, and \(n_0\) is the initial amount of moles of a compound which was added. To determine whether the concentrations of the components during the reaction are below or above their solubilities, the concentrations should be compared to the actual amounts. The actual amounts can be calculated using molar mass balances. The mass balances for Amox and HPG in a batch reactor are:

\[
\frac{dn_{\text{Amox}}}{dt} = (r_{\text{synth}} - r_{\text{prodhyd}}) \cdot V
\]  

(6)
\[
\frac{dn_{\text{HPG}}}{dt} = (r_{\text{prodhydr}} + r_{\text{subhydr}}) \cdot V
\]  

in which \( r \) is the rate of synthesis or hydrolysis and \( V \) is the reactor volume, which is assumed to be constant. AEH catalyzes three reactions, i.e. product synthesis (Figure 2, reaction II), substrate hydrolysis (Figure 2, reaction III) and product hydrolysis (Figure 2, reaction IV). For these reactions the following simplified (mass action based) enzymatic rate equations can be formulated:

\[
r_{\text{synth}} = k_{\text{synth}} \cdot c_{\text{HPGM}} \cdot c_{\text{APA}} \cdot c_{\text{enzyme}}
\]  

\[
r_{\text{subhydr}} = k_{\text{subhydr}} \cdot c_{\text{HPGM}} \cdot c_{\text{enzyme}}
\]  

\[
r_{\text{prodhydr}} = k_{\text{prodhydr}} \cdot c_{\text{Amax}} \cdot c_{\text{enzyme}}
\]

in which \( k \) is the rate constant. The stoichiometric balances for APA and HPGM are:

\[
n_{\text{APA}} = n_{\text{APA,0}} - n_{\text{Amax}}
\]

\[
n_{\text{HPGM}} = c_{\text{HPGM}} \cdot V \\
= \left( c_{\text{Amax}}^- + c_{\text{HPG}}^- + c_{\text{APA}}^- + c_{\text{PAA}}^- + c_{\text{PenG}}^- + c_{\text{inorganic anion}}^- - c_{\text{inorganic cation}}^- \right) \cdot V
\]

In equation 12, the concentration of HPGM is calculated from a charge balance. The rate constants of synthesis, substrate and product hydrolysis can be determined from initial rate measurements.

6.3 Materials and methods

6.3.1 Enzyme

Purified \( \alpha \)-amino acid ester hydrolase (EC 3.1.1.43) solution from Acetobacter pasteurianus ATCC 9325 was obtained from DSM (Delft, The Netherlands).
6.3.2 Enzyme activity

The activity of the AEH solution was 25.2 U/l. Enzyme activity was determined by adding a known amount of enzyme solution to a suspension of 6.26 g HPGM and 6.48 g APA in 100 ml water. The enzymatic synthesis reaction was performed at 25 °C while stirring. After 60 and 90 min a 100 µl sample of the suspension was taken and dissolved in 2.5 ml of a solution of 5.12 g K₂HPO₄.3H₂O, 2.96 g KH₂PO₄ in 6.4 l water and 3.2 l acetonitrile to stop the enzymatic reaction. Concentrations were determined by HPLC.

1 Unit is defined as the amount of enzyme necessary to produce 1 g Amox per hour under these conditions.

6.3.3 Materials

D-\(p\)-Hydroxyphenylglycine methyl ester (HPGM, 99.0% pure), 6-aminopenicillanic acid (APA, 98.5% pure), and penicillin G potassium salt (Pen G, 99.0% pure) were obtained from DSM (Geleen, The Netherlands). Phenylacetic acid (PAA, 99% pure) was obtained from Fluka Chemica. All other compounds used were analytical grade.

6.3.4 Enzymatic reactions

Kato et al. (1980) found that AEH from \textit{X. citri} (which is according to Takahashi et al. (1974) very similar to AEH from \textit{A. pasteurianus}) has a pH optimum between 6 and 7. Therefore we have chosen pH 6.2 for all experiments. Due to the limited availability of the enzyme, reaction volumes were minimised to 500 µl. Enzymatic reactions were performed in Eppendorf reaction vessels at 25 °C in a thermostated shaker (Eppendorf Thermomixer Compact). Stock solutions of APA and HPGM were prepared by adding the required amounts of APA and HPGM to demineralised water. Solutions of the additives were adjusted to pH 6.2 with either 0.5 M KOH, 1 M HCl or ammonia solution (25% solution). Experiments were started by adding 200 µl AEH solution to 50 µl additive solution and 250 µl APA/HPGM stock solution. The pH was checked regularly during the reaction and the pH was adjusted to pH 6-6.4 if necessary by manually adding solid HPGM. During the course of the reaction (normally 1 h) 40 µl samples were taken. The enzymatic reaction in the samples was stopped by dilution of the samples in 960 µl solution of 5.12 g K₂HPO₄.3H₂O, 2.96 g KH₂PO₄ in 6.4 l water and 3.2 l acetonitrile. A blank reaction
was run in which AEH solution was replaced by water to determine chemical hydrolysis of HPGM.

Enzymatic Amox hydrolysis was measured as described for the synthesis reaction except a saturated Amox solution, which was adjusted to pH 6.2 with solid HPGM, was used instead of the APA/HPGM stock solution. Experimental details of all experiments are presented in Tables II and III.

6.3.5 Analysis

Amox, APA, HPGM and HPG were identified and analysed by HPLC using a Waters system with a Zorbax SB-C<sub>18</sub> 4.6×75 mm 3.5-μm column, thermostated at 33 °C, and a Waters 996 PDA detector at 230 nm, a Waters 910 Wisp 10-μl injector, and using a Waters 590 pump with a flow of 1 ml·min<sup>-1</sup>. The eluens was composed of 5% (v/v) MeOH in water and contained 70 mM sodium phosphate buffer of pH 3.

6.3.6 Simulations

Simulations were performed with the program Psi (Boza, Nuenen, The Netherlands), which calculated the amount of moles of HPGM to be added (to control the pH) by iteration at every integration time step.

6.4 Results and discussion

6.4.1 Enzymatic reactions

To investigate the influence of the ionic strength and the presence of different additives, such as PAA, Pen G and inorganic salts on the performance of AEH in the synthesis reaction of Amox, different conditions have been tested (see Table II). Examples of results from typical experiments for the enzymatic synthesis of Amox in the absence of any additive (experiment 1) and in the presence of 25 mM PAA (experiment 11) are given in Figure 4.
Figure 4  Example of two Amox synthesis reactions at 25 °C: A. Synthesis of Amox without any additives (Table II, experiment 9), B. Synthesis of Amox in the presence of 25 mM PAA (Table II, experiment 11). Symbols: ♦ Amox; ○ HPG; ▲ HPGM; ■ APA. Lines are model predictions.

In the graphs the data points of HPGM are scattered, which was caused by the addition of HPGM to adjust the pH to 6.2. As the volume in which the enzymatic reaction was performed was small (500 µl) due to the limited availability of the enzyme, only very small quantities of solid HPGM had to be added to adjust the pH. This addition of HPGM was performed manually (i.e. batch-wise), causing fluctuations in HPGM concentration, while in the model HPGM is added continuously. Dilution errors are visible in both graphs (at 20 min in Figure 4a and at 50 min in Figure 4b), showing lower concentrations for all components in those samples than can be expected from the concentrations in the other samples. In both experiments, Amox and HPG were formed, while the concentrations of APA and HPGM decreased during the experiment. In the presence of PAA, the amount of synthesis of Amox was significantly lower and more hydrolysis of HPGM occurred than in the absence of PAA. Apparently, the presence of PAA has a negative influence on the performance of AEH, i.e. the S/H ratio. For all experiments acceptable mass balances were obtained (>90%) for the nucleus, but for the side chain no mass balances could be obtained as the amount of HPGM added during the measurements was not determined.

From the results of experiments described in Table II, the rate constants for synthesis ($k_{synth}$) and substrate hydrolysis ($k_{subhydr}$) were determined. Rate constants were calculated using equations 8-9 and the determined production/consumption rates (i.e. Amox production rate for $k_{synth}$ and HPG production rate for $k_{subhydr}$ (corrected for
chemical hydrolysis of HPGM) in the linear part of the reaction (i.e. at least up to 40 min). Values of all rate constants are presented in Table II.

**Table II** Initial conditions of Amox synthesis experiments with 10.1 U/l AEH at pH 6.2 and 25 °C.

<table>
<thead>
<tr>
<th>Exp.</th>
<th>$c_{\text{APA},0}$ (mM)</th>
<th>$c_{\text{HPGM},0}$ (mM)</th>
<th>special condition</th>
<th>acid/base for adjusting initial pH</th>
<th>$I_0$ (mM)</th>
<th>$k_{\text{synth}}$ (10^{-3} l/min U^{-1})</th>
<th>$k_{\text{aldehyde}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>49</td>
<td>48</td>
<td>none</td>
<td>ammonia</td>
<td>47</td>
<td>0.19</td>
<td>4.5</td>
</tr>
<tr>
<td>2</td>
<td>35</td>
<td>38</td>
<td>half amount of AEH</td>
<td>none</td>
<td>35</td>
<td>0.25</td>
<td>3.9</td>
</tr>
<tr>
<td>3</td>
<td>36</td>
<td>31</td>
<td>none</td>
<td>ammonia</td>
<td>32</td>
<td>0.38</td>
<td>7.0</td>
</tr>
<tr>
<td>4</td>
<td>32</td>
<td>35</td>
<td>none</td>
<td>ammonia</td>
<td>38</td>
<td>0.26</td>
<td>4.3</td>
</tr>
<tr>
<td>5</td>
<td>39</td>
<td>36</td>
<td>none</td>
<td>ammonia</td>
<td>44</td>
<td>0.17</td>
<td>5.4</td>
</tr>
<tr>
<td>6</td>
<td>50</td>
<td>14</td>
<td>ammonia</td>
<td>ammonia</td>
<td>47</td>
<td>0.26</td>
<td>3.6</td>
</tr>
<tr>
<td>7</td>
<td>93</td>
<td>15</td>
<td>ammonia</td>
<td>ammonia</td>
<td>52</td>
<td>0.11</td>
<td>2.2</td>
</tr>
<tr>
<td>8</td>
<td>50</td>
<td>48</td>
<td>2.5 mM PAA</td>
<td>ammonia</td>
<td>49</td>
<td>0.19</td>
<td>4.1</td>
</tr>
<tr>
<td>9</td>
<td>45</td>
<td>44</td>
<td>5 mM PAA</td>
<td>ammonia</td>
<td>46</td>
<td>0.20</td>
<td>5.2</td>
</tr>
<tr>
<td>10</td>
<td>46</td>
<td>44</td>
<td>25 mM PAA</td>
<td>ammonia</td>
<td>56</td>
<td>0.12</td>
<td>5.8</td>
</tr>
<tr>
<td>11</td>
<td>51</td>
<td>49</td>
<td>25 mM PAA</td>
<td>KOH</td>
<td>61</td>
<td>0.12</td>
<td>5.9</td>
</tr>
<tr>
<td>12</td>
<td>80</td>
<td>79</td>
<td>50 mM PAA</td>
<td>ammonia</td>
<td>102</td>
<td>0.04</td>
<td>4.4</td>
</tr>
<tr>
<td>13</td>
<td>56</td>
<td>51</td>
<td>5 mM Pen G</td>
<td>KOH</td>
<td>54</td>
<td>0.13</td>
<td>4.6</td>
</tr>
<tr>
<td>14</td>
<td>61</td>
<td>48</td>
<td>25 mM Pen G</td>
<td>KOH</td>
<td>65</td>
<td>0.10</td>
<td>5.5</td>
</tr>
<tr>
<td>15</td>
<td>51</td>
<td>46</td>
<td>50 mM KH₂PO₄/K₂HPO₄</td>
<td>none</td>
<td>87</td>
<td>0.16</td>
<td>8.5</td>
</tr>
<tr>
<td>16</td>
<td>51</td>
<td>50</td>
<td>50 mM NaCl</td>
<td>none</td>
<td>99</td>
<td>0.17</td>
<td>6.4</td>
</tr>
<tr>
<td>17</td>
<td>50</td>
<td>49</td>
<td>25 mM (NH₄)₂SO₄</td>
<td>ammonia</td>
<td>111</td>
<td>0.08</td>
<td>6.6</td>
</tr>
</tbody>
</table>

The ionic strength was calculated using the following equation:

$$ I = \frac{1}{2} \cdot \sum m_i \cdot (z_i)^2 $$

$$ = \frac{1}{2} \cdot (c_{\text{APA}} \cdot (-1)^2 + c_{\text{HPGM}} \cdot (1)^2 + c_{\text{Amox}} \cdot (-1)^2 + c_{\text{HPG}} \cdot (-1)^2 + c_{\text{add}} \cdot (-z)^2 + c_{\text{add}} \cdot (z)^2) $$

in which $m_i$ is the molality and $z_i$ is the charge of component $i$. In this equation add can be the additives such as PAA, Pen G and the inorganic salt. If acid or base was
added to adjust the initial pH another term was added to equation 13 (c_{inorganic}^- or c_{inorganic}^+).

To check for possible effects of the enzyme concentration (e.g. multimer formation of the enzyme) and enzyme inactivation on the enzymatic reactions performed by AEH, an experiment was performed using only half the normal amount of AEH solution (experiment 2). The Amox and HPG production rates in this experiment were halved, excluding such complications. In experiments 3 to 7, the ionic strength during the reaction was increased by changing the initial amounts of APA and HPGM. Generally, \( k_{synth} \) decreased upon increasing ionic strength, while \( k_{subhyd} \) seemed more or less constant. The results of the experiments in the presence of PAA and Pen G (experiments 8 to 14), showed that an increase of the concentration of PAA and Pen G caused a decrease of \( k_{synth} \) (also in these experiments \( k_{synth} \) decreases as the ionic strength increases, while \( k_{subhyd} \) seems more or less constant). Apparently, in these experiments the synthesis reaction of Amox is slower, resulting in more HPGM available for substrate hydrolysis. The experiments in the presence of the inorganic salts (experiments 15 to 17), however, show different results. Only in the presence of \((NH_4)_2SO_4\) \( k_{synth} \) is decreased, while for the other salts this rate constant is comparable to the rate constant of the base case experiment (experiment 1) although the ionic strength in all three experiments is much higher than in the base case. For all the tested inorganic salts \( k_{subhyd} \) seems slightly increased.

![Graph A](image)

**Figure 5** \( k_{synth} \) and \( k_{synth}/k_{subhyd} \) as a function of ionic strength at 25 °C. Symbols: ♦ Data from experiments 1-14; △ potassium phosphate; ◊ NaCl; X \((NH_4)_2SO_4\). The lines are exponential regression lines through the data from experiments 1-14, \( k_{synth} = 0.71 e^{-29.4} (R^2=0.83) \), \( k_{synth}/k_{subhyd} = 165 e^{-30.4} (R^2=0.81) \).
In Figure 5A, $k_{\text{synth}}$, calculated from initial rate experiments, is given as a function of the initial ionic strength in the reaction vessel. As the results of $k_{\text{subhyd}}$ (not shown) showed more scattered results, $k_{\text{synth}}/k_{\text{subhyd}}$ is given as a function of ionic strength (Figure 5B). From graphs 5A and 5B follows that especially $k_{\text{synth}}$ is affected (Figure 5A), whereas $k_{\text{subhyd}}$ is not significantly influenced by the ionic strength. Apparently at low ionic strength the synthesis reaction is favoured to the substrate hydrolysis. In experiments 1-14 mainly organic ions contribute to the ionic strength and these seem to have a similar effect on the enzyme. The inorganic salts seem to influence AEH in a different way. Kato et al. (1980) also found that by decreasing the ionic strength by omitting phosphate buffer in the experiments, synthesis of Amox by AEH from X. citri could be improved. They found that both NaCl and phosphate inhibit AEH in the synthesis reaction of Amox. This is also visible in our results: for both $k_{\text{synth}}$ and $k_{\text{subhyd}}$, the results of the inorganic salts NaCl, (NH$_4$)$_2$SO$_4$ and phosphate are not following the general correlation which was observed for PAA and Pen G. Apparently, the nature of the salts that contribute to the ionic strength influences the enzyme kinetics. Another reason for the deviation with the inorganic salts may be that the ionic strength was relatively high in those cases. According to Kasche et al. (1984), ionic strength influences the association reaction between oppositely charged reactants and ionic residues in the active site of the enzyme. The origin of the influence of the ionic strength on the selectivity of AEH is not known and ionic strength is only a provisional explanation of the observed effects. Therefore, an exponential regression line was fitted through the experimental data of all experiments except the experiments with inorganic salts. As mentioned in the Introduction, it is very attractive to reduce the salt production in the biotransformation of Pen G to Amox. Replacement of inorganic base by HPGM to adjust the pH would not only reduce the salt production, but also improve the synthesis reaction as the ionic strength will be lower than in the presence of those extra inorganic salts (Kasche, 1996).

The rate of Amox hydrolysis by AEH was studied as well (see Table III). The rate constant for product hydrolysis was determined using the measured decrease in Amox concentration and equation 10. The value of $k_{\text{prodyh}}$ in Table III shows that AEH is capable of hydrolyzing Amox, but the rate of this enzymatic reaction is very low. Based on these two experiments, $k_{\text{prodyh}}$ seems to be lower at higher ionic strength than at low ionic strength. Because $k_{\text{prodyh}}$ is much smaller than $k_{\text{subhyd}}$ we did not study this phenomenon in further detail. For penicillin acylase, $k_{\text{prodyh}}$ equaled $k_{\text{subhyd}}$ (Diender et al., 2000).
Table III  *Initial conditions of Amox hydrolysis experiments with 10.1 U/l AEH at pH 6.2 and 25 °C.*

<table>
<thead>
<tr>
<th>Exp.</th>
<th>(c_{Amox,0}) (mM)</th>
<th>special condition</th>
<th>acid/base for adjusting initial pH</th>
<th>(I_0) (mM)</th>
<th>(k_{prod-hyd}) (10^3 l-min^-1·U^-1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>18</td>
<td>4.4</td>
<td></td>
<td>HPGM</td>
<td>2</td>
<td>0.92</td>
</tr>
<tr>
<td>19</td>
<td>4.4</td>
<td>25 mM PAA</td>
<td>HCl+HPGM</td>
<td>27</td>
<td>0.30</td>
</tr>
</tbody>
</table>

6.4.2 Simulations

In the model \(k_{synth}\) and \(k_{sub-hyd}\) were calculated with the equations for the exponential regression lines and the calculated ionic strength and \(k_{prod-hyd}\) with the value of 0.92·10^3 l·min^-1·U^-1. Results of the simulations of the concentrations and the ionic strength during experiments 1 and 11 are shown in Figure 4. Simulations of the other experiments are comparable to these examples. As can be seen in Figure 4, the model describes the concentrations during the reaction quite well. During the experiments, the decrease of ionic strength was equal to the decrease of the concentrations of APA and HPGM. For an independent check on the model, a full conversion was carried out (see Figure 6A).

![Figure 6](image)

**Figure 6**  *Course of full conversion reaction for the synthesis of Amox starting from 47 mM APA and 52 mM HPGM with AEH at pH 6.2 and 25 °C using HPGM for titration. A. Original data. B. Predicted data are corrected for a dilution error. Symbols: ♦ Amox; ○ HPG; ▲ HPGM; ■ APA. Lines through the data points are model predictions.*
The yield of Amox on APA was 81% in this experiment. The model predicts some concentrations (APA and HPGM) quite well, whereas for other concentrations (Amox and HPG) only the trends are predicted. A dilution error may have occurred in the first sample (i.e. at the start of the experiment). As this sample provided the initial concentrations of APA and HPGM in the simulations, these concentrations might be too high or low, and in this way lead to wrong predictions of the concentration profiles for Amox and HPGM. If both initial concentrations of APA and HPGM would be only 90% of the measured value due to a dilution error (like at 275 min), the model would predict the concentrations during the reaction much better (Figure 6B).

As this experiment shows, the course of the enzymatic synthesis of Amox in the absence of inorganic salts can be described reasonably well by the model. A high ionic strength decreases the synthesis of Amox and increases the hydrolysis of HPGM. The model can be used to find optimal conditions for Amox production. In Figure 7 is shown that using KOH instead of HPGM to control the pH is negative for the yield.

![Graph showing the course of full conversion reaction for the synthesis of Amox starting from 47 mM APA and 86 mM HPGM with AEH at pH 6.2 and 25 °C using KOH for titration.](image)

**Figure 7** Prediction of course of full conversion reaction for the synthesis of Amox starting from 47 mM APA and 86 mM HPGM with AEH at pH 6.2 and 25 °C using KOH for titration.

The simulations in this graph are at the same conditions as used for Figure 6B, but KOH is used for titration instead of HPGM and the total amount of HPGM is added initially in one portion. When controlling the pH with HPGM higher yields are calculated (88% based on APA and 48% based on HPGM) than by using KOH (53% based on APA, 29% based on HPGM). In the latter case, the ionic strength is initially higher and remains so during the conversion. Feeding of HPGM and thereby
controlling the pH is therefore an advantageous option to achieve higher yields of Amox. In combination with the model for Pen G hydrolysis in two-phase water/organic solvent systems (Diender et al., submitted) optimal conditions can be found for the shortcut route from Pen G to Amox (see Figure 3).

6.5 Conclusions

Amox can be synthesised from APA and HPGM with the enzyme α-amino acid ester hydrolase (AEH) from *Acetobacter pasteurianus* ATCC 9325. The influence of the ionic strength, due to the presence of reactants, on the performance of AEH has been studied. The rate constant for the synthesis reaction, $k_{\text{synth}}$, is affected by the ionic strength, whereas the rate constant for HPGM hydrolysis, $k_{\text{subhyd}}$, is not significantly influenced. The influence of other molecules than the substrate and product, e.g. inorganic salts for pH adjustment or contaminants such as Pen G and PAA, on the performance of AEH has been studied as well. The influence of Pen G and PAA was similar and correlated to the ionic strength. The concentrations of these compounds should be kept as low as possible (< 5 mM). The influence of the studied inorganic salts (potassium phosphate, NaCl and (NH₄)₂SO₄) gave more scattered results. A quantitative kinetic model was based on enzymatic conversion rates (described as a function of ionic strength), pH-dependent solubilities and stoichiometric, macroscopic and charge balances. The model predicted the concentrations during the course of the experiments in the absence and presence of PAA and Pen G reasonably well. At low ionic strength higher yields of Amox will be obtained, while at high ionic strength the unwanted hydrolysis of HPGM is favoured over synthesis. Therefore, the synthesis reaction of Amox should be done preferably in the absence of PAA and Pen G and by feeding HPGM instead of an inorganic base for pH control.

6.6 Acknowledgements

Celine Mulder from DSM (Delft) is acknowledged for her assistance with the experiments.
### 6.7 Notation

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
<th>Unit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amox</td>
<td>amoxicillin</td>
<td></td>
</tr>
<tr>
<td>APA</td>
<td>6-aminopenicillanic acid</td>
<td></td>
</tr>
<tr>
<td>HPG</td>
<td>D-(p-)hydroxyphenylglycine</td>
<td></td>
</tr>
<tr>
<td>HPGM</td>
<td>D-(p-)hydroxyphenylglycine methyl ester</td>
<td></td>
</tr>
<tr>
<td>MeOH</td>
<td>methanol</td>
<td></td>
</tr>
<tr>
<td>PAA</td>
<td>phenylacetic acid</td>
<td></td>
</tr>
<tr>
<td>Pen G</td>
<td>penicillin G</td>
<td></td>
</tr>
</tbody>
</table>

- \( c \) dissolved concentration \( (\text{mol}\cdot\text{L}^{-1}) \)
- \( F \) fraction \( (-) \)
- \( I \) ionic strength \( (\text{mol}\cdot\text{L}^{-1}) \)
- \( k \) rate constant \( (\text{mol}^{-1} \cdot \text{L}^{2} \cdot \text{s}^{-1} \cdot \text{U}^{-1} \text{ or } \text{L}^{-1} \cdot \text{s}^{-1} \cdot \text{U}^{-1}) \)
- \( K_a \) dissociation constant \( (\text{mol}\cdot\text{L}^{-1}) \)
- \( m \) molality \( (\text{mol}\cdot\text{kg}^{-1}) \)
- \( n \) total amount of moles \( (\text{mol}) \)
- \( r \) synthesis or hydrolysis rate \( (\text{mol} \cdot \text{L}^{-1} \cdot \text{s}^{-1}) \)
- \( S \) solubility \( (\text{mol}\cdot\text{L}^{-1}) \)
- \( t \) time \( (\text{s}) \)
- \( V \) volume \( (\text{l}) \)
- \( z \) charge \( (-) \)

*Sub- or superscript*

- 0 initial
- ad added
- synth synthesis
- subhydr substrate hydrolysis
- prodhydr product hydrolysis

### 6.8 References


95
Feasibility of a one-pot shortcut route from penicillin G to amoxicillin in anhydrous organic solvent

Abstract
A new one-pot shortcut route for the biotransformation of penicillin G into amoxicillin in anhydrous organic solvent is presented. In this shortcut route, penicillin G is transamidated with a catalytic amount of ammonia to form phenylacetamide and 6-aminopenicillanic acid. In a second, simultaneous reaction, 6-aminopenicillanic acid is coupled to hydroxyphenylglycine amide to produce amoxicillin and to regenerate ammonia. In the absence of water, no enzymatic hydrolysis of the activated side chain can occur, and a stoichiometric conversion of penicillin G to amoxicillin might be achievable. A quantitative model is presented for the prediction of the equilibrium conversion of penicillin G to amoxicillin via this one-pot shortcut route in anhydrous organic solvent. A thermodynamical cycle was designed to calculate the yield. The model predicted a very attractive yield of 99.2% yield of amoxicillin in anhydrous butyl acetate starting from 0.1 mol·kg\(^{-1}\) penicillin G, 0.1 mol·kg\(^{-1}\) hydroxyphenylglycine amide and 0.001 mol·kg\(^{-1}\) ammonia. Activity measurements showed that cross-linked enzyme of penicillin acylase remains active in anhydrous butyl acetate during 1 day, but does not catalyse the synthesis of amoxicillin. Calculations show that enzymatic reaction rates in anhydrous butyl acetate are probably too slow due to low solubilities of the substrates. Therefore, the one-pot shortcut route is not feasible in anhydrous butyl acetate, but the situation might be different in other organic solvents.

This chapter was submitted for publication.
7.1 Introduction

Previously it has been described how a two-step enzymatic shortcut route from penicillin G (Pen G) to amoxicillin (Amox) can be performed (Diender et al.\(^1\), submitted; Diender et al.\(^2\), submitted). After fermentation, Pen G is extracted from the fermentation broth by an organic solvent, e.g. butyl acetate or amyl acetate. The extracted Pen G in the organic phase can be directly added to an enzymatic reactor (containing an aqueous phase with the enzyme penicillin acylase) and can be hydrolysed efficiently in the resulting two-liquid-phase system by extractive catalysis. One of the products of this hydrolysis reaction, phenylacetic acid (PAA), is extracted to the organic phase (depending on the pH), while the second product, 6-aminopenicillanic acid (APA) stays in the aqueous phase. A convenient pH control agent is hydroxyphenylglycine methyl ester (HPGM), which acts as a base in the aqueous phase. This results in an aqueous phase containing APA and HPGM (Diender et al.\(^1\), submitted).

By adding an additional amount of activated side chain (e.g. HPGM) and the enzyme α-amino acid ester hydrolase to the aqueous phase, the successive enzymatic reaction can be performed in which Amox is formed. Advantages of this shortcut route are 1. the number of process steps can be reduced; 2. APA formed in the hydrolysis reaction does not need to be crystallised inbetween the enzymatic reactions, which decreases the losses of APA during downstream processing; 3. a reduction in salt production can be achieved as the number of pH shifts (for the enzymatic reactions and crystallisation steps) can be decreased (Diender et al.\(^2\), submitted). A disadvantage, however, is that part of the activated side chain is hydrolysed enzymatically in the aqueous environment. An important parameter in the kinetically controlled reactions, such as the Amox synthesis reaction, is the so-called synthesis/hydrolysis ratio \((S/H\), the molar ratio of produced Amox and the hydrolysed side-chain donor\). The \(S/H\) ratio is influenced by process conditions, e.g. pH, temperature and ionic strength (Kasche, 1986). The side product hydroxyphenyl glycine (HPG) may have to be reconverted into HPGM which can be used again for the synthesis reaction.

If the synthesis reaction could be performed in the absence of water, the \(S/H\) ratio would be infinite, as there is no water available for substrate and product hydrolysis. A possible one-pot shortcut route from Pen G to Amox in anhydrous organic solvent may be based on the following reactions:
Pen G + NH$_3$ $\leftrightharpoons$ APA + PA-amide

APA + HPGA $\leftrightharpoons$ Amox + NH$_3$ +

Pen G + HPGA $\leftrightharpoons$ Amox + PA-amide

A solution of Pen G acid is amidated with a catalytic amount of ammonia to form APA and phenylacetamide (PA-amide). Formed APA is coupled to hydroxyphenyl glycine amide (HPGA) in the synthesis reaction of Amox. (Alternatively, phenylglycine amide can be used in the synthesis of ampicillin.) Because APA is used in the second reaction, the first reaction equilibrium is shifted to the product side and reaches a higher extent of conversion. In addition, if the solubility concentration of the products is exceeded, crystallisation of those products might occur. This could also improve the yield considerably. In the second reaction, ammonia is formed which can be used again for the amidation reaction, making it possible to achieve a stoichiometric conversion. As both reactions are performed in anhydrous organic solvent, no activated side chain is hydrolysed. Recycling of the activated side chain is no longer required and an extra reduction in the number of process steps can be achieved.

There are two types of obstacles that might exclude the one-pot shortcut route: thermodynamic reasons (the reaction equilibria are unfavourable) and kinetic reasons (the available enzymes are intrinsically not active and stable, or the solubility of the reactants is too low to obtain reasonable rates).

Numerous examples of enzymatic synthesis reactions in anhydrous organic solvents have been described (e.g. Tawaki and Klibanov, 1993; Partridge et al., 1996; Litjens et al., 1999). So far the reactions of the aforementioned shortcut route have not been described in anhydrous media and we have found no reports of suitable enzyme activity and stability in anhydrous organic solvent. However, the work of some groups on enzyme stabilisation is very promising (Fernandez-Lafuente et al., 1999; Cao et al., 2000; Ebert et al, 1996; Partridge et al., 1996). The aim of this paper is to calculate if the shortcut route is thermodynamically feasible and to check if available enzymes are active at the conditions required for the shortcut route from Pen G to Amox.
7.2 Theory

In order to predict the equilibrium position of the one-pot shortcut route, the equilibrium constants of the contributing reactions will be calculated via thermodynamical cycles. Using a thermodynamical cycle, the equilibrium constant in the organic phase will be calculated from the equilibrium constant in water.

7.2.1 Reaction equilibria in water

The equilibrium of amidation reaction of Pen G in aqueous systems can be described by the apparent equilibrium constant \( K_{\text{eq,app}}^{\text{aq}} \) and the concentrations of the reactants (equation 1).

\[
K_{\text{eq,app}}^{\text{aq}} = \frac{c_{\text{PA-amide}}^{\text{aq}} \cdot c_{\text{APA}}^{\text{aq}}}{c_{\text{PenG}}^{\text{aq}} \cdot c_{\text{NH}_3}^{\text{aq}}} \quad (1)
\]

This equilibrium is a function of pH. The apparent equilibrium constant can be described as a function of a reference reaction constant and the fractions of the reactants at a certain pH. The following reference reaction is chosen:

\[
\text{Pen G}^0 + \text{NH}_3 \rightleftharpoons \text{APA}^{+} + \text{PA-amide}
\]

Then the apparent reaction constant is as follows:

\[
K_{\text{eq,app}}^{\text{aq}} = K_{\text{eq,ref}}^{\text{aq}} \cdot \frac{F_{\text{PenG}^0} \cdot F_{\text{NH}_3}}{F_{\text{APA}^{+}}^{\text{aq}}} \quad (2)
\]

in which \( K_{\text{eq,ref}}^{\text{aq}} \) is the pH-independent reference reaction constant in water and \( F_{\text{PenG}^0}, F_{\text{NH}_3} \) and \( F_{\text{APA}^{+}} \) are the fractions of the uncharged/zwitterionic species of Pen G, ammonia and APA, respectively. The fractions \( F_{\text{PenG}^0}, F_{\text{NH}_3} \) and \( F_{\text{APA}^{+}} \) are calculated from
\[ F_{\text{PenG}^+} = \frac{1}{1 + \frac{K_{a,\text{PenG}}}{c_{H^+}}} \]  

(3)

\[ F_{\text{NH}_3} = \frac{1}{1 + \frac{c_{H^+}}{K_{a,\text{NH}_3}}} \]  

(4)

\[ F_{\text{APA}^{++}} = \frac{1}{1 + \frac{K_{a,\text{APA}}}{c_{H^+} + \frac{c_{H^+}}{K_{a,\text{APA}}}}} \]  

(5)

in which \( c_i \) is the concentration and \( K_{a,i} \) is the dissociation constant of component \( i \) (Tewari and Goldberg, 1988).

As no experimental data are available for the equilibrium of the amidation reaction, it can be calculated from two known sub-reactions, i.e. the hydrolysis of Pen G and the amidation of PAA (see Figure 1, reactions 1a and 1b).

\[
\begin{align*}
\text{Pen G}^0 + \text{H}_2\text{O} & \rightleftharpoons \text{APA}^{++} + \text{PAA}^0 \quad (1a) \\
\text{PAA}^0 + \text{NH}_3 & \rightleftharpoons \text{PA-amide} + \text{H}_2\text{O} + \\
\text{Pen G}^0 + \text{NH}_3 & \rightleftharpoons \text{APA}^{++} + \text{PA-amide} \quad (1)
\end{align*}
\]

\[
\begin{align*}
\text{APA}^{++} + \text{HPG}^{++} & \rightleftharpoons \text{Amox}^{++} + \text{H}_2\text{O} \quad (2a) \\
\text{HPGA}^0 + \text{H}_2\text{O} & \rightleftharpoons \text{HPG}^{++} + \text{NH}_3 + \\
\text{APA}^{++} + \text{HPGA}^0 & \rightleftharpoons \text{Amox}^{++} + \text{NH}_3 \quad (2)
\end{align*}
\]

**Figure 1**  *Schematic overview of reference reactions and sub-reactions of shortcut route.*

The apparent reaction equilibrium constant then is as follows
\[ K_{\text{eq}1,\text{app}} = K_{\text{eq}1,\text{ref}} \cdot K_{\text{eq}1b,\text{ref}} \cdot \frac{F_{\text{PenG}^5} \cdot F_{\text{NH}_3}}{F_{\text{APA}^+}} \] (6)

The second reaction of the shortcut route, the synthesis of Amox from APA and HPGA can also be subdivided into two other reactions, i.e. the hydrolysis of HPGA and the synthesis of Amox from APA and HPG (Figure 1, reactions 2a and 2b). Analogues of equations 1-6 can be derived for these reactions.

### 7.2.2 Partitioning

In two-phase organic/aqueous systems, components will partition between both phases. For weak electrolytes, mainly the uncharged species will partition to the organic phase. The (overall) apparent partition coefficient of a monovalent acid i is defined as

\[ K_{p,i} = \frac{c_{i}^{\text{org}}}{c_{i}^{\text{aq}}} = \frac{c_{i}^{\text{org}}}{c_{i}^{\text{aq}} + c_{i}^{\text{eq}}} \] (7)

The aqueous concentrations in the denominator can be calculated with equations similar to equations 3 to 5 and the measured aqueous concentrations (e.g. \( c_{i}^{\text{eq}} = F_{i} \cdot c_{i}^{\text{eq}} \)). Hence, the partitioning behaviour depends on the pH. This dependence of the apparent partition coefficient \( K_{p,i}^{\text{app}} \) on the pH can be described as follows:

\[ K_{p,i} = K_{p,i}^{\text{ref}} \cdot F_{i}^{\phi} \] (8)

in which \( K_{p,i}^{\text{ref}} \) is the intrinsic (pH-independent) partition coefficient, and \( F_{i}^{\phi} \) is the fraction of the uncharged species of component i, which can be calculated with equations similar to equations 3 to 5.

### 7.2.3 Reaction equilibria in anhydrous organic solvents

When the enzymatic hydrolysis of Pen G is performed in an anhydrous organic solvent the reference equilibrium constant in the organic solvent \( K_{\text{eq},\text{ref}}^{\text{org}} \) can be calculated as follows:

102
From Pen G to Amox, anhydrous system

\[ K_{eq1,ref}^{org} = K_{eq1,ref}^{eq} \cdot \frac{K_{P,APA}^{ref} \cdot K_{P,PA-\text{amide}}^{ref}}{K_{P,PenG}^{ref} \cdot K_{P,NH_3}^{ref}} \quad (9) \]

As there are no charged compounds assumed to be present in the organic solvent the apparent equilibrium constant will be the same as the reference equilibrium constant:

\[ K_{eq1,app}^{org} = \frac{c_{\text{org}}^{APA} \cdot c_{\text{org}}^{PA-\text{amide}}}{c_{\text{org}}^{PenG} \cdot c_{\text{org}}^{NH_3}} = K_{eq1,ref}^{org} \quad (10) \]

Using the values for the reference equilibrium constants of the reactions in water (Table I) and the reference partition coefficients (see Table II), the value of \( K_{eq,app}^{org} \) for both reactions from the shortcut route can be calculated via the thermodynamical cycles.

7.2.4 Crystallisation

If the equilibrium concentration of a reactant in the organic phase exceeds its solubility in the organic solvent, the reactant may be present in the solid phase and dissolve or crystallise during the reaction. This may be beneficial for the yield if one or more of the products crystallises because this will shift the enzymatic reaction equilibria towards the product side. However, when the equilibrium concentration of the substrates is higher than their solubility, the equilibrium is shifted towards the substrate side.

The solubility of a component i in water can be described as a function of pH by the following equation (Diender et al., 2000):

\[ S_i = \frac{S_i^{ref,\text{aq}}}{F_i} \quad (11) \]

in which \( S_i^{ref,\text{aq}} \) is the solubility of the neutral (or zwitterionic) species of component i. As anionic or cationic species are assumed to be absent in an anhydrous organic phase, the solubility in those systems will be equal to the reference solubility in the organic phase (\( S_i^{org} = S_i^{ref,org} \), see Table II).
For each compound a mass balance can be formulated over the organic and solid phase (equation 12)

$$n_i = c_{i}^{org} \cdot V^{org} + n_i^s$$  \hspace{1cm} (12)

in which $n_i$ is the total amount (dissolved and solid) of moles of compound $i$, $n_i^s$ the amount of moles in the solid phase and $V^{org}$ is the volume of the organic phase.

Stoichiometric balances for the nucleus, the side chain, PAA and ammonia are:

$$n_{Amax} = n_{PenG}^0 - n_{PenG} - n_{APA}$$  \hspace{1cm} (13)

$$n_{HPGA} = n_{HPGA}^0 - n_{Amax}$$  \hspace{1cm} (14)

$$n_{PAamide} = n_{PenG}^0 - n_{PenG}$$  \hspace{1cm} (15)

$$n_{NH_3} = n_{NH_3}^0 - n_{PAamide} + n_{HPGA}^0 - n_{HPGA}$$  \hspace{1cm} (16)

in which $n_i^0$ is the initial amount of component $i$.

If the equilibrium concentration of a component is higher than its solubility, the dissolved concentration of compound $i$ ($c_i^{org}$) in equation 10 equals its solubility. Using the initial amounts of Pen G, HPGA and NH$_3$, the mass balances, stoichiometric balances and equation 9, the yield of Amox on Pen G can be calculated.

7.3 Materials and methods

7.3.1 Materials

Penicillin acylase (EC 3.5.1.11) from E. coli is an aqueous suspension of cross-linked enzyme crystals (SynthaCLEC-PA, Altus Biologics Inc., Cambridge, MA, USA). Penicillin G potassium salt (Pen G-K salt, 99.0% pure), D-p-
hydroxyphenylglycine amide (HPGA, 99.0% pure), and 6-aminopenicillanic acid (APA, 98.5% pure) were obtained from DSM (Geleen, The Netherlands). Butyl acetate (BuAc) was obtained from Aldrich (99.7% pure, HPLC-grade). Phenylacetamide (PA-amide) was a kind gift from L. van Langen (Delft University of Technology). BuAc was dried over 3 Å molecular sieves before use.

7.3.2 Solubility measurements

Solubilities of Amox, HPGA, APA and PA-amide in anhydrous BuAc were measured by adding an excess amount of the components to BuAc. This suspension was stirred overnight at 35 °C. Samples were filtered over 0.2-μm Nylaflon® nylon membrane filters (Gelman Sciences). Concentrations in the samples were determined by HPLC.

7.3.3 Enzyme activity

Prior to adding the enzyme suspension to the reaction mixture, the original penicillin acylase suspension was centrifuged for 3 min at 13,000 rpm (MSE MicroCentaur) and washed three times with 1 ml of anhydrous BuAc. After the washing step, the centrifuged penicillin acylase crystals were resuspended in a known amount of anhydrous BuAc.

Enzyme activity of penicillin acylase crystals was determined by the hydrolysis of 0.055 mol·kg⁻¹ PA-amide and 0.050 mol·kg⁻¹ H₂O in 5.0 g anhydrous BuAc. The reaction was started by adding 0.5 g penicillin acylase suspension (0.36 g enzyme·g⁻¹ BuAc) and the mixture was stirred at 35 °C. Samples were taken after 44 h, filtered (0.2-μm Nylaflon® nylon membrane filters, Gelman Sciences) and analysed by HPLC.

7.3.4 Enzymatic reaction

Synthesis reaction of Amox was performed by adding 0.10 mol·kg⁻¹ APA and 0.10 mol·kg⁻¹ HPGA to 3.0 g anhydrous BuAc in a closed, thermojacketed vessel at 35 °C equipped with a top stirrer. The reaction was started by adding 0.40 g penicillin acylase crystals (same pretreatment as described in the previous section). After 5 days a sample of the suspension was taken, dissolved in water and analysed by HPLC.
7.3.5 Analysis

APA, Amox, HPGA, PA-amide and PAA were identified and analyzed by HPLC using a Waters System with a Zorbax SB-C$_{18}$ 4.6×75 mm 3.5-μm column, thermostated at 33 °C, and a Waters 996 PDA UV-detector at 230 nm, a Waters 910 Wisp 10-μl injector, and using a Waters 590 pump with a flow of 1 ml·min$^{-1}$. The eluent was composed of 5% (v/v) MeOH in water and contained 70 mM sodium phosphate buffer of pH 3.

7.3.6 Calculations

Calculations were done with the program EQS4WIN (Mathtrek Systems, USA) which is based on the algorithms of Smith and Missen (1991).

7.4 Results and discussion

As mentioned in the introduction, it would be very interesting to perform the one-pot shortcut route in anhydrous organic solvent, as unwanted hydrolysis of the activated side chain and the product is not possible in those systems. It might be possible to achieve a stoichiometric conversion of Pen G into Amox. Based on already known or measured thermodynamic reaction equilibria, reference solubilities and reference partition coefficients the feasibility of the shortcut route in anhydrous organic solvent was studied. The shortcut route was studied using BuAc as organic solvent, because for this solvent already many data were available (see Diender et al. 1, submitted).

7.4.1 Enzyme activity

To check the activity of cross-linked enzyme crystals of penicillin acylase in anhydrous BuAc, the hydrolysis of PA-amide was measured. The amount of water added was only 9% of the water solubility and probably increased the water activity only slightly. After 44 h 37% of PA-amide was converted into PAA and NH$_3$, which is still far from equilibrium according to the equilibrium constant for this reaction (see Table I). Nevertheless, this enzyme apparently is still active under these conditions. The enzyme used showed no hydrolysis of BuAc.
7.4.2 Reference reaction equilibrium constants

Reference reaction equilibrium constants in aqueous systems for Pen G hydrolysis (reaction 1a) and the thermodynamically controlled synthesis of Amox (reaction 2a) were calculated from published data (see Table I) and using equations 2-5.

Table I  Values of reference equilibrium constants of sub-reactions.

<table>
<thead>
<tr>
<th>Reaction</th>
<th>$K_{eq,\text{ref}}^{\text{aq}}$</th>
<th>$K_{eq,\text{ref}}^{\text{org}}$</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1a</td>
<td>0.454</td>
<td>(-)</td>
<td>Tewari and Goldberg (1988)</td>
</tr>
<tr>
<td>1b</td>
<td></td>
<td>0.015$^b$</td>
<td>Litjens et al. (1999)</td>
</tr>
<tr>
<td>2a</td>
<td>68.4</td>
<td></td>
<td>Diender et al. (1998a)</td>
</tr>
<tr>
<td>2b</td>
<td>28.8</td>
<td></td>
<td>Ulijn et al. (submitted)</td>
</tr>
</tbody>
</table>

$^a$ Calculated from published values from mentioned reference.

$^b$ This value is determined from experimental work in MIBK, and is not corrected for the reference partitioning of the reactants.

For the amidation reaction of PAA (reaction 1b), no (apparent) reaction equilibrium constant in aqueous system was found. However, from data from Litjens et al. (1999), a reference reaction equilibrium constant was determined for this reaction in methyl isobutyl ketone (MIBK). For esterification reactions in organic solvent, the type of organic solvent does not have a large influence on the reference reaction equilibrium constant (Valivety et al., 1991; Van Tol et al., 1995; Tewari et al., 1995). For simplicity, we assume that this is also valid for the amidation reaction. As the determined reference equilibrium constant is the equilibrium constant for the organic phase instead of the aqueous phase, the following equation gave the overall equilibrium constant for reaction 1:

$$K_{eq,\text{app}}^{\text{org}} = K_{eq,\text{ref}1a}^{\text{aq}} \cdot \frac{K_{p,\text{APA}}^{\text{ref}} \cdot K_{p,\text{PAA}}^{\text{ref}}}{K_{p,\text{PenG}}^{\text{ref}} \cdot K_{p,\text{H}_2\text{O}}^{\text{ref}}} \cdot K_{eq,\text{ref}1b}^{\text{org}} = 0.015$$  (17)

For the hydrolysis reaction of HPGA (reaction 2b) no equilibrium data have been reported. We have tried to measure the reaction equilibrium in water starting from 100 mM HPG and 90 mM NH$_4$Cl at pH 6.2 and 25 °C, but the equilibrium position was
extremely towards the HPG and NH₃ side, as no synthesis of HPGA could be detected \((K_{eq,2b,\text{ref}} > 0.1)\). Ulijn et al. (submitted) reported an average value for the reference equilibrium constant for the synthesis of amide bonds or related compounds \((\log K_{eq,\text{ref}} = 3.8)\). Their value is based on unionised species. We have used this value to calculate the equilibrium constant for our reference reaction 2b (see Table I) with analogues of equations 2-5. This led to a value of \(K_{eq,2,\text{ref}}^{\text{rel}} = 3.15 \cdot 10^5\).

7.4.3 Reference solubilities

Solubilities of the components in aqueous systems were found in literature (see Table II). Solubilities of the components in organic solvent, necessary for the calculations, were measured in anhydrous BuAc. Results of these solubility measurements are shown in Table II.

Table II  Dissociation constants, reference solubilities in water and anhydrous BuAc, and reference partition coefficients of the components used in the calculations.

<table>
<thead>
<tr>
<th>Compound</th>
<th>(pK_a)</th>
<th>(S_{p,\text{ref}}^{\text{rel}})</th>
<th>(S_{i,\text{ref}}^{\text{rel}})</th>
<th>(K_{p,i}^{\text{ref}})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pen G</td>
<td>2.5/9.0(^a)</td>
<td>(mol·kg(^{-1}))</td>
<td>(mol·kg(^{-1}))</td>
<td>47(^d)</td>
</tr>
<tr>
<td>APA</td>
<td>2.5/4.9(^a)</td>
<td>8·10(^3)(^b)</td>
<td>1.2·10(^4)(^c)</td>
<td>10(^3)(^d)</td>
</tr>
<tr>
<td>PA-amide</td>
<td></td>
<td>3.7·10(^2)(^c)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PAA</td>
<td>4.3</td>
<td></td>
<td></td>
<td>29(^d)</td>
</tr>
<tr>
<td>NH₃</td>
<td>9.2(^f)</td>
<td></td>
<td></td>
<td>3.2·10(^{-2})(^e)</td>
</tr>
<tr>
<td>HPGA</td>
<td>7.0(^c)</td>
<td>1.6·10(^2)(^c)</td>
<td>3·10(^{-5})(^c)</td>
<td>2·10(^{-3})(^h)</td>
</tr>
<tr>
<td>Amox</td>
<td>2.9/7.4(^a)</td>
<td>9.1·10(^{-3})(^b)</td>
<td>8·10(^{-5})(^c)</td>
<td>1·10(^{-2})(^h)</td>
</tr>
<tr>
<td>H₂O</td>
<td>55</td>
<td>0.585(^8)</td>
<td></td>
<td>1.1·10(^{-2})(^h)</td>
</tr>
</tbody>
</table>

\(^a\) Diender et al. (1998b), \(^b\) Diender et al. (2000), \(^c\) Measured in this study, \(^d\) Diender et al. (Submitted), \(^e\) Leo et al. (1971), \(^f\) Weast et al. (1989), \(^g\) Riddick et al. (1986), \(^h\) values were calculated:

\[
K_{p,i}^{\text{ref}} = \frac{S_{i,\text{ref}}^{\text{rel}}}{S_{p,\text{ref}}^{\text{rel}}}
\]

As can be seen from the results solubilities of most components of the shortcut route are very low (except for PA-amide they are less than 1 mmol·kg\(^{-1}\)). Undissociated Pen G acid is very soluble in BuAc, therefore its solubility in BuAc was not determined. The model does not take into account that ion pairs or precipitating salts of cationic
ammonia or HPGA and anionic Pen G may be formed. In a previous study (Diender et al., submitted) model predictions were correct when leaving out such salts.

7.4.4 Reference partition coefficients

Reference partition coefficients were taken from literature or estimated from the solubilities in aqueous and organic solvents ($K_{p,i}^{\text{ref}} = S_i^{\text{ref,org}} / S_i^{\text{ref,aq}}$). Data for the reference partition coefficients are shown in Table II. No data were available for ammonia in BuAc, but for partitioning of NH₃ in diethyl ether ($\log K_p^{\text{ref}} = -1.96$) and for sec-butanol ($\log K_p^{\text{ref}} = -1.09$) have been published (Leo et al., 1971). For simplicity, we assumed that the value for NH₃ in BuAc is in between the values obtained for these two organic solvents as the hydrophobicity and polarity of BuAc is in between those of both organic solvents.

7.4.5 Predictions

The yield of Pen G to Amox via the one-pot shortcut route can be calculated as a function of the initial amounts of the substrates and based on the values from Table I and II. In Table III, calculated yields are given for the overall shortcut route, starting from 0.1 mol·kg⁻¹ of Pen G, 0.1 mol·kg⁻¹ of HPGA and 0.001 mol·kg⁻¹ of NH₃, and for the two separate reactions, starting from 0.1 mol·kg⁻¹ of each substrate.

As can be seen in these results a high yield of Amox (99.2%) might be achieved with the one-pot shortcut route. For the separate synthesis reaction from APA and HPGA even a 100% yield may be achieved, while for the separate amidation of Pen G a yield of only 18% is calculated. Apparently, the synthesis reaction in combination with the crystallisation of the products of this reaction pulls the thermodynamic equilibrium of the amidation of Pen G completely towards the product side.

To get a better insight in the model, in particular the influence of the parameters on the thermodynamical feasibility of the shortcut route in anhydrous BuAc, the values of the reference equilibrium constants in organic solvents were increased or decreased by a factor 100. Results of those changes on the equilibrium yields are shown in Table III. If $K_{\text{eq},\text{ref}}^{\text{org}}$ would be a factor 100 higher than the calculated value, the yield of the separate amidation reaction would increase to 88.5%, which would improve the yield of the shortcut route from 99.2 to 100%. If $K_{\text{eq},\text{ref}}^{\text{org}}$ would be a factor 100 lower, the overall yield of the shortcut route would
decrease to 32%, due to the more unfavourable equilibrium position of the amidation reaction.

Table III  Calculated equilibrium yields for reaction 1 and 2 and overall shortcut route. Several parameters have been changed in the model to test the sensitivity.

<table>
<thead>
<tr>
<th>Changed parameter</th>
<th>Yield* (%)</th>
<th>Yield* (%)</th>
<th>Yield* (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Reaction 1 (0.1 M Pen G + 0.1 M NH₃)</td>
<td>Reaction 2 (0.1 M APA+0.1 M HPGA)</td>
<td>Overall shortcut route (0.1 M Pen G + 0.1 M HPGA + 0.001 M NH₃)</td>
</tr>
<tr>
<td>None (base case)</td>
<td>18.0</td>
<td>100.0</td>
<td>99.2</td>
</tr>
<tr>
<td>100·(K_{\text{org}}^{\text{eq}1,\text{ref}})</td>
<td>88.5</td>
<td>100.0</td>
<td>100.0</td>
</tr>
<tr>
<td>0.01·(K_{\text{org}}^{\text{eq}1,\text{ref}})</td>
<td>0.3</td>
<td>100.0</td>
<td>32.0</td>
</tr>
<tr>
<td>100·(K_{\text{org}}^{\text{eq}2,\text{ref}})</td>
<td>18.3</td>
<td>100.0</td>
<td>100.0</td>
</tr>
<tr>
<td>0.01·(K_{\text{org}}^{\text{eq}2,\text{ref}})</td>
<td>18.0</td>
<td>99.9</td>
<td>31.4</td>
</tr>
<tr>
<td>100·S(^b)</td>
<td>1.9</td>
<td>99.9</td>
<td>97.8</td>
</tr>
<tr>
<td>0.01·S(^b)</td>
<td>98.9</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

* Yield is calculated as \(n_{\text{APA,final}}/n_{\text{PenG,initial}}\) (for reaction 1), \(n_{\text{Amox,final}}/n_{\text{APA,initial}}\) (for reaction 2) and \(n_{\text{Amox,final}}/n_{\text{PenG,initial}}\) (overall reaction).

\(^b\) Solubilities of all components in the organic phase are increased or decreased by a factor 100.

Increasing \(K_{\text{org}}^{\text{eq}2,\text{ref}}\) with a factor of 100 would also increase the yield of the shortcut route from 99.2% to 100%. A decrease of the reference equilibrium constant of the synthesis reaction however, would decrease the yield of the shortcut route to 31.4 %, while the separate synthesis reaction still has a yield of 99.9%. Apparently, the driving force which is exerted by the synthesis reaction on the amidation reaction is decreased considerably. Increasing the catalytic amount of NH₃ would not improve the yield, as the concentration of NH₃ is not present in the overall apparent equilibrium constant:

\[
K_{\text{org,app}}^{\text{eq}} = \frac{C_{\text{Amox}}^{\text{org}} \cdot C_{\text{PA-amide}}^{\text{org}}}{C_{\text{PenG}}^{\text{org}} \cdot C_{\text{HPGA}}^{\text{org}}}
\]  

(18)
An increase or decrease of the solubilities of all reactants by a factor 100 does not have much influence on the equilibrium yields which can achieved via the shortcut route.

As the model predicts conversions based only on thermodynamic equilibria, it does not include any enzyme kinetics. Although the shortcut route may be feasible from a thermodynamic point of view, kinetically it may still be problematic as enzymes are mostly very slow in anhydrous organic solvents. Therefore, we have tested the synthesis of Amox from APA and HPGA in anhydrous BuAc at 35 °C, as this separate reaction gave the best results in the calculations and the experiment was the easiest one to perform. After 5 days, no synthesis of Amox was detected. This might be caused by kinetic limitations of the enzyme or by low dissolution rates of APA and HPGA. A too low water activity under the conditions of Amox synthesis may have caused a low enzyme activity. To check whether kinetic limitations of the enzyme or the low dissolution rates of the substrates can explain the obtained result some simple calculations were done. Assuming that the synthesis rate is \( r_{\text{synth}} = k_{\text{synth}} \cdot c_{\text{APA}} \cdot c_{\text{HPGA}} \), and \( k_{\text{synth}} \) is independent of the solvent then:

\[
\frac{r_{\text{org}}}{r_{\text{aq}}} = \frac{S_{\text{APA}}^{\text{org}}}{S_{\text{APA}}^{\text{aq}}} \cdot \frac{S_{\text{HPGA}}^{\text{org}}}{S_{\text{HPGA}}^{\text{aq}}} = \frac{1}{67} \cdot \frac{1}{533} = \frac{1}{35711}
\]  

(19)

If in water the conversion would reach equilibrium in 1 h, then it would take 35711 h (i.e. 4 years), to reach equilibrium in BuAc. Based on these calculations, the shortcut route is not feasible due to slow kinetics of the available enzymes. However, when the solubilities of APA and HPGA in organic solvent would be higher (by choosing another organic solvent), the conversion time could reduce considerably. For example, if the solubilities of APA and HPGA in the organic solvent would be 10\(^{-3}\) mol·kg\(^{-1}\), the time to reach conversion would be 128 h (i.e. 5 days). However, if it is assumed that enzymatic rates are dependent on substrate activities rather than on substrates concentrations (Wolff et al., 1997), no such retardation is expected. The other explanation, kinetic problems due to low dissolution rates was checked as well. In the experiment 0.068 g of APA was added to 3 g of BuAc. These APA crystals have a volume of 4.9·10\(^{-3}\) l (density of APA crystals is 1400 kg·m\(^{-3}\), Cambridge Protein Databank). If we assume the crystals to have a cylinder shape with a length of 20 µm and a diameter of 3.3 µm (these values are average values obtained by visual inspection by microscopy), the volume-specific area of the crystals (\( a \)) is 1.3·10\(^{3}\) m\(^{-2}\).
In the experiment the total area of crystals is then 6.4·10^{-2} \text{ m}^2 (= 4.9·10^{-5}·1.3·10^{3}). The maximal dissolution rate (\(\phi_{\text{max}}\)) can be calculated as follows:

\[
\phi_{\text{max}} = k_l \cdot A \cdot S_{\text{APA}}^\text{or} = 1.5 \cdot 10^{-7} \text{ g s}^{-1} \tag{20}
\]

with \(k_l\) as the estimated mass transfer coefficient (10^{-4} \text{ m s}^{-1}, Wolff et al., 1997). According to these simple calculations 0.064 g APA could have dissolved in 5 days, which is 93% of the initial amount. The amount of dissolved APA is dependent on the crystal size: if the crystals would be 100 \mu m long and have a diameter of 16.7 \mu m, then still 18% of the initial amount could have dissolved. These amounts should have been sufficient for some synthesis of Amox to be detectable in the experiment. Most probably, the dissolution rate of the substrates does not limit the feasibility of the shortcut route.

As shown by the calculations on the feasibility of the one-pot shortcut route, an increase of the solubilities by a factor 100 does not have much influence on the yield that can be achieved (see Table III). However, this increase has a major impact on the enzyme kinetics if the synthesis rate is proportional to the solubilities in the organic solvent (see equation 19). Further research should focus on improving enzyme kinetics and finding a suitable organic solvent in which the solubilities of the reactant are sufficiently low to allow the one-pot shortcut route to be thermodynamically feasible, but also sufficiently high to avoid kinetic limitations of the enzyme. This should include a study to select the proper water activity. The model can be used as a tool for the selection of such a suitable organic solvent.

7.5 Conclusions

A one-pot shortcut route for the biotransformation of Pen G into Amox in anhydrous organic solvent has been presented. In this shortcut route, Pen G is amidated with ammonia to form PA-amide and APA, which can be coupled to HPGA to produce Amox and ammonia. A quantitative model has been presented for the prediction of the conversion of Pen G to Amox via the shortcut route in anhydrous organic solvent. A thermodynamic cycle was designed to calculate the yield. This cycle includes reaction equilibria in water, solubilities of the reactants in water and organic solvent, and partition coefficients of the reactants. The model predicted that a
99.2% yield of Pen G to Amox in anhydrous BuAc might be achieved, starting from 0.1 mol·kg⁻¹ Pen G, 0.1 mol·kg⁻¹ HPGA and 0.001 mol·kg⁻¹ NH₃. For the synthesis reaction of Amox directly starting from 0.1 mol·kg⁻¹ APA and 0.1 mol·kg⁻¹ HPGA, a 100% yield was predicted. This synthesis reaction was tested experimentally, but no synthesis of Amox was observed. Probably this is caused by kinetic limitations of the enzyme under these conditions. Still, penicillin acylase cross-linked enzyme crystals showed activity in the hydrolysis of PA-amide in anhydrous BuAc. The one-pot shortcut route from Pen G in anhydrous BuAc is thermodynamically very attractive, but calculations show that enzymatic reaction rates in anhydrous butyl acetate are probably too slow due to low solubilities of the substrates. Therefore, the one-pot shortcut route is not feasible in anhydrous butyl acetate, but the situation might be different in other organic solvents.

7.6 Acknowledgements

The section Organic Chemistry and Catalysis from Delft University of Technology are kindly acknowledged for providing phenylacetamide.

7.7 Notation

<table>
<thead>
<tr>
<th>Term</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amox</td>
<td>amoxicillin</td>
</tr>
<tr>
<td>APA</td>
<td>6-aminopenicillanic acid</td>
</tr>
<tr>
<td>BuAc</td>
<td>butyl acetate</td>
</tr>
<tr>
<td>HPGA</td>
<td>D-(p-)hydroxyphenylglycine amide</td>
</tr>
<tr>
<td>NH₃</td>
<td>ammonia</td>
</tr>
<tr>
<td>PAA</td>
<td>phenylacetic acid</td>
</tr>
<tr>
<td>PA-amide</td>
<td>phenylacetamide</td>
</tr>
<tr>
<td>Pen G</td>
<td>penicillin G</td>
</tr>
<tr>
<td>a</td>
<td>volume-specific area</td>
</tr>
<tr>
<td>c₁</td>
<td>dissolved concentration</td>
</tr>
<tr>
<td>F</td>
<td>fraction</td>
</tr>
<tr>
<td>k₁</td>
<td>mass transfer coefficient</td>
</tr>
<tr>
<td>Kₐ</td>
<td>dissociation constant</td>
</tr>
</tbody>
</table>

(m²·m⁻³) (mol·l⁻¹) (⋅) (m·s⁻¹) (mol·l⁻¹)
\[ K_{eq} \quad \text{enzymatic equilibrium constant} \quad (\text{mol}^{-1}\text{l}) \]
\[ K_p \quad \text{partition coefficient} \quad (-) \]
\[ n_i \quad \text{total amount of moles} \quad (\text{mol}) \]
\[ S \quad \text{solubility} \quad (\text{mol}^{-1}\text{l}) \]
\[ V \quad \text{volume} \quad (\text{l}) \]

Sub- or superscript

0 \quad \text{initial condition} \\
app \quad \text{apparent} \\
aq \quad \text{aqueous} \\
i \quad \text{compound i} \\
org \quad \text{organic} \\
ref \quad \text{reference} \\

7.8 References


Diender\textsuperscript{1} MB, Straathof AJJ, Van der Does T, Ras C, Heijnen JJ. Equilibrium modelling of extractive enzymatic hydrolysis of penicillin G with concomitant 6-aminopenicillanic acid crystallisation. Submitted.

Diender\textsuperscript{2} MB, Straathof AJJ, Van der Does T, Heijnen JJ. Modeling the influence of ionic strength on \(\alpha\)-amino acid ester hydrolase in the salt-free production of amoxicillin. Submitted.


Tawaki S, Klibanov AM. 1993 Chemoselectivity of enzymes in anhydrous media is strongly solvent dependent. Biocatal 8:3-19.


Ulijn RV, Moore BD, Janssen AEM, Halling PJ. When is precipitation driven biosynthesis feasible? Submitted.


Summary
New process concepts for the enzymatic synthesis of amoxicillin from penicillin G

Traditionally, the conversion of penicillin G (Pen G) into semisynthetic β-lactam antibiotics was only performed chemically. In these chemical processes, much waste is produced. Also, halogenated organic solvents and energy-consuming sub-zero reaction temperatures are used. Nowadays, semisynthetic antibiotics may be produced via biocatalytic processes. These biocatalytic processes are performed in water at ambient or slightly elevated temperatures and less auxiliary chemicals are used, which reduces the waste production. This thesis describes new enzymatic process concepts for the production of the semisynthetic antibiotic amoxicillin (Amox), with the emphasis on the enzymatic coupling reaction of the β-lactam nucleus 6-aminopenicillanic acid (APA) and a side chain. These new processes should lead to a further reduction in waste production.

The simplest strategy for the enzymatic synthesis of Amox is the thermodynamically-controlled synthesis from APA and hydroxyphenylglycine (HPG). The thermodynamic reaction equilibrium of this synthesis was determined in order to study the feasibility of a suspension-to-suspension conversion in aqueous solvent. In suspension-to-suspension conversions, the substrate(s) and the product(s) are mainly present as undissolved particles, whereas the enzymatic reaction takes place in the solvent. In aqueous solution, the synthetic yield of Amox remains lower than the solubility of Amox. Therefore, a suspension-to-suspension synthesis in aqueous solution is not feasible.

Synthetic yields in enzymatic condensation reactions (such as the synthesis reaction of Amox) can often be improved by using organic solvents in monophasic systems. A model was developed for the prediction of reaction equilibria in cosolvent-water mixtures. This model is based on the equilibrium constant in water and the solvent-mixture-dependent apparent dissociation constants of all reactants in those mixtures. According to calculations, addition of organic cosolvents improves the apparent equilibrium constant of amoxicillin synthesis considerably, but probably not the synthetic yield, due to solubility restrictions of the reactants. Therefore, a suspension-to-suspension synthesis of Amox in cosolvent-water mixtures is most probably not feasible either.

A well-known strategy for the synthesis of Amox is the kinetically-controlled reaction in which APA is coupled to an activated side chain such as hydroxyphenylglycine methyl ester (HPGM) or hydroxyphenylglycine amide (HPGA). High yields
on nucleus are possible, but part of the activated side chain is hydrolysed enzymatically and is not coupled to the nucleus. Also, produced Amox can be hydrolysed enzymatically. To suppress these unwanted hydrolysises the reaction can be performed using the suspension-to-suspension concept. In a suspension-to-suspension process different sub-processes take place, such as dissolution and crystallisation of the reactants, enzymatic synthesis of the product and undesired hydrolysis of the substrate(s) and/or product(s). A quantitative model was developed for predicting the pH and concentrations of the reactants during suspension-to-suspension conversions by the enzyme penicillin acylase. This model predicts the course of the concentrations reasonably well and gives excellent insight into the different sub-processes taking place during the suspension-to-suspension process. Therefore, the model can be used as a tool to optimise this type of conversions.

In the present downstream processing of Pen G, Pen G is extracted from the fermentation broth with an organic solvent and is purified as a potassium salt via a number of back-extraction and crystallisation steps. After purification, Pen G is hydrolysed by the enzyme penicillin acylase to APA, which is used for the synthesis of semisynthetic penicillins after several crystallisation and washing steps. In each process step salts are formed due to pH shifts. A two-step shortcut route is presented in which the number of process steps, the losses of APA during downstream processing and the salt formation may be reduced. In the first step of this two-step shortcut route, acidic Pen G in the organic phase after extraction from the fermentation broth is directly added to an aqueous immobilised enzyme suspension and is hydrolysed in an aqueous/organic solvent two-phase system by extractive catalysis.

Conversions higher than 90% were achieved at low pH (3-4). This was not only a result from extraction of phenylacetic acid (PAA), but also from crystallisation of APA. A model was developed to predict the equilibrium conversion of Pen G in this water-organic solvent two-phase batch system. The model incorporates the partitioning behaviour of the reactants, the enzymatic equilibrium and the crystallisation of APA. The model predicted the conversion of Pen G quite reasonably for different initial values of pH, Pen G concentrations and phase volume ratio.

The pH that is required for the reactive extraction can be set by adding HPGM instead of an inorganic base. Then the aqueous phase resulting from the hydrolysis of Pen G in water-organic solvent two-phase system contains mostly APA and HPGM. This can be used directly for the second step of the shortcut route, the synthesis
reaction of Amox, by addition of the enzyme and an additional amount of HPGM. So far, this second step has not been possible, as one of the products from the Pen G hydrolysis reaction, PAA, essentially inhibits the catalytic activity of penicillin acylase for the synthesis of Amox. From literature is known that the enzyme α-amino acid ester hydrolase (AEH) from *Acetobacter pasteurianus* ATCC 9325 is able to catalyze the synthesis reaction in the presence of PAA. Therefore, this enzyme was used to catalyze the synthesis reaction of the shortcut route in the presence of PAA.

The influence of the presence of unextracted PAA and unhydrolysed Pen G on the synthesis of Amox by the enzyme AEH were studied. The influence of PAA and Pen G was similar and correlated to the ionic strength, showing in general a negative effect on the S/H ratio. A quantitative model was developed for predicting the concentrations of the reactants during the synthesis reaction of Amox from APA and HPGM in the presence and absence of PAA and Pen G. The model was based on enzyme kinetics (described as a function of the ionic strength), pH-dependent solubilities, and stoichiometric, mass and charge balances. The model described the synthesis of Amox and the undesired hydrolysis of HPGM reasonably. As decreasing the ionic strength in the reactor will increase the yield of Amox, the synthesis reaction should be done preferably in the absence of PAA and Pen G and by using HPGM instead of an inorganic base for pH control.

Finally, the concept of a new one-pot shortcut route for the biotransformation into Amox in anhydrous organic solvent is presented. In this shortcut route Pen G is amidated with a catalytic amount of ammonia to form phenylacetamide (PA-amide) and APA. In a second, simultaneous reaction this APA is coupled to HPGA to produce Amox and regenerate the ammonia. If water is absent, enzymatic hydrolysis of the activated side chain can not occur, and stoichiometric conversion of Pen G to Amox might be achievable. A quantitative model is presented for the prediction of the equilibrium conversion of Pen G to Amox via this one-pot shortcut route in anhydrous organic solvent. A thermodynamic cycle is formulated in order to calculate the yield in anhydrous organic solvent. The cycle is based on thermodynamic reaction equilibria in aqueous systems and partition coefficients and solubilities of the reactants. Calculations show that the one-pot shortcut route is thermodynamically very attractive, but probably due to kinetical limitations of the enzyme in anhydrous organic solvent not feasible (yet).
Samenvatting
Samenvatting

Nieuwe procesconcepten voor de enzymatische synthese van amoxicilline vanuit penicilline G

Traditioneel werden omzettingen van penicilline G (Pen G) tot semisynthetische β-lactam antibiotica alleen op chemische wijze uitgevoerd. In deze chemische processen wordt veel afval geproduceerd, er wordt gebruik gemaakt van gehalogeneerde organische oplosmiddelen en bovendien zijn temperaturen beneden 0°C noodzakelijk. Tegenwoordig is het mogelijk om semi-synthetische antibiotica te produceren met behulp van biokatalytische processen. Deze biokatalytische processen worden uitgevoerd bij kamertemperatuur of iets verhoogde of verlaagde temperaturen en er worden minder chemicaliën gebruikt, wat de afvalproductie reduceert. In dit proefschrift worden nieuwe procesconcepten beschreven voor de enzymatische produktie van het semi-synthetische antibioticum amoxicilline (Amox), waarbij de nadruk ligt op de enzymatische koppeling van de β-lactam-kern 6-aminopenicillaanzuur (APA) en een zijketen. Deze nieuwe procesconcepten zouden een verdere reductie van de afvalproductie kunnen opleveren.

De meest eenvoudige strategie voor de enzymatische synthese van Amox is de thermodynamisch gecontroleerde synthese vanuit APA en hydroxyphenylglycine (HPG). Het thermodynamische reactie-evenwicht van deze synthese is bepaald om de haalbaarheid van een suspensie-naar-suspensie-omzetting in waterig milieu te onderzoeken. In suspensie-naar-suspensie-omzettingen zijn één of meer van de substraten en één of meer van de produkten voornamelijk aanwezig als onopgeloste deeltjes, terwijl de enzymatische reactie plaatsvindt in het oplosmiddel. In waterig milieu is de evenwichtsconcentratie aan Amox lager dan de oplosbaarheid van Amox, waardoor de suspensie-naar-suspensie-omzetting in waterig milieu niet haalbaar bleek.

In enzymatische condensatie-reacties (zoals de synthese-reactie van Amox) kan de opbrengst vaak verbeterd worden door gebruik te maken van organische oplosmiddelen in éénfase systemen. Er is een model opgesteld om reactie-evenwichten in mengsels van water-mengbare organische oplosmiddelen en water te voorspellen. Dit model is gebaseerd op de reactie-evenwichtsconstante in water en de schijnbare dissociatie-constanten van alle reactanten in dergelijke mengsels. Er is berekend dat het toewijgen van water-mengbare organische oplosmiddelen weliswaar de schijnbare evenwichtsconstante van de synthese van Amox aanzienlijk verbetert, maar waarschijnlijk niet de opbrengst, vanwege beperkingen in de oplosbaarheid van
Samenvatting

de reactanten. Hierdoor is de suspensie-voor-suspensie-omzetting voor de synthese van Amox in mengsels van watermengbare organische oplosmiddelen en water waarschijnlijk evenmin haalbaar.


Omzettingen hoger dan 90% werden behaald bij lage pH (3-4). Dit was niet alleen het resultaat van de extractie van fenylazijnzuur (PAA), maar ook van de
kristallisatie van APA. Er is een model ontwikkeld om de evenwichtsomzetting van Pen G in deze organische oplosmiddel/water batch-systemen te voorspellen. In het model is het partitiedrag van de reactanten, het enzymatische evenwicht en de kristallisatie van APA opgenomen. Het model voorspelde de omzetting van Pen G redelijk goed voor verschillende initiële waarden van de pH, de initiële Pen G concentratie en de fasevolume-ratio.

De pH die nodig is voor de reactieve extractie kan worden ingesteld door het toevoegen van HPGM in plaats van anorganische base. De waterfase die overblijft na de hydrolyse van Pen G in het bovengenoemde systeem bevat hoofdzakelijk APA en HPGM. Deze waterfase kan direct gebruikt worden voor de tweede stap van de kortsuitroute, de synthese van Amox, door het toevoegen van enzym en een extra hoeveelheid HPGM. Tot nu toe is het niet mogelijk geweest om deze tweede stap uit te voeren, aangezien één van de produktten van de Pen G hydrolyse, PAA, de katalytische activiteit van penicilline-acylase remt in de Amox-synthesereactie. Uit literatuur is gebleken dat het enzym α-aminozuuresterhydrolase (AEH) van Acetobacter pasteurianus ATCC 9325 de synthese-reactie kan uitvoeren in de aanwezigheid van PAA. Daarom is gebruik gemaakt van dit enzym om de synthese-reactie van de kortsuitroute in aanwezigheid van restanten PAA uit te kunnen voeren.

De invloed van de aanwezigheid van het niet-geëxtraheerde PAA en niet-gehydrolyseerde Pen G op de synthese van Amox door AEH is onderzocht. De invloed van beide componenten bleek hetzelfde te zijn en deze werd gerelateerd aan de ionsterkte. Een kwantitatief model is ontwikkeld om de concentraties van de reactanten tijdens de synthese van Amox uit APA en HPGM in de aan- en afwezigheid van PAA en Pen G te voorspellen. Het model was gebaseerd op enzymkinetiek (die beschreven is als een functie van de ionsterkte), pH-afhankelijke oplosbaarheden en stoichiometrische-, massa- en ladingsbalansen. Het model beschreef de synthese van Amox en de ongewenste hydrolyse van HPGM redelijk. Aangezien een toenemende ionsterkte in de reactor de opbrengst aan Amox zal verminderen kan de synthese-reactie het beste worden uitgevoerd in afwezigheid van PAA en Pen G en door HPGM te gebruiken voor pH-regeling in plaats van een anorganische base.

Tenslotte is er een concept beschreven voor een nieuwe éénpots kortsuitroute voor de biotransformatie van Pen G naar Amox in watervrij organisch oplosmiddel. In deze kortsuitroute wordt Pen G ge-amideerd met een katalytische hoeveelheid ammonia waarbij fenylacetamide (PA-amide) en APA worden gevormd. In een
tweede simultane reactie wordt het gevormde APA gekoppeld aan HPGA voor de
produktie van Amox, waarbij ammonia geregenerereerd wordt. Als water afwezig is kan
de enzymatische hydrolyse van de geactiveerde zijketen niet plaatsvinden en is het
mogelijk een stoïchiometrische omzetting van Pen G naar Amox te bereiken. Een
kwantitatief model is beschreven om de evenwichtsomzetting van Pen G naar Amox
via deze éénpots kortsluitroute in watervrij organisch oplosmiddel te voorspellen. Een
thermodynamische cyclus is opgesteld, gebaseerd op de thermodynamische reactie-
evenwichten in water en de partietiecoëfficiënten en oplosbaarheden van de reactanten.
Berekeningen tonen aan de éénpots kortsluitroute thermodynamisch erg aantrekkelijk
is, maar waarschijnlijk zijn er kinetische beperkingen van het enzym in watervrije
organische oplosmiddelen en is de kortsluitroute (nog) niet haalbaar.
Curriculum vitae

Marjon Brigitte Diender

geboren 14 juni 1973 te Zwolle.

1986-1991
Gymnasium aan het Thomas à Kempis College te Zwolle

1991-1996
Bioprocestechnologie aan de Landbouwuniversiteit Wageningen.
Oriëntatie: Toegepaste biokatalyse.
Eerste afstudeeropdracht uitgevoerd aan de sectie Industriële Microbiologie van de Landbouwuniversiteit Wageningen.
Tweede afstudeeropdracht uitgevoerd bij het Laboratorium voor Produkt- en Procesontwikkeling van het Rijksinstituut voor Volksgezondheid en Milieuhygiëne, B Lithoven.
Stage uitgevoerd aan de vakgroep Microbiologie van de Universiteit van Stellenbosch, Zuid-Afrika.

1996-2000
Promovenda bij de faculteit Toegepaste Natuurwetenschappen van de Technische Universiteit Delft.
Werkzaam onder leiding van prof.dr.ir. J.J. Heijnen van het Kluyver Laboratorium voor Biotechnologie.

Vanaf 20 november 2000 werkzaam bij de afdeling Spreads and Cooking Products Category, Unilever Research te Vlaardingen.
Dankwoord

Nu aangekomen bij de eindstreep van mijn promotieperiode wil ik graag een aantal mensen bedanken, aangezien het werk dat in dit boekje is beschreven natuurlijk niet door mij alleen is gedaan maar door vele mensen binnen en buiten het Kluyver laboratorium.

Allereerst natuurlijk mijn begeleiders Sef Heijnen en Adrie Straathof, jullie hebben mij de kans en ruimte gegeven om dit onderzoek uit te voeren. Jullie beider kritische blik, brede kennis en goede suggesties hebben mij altijd erg geholpen. Mijn dank gaat ook uit naar Thom van der Does van DSM Anti-infectives, voor alle discussies en goede suggesties tijdens de werkbesprekingen en Chemferm bijeenkomsten. Door jouw inbreng is dit project niet alleen een wetenschappelijke exercitie geworden. Verder wil ik alle partners uit het Chemfermproject bedanken voor hun inbreng in mijn project.

Ik wil graag DSM en het Ministerie van Economische Zaken (via Senter) bedanken voor de financiële steun en voor het mij voorzien van de nodige chemicaliën en enzymen. Ook Luuk van Langen en Lin-Qiu Cao van Organische Chemie en Katalyse wil ik bedanken voor een aantal chemicaliën en enzymenpreparaten (CLEC’s en CLEA’s).

Cor Ras en Max Zomerdijk voor alle HPLC-analyses die jullie gedurende de mijn promotieperiode hebben uitgevoerd. Vooral Cor heb ik voortdurend lastig gevallen door telkens weer een extra component in mijn mengsels te introduceren. Sjaak Lispens voor het vinden van de juiste apparatuur en Joop Houwers voor zijn technische hulp bij de pH-stats en Biowatch. Hans Kemper en Marcel van den Broek, mijn computerhelden, voor als mijn computer voor de zoveelste keer weer eens rare dingen deed.

Vooral bij het onderzoek met het befaamde α-aminozuuresterhydrolase enzym hebben vele mensen meegeholpen. Met name wil ik hier Ko Vinke en Rob Kersten noemen die mij geweldig hebben geholpen met de fermentatie van de Acetobacter pasteurianus voor de productie van het enzym. Ook Marijke Luttik voor haar hulp bij de opwerking van het enzym bij de initiële experimenten met dit enzym. Verder wil ik Celine Mulder van DSM Anti-infectives bedanken voor de uiteindelijke experimenten die ik samen met haar heb uitgevoerd.
Dankwoord

Ik wil ook graag mijn afstudeerstudente Sonja Lie en researchpraktikantes Josephine Treijtel en Daniëlle Hornemann bedanken voor al het werk dat zij voor mijn project hebben uitgevoerd. Ook al zijn hun resultaten niet voor publikaties gebruikt, ze hebben mij wel het nodige inzicht gegeven voor mijn verdere onderzoek.

Dan ben ik aangekomen bij de mindere wetenschappelijke, maar niet minder belangrijke kant van het promoveren, namelijk de sociale kant. Hiervoor wil ik specifiek mijn kamergenoten van het ‘kipenhok’ bedanken, Susanne, Luis en Bénédicte. Zonder jullie non-stop afleiding had ik waarschijnlijk niet volgehouden (of was ik in drie jaar klaar geweest). Verder natuurlijk ook de rest van het Kluyverlab voor de vele gezellige gesprekken in de wandelgangen en in ’t Keldertje.

Zonder de postduifservice van Susanne en Eckhard van vele formulieren en andere post tussen mij en het lab en de pedel zou ik waarschijnlijk in de problemen zijn gekomen met alle officiële zaken.

Dan tenslotte natuurlijk nog het thuisfront, pa en ma, jullie hebben maanden het beeld moeten missen omdat ik die zonodig op mijn voorkant wilde en er maar geen foto van maakte. Tenslotte nog Herr Doktor Jochen, al heb jij er niet zo veel aan gedaan om die titel te verdienen....