New Strategies for the Optimisation of N-Acetylneuraminic Acid Synthesis

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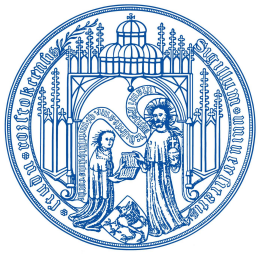

vorgelegt von

Vera Zimmermann, geb. am 31. Oktober 1979 in Lemgo

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Gutachter: Prof. Dr. Udo Kragl, Universität Rostock
Prof. Dr. Durda Vasić-Rački, Universität Zagreb
Prof. Dr. Christian Vogel, Universität Rostock

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Mein ganz besonderer Dank gilt meinen Eltern, Mona und René für ihre Liebe, ihre Aufmerksamkeit und den Rückhalt, den sie mir geben.

meinen Eltern gewidmet

Die Endlosigkeit des wissenschaftlichen Ringens sorgt unablässig dafür,
daß dem forschenden Menschengeist seine beiden edelsten Antriebe
erhalten bleiben und immer wieder von neuem angefacht werden:
die Begeisterung und die Ehrfurcht.

Max Planck

Abstract

New Strategies for the Optimisation of N-Acetylneuraminic Acid Synthesis

In this work, a model describing the complete enzyme catalysed synthesis of N-acetylneuraminic acid (Neu5Ac) from N-acetyl-D-glucosamine (GlcNAc) is presented. It includes the combined reaction steps of epimerisation from GlcNAc to N-acetyl-D-mannosamine (ManNAc) and the aldol condensation of ManNAc with sodium pyruvate yielding Neu5Ac. The model is expedient to predict the reaction course for various initial and feed concentrations and therefore to calculate reaction times and yields. Using the model, an optimisation of reaction conditions in consideration of different targets is possible.

Furthermore, a new method for the purification of N-acetylneuraminic acid (Neu5Ac) is presented. The reactive extraction with phenylboronic acid (PBA) and trioctylmethylammonium chloride (TOMAC) efficiently extracts the compound. The extraction system was characterised in detail by equilibrium studies for various solvents, pH values and carrier and substrate concentrations. The results obtained for pure compounds could be assigned to mixtures and reaction solutions. For the extraction a two step mechanism is assumed and the ion exchange at the interface was determined to be the rate limiting step. Kinetic measurements were performed in a Lewis-type stirred cell. A kinetic model describing the extraction was developed and combined with the model describing the reaction to simulate the integration of reaction and extraction. The integrated process was simulated with varying substrate concentrations, carrier concentrations and phase ratios. The model is feasible to analyse and evaluate integrated product removal as well as separately operated reaction and extraction.

Contents

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Tables listing the kinetic constants for reaction and extraction are included in the respective publications.

Nomenclature

Abbreviation	
ATP	Adenosine triphosphate
D2EHPA	Di(2-ethylhexyl) phosphoric acid
GlcNAc	*N*-Acetyl-D-glucosamine
Hept.	Heptane
Hex.	Hexane
HPLC	High performance liquid chromatography
IC	Ion chromatography
ISPR	*in situ* product removal
ManNAc	*N*-Acetyl-D-mannosamine
MTBE	Methyl-*tert*-butylether
Neu5Ac	*N*-Acetyl-D-neuraminic acid
Oct.	Octanol
PBA	Phenylboronic acid
PVR	Phase volume ratio
Pyr	Pyruvic acid or sodium pyruvate
STY	Space-time yield
TOMAC	Trioctylmethylammoniumchloride

Table 0.1.: List of abbreviations. Additional abbreviations used in the included publications are listed in the respective publication.

Symbol		**Unit**
A	Symbol for Carbohydrates (GlcNAc, ManNAc or Neu5Ac)	
Cl^-	Chloride ion	
D	Distribution coefficient	–
K_A	Dissociation constant of an acid	mol l^{-1}
K_M	Michaelis Menten constant	mol l^{-1}
Q^+	Carrier cation	
σ_+/σ_-	Parameter to calculate the ionic form of an amino acid (defined by equation 4.1)	–
T	Temperature	°C
U	Enzyme activity	μmol l^{-1} min^{-1}
v	reaction velocity	mol l^{-1} min^{-1}
V	Volume	l
Indices		
0	initial	
aq	aqueous phase	
f	feed	
org	organic phase	

Table 0.2.: List of symbols. Additional symbols used in the included publications are explained therein.

1. Introduction

Sustainable development is a task all parts of society have to contribute to. The chemical industry has commited its responsibility concerning a sustainable development to meet the needs of the present without compromising the ability of future generations to thrive. There are several approaches to make production processes in chemical industries more sustainable. One approach is the application of biotechnology for the production of new products or to replace old production processes or single reaction steps [Liese 2002]. Biocatalytic processes have several advantages compared to classical chemical processes. Due to moderate reaction conditions and high selectivity the energy demand and amount of waste are relatively low.

Despite these advantages biocatalytic processes are often not competitive compared with classical processes. These classical processes have been developed and optimised over decades while biotechnology and its application at industrial scale is comparatively young. Therefore biotechnology has a lot of catch up to do when it comes to the enhancement of catalysts, process engineering and development of downstream processing methods to recover the often low-concentrated products from aqueous solutions. A number of different factors in all parts of a production process influence the overall efficiency of the process. Figure 1.1 shows a diagram with some of the parameters that have to be considered when a process has to be developed or optimised. The figure shows two exemplary processes with different limiting factors. In process 1 (red line) the reaction gives high yields, but a lot of the product is lost due to inefficient downstream processing. In process 2 (blue line) the downstream processing is quite efficient, but the reaction kinetics and thermodynamics are unfavourable and the reaction produces a lot of waste, which can result in very high costs for disposal. When optimising a process, all parameters that can be influenced should be considered,

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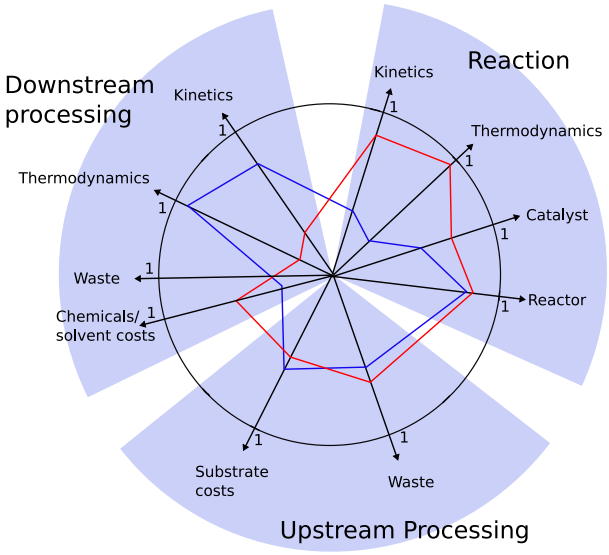

Figure 1.1.: Parameters that influence the overall efficiency and costs of a production process. 1 means best performance concerning this parameter.

which means that in a ray diagramm the area within a curve should be maximised on basis of detailed knowledge of the single parameters. The single parameters may be weighted differently cosidering their impact on the overall process and the target of optimisation.

Often the limiting factor in biocatalytic processes is low product concentraton due to inhibition of the catalyst or thermodynamic equilibrium. In both cases, the recovery of the product is complicated. Especially when only low yields are obtained because of unfavourable thermodynamic equilibrium, the solution contains large amounts of educts and possible byproducts next to the desired product. The downstream processing in such cases is especially elaborate when isolated enzymes are used as catalysts, because educt and product of the reaction are very similar, as mostly only one reaction step is catalysed. In whole-cell biocatalysis nutrients are converted into the desired product by the cell metabolism and educt (nutrient) and product have normally no structural similarity. New approaches to face these problems are the integration of reaction and downstream processing or the design of cascade reactions to shift thermodynamic equillibria or directed evolution to improve the employed enzymes or cells.

For the optimisation of (bio-)processes it is necessary to have detailed knowledge of reaction kinetics, thermodynamic equillibria, inhibitions of the (bio-)catalysts, etc.

Due to the progress in computer performance it is possible to develop models for reaction and downstream processing and simulate the processes under varying conditions quite fast. The use of modelling and reaction engineering is of great importance for the improvement of biocatalytic processes and both methods have to work in concert as (kinetic) modelling deals with the chemical processes while technical equipment and implementantion are subject to reaction engineering. Aim of all process optimisations should be better atom efficiency, improved E-factor, higher ecoefficiency and therefore less waste, lower production costs and improved environmental friendliness [Sheldon 2007, Kussi et al. 2000, Biwer and Heinzle 2001].

An interesting group of biocatalytic reactions are the diastereo- und enantioselective aldol condensations of an aldehyde and a ketone or two aldehydes catalysed by aldolases (aldehyd-lyases, E.C. 4.1.2.x). The products are valuable building blocks in the emerging field of glycotechnology [Faber 2004, Huang et al. 2007, Yu et al. 2006]. Unfortunately, the large-scale production is often not very economical due to an unfavourable equilibrium of the catalysed reaction. Therefore the application of new tools and methods for process optimisation like modelling (to find the best operating conditions) or the concurrent removal of product during reaction (to shift the equilibrium) are of high interest dealing with these reactions.

One of the most interesting aldolases is *N*-acetylneuraminic acid aldolase (E.C. 4.1.3.3) because it catalyses the synthesis of *N*-acetylneuraminic acid, an amino sugar often linked to oligosaccarides in the terminal position in mammalian cells. Neuraminic acid and its derivatives are interesting compounds in various therapeutical applications (compare chapter 3).
This work deals with the enzyme catalysed production of *N*-acetylneuraminic acid from *N*-Acetyl-D-Glucosamine. Neuraminc acid is the major building block for an drug against influenza called Zanamivir. Zanamivir is marketed as Relenza by GlaxoSmithKline. The increasing demand for Relenza can be expressed by the sales figures for 2006 and 2007 presented in figure 1.2.

The chemical synthesis of *N*-acetylneuraminic acid from non-carbohydrate sources requires up to fifteen steps [Banwell et al. 1998]. On industrial scale, neuraminic acid is produced enzymatically in two steps from *N*-acetylglucosamine, which is the

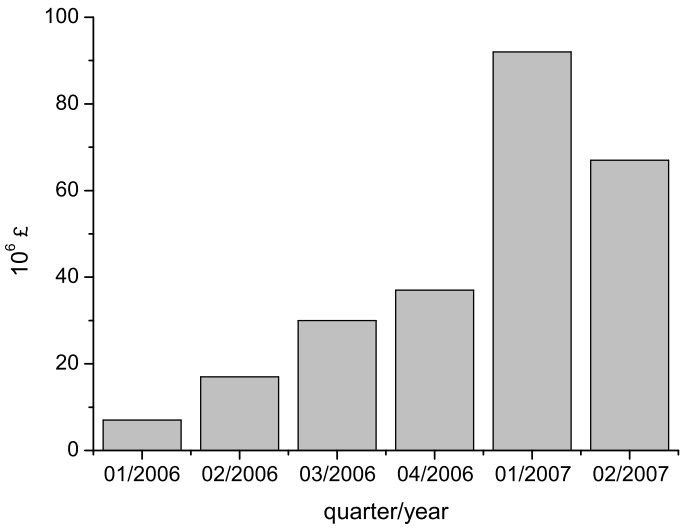

Figure 1.2.: Sales of the influenza agent Relenza in 2006 and 2007 [GlaxoSmithKline 2007]

monomer of chitin, one of the worlds biggest renewable carbohydrate resources. *N*-Acetylglucosamine can be obtained from chitin by acid hydrolysis of shrimp shells. The first step of neuraminic acid synthesis is the epimerisation of *N*-acetylglucosamine to *N*-acetylmannosamine. In the second step *N*-acetylmannosamine reacts with sodium pyruvate to give neuraminic acid. Both steps suffer from unfavourable thermodynamic equilibria, but fortunately they can be performed in one pot as both enzymes work under similar conditions. An improvement of the process yield or reduction of the usually applied excess of sodium pyruvate would lead to better atom efficiency, reduced waste and reduced production costs. The approach pursued here is the improvement of the process by detailed kinetic analysis and subsequent process simulation of the two-step enzymatic reaction and downstream processing by reactive extraction.

2. Objectives of this work

The synthesis of *N*-acetylneuraminic acid from *N*-acetyl-D-glucosamine is a good example for a biocatalytic process used at industrial scale today for production of valuable fine chemicals. Most parameters of the process are well known and, despite the unfavourable equilibria involved, it is suitable to produce neuraminic acid at large scale. But this is mostly due to the fact that neuraminic acid is a high priced building block for the pharmaceutical industry.

Further optimisation of the process should not, as done before, focus on separate parts of the process like upstream processing, reaction or downstream processing, but consider the interaction of these process parts. In this special case the optimisation of upstream processing is barely possible as GlcNAc can be won by simple hydrolisation of shrimp shells and is quite cheap. But optimisation of reaction and downstream processing, which is the most cost intensive part of the process today, should be considered. As mentioned before, many parameters of the neuraminic acid synthesis are already known and optimisation by change in substrate concentration and ratios, temperature shift and variation of the pH value has already been investigated. Next to directed evolution of enzymes kinetic modelling and reaction engineering are very important tools for the optimisation of biocatalytic processes. Aim of this work was the optimisation of neuraminic acid synthesis by kinetic modelling and simulation.

Objectives of this work were:

- **Kinetic modelling of the neuraminic acid synthesis.** This part includes the determination of kinetic parameters for both enzymes and verification of the applied model. The model should be used to simulate the reaction under various conditions and in different reactor types. The simulations should allow parameter optimisation and analysis of reaction conditions. The simulation

results should show how the reaction can be performed in such a way that downstream processing is facilitated. The results of optimisation are presented in the publication in section 6.1.

- **Development of a new downstream processing method.** Until now neuraminic acid is separated from the reaction solution by chromatographic methods and/or precipitation with glacial acetic acid. Both methods suffer from bad atom efficiency either because of high solvent consumption or the large amount of acetic acid required. Nevertheless there were no new separation methods developed during the last twenty years. Aim of this work was to investigate if the separation of neuraminic acid from the other compounds involved in the reaction on basis of the reaction of polyols with boronates *via* reactive extraction is possible. A method for reactive extraction of neuraminic acid with phenylboronic acid and trioctylmethylammonium chloride is presented in the publication in section 6.2.

- **Kinetic modelling of downstream processing.** For the developed separation method a kinetic model should be developed and utilised to simulate separation under varying conditions. The chosen conditions should refer to the results of the simulations performed for the reaction. A kinetic model for the developed extraction and simulations of integrated reaction and extraction are presented in section 6.3.

3. Synthesis of N-Acetylneuraminic acid

3.1. Enzymatic synthesis of N-acetylneuraminic acid

N-Acetylneuraminic acid is an important building block for pharmaceuticals. In nature this amino sugar is terminally connected to glycoproteins and glycolipids. Neuraminic acid is of great biological importance, as these glycoproteins and -lipids are involved in in many cellular recognition processes, e. g. as receptors for hormons, enzymes and viruses [Schauer 1982]. *N*-Acetylneuraminic acid is of great interest for many pharmaceutical applications, especially as basic structure for neuraminidase inhibitors which are applied for the therapy and prevention of influenza. These pharmaceuticals are structurally related to Zanamivir, the active ingredient in Relenza [Maru et al. 2002, Zürcher et al. 2006]. It is interesting that this group of drugs was designed rationally on basis of the knowledge of the mechanism of the enzyme influenza virus sialidase [von Itzstein et al. 1993, De Clercq 2006]. Therefore it is one

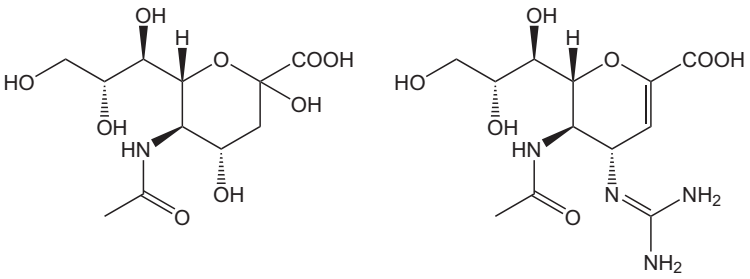

Figure 3.1.: Structure of *N*-acetylneuraminic acid (Neu5Ac) and zanamivir, a pharmaceutical produced from Neu5Ac

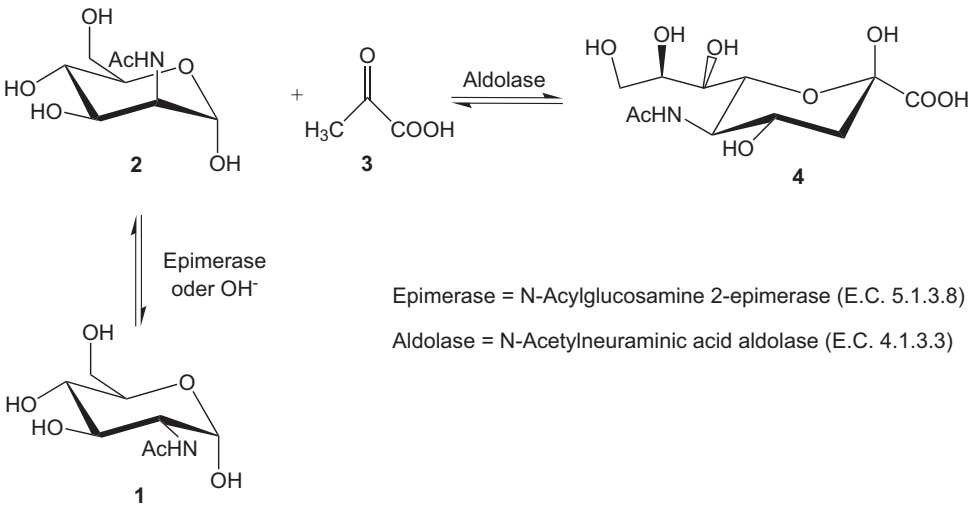

Figure 3.2.: Synthesis of *N*-acetylneuraminic acid from *N*-acetylglucosamine

of the few examples where this has been achieved despite many attempts over the last three decades.

On industrial scale, *N*-acetylneuraminic acid (Neu5Ac) is produced in two steps from *N*-acetyl-D-glucosamine (GlcNAc). The first step is the epimerisation of *N*-acetyl-D-glucosamine to *N*-acetyl-D-mannosamine (ManNAc), which can be catalysed by hydroxid ions (pH>9) or enzymatically. Usually, the enzymatic catalysis is preferred because this way the epimerisation can be performed in one pot with the second reaction step, the enzyme catalysed aldol condensation of ManNAc with sodium pyruvate (figure 3.2).

N-Acetyl-D-glucosamine is the monomer of chitin, which is the main component of the cell walls of fungi and the exoskeletons of arthropods, like insects and crustaceans. On a quantity basis chitin is, after cellulose, the second largest renewable resource[1] [Breitmaier and Jung 1995]. *N*-Acetyl-D-glucosamine is obtained by acid hydrolysis of shrimp shells. The epimerisation of GlcNAc to ManNAc is catalysed by several metal hydroxides [Kuhn and Brossmer 1958, Simon et al. 1988] or by the enzyme *N*-acylglucosamine-2-epimerase (E. C. 5.1.3.8). At equilibrium the ratio of GlcNAc and ManNAc is 4:1, i. e. the yield of this reaction is 20 %. For the isolation of ManNAc large amounts of organic solvents and energy to remove water are required [Simon et al. 1988].

[1] World market price of chitin starts at 3 Euro/kg, depending on the chitin quality.

The yield of Neu5Ac obtained by condensation of ManNAc and sodium pyruvate is also limited by the thermodynamic equilibrium. Usually, a two- to ten-fold excess of pyruvate is applied to shift the reaction to the product side [Augé et al. 1990, Simon et al. 1988, Sugai et al. 1995]. This results in a high pyruvate excess present in the solution after reaction. The separation of neuraminic acid from sodium pyruvate is difficult because both compounds have a similiar pK_A-value and are well soluble in water at neutral pH.

3.2. Developments in neuraminic acid synthesis

Until the 1980s neuraminic acid was obtained from natural sources such as edible birds nest or *Escherichia coli* cell walls [Schauer 1982] as the chemical synthesis is complicated [Benzingnguyen and Perry 1978, Danishefsky and DeNinno 1986]. During the 1980s first the aldolase and later the epimerase became available for reactions at lab scale due to the progress in biotechnology techniques, especially genetic engineering. The use of enzymes to synthesise Neu5Ac from ManNAc and Pyr or later GlcNAc and Pyr was the first milestone in the development of neuraminic acid synthesis. The enzyme *N*-acetylneuraminic acid aldolase was first described by Comp and Roseman in 1958, but its first application in neuraminic acid synthsis was not until the early 1980s, performed by Claudine Augé. The enzymatic synthesis of neuraminic acid from ManNAc and pyruvate was subject to several research activities during the 1980s, including the identification of new enzyme sources [Uchida et al. 1984] and immobilisation of the aldolase [Bednarski et al. 1987, Augé et al. 1985]. Efforts to improve reaction conditions to give higher yields or improve the atom efficiency dealt mostly with variation of the pyruvate excess [Kim et al. 1988, Augé et al. 1990, Sugai et al. 1995]. In 1965 Ghosh and Roseman purified *N*-acylglucosamine 2-epimerase from hog kidney and studied some of its kinetic properties. The epimerase was readiliy available a few years later than the aldolase, but also very important because the epimerisation from GlcNAc to ManNAc could be performed under mild conditions and large quantities of metal hydroxides could be avoided. Today the epimerase is prepared by overexpression in *Escherichia coli* [Maru et al. 1996, Wang and Lee 2006].

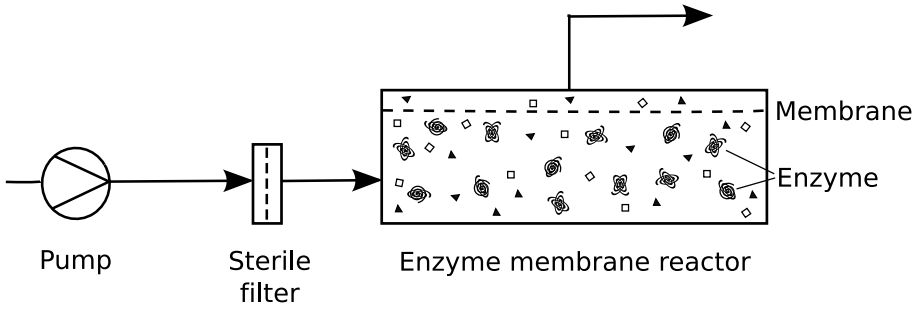

Figure 3.3.: Neu5Ac synthesis in an enzyme membrane reactor [Kragl et al. 1991].

Since the beginning of the 1990s there were other approaches to improve the synthesis. An important step was the combination of enzyme catalysed epimerisation and aldol condensation in one pot [Kragl et al. 1991]. This way, the isolation of ManNAc, which consumed large amounts of organic solvents like isopropanol [Simon et al. 1988], was avoided and the waste produced in the overall process was reduced. Furthermore, Kragl *et al.* performed the reaction in a continuously operated enzyme membrane reactor, (figure 3.3). The reactor allowed to retain the enzymes in the reactor and therefore recycle them. This was the first time that the neuraminic acid synthesis was performed in an continuously operated reactor. GlcNAc and pyruvate were fed to the reactor in a 2:1 ratio and the yield of the process was 28 % referring to GlcNAc.

Another approach was published by Sugai *et al.* in 1995. The time needed for the epimerisation of GlcNAc was shortened by application of an saturated calcium hydroxide solution to catalyse this step. The aldol condensation was improved by introduction of another enzymatic step in which the excess sodium pyruvate is decomposed by a pyruvate decarboxylase. Referring to ManNAc the yield of the process was 60 %, but only 7.6 % referring to GlcNAc. The great advantage of this process is the enzymatic decompositon of pyruvate to acetaldehyde and carbondioxide because the isolation of neuraminic acid is facilitated.

Further approaches were the accumulation of ManNAc after hydroxide catalysed epimerisation [Mahmoudian et al. 1997] or the step-wise dosing of pyruvate after the reaction reached equilibrium [Maru et al. 1998]. Other groups performed the two-step synthesis in one pot under varying conditions [Ohta and Tsukada 1995, Blayer et al. 1999] and obtained yields between 31 % and 51 %.

3.3. Optimisation of neuraminic acid synthesis by kinetic analysis and process engineering

Since the beginning of this century there were few new approaches to improve the synthesis of neuraminic acid. In 2004 Lee *et al.* tried to shift the equilibrium to the product side by a change in reaction temperature [Lee et al. 2004]. As the temperature has to be lowered, the reaction velocity decelerates and the reaction time has to be extended, which results in lower space-time yields.

The most important reason for the decelaration of reaction optimisation is that the classical tools are mostly exploited. As for many other bioprocesses new tools like bioinformatics, directed evolution and improved process engineering should be employed [Kussi et al. 2000, Braiuca et al. 2006]. Further approaches to improve bioprocesses and bypass their disadvantages are the integration of reaction and downstream processing [Freeman et al. 1993] or the development of cascade reactions, a principle taken over from nature [Bruggink et al. 2003].

The first detailed analysis of the neuraminic acid production process was published in 1996 by Blayer *et al.*, exemplifying a structured approach to biotransformation process design and presenting operation windows in which the reaction should be performed. The process analysis dealed with neuraminic acid production from GlcNAc by combined base-catalysed epmerisation and enzyme catalysed aldol condensation with analdolase from *Escherichia coli*. Parameters included in in this approach are compound solubility, enzyme inhibitions and enzyme stability at different pH values. Optimisation targets were yield of the single reaction steps, yield of the overall process and minimisation of the Neu5Ac/Pyr ratio because of facilitated downstream processing. Figure 3.4 shows the operation window for ManNAc and pyruvate concentrations in the reactor defined by Blayer *et al.*. From their analyis by rational approach Blayer *et al.* conclude that the integration of base catalysed epimerisation and enzyme catalysed condensation is difficult due to enzyme stability and pyruvate degredation. They suggest integration of product removal to improve the yield of the process but eliminate Neu5Ac crystallisation because of the necessary low pH value and suggest ion exchange chromatography as an alternative. Furthermore, the feeding

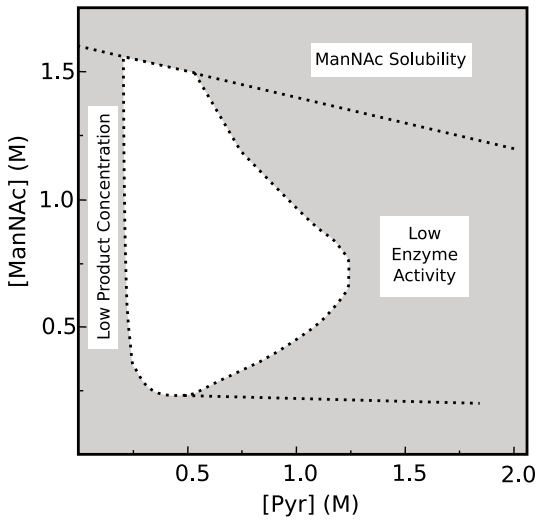

Figure 3.4.: Neu5Ac synthesis process limits as a function of ManNAc and pyruvate reactor concentrations. The shaded area indicates inefficient operation (Figure taken from [Blayer et al. 1996]).

of pyruvate during reaction is recommended due to enzyme inhibition at high pyruvate concentrations.

Unlike the rational approach presented before, kinetic modelling was chosen to analyse and optimise the synthesis of neuraminic acid in the work presented here (compare section 6.1). Kinetic modelling can be utilised to find optimal operation conditions and improve process yields [Vasic-Racki et al. 2003]. The developed kinetic model was used to simulate the two-step enzymatic reaction with various substrate and catalyst concentrations in different types of reactors. A model used for such simulations has to be valid over a wide range of substrate and catalyst concentrations. The model employed here was developed in 1992 by U. Kragl [Kragl 1992]. All parameters of the model were fitted again to describe the behaviour of the enzymes used in this work and the model was validated to show that it is adequate to describe the reaction in the examined concentration ranges.

The model was used to optimise the catalyst ratio between epimerase and aldolase (compare figure 2 on page 602 in publication 6.1). This way both enzymes can be applied with the optimal activity thus saving catalyst costs in the process. Furthermore, the process was simulated in fed-batch reactors with pyruvate (as suggested by Blayer *et al.*) and/or GlcNAc fed to the reactor. Simulations showed that the

same space-time yield of Neu5Ac can be obtained with pyruvate or GlcNAc excess in fed-batch processes (compare figure 3 on page 603 in publication 6.1), which is interesting for downstream processing because of the difficult separation of Neu5Ac/pyruvate mixtures.

Moreover, the findings gained from kinetic analysis were applied to the neuraminic acid production process immediately in this work. After analysis of the dependence of space-time yield on catalyst concentrations and ratios, all further experiments and simulations were performed with the optimised catalyst ratio. Furthermore a fed batch process on larger scale was performed with the optimised initial and feed concentrations determined by simulation prior to the experiment.

4. Extraction of Carbohydrates

4.1. Reactive extraction

4.1.1. Basic principle

Reactive extraction is a method to remove hydrophilic compounds from aqueous solutions. The hydrophilic compound is modified by a reversible chemical reaction to increase its solubility in organic solvents. The different types of reactive extraction rely on three basic mechanisms [Bart 2001, Blass and Sluyts 2002]:

- **Solvating and/or chelating reactions** Solvation reagents are solvents with either carbon or phosphor-bound oxygen or other electron donating atoms. Chelating agents are also compounds with electron donating atoms like oxygen or nitrogen. These agents stabilise the extracted compound in the organic phase by formation of stable six-membered rings. Examples for common chelating agents are dioximes, hydroximes or diketones.
- **Cation exchange** Common ion exchangers for the extraction of cations are phosphoric acid derivatives like D2EHPA
- **Anion exchange** For anion exchange mostly polyalkylammounium salts and aliphatic amines are used as carriers.

Reactive extraction was first used in hydrometallurgy to selectively remove metal ions from diluted solutions [Cox 1992]. Since the beginning of the 1980s is is used more and more for the extraction of organic compounds, especially organic acids like amino acids [Uddin et al. 1990], lactic acid [Joglekar et al. 2006] and penicillins

[Likidis and Schügerl 1987, Patnaik 1992]. If organic acids are supposed to be extracted, the mechanism and the applied carrier compound depend on the pH value of the aqueous solution. At pH values above its pK_A value the acids exists in the anionic form and anion exchange carriers are applied [Scarpello and Stuckey 2000, Pursell et al. 2003b, Hano et al. 1991]. At pH values lower than their pK_A acids are neutral and, if extraction is possible, are extracted via solvating, chelating or similar mechanisms. Marták *et al.* extracted lactic acid with phosphonium ionic liquids from acidic solutions and proposed a H-bonding mechanism [Marták and Schlosser 2006]. Due to their zwitterionic character amino acids are an exeption. Depending on the pH value they exist in cationic, anionic or neutral form and are therefore extracted via the corresponding mechanism [Rüffer et al. 2004].

The mechanism of ion exchange is a so called 'countertransport mechanism'. The carrier is soluble in the organic phase because the charge of the ion pair is shielded by the hydrophobic parts of the molecule. During extraction the small hydrophilic an- or cation is exchanged with the extracted compound at the liquid-liquid interface. Compound and carrier counterion form a new ion pair which is transported into the organic phase.

4.1.2. Industrial applications of reactive extraction processes

The principle of reactive extraction was first used in industry for the extraction of metal ions. The selective removal of metal ions from dilute solutions was developed in the U.S. "Manhattan"-project in the 1940s for the production of uranium and applied at large scale since 1942 [Cox and Rydberg 2004]. In the 1950s and early 1960s the principle was applied in purification and separation processes of metals in non-nuclear industry as well. Today the production of copper is the largest process in which reactive extraction is applied. About 15 % of the primary copper production in the western world is produced by sulfuric acid leaching of copper ore, enrichment of copper by reactive extraction and subsequent electrowinning.

Today a large number of different carriers for the extraction of copper are available. Largest distributers are Henkel with its LIX series and Allied Signal with MOC. The

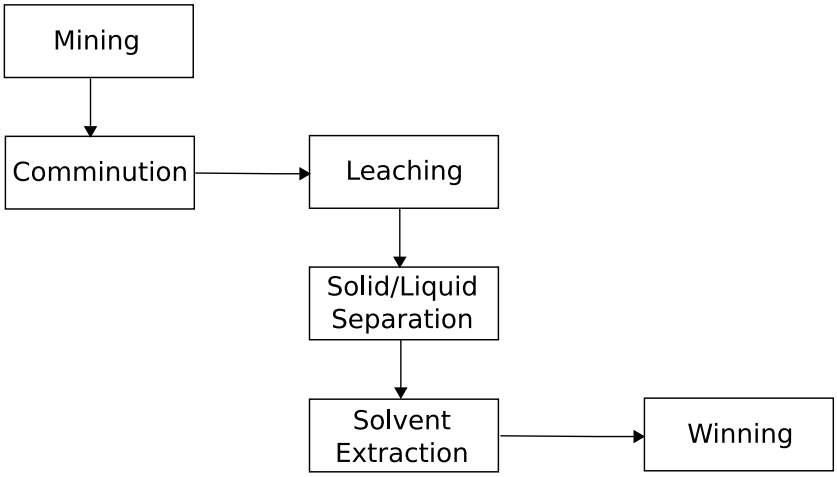

Figure 4.1.: Hydrometallurgical flow sheet using solvent extraction (taken from [Cox 2004]).

first carrier distributed by Henkel was an oxime called LIX 64. In 1964 the first copper extraction plant was commissioned and this carrier was used. The carriers used today are salicylaldoximes and ketoximes [Lossin 2002, Cognis 2008a]. Figure 4.2 shows the structure of a carrier from Henkels LIX series.

Another important application is the purification of waste water streams. More than 99 % of the nitrate, 90 % of the chloride and 85 % of the sulfonic acid contained in the waste water stream from a ammonium sulfate cristallisation unit in a caprolactam plant can be removed by reactive extraction in three stages [Verbueken et al. 1989]. Biological treatment of the waste water from a polycarbonate plant can be avoided when the contained traces of phenol and bisphenol are removed by reactive extraction with trioctylamine [Wang and Liu 1997].

An example for the reactive extraction of organic compounds at industrial scale is

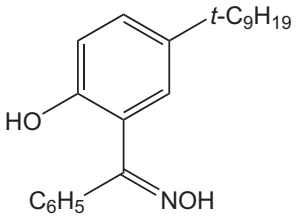

Figure 4.2.: Structure of LIX65N, a carrier used for copper extraction [Tyman and Iddenten 2005].

the production of citric acid. Citric acid is produced by fermentation with *aspergillus niger*. Usually, the product is separated from the fermentation broth by precipitation with calcium hydroxide and subsequent dissolving with sulfuric acid, but this approach produces more than one ton of gypsum per ton of product. An alternative separation method is the extraction with trilaurylamine. After extraction the carrier and product can be separated by heating the solution and carrier and organic solvent can be recycled [Lopez-Garcia 2002, Kragl 2005]. It is envisaged that further processes including reactive extraction will be developed for the purification of valuable organic compounds for the pharmaceutical, agrochemical or cosmetic industry.

4.1.3. Models describing liquid-liquid phase transfer

Different theories have been developed to describe physical extraction. The most important theories are

- Two-film theory
- Penetration model
- Surface renewal
- Boundary layer

The two-film theory by Whitman is the oldest of the common theories and mostly used as basis for kinetic mass transfer models. It was first developed to describe liquid-gas mass transfer [Whitman and Keats 1922, Whitman 1923] and was later adopted for description of liquid-liquid mass transfer. In this theory it is assumed that the bulk phases are well mixed and adjacent to the interface between the phases theres a thin film on each side in which transport only takes place by diffusion (figure 4.3). In these layers the liquid phases are considered to be completely stagnant.

When dealing with reactive extraction processes next to diffusion also the various chemical reactions taking place in the system have to be taken into account. Possible steps of the overall extraction are, to name only the important ones:

- Diffusion to/from the interface.
- Interfacial adsorption/desorption processes.

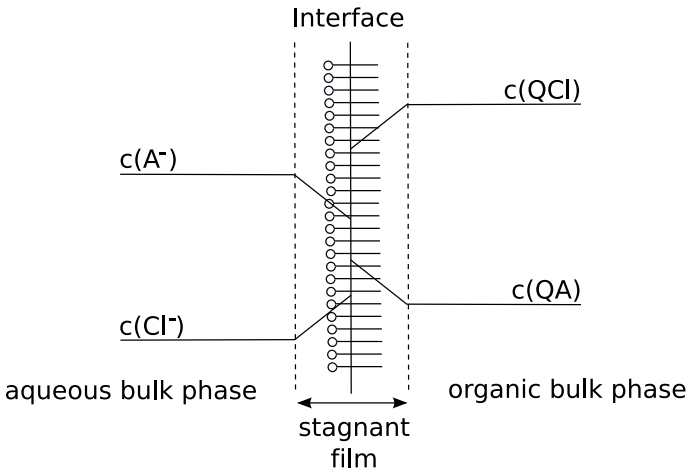

Figure 4.3.: Interfacial diffusion films according to the two-film theory. At the interface an adsorbed layer of ion exchanger is shown and concentration profiles of the reactants are indicated (reaction at the interface: $A^-_{aq} + QCl_{org} \leftrightharpoons Cl^-_{aq} + QA_{org}$).

- The aggregation or dissociation of the organic ion exchanger.
- The removal of solvating water molecules by an organic compound.
- The ion exchange reaction at the interface.
- The competitive reaction of another solute.

Depending on the investigated system usually only the most important steps like diffusion, reaction at the interface and often also coextraction of other solutes, especially hydroxide ions, are taken into account. The overall mass transfer can take place under a chemical reaction controlled regime, a diffusional controlled regime or a mixed regime. The regime evaluation is only possible in a stirred cell (compare figure 2 in section 6.3). This type of cell was introduced by Lewis [Lewis 1954] and subsequently modified by other groups such as Nitsch [Bauer et al. 2002, Sacher and Nitsch 2006]. In such a cell both phases can be stirred independently without disturbing the planar interface with known dimensions. Kinetic measurements are usually performed under a chemical reaction controlled regime, which can be ensured by careful selection of the stirring speed [Bart 2001, Danesi 2004]. A good overview over the development and different types of Lewis cells was given by Hanna and Noble in 1985.

Name	Structure	Solubility	Source
Alamine 336	Tertiary amine	<5 ppm	[Cognis 2008b]
Aliquat 336 (TOMAC)	Quarternary ammonium salt	0.12g/100g	[Cognis 2008b]
Amberlite LA-1	Secondary amine	15 ppm	[Kunin 1962]
D2EHPA	Di(2-ethylhexyl) phosphoric acid	100 ppm	[Kunin 1962]

Table 4.1.: Solubility in water of typical commercial ion exchangers used in reactive extraction.

When reactive extraction processes are modelled, the following assumptions are usually made:

- Physical extraction is negligible because the extracted compound is hydrophilic.
- The ion exchanger does not dissolve in the aqueous phase and therefore the ion exchange reaction takes place at the interface.
- The amphophilic ion exchanger adsorps at the interface which can be described by an adsorbtion isotherm.
- Dissolving of solvents in the other phase is negligible and no solubility enhancing effects occur.

The assumptions are based on the chemical and physical properties of the solvents, carriers and compounds to be extracted. The solubilities in water of some common ion exchange carriers are given in table 4.1.

During the last decades several kinetic models for reactive extraction with ion exchangers have been developed. Naturally, because the first ion exchange reactions were those of metal ions, also the first kinetic models were developed for metal ion extraction. A good overview was given by Danesi and Chiarizia in 1980 [Danesi and Chiarizia 1980]. Since the 1980s the reactive extraction of organic acids was subject to investigations of several groups and models were developed for pharamceutical interesting substances like amino acids or penicillin [Reschke and Schügerl 1984, Haensel et al. 1986, Uddin et al. 1990, Uddin et al. 1992, Chan and Wang 1993, Bora et al. 1997]. Since the late 1990s the coextraction of hydroxide ions and other solutes and the influence of coextraction on the kinetic models was considered [Tamada and King 1990, Scarpello and Stuckey 2000, Pursell et al. 2003a]. Latest research deals with the anal-

ysis of complex matrices like fermentation broth or the separation of chiral compounds via reactive extraction [Pursell et al. 2004, Steensma et al. 2007].

An interesting model was published by Chan and Wang in 1993 for the extraction of phenylalanine and glutamic acid with different carriers from alkaline as well as acidic solutions. The model takes the pH value of the aqeous phase and therefore the extractable, that is the ionic, fraction of the respective amino acid into acount via a parameter σ. For acidic solutions the parameter is specified as σ_+, for alkaline solutions as σ_-

$$\begin{aligned} \sigma_+ &= 1 + \frac{K_{A1}}{[H^+]} + \frac{K_{A1}K_{A2}}{[H^+]^2} + \frac{K_{A1}K_{A2}K_{A3}}{[H^+]^3} \\ \sigma_- &= \frac{[H^+]^2}{K_{A1}K_{A2}} + \frac{[H^+]}{K_{A2}} + 1 + \frac{K_{A3}}{[H^+]} \end{aligned} \tag{4.1}$$

By using equation 4.1 the concentration of the ionic species of the amino acid can then be calulated from the measured overall concentration and the pH value of the aqueous phase. In this model the overall extraction process is divided into five steps which are

1. Diffusion of the reactants from the bulk phase to the interface
2. Adsorption of the ion exchanger at the interface according to the Langmuir model
3. Ion exchange reaction
4. Desorption of the amino acid-ion exchanger ion pair from the interface
5. Diffusion of the products into the bulk phases

For this mechanism Chan and Wang derived a general equation for the interfacial flux J which can be simplified for three cases. In the first case, the extraction rate is limited by aqueous phase film diffusion, in the second case it is limited by organic phase film diffusion or, as in the third case, the rate is limited by the the interfacial reaction.

In this work (see publication 6.3) the model published by Chan and Wang was modified and used to describe the reactive extraction of GlcNAc, ManNAc, pyruvate and Neu5Ac with phenylboronic acid and trioctylmethylammonium chloride described in section 4.2.2. The modifed model is the first kinetic model for the reactive extrac-

tion with this combination of phenylboronic acid and TOMAC as ion exchanger. Our investigations led to the assumption of a two-step mechanism. In the first step, the phenylboronic acid, which is dissolved in the aqueous phase, reacts with the carbohydrate. The second step is the extraction of the formed anion with TOMAC via ion exchange. For the mathematical description of this mechanism the parameter σ was modified and now describes the fraction of carbohydrate that has reacted with phenylboronic acid and can therefore be extracted with TOMAC.

4.2. Reactive extraction of carbohydrates

4.2.1. Development of different carrier systems

The extraction of carbohydrates from aqueous solutions is difficult. Compared to metal ions they are relatively big and because of the hydroxy groups they are very hydrophilic. In contrast to organic acids, for which several extraction methods are known, they do not have ionic groups and therefore extraction by ion exchange is not possible. Nevertheless, during the last decade, some carrier/solvent systems for the extraction of carbohydrates have been developed. In most publications the transport of carbohydrates through liquid membranes is investigated due to the importance of these systems for the understanding of biological systems. Often boronic acid dervatives are used as carriers in these membranes [Shinkai et al. 1991, Westmark et al. 1996, Karpa et al. 1997]. Other systems are rare. The efficient transport of saccarides mediated by a cyclodextrin dimer was reported by Ikeda *et al.* in 2003, while Hameister and Kragl showed that amines can be efficient carriers for some carbohydrates [Hameister and Kragl 2006]. Another possibility to extract carbohydrates from aqueous media is the use of reversed micelles [Kida et al. 1996].

4.2.2. Extraction of carbohydrates with PBA and TOMAC

A well known carrier used for reactive extraction is triooctylmethylammonium chloride (TOMAC), also known as Aliquat 336®. TOMAC was first used for the extraction

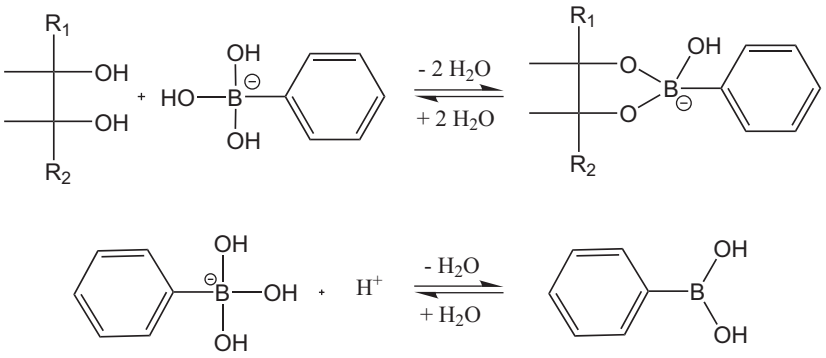

Figure 4.4.: Reaction of diols with phenylboronic acid and formation equilibrium of boronates

of metal ions [Cox 1992, Bagreev et al. 1978], later it was applied in the extraction of amino acids, especially phenylalanine [Haensel et al. 1986, Uddin et al. 1992]. It is not possible to extract carbohydrates with TOMAC to a extend worth mentioning, probably because they are usually uncharged in aqueous solutions and therefore cannot form an ion pair with the ion exchanger in the organic phase. Carbohydrates have to be modified before they can be extracted by ion exchange with TOMAC. One possible reaction to modify carbohydrates is the well known reaction of polyols with phenylboronic acid (PBA). Polyols, which are compounds with more than one hydroxy group, react with boronates, the anions of phenylboronic acid derivatives, under elimination of water as presented in figure 4.4 [Lorand and Edwards 1959, Kuivila et al. 1954]. The equilibrium of the reaction is influenced by the constitution of the polyol, the constitution of the boronate and the pH value of the solution [Sienkiewicz and Roberts 1980]. Among others, the equilibrium of the reaction between phenylboronic acid (and some derivatives) and different carbohydrates was investigated by Barker *et al.* in 1973 and later by Westmark *et al.* in 1996. Westmark *et al.* investigated the reactivity of different carbohydrates with PBA and stated that carbohydrates with 1,3-*trans* hydroxy groups react best with PBA, followed by carbohydrates with 1,2-*cis* located hydroxy groups. Carbohydrates with other configurations react to a smaller extend. Because of the different reactivities PBA is used as ligand in adsorption and chromatography materials to separate polyols and carbohydrates [Wulff 1982, Lee 1990].

In the 1990s the transport of carbohydrates through liquid membranes was investigated intensively because the cross-membrane transport of biological active substances, like

saccharides, is of great interest for the understanding of biological systems. Often phenylboronic acid and its dervatives were used as carriers in these systems. An overview over the separation of sugars by use of liquid membranes and boronic acid carriers was given by B. D. Smith in 1996.

Unfortunately, the neutral carbohydrate-PBA complexes are subject to hydrolisation and only the anionic complexes are stable in the presence of water. The anionic complexes cannot be extracted because of their charge. It is necessecary to add an ion exchanger to extract these compounds to an organic phase. The combination of PBA and a quaternary ammounium salt as ion exchanger for the transport of carbohydrates and other molecules of biological interest through liquid membranes was used by several groups [Morin et al. 1994, Schroen et al. 2002, Lye and Woodley 1999]. Takeuchi *et al.* [Takeuchi et al. 1996] even combined the phenylboronic acid and the ion exchanger in one molecule and extracted α-*p*-nitrophenyl-D-glucopyranoside to 1,2-dichlorethane as organic phase. The combination of PBA and TOMAC for the extraction of carbohydrates was then applied by Matsumoto *et al.* in 2005 for the extraction of several common sugars like glucose, xylose and mannose. All work up to this point dealt with small carbohydrate concentrations and mostly common sugars like glucose, mannose and fructose because it was meant to investigate transport processes in biological systems.

The first work with the aim of applying this extraction system at industrial scale was published by Griffin and Shu in 2004. Scope of this work was the purification and concentration of sugars from hemicellulose hydrolysates. Five types of boronic acid in combination with TOMAC and different industrial organic solvents (Shellsoll® 2046, Exxal® 10) were tested for the extraction of fructose, glucose, sucrose and xylose. TOMAC was applied in excess compared to boronic acid to enhance its solubility. Trials with pure single sugar solutions showed that, with all carrier combinations and concentrations, either fructose or glucose gave the greatest extraction. Furthermore it was shown that the extracted amount of sugar depends strongly on the right combination of phenylboronic acid derivative and organic solvent. The extraction of bagasse hydrolysates was performed with naphthalene-2-boronic acid which gave the best results for extraction of xylose in the trials. The extraction isotherm for xylose at two different buffer concentrations is given in figure 4.5. The carbohydrates were recovered

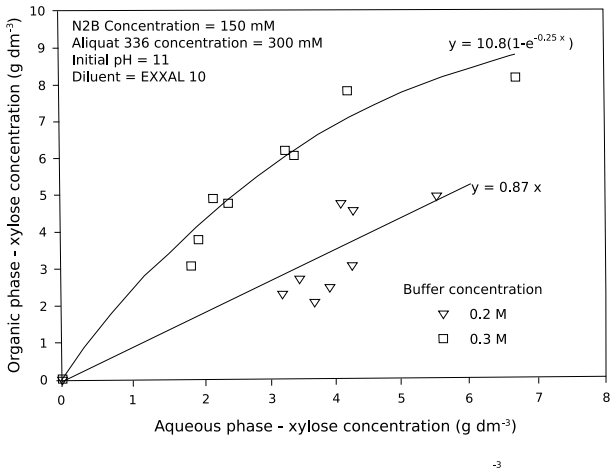

Figure 4.5.: Extraction isotherms for xylose from bagasse hydrolysates at different buffer concentrations as published by Griffin and Shu in 2004.

from the organic phase by stripping with aqueous hydrochloric acid. The stripping solution contained less than 7 % of undesired impurities contained in the originial hydrolysate, but other sugars present in large amounts like arabinose were not considered [Griffin and Shu 2004, Griffin 2005].

The application of PBA and TOMAC to separate neuraminic acid from pyruvate, GlcNAc and ManNAc is the first time this system is used as a downstream processing method for a biocatalyic process (see publication in section 6.3). The extraction of all compounds was investigated at different concentrations and ratios of substrate and carriers, pH values and with a variety of organic solvents. In dilute mixtures of the

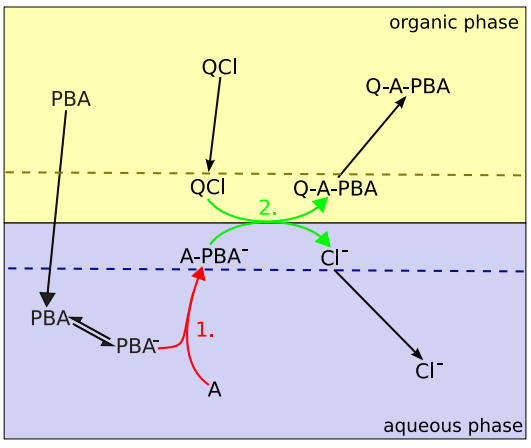

Figure 4.6.: Assumed mechanism: The extraction of carbohydrates (A) with PBA/TOMAC proceeds in two steps.

carbohydrates a selectivity of up to 13 for neuraminic acid is found. For the extraction a two-step mechanism was assumed. In the first step, the tetrahedral boronic acid anion, which is formed in the aqueous phase reacts with the carbohydrate and the formed complex is extracted in an ion exchange reaction with TOMAC to the organic phase. A scheme of the assumed mechanism is presented in figure 4.6. The structure of the PBA-Neu5Ac complex was published by Otsuka *et al.* in 2003. On the basis of detailed equilibrium studies the equilibrium constants for the single reaction steps as well as the overall process were determined.

5. Integrated product removal in biocatalysis

As mentioned before, one problem of some biocatalytic processes is the low concentration of product after reaction (compare chapter 1). One possibility to face this problem is the integration of downstream processing during reaction (*in situ* product removal: ISPR). By ISPR the product is already removed from the reaction solution during reaction. ISPR allows the improvement of productivity and yield of biocatalytic processes because of the following items [Freeman et al. 1993]:

- Inhibition of the biocatalyst can be avoided.
- Unfavourable thermodynamic equilibria can be shifted to the product side.
- Loss of product by consecutive reaction or decomposition can be suppressed.
- The number of downstream processing steps may be reduced.

Furthermore, the product should be accumulated to simplify later isolation. The separation of the product during reaction can be achieved by different methods like adsorption, extraction, membrane filtration or pervaporation. These methods are applied in conventional downstream processing as well, but during ISPR the separation is carried out simultaneously with the reaction. Normally, catalysts, remains of the substrates and side products are seperated in subsequents steps [Lye and Woodley 1999]. Some of these energy and solvent consuming steps may be avoided by introduction of an ISPR. ISPR can be applied in batch as well as in continuous processes (figure 5.1).

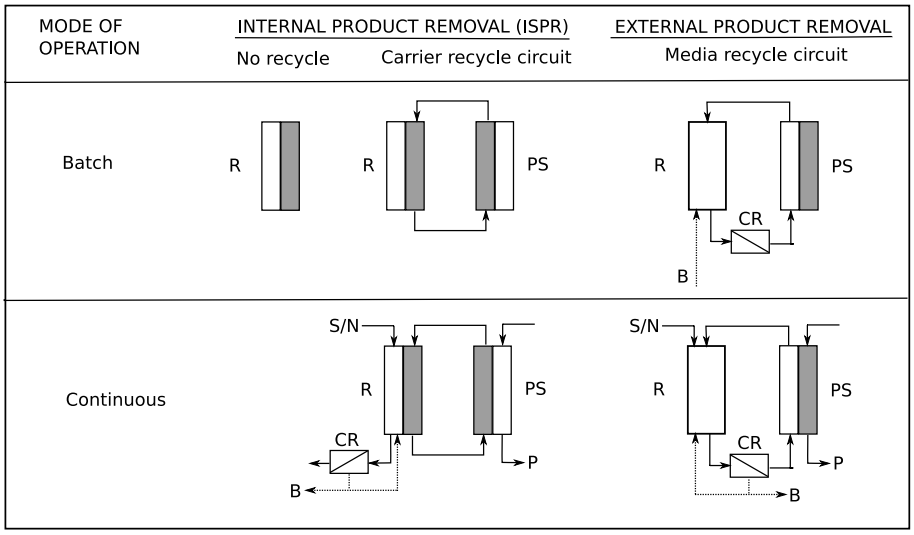

Figure 5.1.: Options for direct product removal from a bioreactor (taken from [Freeman et al. 1993]). The diagrammatic representations of reactors and product stripping units do not inidicate flow patterns within them or the nature of the carrier. (B) Option for the bleeding of cellular catalyst, (CR) cell retention device, (P) product bleed, (PS) product stripping unit, (R) reactor, (S/N) substrate or nutrient feed.

On the other hand, introduction of an ISPR demands investment in research, know-how and especially technical equipment. Therefore ISPR may be applied in production processes for value added products like pharmaceutics or food additives rather than in the production of bulk chemicals [Freeman et al. 1993].

Most applications of ISPR indicate that there are two major limitations, especially in enzyme catalysed reactions. These limitation that reduce economic efficiency are due to the chemical similar structures of substrates and products in enzymatic transformations. The separation methods are either not selective enough so that also the educts are removed from the reaction solution or the capacity of the phases, like adsorption materials, is to low and the influence of ISPR on the equilibrium is too low to improve the yield of the process [Lye and Woodley 1999, Straathof 2003]. There are different approaches to improve the separation methods. The develompent of new methods and materials could improve the selectivity and capacity, or exact analysis and optimisation of reaction parameters by reaction engineering can improve the overall process.

In biotechnology, ISPR is mostly used to improve fermentation processes. During fermentation short chain alcohols like methanol or ethanol or small organic acids are

removed from the fermentation broth by pervaporation, extraction or electrodialysis. The removed compounds would otherwise inhibit the microorganisms and slow down or stop the fermentation process. In fermentation processes such byproducts can be removed quite easily because their properties are not very similar to those of the educts (carbohydrate sources) and products. Exemplarily the production of L-phenylalanine with integrated reactive extraction may be presented. During the fermentative fed-batch process the product is removed from the reaction solution by extraction with kerosene an D2EHPA in a bypass to the reactor. The bypass is used to avoid inhibition of the microorganisms by the organic solvent and to introduce a filtration step so that the extracted solution does not contain enzymes and proteins. With the integrated extraction the relative glucose/L-phenylalanine yield could be increased by 28 % compared to the non-ISPR process [Rüffer et al. 2004]. The process is a good example for the process enhancement that can be achieved by integration of reaction and downstream processing on the one hand and the increased requirements concerning technical equipment on the other hand. A good overview on microbial biotransformations with integrated product removal was given by Stark and von Stockar in 2003.

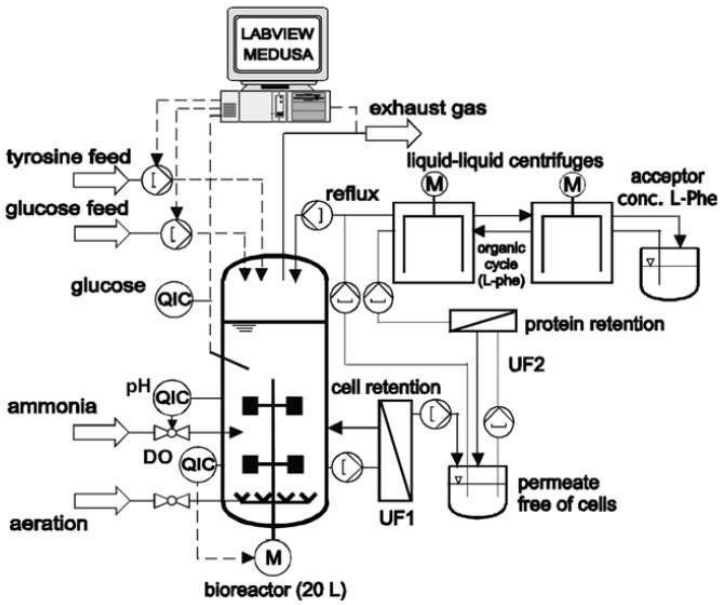

Figure 5.2.: Experimental set-up of the total ISPR process using two liquid-liquid centrifuges for reactive extraction of L-phenylalanine in cell- and protein-free permeate drained from the ultrafiltration module (UF1) installed in the bypass of the 20 l-bioreactor (figure taken from Rüffer *et al.*, 2004)

In enzyme catalysed processes the integration of a downstream processing step is much more difficult than in fermentation processes because educts and product of the reaction are chemically very similar. Especially the selective removal of carbohydrates from aqueous solutions is difficult due to their high hydrophilicity and structural similarity. Mostly the reaction of boronates with carbohydrates (compare section 4.2.2) is utilised for integrated product removal approaches.

A good example for the problems that occur when product removal is integrated with enzyme catalysed biotransformations was given by Chauhan *et al.* in 1997. Chauhan *et al.* investigated different methods of product removal (complexation with soluble, insoluble and immobilised boronates) for the enzymatic synthesis of L-erythrulose from glycoaldehyde and β-hydroxypyruvate using a transketolase. In this case ISPR was supposed to prevent the product from consecutive reaction. Systematic investigations of chemical properties and stabilities revealed that soluble complex formation was not suitable as product removal because the enzyme was inhibited by the soluble boronates. Immobilised boronates were chosen for ISPR although this method works best at alkaline pH-values where the product is not stable. In a batch reaction with ISPR 69 % yield were achieved after five hours, compared to 70 % yield without ISPR. Although the product removal with imobilised boronates seemed to be suitable for the system, the yield could not be improved, probably due to unspecific substrate binding to the adsorption material. To avoid unspecific binding the reaction was carried out with substrate feed (fed-batch process) as well, the yield achieved with ISPR was even lower than without ISPR.

Another enzyme catalysed type of reaction involving carbohydrates that is interesting for ISPR are transglycosylations. In this type of reaction a glycosidically bound sugar is transferred to another hydroxyl group. Usually the yield of such reactions are quite low (around 10 %) due to competing hydrolysis of the product. In 2001 Ahmed *et al.* published a method for ISPR for the synthesis of phenyl α-maltoside from 2-phenyl α-D-glucoside catalysed by a α-glucosidase (figure 5.3). The adsorption of the product to Affi-Gel 601® containing boronate ligands was performed concurrent with reaction. Although the optimum pH value for reaction did not correspond with the one for adsorption of the product as well, the yield of the process could be improved by 25 % compared to the non-ISPR reaction.

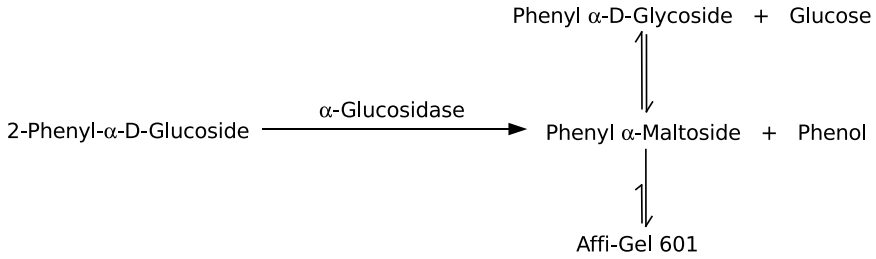

Figure 5.3.: Scheme of phenyl α-maltoside synthesis by transglycosylation including hydrolysis of the product and integrated product removal by adsorption to Affi-Gel (figure taken from [Ahmed et al. 2001]).

The examples given show that the introduction of an ISPR is elaborate and often the conditions of reaction and separation are not concordant. In the work presented here (see publication 6.3) we developed a model to describe the synthesis of neuraminic acid from GlcNAc and ManNAc and a model describing the reactive extraction of all compound to an organic phase. Combination of both models enabled us to simulate integrated product removal. The advantages of modelling ISPR processes are that the process can be simulated under various conditions in short time and, furthermore, the efficiency can be predicted without performing several experiments. Simulations can show quite fast if the selectivity of a method is high enough to countervail the increased requirements concerning technical equipment.

6. Publications

In this chapter three publications dealing with the optimisation of the enzyme catalysed neuraminic acid production process are presented. For each article a short summary and contibutions of the authors are given. For better understanding and classification of the single articles, the part of the process which was obtimised in the respective article, is marked by a red frame in a diagramm showing the different parameters of process optimisation (referring to scheme 1.1 in the introduction of this thesis).

As stated in chapter 2, future process optimisation should not, as done before, focus on separate parts of the process like upstream processing, reaction or downstream processing, but consider the interaction of these process parts. The optimisation performed here exceeds former approaches because

- kinetic models for reaction *and* the developed downstream processing method were developed
- different selectivities of possible applied purification methods were considered during simulation of the reaction (compare section 6.1)
- the developed models were used for not only simulation of the single reactions steps but also concurrent reaction and extraction and analysis of the interactions (compare section 6.3).

The results are summarized in chapter 7 and discussed in detail in chapter 8.

6.1. Modelling the reaction course of N-acetylneuraminic acid synthesis from N-acetyl-D-glucosamine – new strategies for the optimisation of neuraminic acid synthesis

V. Zimmermann, H.-G. Hennemann, T. Daussmann, U. Kragl, *Applied Microbiology and Biotechnology* **2007**, 76, 597-605.

Summary and classification

This article presents a kinetic model for the two-step enzymatic synthesis of *N*-acetyl-neuraminic acid. The model is used to simulate the reaction under various conditions [1]

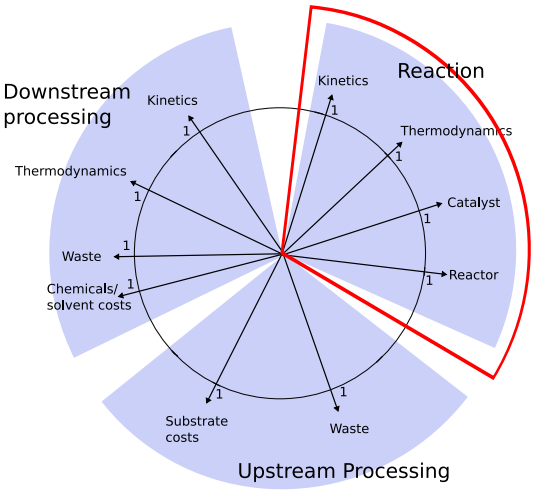

Figure 6.1.: Parameters that are subject to analysis and optimisation in this article.

and in different reactor types and an operating window for the applied ratio of catalysts is defined. Concerning the optimisation of the neuraiminic acid synthesis, this work deals with determination of reaction kinetics and thermodynamics and the application of the gained knowledge for the optimisation of catalyst consumption and choice of the reactor type (figure 6.1).

Contributions

All kinetic measurements and determination of kinetic constants for the enzymes used during this work were performed by myself. Dr. H.-G. Hennemann contributed to this

publication by performing the fed-batch experiment and providing the data presented in figure 4 on page 603. My own contribution averages out at approximately 90 %.

Appl Microbiol Biotechnol (2007) 76:597–605
DOI 10.1007/s00253-007-1033-6

Modelling the reaction course of *N*-acetylneuraminic acid synthesis from *N*-acetyl-D-glucosamine—new strategies for the optimisation of neuraminic acid synthesis

Vera Zimmermann · Hans-Georg Hennemann · Thomas Daußmann · Udo Kragl

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Abstract In this work, a model describing the complete enzyme catalysed synthesis of *N*-acetylneuraminic acid (Neu5Ac) from *N*-acetyl-D-glucosamine (GlcNAc) is presented. It includes the combined reaction steps of epimerisation from GlcNAc to *N*-acetyl-D-mannosamine (ManNAc) and the aldol condensation of ManNAc with sodium pyruvate yielding Neu5Ac. The model is expedient to predict the reaction course for various initial and feed concentrations and therefore to calculate reaction times and yields. The equilibrium constants calculated from the kinetic constants via the Haldane relationship correspond with experimental values very well (0.26 calculated and 0.24 experimental value for the epimerisation, 27.4 l mol^{-1} calculated and 28.7 l mol^{-1} experimental for the aldol condensation). The actual relevance of the model is shown by a scale-up. Using the model, an optimisation of reaction conditions in consideration of different targets is possible. Exemplarily, it is presented how the optimal ratio of the two enzymes in the reaction can be determined and how the composition of the reaction solution in a fed-batch reactor can be designed to meet downstream processing needs.

V. Zimmermann · U. Kragl (✉)
Abteilung für Analytische, Technische und Umweltchemie,
Universität Rostock,
Albert-Einstein-Str. 3a,
18059 Rostock, Germany
e-mail: udo.kragl@uni-rostock.de

H.-G. Hennemann · T. Daußmann
Julich Chiral Solutions GmbH,
Prof.-Rehm-Str. 1,
52428 Jülich, Germany

Introduction

The synthesis of *N*-acetylneuraminic acid (Neu5Ac) still attracts growing attention due to increased need for this compound in pharmaceutical industry (Samland and Sprenger 2006; von Itzstein et al. 1993). In spite of this growing need there was almost no advancement of the synthesis during the recent years, although a lot of problems like the unfavourable equilibrium, a high amount of waste per kilogram of product and difficult downstream processing are encountered in the process. The process consists of two reaction steps (Fig. 1). In a first step, *N*-acetyl-D-glucosamine (GlcNAc) is converted to *N*-acetyl-D-mannosamine (ManNAc). In the second step, ManNAc reacts with pyruvic acid to form the desired product. The epimerisation of GlcNAc to ManNAc catalysed by strong bases is long known (Kuhn and Brossmer 1958). Unfortunately, this reaction yields only 20% of ManNAc and large amounts of bases to catalyse the reaction as well as organic solvents for purification of the product are needed (Simon et al. 1988). Neu5Ac was first synthesised from ManNAc in 1958 by Comb and Roseman (1958). The reaction is catalysed by *N*-acetylneuraminic acid aldolase and suffers from an unfavourable equilibrium as well (K=28.7 l mol^{-1}). In large-scale production the product is usually purified by precipitation with glacial acetic acid (Maru et al. 1998). A first milestone facilitating the synthesis, reducing waste and improving the eco-efficiency was the use of biocatalysis to convert GlcNAc to ManNAc. The epimerisation is catalysed by the enzyme *N*-acylglucosamine-2-epimerase (E.C. 5.1.3.8), which was fist described by Ghosh and Roseman (1965).

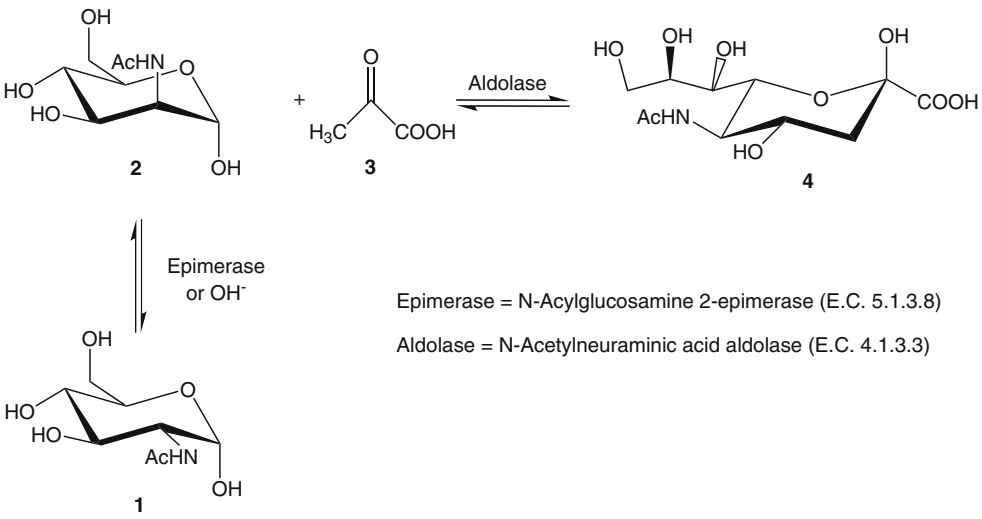

Fig. 1 Synthesis of *N*-Acetylneuraminic acid from GlcNAc in two steps

During the 1980s, Neu5Ac was still gained from natural sources like edible bird's nest or cow's milk (Schauer 1982) because the chemical synthesis is complex (Benzingnguyen and Perry 1978; Carroll and Cornforth 1960; Danishefsky and DeNinno 1986) and enzymatic synthesis was still investigated on laboratory scale. These investigations dealt with immobilisation (Augé et al. 1984) and membrane enclosing (Bednarski et al. 1987) of the aldolase, and new sources for the enzyme were opened up (Uchida et al. 1984). In this period, most approaches to improve the reaction were based on raising the amount of pyruvate added to the reaction mixture to shift the equilibrium towards the product side (Kim et al. 1988; Simon et al. 1988). The equilibrium yield varies between 27% at equal GlcNAc and pyruvate initial concentrations and 85% at a tenfold pyruvate excess referring to GlcNAc (initial GlcNAc concentration 0.1 mol l^{-1}). Unfortunately, a high pyruvate excess increases the complexity of later downstream processing. Neuraminic acid and pyruvic acid have the same pK_a value and can only be separated by elaborate chromatographic procedures or precipitation of Neu5Ac with glacial acetic acid. A large excess of pyruvate complicates these separations even more. The next great improvement was the combination of both reaction steps in one pot and the continuous production of Neu5Ac in the enzyme membrane reactor (Kragl et al. 1991a, b). Then there were great efforts to enhance productivity and efficiency of the process. Maru et al. (1998) fed pyruvate to the reaction solution when equilibrium was reached and obtained Neu5Ac in 77% yield and with a productivity of 1.8×10^{-3} mol l^{-1} h^{-1}. Mahmoudian et al. (1997) did not perform the reaction in one pot but tried to improve the process by enrichment of ManNAc after base-catalysed epimerisation. Other groups (Blayer et al. 1999; Ohta and Tsukada 1995) combined the base-catalysed epimerisation with the enzymatic aldol condensation and obtained Neu5Ac in yields from 31 to 51%. Because of improved genetic engineering and overexpression methods, the enzymes, at least the aldolase, were readily available in large amounts a few years later (Maru et al. 1996, 2002) and therefore, also, large-scale approaches were possible (Dawson et al. 2000; Maru et al. 1998). However, since the beginning of this century, there were no auspicious approaches for further optimisation of the process. In 2004, Lee et al. tried to improve yield by applying a temperature shift at expense of reaction time (Lee et al. 2004). The main reason for this deceleration of process improvement is that there are hardly any more possibilities left to improve the reaction by classical methods like optimisation of reaction conditions and substrate concentrations. Influencing the equilibrium of the aldol reaction by substrate feed, temperature shift or pH has readily been investigated, with hardly any improvements applicable for large-scale production. In fact, today, neuraminic acid is produced the same way as 15 years ago, i.e. in a classical batch process with considerable pyruvate excess and precipitation of the product with glacial acetic acid. Lye et al. (2002) stated that the use of new tools like directed evolution, bioinformatics and process modelling is necessary to implement more biocatalytic reactions in industrial scale. Concerning neuraminic acid synthesis, developments during the recent years concentrated on metabolic engineering (Koizumi et al. 2001, 2005; Tabata et al. 2002; Wang and Lee 2006; Wong and Lin 2002). The next step in enhancing this process is the optimisation with help of reaction engineering tools and modelling, whereas the later downstream processing has to be kept in mind all the time.

In this article we present new strategies for optimisation of neuraminic acid synthesis, a kinetic model suitable to describe batch and continuous flow reactors and first results of pilot-scale semi-continuous production of neuraminic acid.

Materials and methods

Chemicals

Sodium pyruvate was obtained from Fluka (Buchs, Switzerland), *N*-acetyl-D-mannosamine from Sigma-Aldrich (Steinheim, Germany). *N*-acetyl-D-glucosamine and *N*-acetylneuraminic acid were provided by Julich Chiral Solutions as well as *N*-acyl-D-glucosamine epimerase (E.C. 5.1.3.8) and *N*-acetylneuraminic acid aldolase (E.C. 4.1.3.3).

Enzyme assays

The activities of both enzymes were determined using the following assays: Epimerase, 20 mM GlcNAc, 2 mM $MgCl_2$, 2 mM adenosine triphosphate (ATP), 15 mM phosphate buffer, 37°C, pH 7.0, ca. 10 U ml^{-1}.

Aldolase, 20 mM Neu5Ac, 15 mM phosphate buffer, 37°C, pH 7.0, ca. 20 U ml^{-1}.

In both cases the vials were shaken in an Eppendorf thermo mixer comfort at 1,000 rpm, and periodically, aliquots were withdrawn and diluted in 0.1 mol l^{-1} sulphuric acid (1:10). Samples were analysed by high-performance liquid chromatography (HPLC). One unit is defined as the conversion of 1 μmol substrate per minute.

Kinetic constants

The K_M value for GlcNAc (epimerase) was obtained by determination of initial reaction rates for various substrate concentrations at a constant temperature of 25°C and a pH of 7.5. Substrate concentrations were varied between 0.007 and 0.197 mol l^{-1}, while the enzyme concentrations remained constant (0.12 U ml^{-1}). Periodically, aliquots of 100 μl were withdrawn and diluted 1:10 in sulfuric acid (pH 1). The samples were analysed by HPLC. All other constants, except inhibition constants, were derived by fitting the parameters to several reaction courses at various conditions.

Reaction conditions

Reactions were carried out in water at 25°C and pH 7.5. $MgCl_2$ and ATP were added in catalytic amounts (2 mM) as cofactors for the epimerase. The vials were shaken in an Eppendorf thermo mixer at 1,000 rpm. The pH was controlled manually during reaction. In the epimerase catalysed reaction, GlcNAc concentrations were varied between 0.05 and 0.88 mol l^{-1}. In the second reaction, ManNAc concentrations were varied between 0.04 and 0.57 mol l^{-1}, pyruvate between 0.26 and 1.7 mol l^{-1} and, investigating the reverse reaction, Neu5Ac concentrations were varied between 0.08 and 0.42 mol l^{-1}. Samples were diluted in 0.1 mol l^{-1} sulfuric acid, the dilution factor depending on initial concentrations, and monitored by HPLC.

High-performance liquid chromatography

The concentrations of GlcNAc, ManNAc, Neu5Ac and pyruvate were determined by HPLC measurement using a Biorad Aminex HPX-87H analytical column. Samples were eluted at 65°C with 0.006 mol l^{-1} sulphuric acid at 0.8 ml min^{-1} and monitored with refractive index and UV at 203 nm.

Liquid chromatography-mass spectrometry

HPLC-mass spectrometry (MS) analysis was performed using a Rezex-Monosaccharide H column (Phenomenex) with a mobile phase flow rate of 0.4 ml min^{-1} and MS determination by LCQ-Advantage (Thermo Fisher Scientific). A mixture of 90% water and 10% formic acid (1%) was used as mobile phase. Samples were diluted in water or formic acid. Five microlitres were applied to the column and identified by measurement of positive and negative ions within electrospray ionization mode between 70–2000 amu.

Modelling

All calculations, e.g. parameter fitting and solving the system of differential equations, were performed using MatLab 7.1.0.246 (R 14).

Results

Kinetic model

To model the reaction course of the neuraminic acid synthesis, the kinetics of every reaction step were determined. The reactions taking place simultaneously during the synthesis can be distinguished in Fig. 1. The enzymatic epimerisation of *N*-acetyl-D-glucosamine **1** is coupled with the aldolase-catalysed reaction of *N*-acetyl-D-mannosamine **2** with pyruvate **3** to yield neuraminic acid **4**. The reaction kinetics for both reactions were determined by initial rate

Table 1 Kinetic constants

Constant	Value
K_M^{GlcNAc}	$(1.76\pm0.26)\times10^{-2}$ mol l^{-1}
$K_M^{ManNAc,e}$	$(9.93\pm1.14)\times10^{-2}$ mol l^{-1}
A_V^{GlcNAc}	$(5.31\pm0.74)\times10^{-7}$ U l^{-1}
A_V^{ManNAc}	$(1.16\pm0.33)\times10^{-5}$ U l^{-1}
$K_i^{Pyr,e}$	0.146 ± 0.019 mol l^{-1}
$K_i^{Neu5Ac,e}$	0.719 ± 0.158 mol l^{-1}
K_M^{Pyr}	$(9.41\pm0.91)\times10^{-2}$ mol l^{-1}
K_M^{ManNAc}	$(1.31\pm0.15)\times10^{-2}$ mol l^{-1}
K_M^{Neu5Ac}	$(4.26\pm0.80)\times10^{-2}$ mol l^{-1}
A_V^f	$(7.51\pm1.11)\times10^{-8}$ U l^{-1}
A_V^r	$(1.05\pm0.05)\times10^{-7}$ U l^{-1}
K_i^{Pyr}	$(8.49\pm1.06)\times10^{-3}$ mol l^{-1}
K_i^{ManNAc}	$(1.19\pm0.91)\times10^{-2}$ mol l^{-1}
K_V	0.035 l mol^{-1}

measurements and progress curve analysis. The values for all constants are summarised in Table 1.

Epimerisation

The kinetics of the epimerisation of GlcNAc to ManNAc and the reverse reaction can be described with one single equation:

$$v_{Epi} = \frac{E_{epi}\left(\frac{A_V^{GlcNAc}\cdot[GlcNAc]}{K_M^{GlcNAc}} - \frac{A_V^{ManNAc}\cdot[ManNAc]}{K_M^{ManNAc,e}}\right)}{1+\frac{[GlcNAc]}{K_M^{GlcNAc}}+\frac{[ManNAc]}{K_M^{ManNAc,e}}+\frac{[Pyr]}{K_i^{Pyr}}+\frac{[Neu5Ac]}{K_i^{Neu5Ac,e}}} \quad (1)$$

The K_M value for GlcNAc was obtained by determination of initial reaction rates for various substrate concentrations at a constant temperature of 25°C and a pH of 7.5. The K_M value for ManNAc and the volume-specific activities were derived by fitting the parameters to several reaction courses. The initial GlcNAc concentration varied between 0.05 and 0.2 mol l^{-1}.

As both reactions steps are combined in one pot, inhibitions of the epimerase by the components of the second reaction step, that is sodium pyruvate and *N*-acetylneuraminic acid, are taken into account as well. Inhibitions were determined by performing the assay with different amounts of inhibitor added. Usually, competitive inhibition is described with the following modified Michaelis-Menten equation:

$$v = \frac{V_{max}[S]}{K_M\left(1+\frac{[I]}{K_i}\right)+[S]} \quad (2)$$

This equation can be applied here because the inhibitions were determined by initial rate analysis, so it is not necessary to consider the reverse reaction. The equation can be rewritten as

$$\frac{v_0}{v} = 1+\frac{(K_M/K_i)}{K_M+[S]}[I] \quad (3)$$

where $[S]$ and $[I]$ are the concentrations of the substrate and the inhibitor, respectively, and v_0 is the reaction rate at $[I] = 0$. The inhibition constant K_i^{Pyr} was calculated and found to be 0.146 mol l^{-1}. The inhibition constant for neuraminic acid was determined in the same manner and found to be 0.687 mol l^{-1}.

At equilibrium, the reaction velocity decreases to 0 and the relationship between kinetic parameters and the equilibrium constant can be described by the Haldane relationship (Cornish-Bowden 2004):

$$K_{eq}^{epi} = \frac{A_V^{GlcNAc}\cdot K_M^{ManNAc}}{A_V^{ManNAc}\cdot K_M^{GlcNAc}} \quad (4)$$

The equilibrium constant calculated via this equation is 0.26 ± 0.09, which corresponds well with the experimental value of 0.24 ± 0.02. The standard deviation of the calculated value may seem quite high. This is due to the fact that the kinetic parameters used to calculate the equilibrium constant already have standard deviations of about 10–15% themselves because of normal variance in experimental points and HPLC measurements. For this calculation, the inhibitions by sodium pyruvate and neuraminic acid were not taken into account because it refers to the epimerisation without combination with the second reaction step.

Neu5Ac synthesis from ManNAc

Mechanistic investigations were not subject of this work, but for the aldolase, an ordered bi-uni mechanism has been described (Baumann et al. 1989). This mechanism was assumed for the enzyme used here as well. In this case, the kinetic behaviour of the neuraminic acid aldolase can be described by the following equation:

$$v_{ald} = \frac{E_{ald}\left(\frac{A_V^f\cdot[ManNAc]\cdot[Pyr]}{K_i^{Pyr}\cdot K_m^{ManAc}} - \frac{A_V^r\cdot[Neu5Ac]}{K_m^{Neu5Ac}}\right)\cdot\frac{1}{1+\frac{[Pyr]_0+[ManNAc]_0+[GlcNAc]_0+[Neu5Ac]_0}{K_V}}}{1+\frac{[Pyr]}{K_i^{Pyr}}+\frac{K_m^{Pyr}\cdot[ManNAc]}{K_i^{Pyr}\cdot K_M^{ManNAc}}+\frac{[ManNAc]\cdot[Pyr]}{K_i^{Pyr}\cdot K_M^{ManNAc}}+\frac{[Neu5Ac]}{K_M^{Neu5Ac}}+\frac{[Neu5Ac]\cdot[ManNAc]}{K_i^{ManNAc}\cdot K_M^{Neu5Ac}}} \quad (5)$$

The K_M value for neuraminic acid and the activity factor A_V^f for the reverse reaction were obtained by initial rate analysis and found to be $(4.08\pm0.75)\times10^{-3}$ mol l^{-1} and $(1.05\pm0.05)\times10^{-7}$, respectively (data not shown). The K_M values for ManNAc and pyruvate, the activity factor for the forward reaction as well as the inhibition constants for pyruvate and ManNAc were obtained by reaction progress analysis. The constants are summarised in Table 1.

Comparison of experimental and modelled reaction course showed that at high substrate concentrations, the reaction course is much slower than described by the model. This could be due to the increasing viscosity of the solution. To take this into account, a factor including the sum of initial concentrations was applied. Therefore an inhibition constant related to the initial concentrations K_V was defined. At small initial concentrations, the factor is very small and does not influence the modelled reaction course. At high initial concentrations it grows larger and takes the slower reaction rate found in experiments into account. This additional term also includes the initial GlcNAc concentration so that the observed slight inhibition by GlcNAc is included in K_V as well. With this modified equation it is possible to calculate the concentrations of all reaction components during reactions as well as the equilibrium conversion.

In the same manner as before, it is possible to calculate the equilibrium constant from the kinetic constants using the Haldane relationship, which is slightly modified in this case:

$$K_{eq}^{ald} = \frac{A_V^f \cdot K_M^{Neu5Ac}}{A_V^r \cdot K_i^{Pyr} \cdot K_M^{ManNAc}} \quad (6)$$

The calculated value of 27.4 ± 8.1 l mol^{-1} correlates with the experimental value of 28.7 ± 10.4 l mol^{-1} very well. For the standard deviation, hold the same arguments as given for the epimerisation.

Kinetic model for both reaction steps

We further combined the kinetic equations for all reaction steps to model the reaction course. The pH of the solution was maintained constant during reaction. Hence the model was derived for a constant pH and reactive forms of the components, and hence dissociation constants for acidic groups are not considered. The HPLC analysis applied allowed the determination of components **1**–**4** concurrently, and the measured reaction courses were concordant with the model. The overall equilibrium constant $K_{eq}^{epi,ald}$ can be calculated from the equilibrium constants for the single reaction steps derived from the Haldane relationship and is 7.12 ± 3.25 l mol^{-1}.

As presented in Table 1, most parameters have a standard deviation between 10 and 15%, which is acceptable for constants based on experimental values derived via HPLC measurements. The deviation of K_i^{ManNAc}, which was derived by fitting some parameters to reaction courses, is significantly higher. This shows that a change in K_i^{ManNAc} does not significantly influence the model.

Optimisation and reaction engineering

Having developed a model that describes the complete reaction course of the neuraminic acid synthesis from GlcNAc including all parameters necessary to describe a system consisting of an equilibrium and a consecutive reaction, we considered an optimisation of the reaction. Although the determination of kinetics including inhibitions and other effects might seem elaborate, model building is a useful tool for reactor design and parameter optimisation (Vasic-Racki et al. 2003) and can clearly help shorten development times. This effort becomes more and more worthwhile, the cheaper sufficient calculating capacities get. About 15 years ago, a normal desktop PC worked about 30 min to solve a system of kinetic differential equations describing a reaction with four components. Today, these calculations take only seconds. This progress enables us, once the kinetics are determined, to simulate reaction courses for various parameter combinations in short time, and in doing so, gain knowledge and insight in factors influencing equilibria, conversion etc. much faster than by performing experiments. Therefore reaction modelling will be utilised also for bioprocesses more and more. The model developed here enables us to calculate the time needed to reach equilibrium and the conversion for different initial substrate concentrations and ratios as well it is possible to calculate the reaction course in different reactor types with different substrate feeds, e.g. GlcNAc and/or pyruvate feed. While performing these calculations, it is necessary to define a clear target. Such targets could be the maximisation of equilibrium yield, the shortening of reaction time, the minimisation of catalyst consumption or the ratio of the different reaction components at the end of the reaction. The latter is especially interesting concerning downstream processing.

Our calculations showed that in batch reactions the equilibrium conversion for the coupled reaction is 27% referring to GlcNAc at equal initial concentrations of GlcNAc and pyruvate. Employing a tenfold pyruvate excess, the equilibrium conversion reaches 85% referring to GlcNAc, which means only 8.5% referring to pyruvate (simulated with initial GlcNAc concentration of 0.1 mol l^{-1}). Not only the improvement of conversion is a classic target of optimisation of bioprocesses, but also the minimisation of catalyst consumption. Due to the progress in genetic engineering for effective enzyme production, many enzymes have become a lot cheaper over the last decade.

Nevertheless catalysts are an important cost factor in bioprocesses. Figure 2 illustrates the space–time yield of Neu5Ac achievable in a batch reactor at different epimerase and aldolase concentrations.

From the figure, it can be seen that, for optimal catalyst usage, it is necessary to apply the catalysts in the right ratio. At a fixed aldolase activity, it is useful to apply an epimerase activity that ensures that the epimerisation is at equilibrium and therefore ManNAc is available as substrate for the aldol reaction in the highest amount possible. Higher activities of epimerase do not lead to increased space–time yields because the aldol reaction is rate limiting, which is illustrated by the slope approaching 0. On the other hand, it is not economic to raise the aldolase concentration more than a certain value at a fixed epimerase concentration because the space–time yield does not increase proportionally with the aldolase concentration. This is illustrated by the figure because the distance between two curves with the same difference in aldolase concentration gets smaller the higher the aldolase concentration is. Thus the applied aldolase is used less effective, the higher its concentration gets. Experimentally, it is quite difficult to estimate the minimum enzyme concentrations and optimal ratios to reach the desired space–time yield. The model enables us to determine these parameters quite fast. The optimal ratio and amount of catalysts depend on enzyme costs, availability and solubility. The dashed straight lines in Fig. 2 mark two borderline cases. The left line marks the optimal ratio in case that the epimerase is much more expensive that the aldolase. With a minimum amount of epimerase, the space–time yield should be as high as possible. This occurs where the slope of the curves turns from very steep to less steep because, at a smaller slope, an increase in epimerase concentration does not lead to a high increase in space–time yield anymore. The right line marks the optimal ratio if the aldolase is much more expensive than the epimerase. In this case, the space–time yield should be as high as possible with a minimum amount of aldolase. This is possible by taking as little aldolase as possible and an amount of epimerase just before the slope of the curve approaches 0. Overall, the area between the straight lines is the range in which the reaction should be performed.

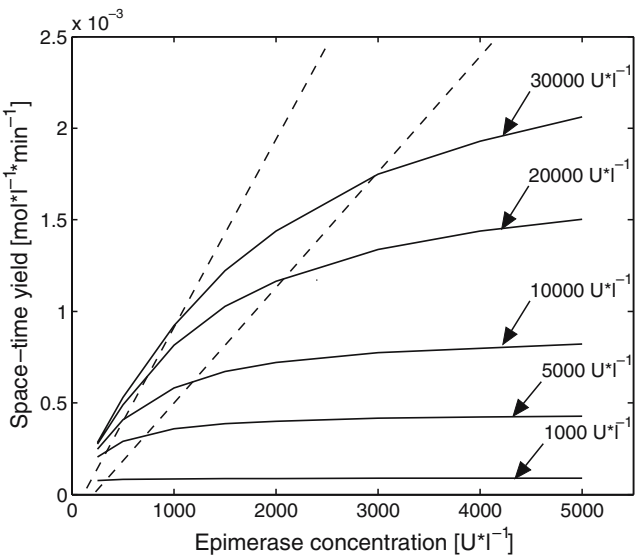

Fig. 2 Calculated space–time yield (at 98% of equilibrium conversion, c_0(GlcNAc)=0.1 mol l^{-1}, c_0(Pyr)=0.3 mol l^{-1}) at different concentrations of epimerase and aldolase. The *curves* are labelled with the aldolase concentration. The *straight lines* define the limits in which the reaction should be performed

In a fed-batch reactor, there are two possibilities to operate the reactor. First, a high amount of additional substrate can be added after the reaction reached equilibrium for the first time to shift the equilibrium towards higher product yield (Maru et al. 1998). There are several disadvantages in this case. First of all, after addition, the substrate concentration is very high, which leads to inhibition of the enzymes. Secondly, the reaction time is quite long because the equilibrium has to be reached several times and, as usual, reaction rates get very slow when the concentrations approach equilibrium. The second approach is the constant feeding of substrate starting shortly after the reaction start. The substrate should be fed in the amount that is consumed by the reaction to keep the reaction far from equilibrium and therefore reach high reaction rates. The disadvantage in this case is the higher operating expense due to additional equipment needed such as pumps and more complex control strategies.

Figure 3 shows the modelled reaction courses for a fed-batch reactor to which GlcNAc and pyruvate are fed simultaneously. Depending on the initial concentrations of the two substrates and their feed concentrations, the concentration ratio of neuraminic acid and GlcNAc or pyruvate at the end of the reaction has changed remarkably, while the conversion stays almost the same: (a) 38% referring to GlcNAc, 63% referring to pyruvate; (b) 63% referring to GlcNAc, 40% referring to pyruvate). The final Neu5Ac concentration is the same in both cases, but in example (a), the ratio of Neu5Ac/Pyr is 1.7 while it is 0.7 in example (b). As mentioned above, this ratio is critical for downstream processing today. The example shows that by reaction modelling, it is possible to optimise the composition of the reaction solution, referring to the requirements of downstream processing, quite fast.

Scale-up

To proof the results obtained by modelling, one of the modelled fed-batch reaction courses was performed at bench-scale (initial volume 0.1 l). Due to the requirements for high productivity at industrial scale reactions, the feed concentrations of GlcNAc and pyruvate were set to 1 and

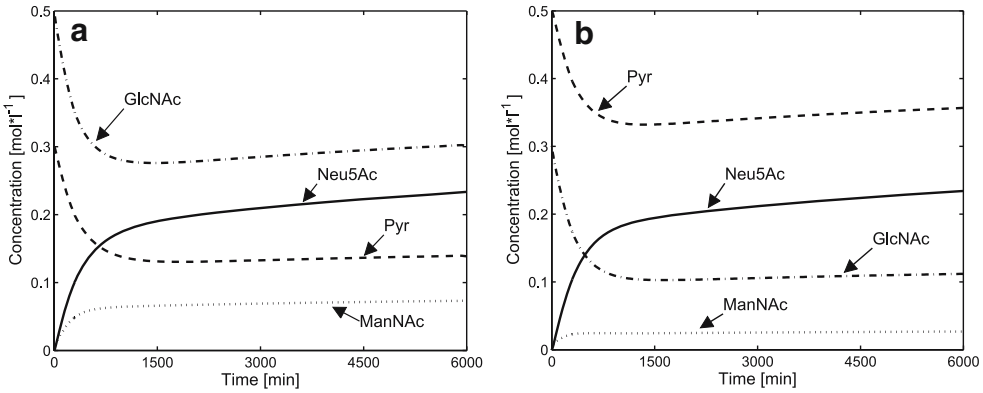

Fig. 3 Fed-batch simulations. Modelled reaction courses, GlcNAc and pyruvate are fed constantly from t=300 min at 0.1 ml min^{-1} (V_0=1 l, 1,500 U epimerase, 24,000 U aldolase) with different feed concentrations. **a** c_0(GlcNAc)=0.5 mol l^{-1}, c_0(Pyr)=0.3 mol l^{-1}, c_f(GlcNAc)=0.8 mol l^{-1}, c_f(Pyr)=0.5 mol l^{-1}. **b** c_0(GlcNAc)=0.3 mol l^{-1}, c_0(Pyr)=0.5 mol l^{-1}, c_f(GlcNAc)=0.5 mol l^{-1}, c_f(Pyr)=0.75 mol l^{-1}

0.9 mol l^{-1}, respectively. Figure 4 shows a good consistency of measured concentrations and modelled reaction course. The only concentration differing remarkably from the calculated data is the pyruvate concentration. The remarkable decline of pyruvate concentration during reaction is due to the formation of di- and trimers, which was approved by LC-MS measurements (data not shown). The formation of parapyruvic acid, a dimer of pyruvic acid, and other (aldol) condensation products and the instability of pyruvic acid as well as its condensation products under alcaline conditions is long known (von Korff 1964; Waldmann et al. 1954; Wolff 1899) and was observed in Neu5Ac synthesis before (Blayer et al. 1999). Furthermore, the condensation is known to be catalysed by Nickel(II) and Zinc(II) (Tallman and Leussing 1969) and therefore may be catalysed by Magnesium(II) as well, which is added to the solution in catalytic amounts as cofactor for the epimerase. The condensation was observed at lab scale reactions before but, as can be expected for self-condensation, only at high pyruvate concentrations (over 0.3 mol l^{-1}) and could be avoided by short reaction times. Another important point why the condensation is more prominent in the fed-batch experiment is that the reaction already starts in the stock solution that is fed to the reactor and has a very high pyruvate concentration (0.9 mol l^{-1}). Interestingly, during the fed-batch experiment, the reaction is not slowing down remarkably. Conclusively, the pyruvate di- or trimers are reactive as well or decompose to the monomer again as this is consumed by the reaction. In the further course, the reaction is becoming slower than predicted with the model due to formation of higher pyruvate oligomeres, which were detected by LC-MS as well. After 74.25 h, the Neu5Ac yield was 41% referring to the total amount of GlcNAc added. The productivity of the process was 3.3×10^{-3} mol l^{-1} h^{-1}. The total amount of pyruvate added equalled the amount of GlcNAc, so no excess of pyruvate was applied. The ratio of Neu5Ac to pyruvate at the end of the reaction was expected to be 1 (compare simulated reaction course) but turned out to be 3 due to the pyruvate condensation. The pyruvate oligomers present in the reaction mixture are probably less interfering during Neu5Ac purification than pyruvate itself because the pK_a value of the condensation products is different from that of pyruvic acid (pK_a=2.5) and Neu5Ac (pK_a=2.6). The dimer parapyruvic acid, which is a dibasic acid, has pK_a values of pK_{a1}=1.7 and pK_{a2}=3.7 (Tallman and Leussing 1969).

Discussion

A model describing the complete reaction course of the neuraminic acid synthesis from *N*-acetyl-D-glucosamine was developed. Therefore detailed kinetic investigations of the two enzymatic reactions were necessary. In addition, the combination of two enzymes in one pot required the investigation of various inhibitions.

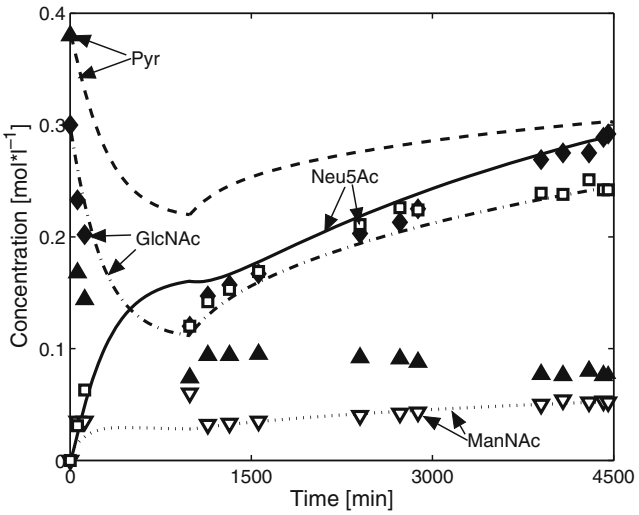

Fig. 4 Performed fed-batch experiment with modelled reaction course (Conditions: V_0=0.1 l, c_0(GlcNAc)=0.3 mol l^{-1}, c_0(Pyr)=0.38 mol l^{-1}, 150 U epimerase, 2,400 U aldolase, c_f(GlcNAc)=1 mol l^{-1}, c_f(Pyr)=0.9 mol l^{-1}, 1.2 ml h^{-1}, feed started after 990 min)

The model was used for the optimisation of the reaction. When optimising such complex reactions, it is necessary to define clear optimisation targets, which can differ according to the costs of the different process elements like substrate costs, operating costs of the reactor and downstream processing. In this work we showed how to calculate an operating window for the reasonable application of the two catalysts, depending on catalyst costs or availability. Furthermore, the reaction course of the two-step synthesis was simulated in a fed-batch reactor with different GlcNAc and pyruvate feed rates and initial concentrations. Prior fed-batch approaches were confined to pyruvate fed when the reaction approached equilibrium (Maru et al. 1998). Compared with this process, the productivity of Neu5Ac synthesis was improved from 1.8×10^{-3} to 3.3×10^{-3} mol l^{-1} h^{-1} in an exemplary fed-batch reaction that was carried out at litre scale. The reaction course was simulated and optimised before reaction, which demonstrates the benefits of reaction modelling for this bioprocess. Based on this study, Julich Chiral Solutions is revising the actual production process to make full use of its proprietary access to the required enzymes.

With further fed-batch simulations, it was demonstrated that reaction modelling allows to design the reaction solution to meet downstream processing needs by variation of feed flow, feed concentrations and initial concentrations. Unlike prior work and optimisation efforts dealing with this system, the approach presented here focuses not only on maximising conversion but utilises kinetic modelling as a tool to analyse the behaviour of the system under various conditions and optimise these conditions for several targets, including not only the conversion but also the requirements of downstream processing, catalyst consumption or reaction time. Due to the progress in computer performance, the calculations are so fast today that the effort of determining the kinetics of enzyme catalysed reactions, including inhibitions in multi-step reactions, is compensated by the benefits of reaction simulations. Thinking about multi-step biocatalytic reactions in particular, whose relevance for industrial production of complex chiral compounds will increase in the future (Bruggink et al. 2003), reaction optimisation without modelling is hardly imaginable due to multiple interactions between compounds of the different reactions steps.

Acknowledgements We would like to thank Dr. W. Ruth for performing as well as interpreting the LC-MS measurements. This work was supported by the scholarship programme of the German Federal Environmental Foundation (Deutsche Bundesstiftung Umwelt).

Appendix

Table 2 Abbreviations and symbols

A_V^{f}	Volume-specific activity for the forward reaction (aldolase), U l^{-1}
A_V^{GlcNAc}	Volume-specific activity for the forward reaction (epimerase), U l^{-1}
A_V^{ManNAc}	Volume-specific activity for the reverse reaction (epimerase), U l^{-1}
A_V^{r}	Volume-specific activity for the reverse reaction (aldolase), U l^{-1}
c_0	Initial concentration, mol l^{-1}
c_{f}	Feed concentration, mol l^{-1}
E_{ald}	Dilution factor (aldolase)
E_{epi}	Dilution factor (epimerase)
GlcNAc	*N*-acetyl-D-glucosamine
K_{eq}	Equilibrium constant
$K_{\mathrm{i}}^{\mathrm{Neu5Ac}}$	Inhibition constant for *N*-acetylneuraminic acid (aldolase), mol l^{-1}
$K_{\mathrm{i}}^{\mathrm{Neu5Ac,e}}$	Inhibition constant for *N*-acetylneuraminic acid (epimerase), mol l^{-1}
$K_{\mathrm{i}}^{\mathrm{Pyr}}$	Inhibition constant for pyruvate (aldolase), mol l^{-1}
$K_{\mathrm{i}}^{\mathrm{Pyr,e}}$	Inhibition constant for pyruvate (epimerase), mol l^{-1}
$K_{\mathrm{M}}^{\mathrm{GlcNAc}}$	Michaelis-Menten-constant for *N*-acetyl-D-glucosamine (epimerase), mol l^{-1}
$K_{\mathrm{M}}^{\mathrm{ManNAc,e}}$	Michaelis-Menten-constant for *N*-acetyl-D-mannosamine (epimerase), mol l^{-1}
$K_{\mathrm{M}}^{\mathrm{Pyr}}$	Michaelis-Menten-constant for pyruvate (aldolase), mol l^{-1}
K_M^{ManNAc}	Michaelis-Menten-constant for *N*-acetyl-D-mannosamine (aldolase), mol l^{-1}
$K_{\mathrm{M}}^{\mathrm{Neu5Ac}}$	Michaelis-Menten-constant for *N*-acetylneuraminic acid (aldolase), mol l^{-1}
K_{V}	Inhibition constant describing the viscosity of the medium, l mol^{-1}
ManNAc	*N*-acetyl-D-mannosamine
Neu5Ac	*N*-acetylneuraminic acid
v	Reaction velocity, mol l^{-1} min^{-1}
V_0	Initial volume, l

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6.2. Reactive extraction of N-acetylneuraminic acid - A new method to recover neuraminic acid from reaction solutions

V. Zimmermann, U. Kragl, *Separation and Purification Technology* **2008**, 61, 64-71.

Summary and classification

This article presents new separation method for neuraminic acid. The reactive extraction with phenlyboronic acid and trioctylmethylammoniumchloride could be an alternative downstream processing method. In this article thermodynamic data are presented (figure 6.2).
[1]

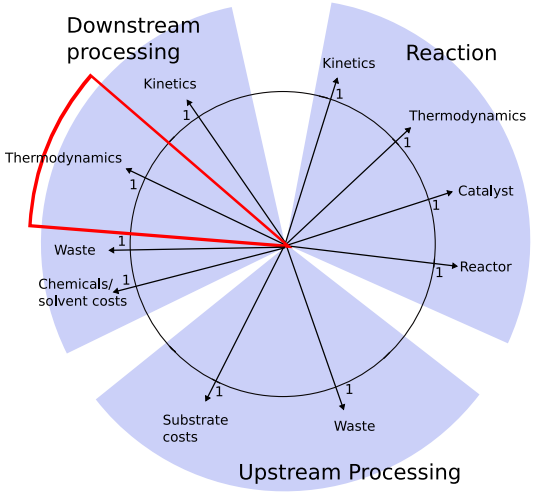

Figure 6.2.: This article deals with thermodynamic data of reactive extraction of Neu5Ac.

Contributions

100 % of the work published here were performed by myself.

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Reactive extraction of *N*-acetylneuraminic acid—A new method to recover neuraminic acid from reaction solutions

Vera Zimmermann, Udo Kragl *

Universität Rostock, Abteilung für Analytische, Technische und Umweltchemie, Albert-Einstein-Street 3a, 18059 Rostock, Germany

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Abstract

A new method for the purification of *N*-acetylneuraminic acid (Neu5Ac) is presented. A mixture of phenylboronic acid (PBA) and trioctylmethylammonium chloride (TOMAC) efficiently extracts the compound, which usually needs to be purified by elaborate chromatographic procedures or precipitation with glacial acetic acid. The presented extraction method is suitable to enrich Neu5Ac referring to pyruvate and the other compounds and facilitate further purification via classical crystallisation or chromatography, thus facilitating the established production process which utilises a large excess of pyruvate. The extraction system was characterised in detail by equilibrium studies for various solvents, pH values and carrier and substrate concentrations. The equilibrium constants determined for the two reaction steps (reaction with phenylboronic acid and reactive extraction) show that the equilibrium of extraction is more important than the extent of reaction with phenylboronic acid. The results obtained for pure compounds could be assigned to mixtures and reaction solutions without difficulty. Furthermore an efficient recovery of the carbohydrates from the organic phase is presented. The treatment of reactions mixtures showed that this system is not only feasible at lab scale and concentrations typical for scientific studies, but also for high substrate concentrations as used in industrial processes. The extraction step was shown to be the limiting factor in the extraction/recovery process.

Keywords: Neuraminic acid; Reactive extraction; TOMAC; Phenylboronic acid; Carbohydrates

1. Introduction

Reactive extraction is a method to remove hydrophilic compounds from aqueous solutions by altering them by a reversible chemical reaction to improve the solubility in an organic solvent. One of the chemical reactions applied for the reactive extraction of ionic species is ion exchange. The organic solvent contains a carrier compound, which consists of a cation with large organic chains and chloride as counterion to neutralise the charge. At the phase interface, the chloride anion is exchanged with the anionic substrate, which forms an ion pair with the cationic carrier and is transported into the organic phase (countertransport mechanism). The chloride moves into the aqueous phase. A common carrier used for reactive extraction is trioctylmethylammonium chloride (TOMAC) also known as Aliquat 336. It is a carrier which was first used to remove metallic ions from aqueous solutions [1,2], but is now also used to for reactive extraction of amino acids [3,4], other acids of low molecular weight [5,6] and even extractive separation of enantiomers [7]. In this work we present a carrier system consisting of TOMAC and phenylboronic acid that is suitable to selectively remove *N*-acetylneuraminic acid (Neu5Ac) from the reaction mixture after its synthesis from *N*-acetylglucosamine (GlcNAc).

The synthesis of *N*-acetylneuraminic acid (Neu5Ac) still attracts growing attention due to the increased need for this compound in pharmaceutical industry [8]. Usually neuraminic acid is produced from *N*-acetyl-D-mannosamine (ManNAc) and sodium pyruvate (Pyr) catalysed by *N*-acetylneuraminic acid aldolase (E.C. 4.1.3.3) or from the ManNAc epimer *N*-acetyl-D-glucosamine (GlcNAc), which is quite cheap and can be obtained by hydrolisation of shrimp shells. The epimerisation of GlcNAc can be catalysed by an inorganic base like sodium or calcium hydroxide or by the enzyme *N*-acyl-D-glucosamine epimerase (E.C. 5.1.3.8). As both enzymes work under similar conditions, the reaction can be performed in one pot [9]. The reaction scheme is shown in Fig. 1.

* Corresponding author. Tel.: +49 381 498 6450; fax: +49 381 498 6452.
E-mail addresses: vera.zimmermann@uni-rostock.de (V. Zimmermann), udo.kragl@uni-rostock.de (U. Kragl).

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Nomenclature

A	carbohydrate (GlcNAc, ManNAc or Neu5Ac)
c_0	initial concentration
Cl^-	chloride anion
GlcNAc	*N*-acetyl-D-glucosamine
Hept.	heptane
Hex.	hexane
ManNAc	*N*-acetyl-D-mannosamine
MTBE	methyl *tert*-butyl ether
Neu5Ac	*N*-acetylneuraminic acid
Oct.	octanol
PBA	phenylboronic acid
PVR	phase volume ratio
Pyr	sodium pyruvate/pyruvic acid
OH^-	hydroxide ion
Q^+	trioctylmethylammonium cation
TOMAC	trioctylmethylammonium chloride

However, both reaction steps suffer from low yields due to the thermodynamic equilibrium. The equilibrium constant of the GlcNAc epimerisation is 0.24, the one of the aldol condensation is 28.7 l/mol [10]. The conversion can be improved by adding an excess of pyruvate, usually 2–10-fold compared to ManNAc [11–13]. The problem with shifting the reaction in this way is the difficulty of downstream processing, based on the fact that pyruvate and neuraminic acid have the same pK_a value (2.5 and 2.6, respectively). These components can only be separated by chromatography, where long columns and large amounts of solvents are needed, or by precipitation of neuraminic acid with large amounts of glacial acetic acid [14].

Therefore, it is still interesting to find new downstream processing methods for one or both steps of the synthesis to improve the overall yield of the process. In this work, we present the results of our work dealing with the selective extraction of neuraminic acid from the reaction mixture.

2. Theory

In basic aqueous solutions *N*-acetylneuraminic acid exists mostly in its ionic form (pK_a = 2.6). Therefore, it could be assumed that it can be extracted to an organic phase via ion exchange with TOMAC. Unfortunately, Neu5Ac is a very hydrophilic compound and the ion exchange does not occur in considerable extent. For the reactive extraction, a further reaction step hast to be inserted to lower the hydrophilicity of the compound. The reaction chosen is the well-known reaction of polyols with phenylboronic acid (PBA).

For the developed extraction of carbohydrates mediated by the carrier-system PBA/TOMAC a two-step mechanism is assumed. This assumption will be supported with further data presented in this paper. First, the carbohydrate reacts with phenylboronic acid to give a negative charged complex (Fig. 2). This kind of reaction of PBA with diols is well-known [15,16]. Like all boronic acids phenylboronic acid (pK_a = 8.8) [17] exists in two forms in the aqueous phase, the uncharged trigonal form and the negative charged tetrahedral form with one OH-group added at the boron centre. Only the latter reacts with carbohydrates to form esters. The amount of PBA existing in the reactive tetrahedral form depends strongly on the pH value of the solution. This reaction step can be described by the following equation:

$$A + PBA^- \rightleftharpoons A - PBA^- + 2H_2O \quad (1)$$

The reaction described is an equilibrium reaction and the equilibrium constant can be defined as follows:

$$K_{PBA,A} = \frac{[A - PBA^-]}{[A][PBA^-]} \quad (2)$$

The concentration of water is assumed to be constant and is included in $K_{PBA,A}$. The structure of the ester between Neu5Ac and a PBA derivative was investigated in detail by Otsuka et al. [18].

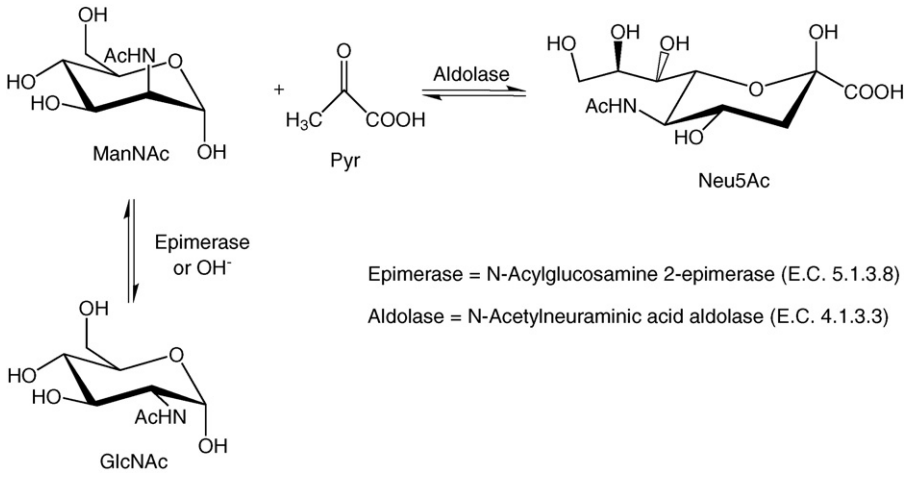

Fig. 1. Synthesis of neuraminic acid from GlcNAc by combination of GlcNAc epimerisation and aldolase catalysed reaction.

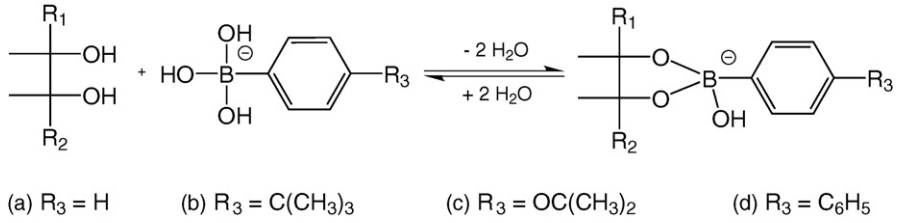

Fig. 2. The reaction of phenylboronate anion with polyols. Different phenylboronic acid derivatives were tested for extraction: (a) phenylboronic acid, (b) *tert*-butylphenylboronic acid, (c) 4-isopropoxyphenylboronic acid, (d) 4-biphenylboronic acid.

In the second step the negative charged complex reacts with TOMAC. The compound consists of the cation trioctylmethylammonium (Q^+) and a chloride anion (Cl^-). Because of the bulky hydrophobic chains of the cation it is possible to dissolve the ion pair in organic solvents. In the reactive extraction step the hydrophilic anion, that means the carbohydrate-phenylboronic acid complex (A-BPA^-) in this case, is exchanged with the chloride anion and the new carrier-PBA-carbohydrate complex (QA-PBA) is transported into the organic phase. This countertransport process which takes place at the interface can be expressed by Eq. (3).

$$A - PBA^- + QCl \rightleftharpoons QA - PBA + Cl^- \quad (3)$$

The equilibrium constant for the extraction process is defined in Eq. (4).

$$K_{ex,A} = \frac{[QA - PBA][Cl^-]}{[A - PBA^-][QCl]} \quad (4)$$

Unfortunately, the carrier does not selectively transport one anion to the organic phase, but every anion species present in the aqueous phase is a possible substrate. Therefore, the coextraction of OH^-, pyruvate and the carbohydrate-PBA complexes of ManNAc and GlcNAc are processes competing with the extraction of neuraminic acid. Eqs. (3) and (4) are valid to describe the ion exchange process and the equilibrium constant for these processes as well.

3. Materials and methods

3.1. Chemicals

Sodium pyruvate was obtained from Fluka (Buchs, Switzerland), *N*-acetyl-D-mannosamine, trioctylmethylammonium chloride, phenylboronic acid and its derivatives from Sigma–Aldrich (Steinheim, Germany). *N*-Acetyl-D-glucosamine and *N*-acetylneuraminic acid were provided by Julich Chiral Solutions (Jülich, Germany). All solvents were obtained from J.T. Baker and of "Baker analyzed" grade (Deventer, Netherlands). All compounds were used without further purification. Enzymes were provided by Julich Chiral solutions. The pH value was adjusted using sodium hydroxide or hydrochloric acid.

3.2. Extraction

3.2.1. Equilibrium data for pure components

For determination of equilibrium data 500 μl of aqueous phase containing the carbohydrate and 500 μl of organic solvent containing TOMAC and phenylboronic acid were mixed on a vortex mixer and the pH of the aqueous phase controlled and adjusted with sodium hydroxide if required. Subsequently, the solution was shaken in a Eppendorf thermomixer comfort at 1000 rpm for 15 min to equilibrate. After equilibration the pH was measured again. Samples were taken from the aqueous phase and diluted in 0.1 mol/l sulphuric acid, the dilution factor depending on initial concentrations, and monitored by HPLC. The distribution coefficient of phenylboronic acid was determined accordingly: 500 μl of water containing different amounts of sodium hydroxide were mixed with 500 μl of 0.1 mol/l PBA/TOMAC in *n*-heptane/1-octanol (85:15) and shaken at 25 °C for 15 min. The equilibrium pH was measured and PBA concentration in the aqueous phase determined by HPLC.

For determination of equilibrium constants experiments were carried out in deionised water and additional to HPLC measurements the chloride concentration was determined by ion chromatography (IC).

For recovery experiments the extraction was performed with 3.5 ml volume per phase. After 15 min 500 μl samples of the organic phase were withdrawn and mixed with 500 μl aqueous solution with different amounts of hydrochloric acid added. After another 15 min mixing the organic phase was removed, the pH of the aqueous phase was measured and HPLC samples withdrawn.

All extraction experiments were performed at a phase volume ratio (PVR) of 1. Each measurement was done in triplicate and the mean and standard deviation were calculated.

3.2.2. Reaction solutions

Reaction solutions were filtered with Pall Macrosep 10 K Omega centrifugal devices at 3300 × *g* to remove enzymes and other components of the cell raw extract. Seven millilitres of the solution were treated with sodium hydroxide and extracted with equal volumes of 0.5 mol/l carrier solution for 5 min. The pH value of the aqueous phase at equilibrium was 10.9. The solution was centrifuged for 5 min to accelerate phase separation. After sample drawing the aqueous phase was removed and replaced with 7 ml of concentrated hydrochloric acid and vortexed for 5 min. After centrifugation the organic phase was removed and

aliquots were taken from the aqueous phase. The pH value of the aqueous phase was 1.0.

3.3. HPLC/IC

The concentrations of GlcNAc, ManNAc, Neu5Ac, pyruvate and PBA were determined by HPLC-measurement using a Biorad Aminex HPX-87H analytical column. Samples were eluted at 65 °C with 0.006 mol/l sulphuric acid at 0.8 ml/min, and monitored with refractive index and UV at 203 nm.

Chloride concentrations were determined by ion chromatography (IC) using a Hamilton PRP-X110S column, a Metrohm 828 IC Dual Suppressor and monitored with an Alltech 550 Conductivity Detector at 35 °C. A buffer containing 1.7 mmol/l sodium hydrogen carbonate, 1.8 mmol/l sodium carbonate and 0.1 mmol/l sodium thiocyanate was used as mobile phase.

4. Results and discussion

4.1. General conditions

To develop a method suitable to extract neuraminic acid from the reaction solution after enzymatic synthesis, the carrier system PBA/TOMAC was used together with an organic solvent.

There are two factors influencing the choice of the organic solvent: First, the carrier compounds have to dissolve properly. All the solvents listed in Fig. 3 dissolve PBA and TOMAC in a concentration of at least 0.1 mol/l, which was used during this investigation. Second, the extracted amount of neuraminic acid should be as high as possible to give the best overall yield. The results of the solvents tested are presented in Fig. 3.

The highest amount of neuraminic acid was extracted when a mixture of *n*-hexane or *n*-heptane with 1-octanol (85:15, v/v) was used as organic solvent. Both solvent mixtures dissolve PBA and TOMAC very well (>0.5 mol/l). For further experiments the mixture of *n*-heptane and 1-octanol was chosen because *n*-hexane is neurotoxic [19,20] and therefore heptane will be easier to handle in a later industrial extraction process.

We next thought about an improvement of the carrier system. The combination of PBA and TOMAC as carrier for the extraction of carbohydrates was investigated before for glucose, mannose and several other common carbohydrates [21–23]. The smaller and more hydrophobic an anion is, the better it is transported to the organic phase with TOMAC as carrier [24,1], but neuraminic acid is a very polar compound because of the various OH-groups it contains. We increased the hydrophobicity of the carbohydrate-phenylboronic acid complex by using different derivatives of phenylboronic acid. The derivatives used were *tert*-butylphenylboronic acid, 4-isopropoxyphenylboronic acid and 4-biphenylboronic acid. The amount extracted of neuraminic acid varied between 30% and 60% at an equilibrium pH of 11. With phenylboronic acid carrying no other substituents 85% of neuraminic acid could be extracted into the organic phase, so it was further used for the process.

An important factor influencing the equilibrium of the system is the pH value, because PBA exists in two forms in the aqueous phase, of which only the ionic form is reactive, and the relative amount of each form depends on the pH of the solution. So the first reaction step of the extraction is pH-depended and therefore the overall extraction as well. Fig. 4 shows the distribution coefficients of GlcNAc, ManNAc, Neu5Ac and pyruvate at different pH values at equilibrium. For further experiments a pH value of 11 was chosen because the amount of extracted neuraminic acid is highest and the selectivity does not depend on the pH very much.

As reported before, *N*-acetylneuraminic acid is not stable at high pH values [25,26]. Fortunately, the extraction process is very fast so that reaction times can be short. After 15 min of extraction at pH 11 86% of Neu5Ac are extracted (47% GlcNAc, 40% ManNAc, 33% Pyr) and only negligible amounts of byproducts are observed by HPLC.

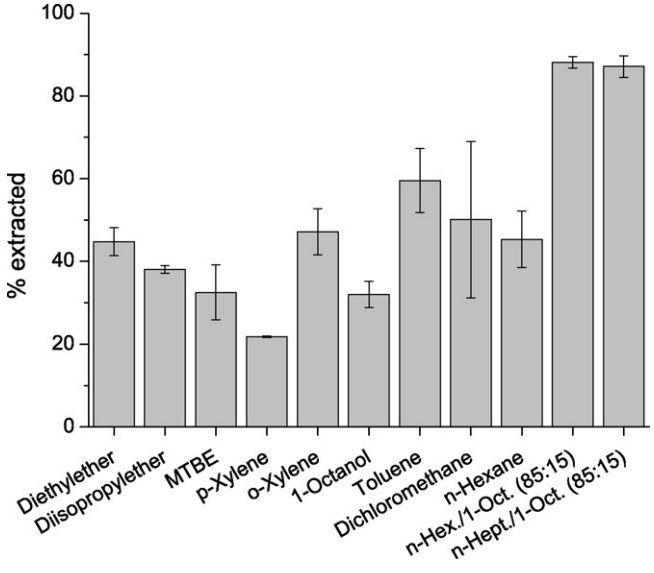

Fig. 3. The amount of neuraminic acid extracted from the aqueous phase at equilibrium with different solvents used as organic phase and PBA/TOMAC used as carrier (T = 25 °C, PVR = 1, c_0(Neu5Ac) = 0.01 mol/l, c_0(PBA/TOMAC) = 0.1 mol/l).

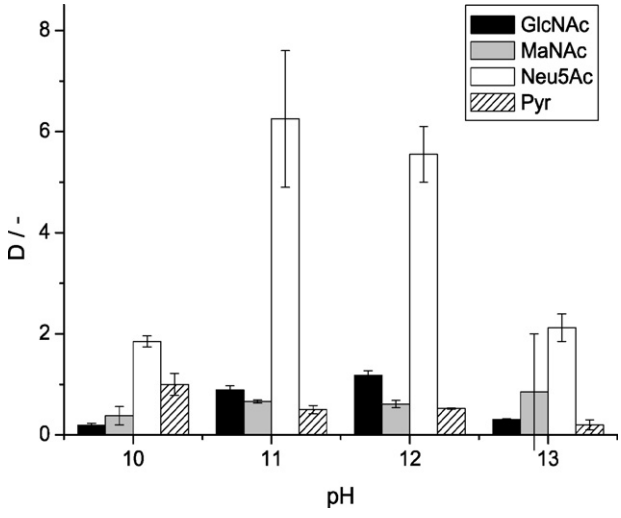

Fig. 4. The amount of carbohydrate extracted from the aqueous phase at equilibrium depends on the pH value of the solution (T = 25 °C, PVR = 1, c_0(substrate) = 0.01 mol/l, c_0(PBA/TOMAC) = 0.1 mol/l).

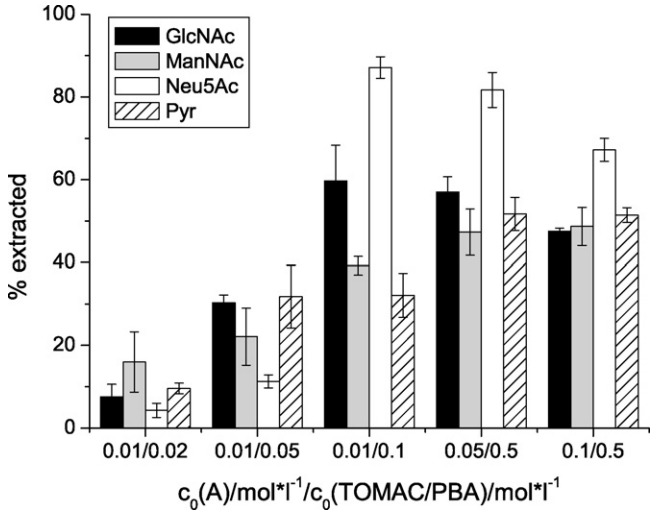

Fig. 5. The amount of substrate extracted from the aqueous phase at equilibrium at different initial substrate and carrier (PBA/TOMAC) concentrations at 25 °C.

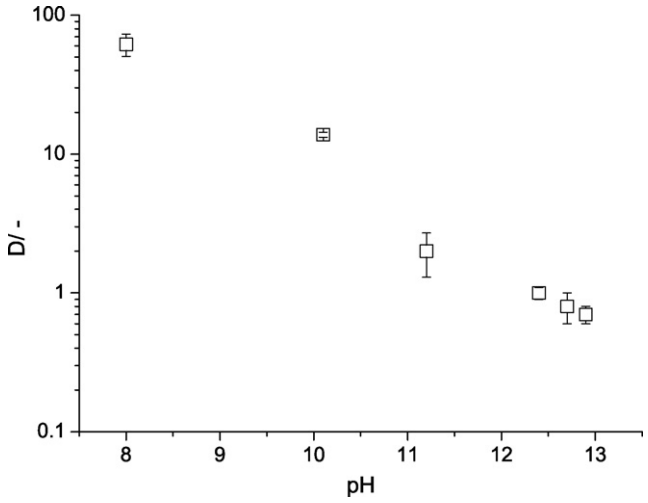

Fig. 6. Distribution coefficient D of PBA is changing with pH (initial TOMAC/PBA concentration was 0.1 mol/l, organic phase: *n*-heptane/1-octanol 85:15, PVR = 1).

Another factor influencing the amount of carbohydrate extracted is the initial carbohydrate concentration, the carrier concentration and the ratio of both. The combination of 0.01 mol/l initial carbohydrate concentration and 0.1 mol/l carrier is not suitable for industrial purpose, because the carbohydrate concentration is much too low. We tested extraction at initial concentration up to 0.1 mol/l carbohydrate and 0.5 mol/l carrier. The results presented in Fig. 5 show that the percentage extracted depends primarily on the ratio of initial carbohydrate and carrier concentration, but rises with increased initial concentrations as well. The increase with increasing carrier excess is probably due to formation of ion clusters with several ion pairs in the organic phase, which leads to better shielding of the ionic charges. The increase with increasing carbohydrate concentration may be due to suppression of OH^--coextraction at high carbohydrate concentrations ascribable to competing equilibria.

4.2. Assumed mechanism

The mechanism assumed for the extraction of carbohydrates is a two-step ion exchange mechanism. Recently, also a H-bonding mechanism has been discussed for the extraction of lactic acid into ionic liquids [27]. We prefer to assume the two-step mechanism described in the theory part because considerable extraction only occurs at high pH values and only in the presence of PBA. The first step, reaction with the anionic form of PBA, is necessary to make the carbohydrates extractable and, as only the anionic form of PBA is reactive, the product of the first reaction step is a carbohydrate-PBA ester with negative charge (compare Fig. 2). This negative charged compound can be extracted by TOMAC *via* ion exchange.

The extraction can only occur at high pH values because the distribution of PBA between organic and aqueous phase depends strongly on the pH as shown in Fig. 6. The distribution coefficient

$$D = \frac{c_{org}}{c_{aq}} \quad (5)$$

(with c_{org} – equilibrium concentration in the organic phase, c_{aq} – equilibrium concentration in the aqueous phase) decreases rapidly with increasing pH, which means that the distribution is shifted to the aqueous phase. This is due to the equilibrium of PBA in its charged and uncharged form in the aqueous phase. The equilibrium depends strongly on the pH value. At pH values over the pK_a of PBA, which is 8.8, the fraction of charged PBA is strongly increasing with increasing pH and as it is soluble much better in the aqueous phase than uncharged PBA, the distribution is shifted to the aqueous phase. A considerable amount of PBA in the charged form is only present in the aqueous phase at pH values of about 10 and higher. As extraction of Neu5Ac, ManNAc and GlcNAc is only observed under these conditions it is very likely that these compounds have to react with PBA to the negative charged ester in the water phase before being extracted by TOMAC into the organic phase in an ion exchange mechanism.

The extraction of Pyr does not depend very much on pH and no reaction with PBA could be observed when mixing the compounds [16]. Therefore, it is assumed that Pyr is extracted directly by TOMAC without prior reaction with PBA.

4.3. Equilibrium constants

Usually, equilibrium constants for an extraction are determined from a plot of [QA][Cl^-] vs. [A][QCl] following Eq. (4) [28]. This is not possible with the PBA reaction prior to extraction because the concentration of the carbohydrate-PBA complex in the aqueous phase cannot be determined. Therefore, an overall equilibrium constant for both reaction steps is defined using Eqs. (2) and (4):

$$K_A^* = K_{ex,A} \times K_{PBA,A} = \frac{[Cl^-][QA\text{-}PBA]}{[QCl][A][PBA^-]} \quad (6)$$

This constant can be determined by plotting [Cl^-][QA-PBA] vs. [QCl][A][PBA^-] (Fig. 7). The chloride, carbohydrate and phenylboronic acid concentrations at equilibrium

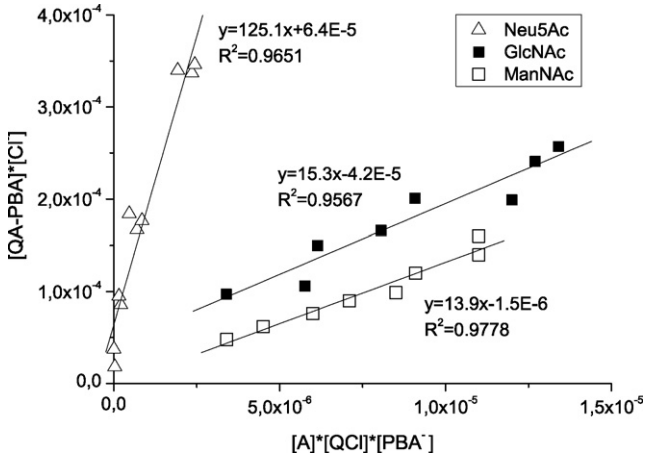

Fig. 7. Determination of overall extraction constant K_A^* via linear plot. The initial carbohydrate concentration was varied between 0.001 and 0.01 mol/l, initial TOMAC/PBA concentration was 0.1 mol/l.

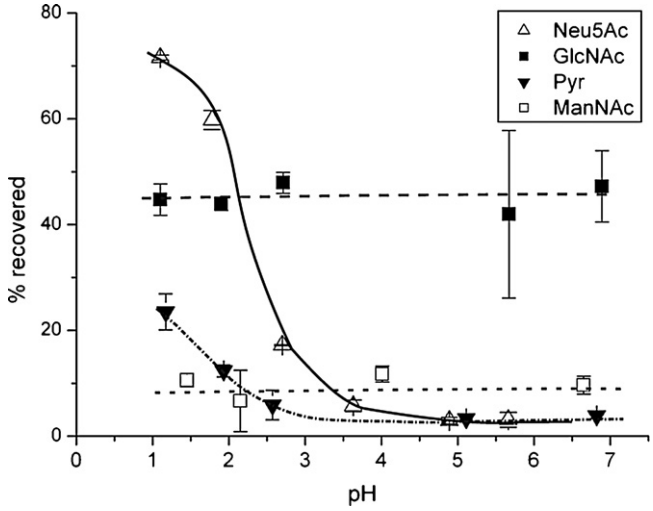

Fig. 8. Amount of carbohydrates and pyruvate recovered after extraction with PBA/TOMAC in *n*-heptane/1-octanol and subsequent reextraction of the organic phase with acidic aqueous solution (initial carbohydrate/pyruvate concentration: 0.01 mol/l, initial PBA/TOMAC concentration: 0.1 mol/l).

were determined by ion chromatography and HPLC. The amount of negative charged PBA was calculated via the Henderson–Hasselbalch-equation and the other concentrations via mass balances. The linearity of the plot proofs the assumption of a 1:1 reaction between the carbohydrate and PBA and with the carrier. The values obtained for K_A^* are 125.1 ± 12.0 l/mol for Neu5Ac, 15.3 ± 1.9 l/mol for GlcNAc and 13.9 ± 1.2 l/mol for ManNAc.

To determine the equilibrium constant for the extraction $K_{ex,A}$, the constant for the reaction of carbohydrate with phenylboronic acid $K_{PBA,A}$ has to be known. The constant can be determined by measuring the change in pH when carbohydrate and PBA are mixed [16]. Using this method the constants were determined to be 15.5 ± 1.8 l/mol, 128.6 ± 14.5 l/mol, and 22.4 ± 0.8 l/mol for GlcNAc, ManNAc and Neu5Ac, respectively. Using Eq. (6), $K_{ex,A}$ can be calculated which gives results of 0.99 ± 0.17 for GlcNAc, 0.11 ± 0.02 for ManNAc and 5.6 ± 0.6 for Neu5Ac. Pyruvate does not react with PBA prior to extraction with TOMAC. The deprotonated form of Pyr present in basic solutions is probably extracted directly by TOMAC like other organic acids [3,29,30]. The equilibrium constant $K_{ex,Pyr}$ can therefore be determined by plotting [Q-A][Cl^-] vs. [A][QCl]. $K_{ex,Pyr}$ is 0.38 ± 0.01 (data not shown).

4.4. Recovery from the organic phase

The carbohydrates can easily be recovered from the organic phase by mixing with a fresh aqueous phase with low pH. As presented in Fig. 8, the amount recovered from the organic phase rises with decreasing pH value for Neu5Ac and pyruvate, probably because of their acidity. The protonation/deprotonation equilibrium seems to be an important factor driving the extraction equilibrium back to the organic phase when the major fraction of the acid is protonated. This may be assumed because the experimental data show that the amount recovered increases strongly at pH values lower that the acids pK_a value. Moreover, it also indicates that the neutral form of neuraminic acid is not extracted into the organic phase, which further supports the assumption of an ion exchange mechanism. The amount of GlcNAc and ManNAc recovered does not change significantly between pH values of 1 and 7. As the amount of Neu5Ac recovered increases dramatically at pH values lower than 2, the selectivity is best at these pH values and therefore the product recovery should be performed under such conditions.

4.5. Reaction solutions

To test the developed extraction process under real conditions neuraminic acid was prepared in a usual batch reaction with twofold excess of pyruvate. At equilibrium, the reaction solution contained 0.12 mol/l GlcNAc, 0.02 mol/l ManNAc, 0.30 mol/l Pyr and 0.13 mol/l Neu5Ac. The solution was extracted with equal volumes of carrier solution. In the first experiment (R1) the carrier concentration was 0.5 mol/l, in the second experiment 1.0 mol/l (R2). Before extraction, the enzymes were removed by ultrafiltration and the pH adjusted to a strong alkaline value with sodium hydroxide. After extraction the aqueous phase was removed and replaced by concentrated hydrochloric acid.

In fed-batch reactions the reaction solution that has to be processed can be designed to meet the requirements of downstream processing better than in batch reactions. A high yield of neuraminic acid can be achieved with an excess of GlcNAc as well [10]. Two model solutions with concentrations according to fed-batch reactions simulated in [10] were prepared. The first solution contained an excess of GlcNAc (0.32 mol/l GlcNAc, 0.05 mol/l ManNAc, 0.20 mol/l Pyr and 0.25 mol/l Neu5Ac), the second, an excess of pyruvate (0.10 mol/l GlcNAc, 0.01 mol/l ManNAc, 0.35 mol/l Pyr and 0.23 mol/l Neu5Ac). The solutions were treated as the reaction solutions, but without ultrafiltration (1.0 mol/l PBA and TOMAC in the organic phase).

In all experiments described the pH value of the aqueous phase varied between 10.9 and 11.5 after extraction and 0.6 and 1 after reextraction. The amounts of each component that were

Table 1
Amount of carbohydrates and pyruvate extracted (extraction) and recovered (recovery) from batch reaction mixtures (R1 and R2, details see text) and model solutions for fed-batch processes (R3 and R4)

	R1			R2		
	c_0 (mol/l)	Extraction (%)	Recovery (%)	c_0 (mol/l)	Extraction (%)	Recovery (%)
GlcNAc	0.12	44.2	23.0	0.12	58.7	39.3
ManNAc	0.02	41.9	13.6	0.02	38.1	29.8
Pyr	0.30	40.1	8.5	0.30	50.2	9.2
Neu5Ac	0.13	31.0	32.2	0.13	47.5	32.9
	R3			R4		
	c_0 (mol/l)	Extraction (%)	Recovery (%)	c_0 (mol/l)	Extraction (%)	Recovery (%)
GlcNAc	0.32	34.2	26.7	0.10	52.5	36.2
ManNAc	0.05	36.6	27.3	0.01	11.5	36.2
Pyr	0.20	54.3	17.6	0.35	60.1	14.8
Neu5Ac	0.25	44.9	32.3	0.23	53.4	42.4

The amounts refer to the initial concentration before the first extraction step.

extracted in the first step and recovered in the second are listed in Table 1.

The results show that less carbohydrate is extracted than in previous experiments, which is due to the high concentrations that are necessary to meet the requirements of industrial production processes. In the experiments R1 and R2 the concentration of all compounds together is 0.57 mol/l while the PBA/TOMAC concentration in the organic phase is 0.5 mol/l and 1.0 mol/l, respectively. Although there is no excess of carrier or not even a two-fold excess, more 30–50% of every compound is extracted. This is more than could be expected from preliminary experiments (compare Fig. 5). Per mole of carrier more substrate is extracted, which means that the extraction process is becoming more efficient at high concentrations. On the other hand, the extraction step is the limiting factor in the overall process, because only 30–40% of neuraminic acid are extracted. Fortunately, the back extraction from the organic phase proceeds with high yields up to 100%. Unfortunately, the carrier concentration cannot be increased unlimited (even if pure TOMAC is used as solvent for the organic phase, the maximum concentration is 2.2 mol/l). Furthermore, although phenylboronic acid and TOMAC are completely miscible with *n*-heptane/1-octanol, the viscosity of the organic phase is strongly increasing with increasing TOMAC concentration. This may lead to mass transport limitations and more complicated handling of the solutions.

Altogether a significant enrichment of Neu5Ac can be achieved. Depending on the different ratios between GlcNAc, ManNAc, pyruvate and Neu5Ac concentration different amounts of the compounds are extracted due to competing equilibria. However, even with 2–3-fold excess of pyruvate in the original solution (R1, R2, R4) 30–40% of Neu5Ac are recovered, while the final solution contains only 9–15% of the original pyruvate amount.

5. Conclusions

A new method for the purification of *N*-acetylneuraminic acid, is presented. The method includes reactive extraction and recovery from the organic phase. Measured equilibrium constants show that the extraction is the limiting step. This disadvantage could be overcome by multistage extraction.

The next step in development of this process could be the integration of reaction and extraction step. Integrated product removal was proposed for this system before [31], due to the unfavourable equilibrium of the reaction. Shifting the equilibrium to Neu5Ac could lead to improved yield and a much better atom efficiency of the process, because a smaller excess of pyruvate would have to be applied. Unfortunately, the conditions of reaction and extraction (pH value) cannot be reconciled to date. But assuming that it is possible to develop an Neu5Ac aldolase working at a pH value of 10.5, e.g. by directed evolution, it would not only be possible to integrate reaction and extraction, but also the base catalysed epimerisation of GlcNAc to ManNAc could be used.

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6.3. Reactive extraction of N-acetylneuraminic acid - Kinetic model and simulation of integrated product removal

V. Zimmermann, I. Masuck, U. Kragl, *Separation and Purification Technology* **2008**, *accepted*.

Summary and classification

This article presents a kinetic model for the description and simulation of the reactive extraction presented in section 6.2. Furthermore, by simulation a process was developed in which the product is enriched in an organic phase concurrent to reaction. It is the second part of this work dealing with the developed new downstream processing method for the Neu5Ac process (figure 6.3).

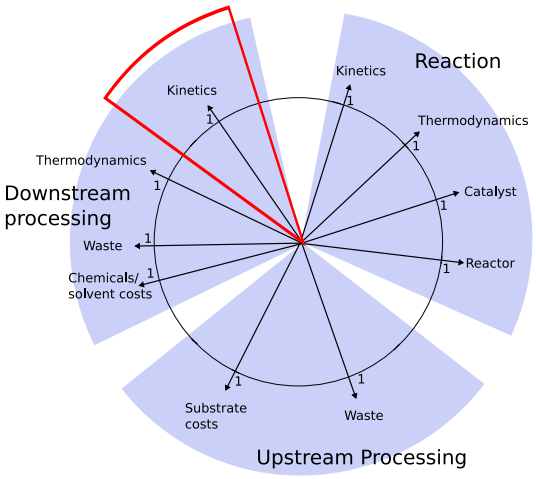

Figure 6.3.: This article presents kinetic data for the reactive extraction of Neu5Ac.

Contributions

In the context of her diploma thesis I. Masuck contributed to this work by performing the experiments to characterise the Lewis-type cell which was used to measure reaction kinetics. Furthermore, she performed the kinetic measurements for *N*-acetyl-D-glucoseamine. The kinetic measurements for all other compounds, the adaption of the kinetic model, including the new definition of the parameter σ according to the assumed reaction mechanism, and all simulations were performed by myself. My contribution to this work is about 85 %.

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Reactive extraction of *N*-acetylneuraminic acid—Kinetic model and simulation of integrated product removal

Vera Zimmermann, Ines Masuck, Udo Kragl*

Universität Rostock, Abteilung für Analytische, Technische und Umweltchemie, Albert-Einstein-Str. 3a, 18059 Rostock, Germany

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ABSTRACT

The reactive extraction of *N*-acetylneuraminic acid with phenylboronic acid (PBA) and trioctylmethylammoniumchloride (TOMAC) is a new method to recover neuraminic acid from reaction solutions. We describe a kinetic model which is suitable to describe the extraction of pure compounds as well as mixtures of the reactants, which are *N*-acetyl-D-glucosamine (GlcNAc), *N*-acetyl-D-mannosamine (ManNAc), sodium pyruvate (Pyr) and neuraminic acid (Neu5Ac). For the extraction a two-step mechanism is assumed and the ion exchange at the interface was determined to be the rate-limiting step. Kinetic measurements were performed in a Lewis-type cell. The first reaction step is included in the model by a parameter σ which describes the extractable amount of carbohydrate. The extraction model is combined with a model describing the enzymatic synthesis of Neu5Ac from GlcNAc to simulate the integration of reaction and extraction. The integrated process was simulated with varying substrate concentrations, carrier concentrations, phase ratios and interfacial area. A batch reaction with concurrent extraction and continuous renewal of the organic phase was developed. Under optimised conditions neuraminic acid is produced with the same space–time yield as without extraction and the product is accumulated in the organic phase, which facilitates further downstream processing. The developed model is feasible to analyse and evaluate integrated product removal as well as separately operated reaction and extraction.

1. Introduction

The synthesis of *N*-acetylneuraminic acid (Neu5Ac) still attracts growing attention due to the increased need for this compound in pharmaceutical industry [1]. Usually neuraminic acid is produced enzymatically from *N*-acetyl-D-mannosamine (ManNAc) and sodium pyruvate catalysed by *N*-acetylneuraminic acid aldolase (E.C. 4.1.3.3) [2] or from the ManNAc epimer *N*-acetyl-D-glucosamine (GlcNAc), which is cheap and can be obtained by hydrolysis of shrimp shells. The epimerisation of GlcNAc can be catalysed by an inorganic base like sodium or calcium hydroxide or by the enzyme *N*-acetyl-D-glucosamine epimerase (E.C. 5.1.3.8). The reaction scheme is shown in Fig. 1.

However, both reactions suffer from low yields due to the thermodynamic equilibrium and a very complicated or rather expensive downstream processing [3,4]. The equilibrium constant of the GlcNAc epimerisation is 0.24, the equilibrium constant for the aldol reaction is 28.7 l/mol [5]. The equilibrium of the aldol reaction is usually shifted to the product side by adding a 5–10-fold excess of sodium pyruvate [6–9], which complicates downstream processing because the product Neu5Ac is difficult to separate from pyruvate as the compounds have similar pK_a values (2.6 and 2.5 respectively). Usually, neuraminic acid is separated by precipitation with glacial acetic acid, if necessary with prior chromatographic treatment [10,8,4].

A new method for the purification of Neu5Ac is the reactive extraction with phenylboronic acid (PBA) and trioctylmethylammonium chloride (TOMAC) [11]. If the extractants are applied in a 10-fold excess, 86% of the Neu5Ac can be extracted. At equilibrium, the extracted amount of the other compounds is much lower (47% of GlcNAc, 40% of ManNAc and 33% of Pyr) and neuraminic acid is enriched in the organic phase.

Considering the drawbacks of Neu5Ac synthesis, it could be useful to integrate extraction and reaction to enrich the product in the organic phase and shift the equilibria towards the product side. The development of a process with integrated product removal has been suggested for the optimisation of neuraminic acid before [12], but has not been realised yet. The integration of downstream processing steps is a tool that is considered to be promising for further improvement of biocatalytic processes to meet the requirements of industrial processes [13,14].

By removal of the product during reaction, inhibitions can be avoided and equilibria can be shifted towards higher product yields [15,16]. The concept has been successfully applied to fermentation

* Corresponding author. Tel.: +49 381 498 6450; fax: +49 381 498 6452.
E-mail address: udo.kragl@uni-rostock.de (U. Kragl).

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Nomenclature

A	compound (GlcNAc, ManNAc, Neu5Ac or Pyr)
c	concentration (mol/l)
Cl^-	chloride anion
D	distribution coefficient
F	flow rate (l/min)
GlcNAc	*N*-acetyl-D-glucosamine
ISPR	*in situ* product removal
M1–M4	abbreviation for different compound mixtures
ManNAc	*N*-acetyl-D-mannosamine
Neu5Ac	*N*-acetylneuraminic acid
PBA	phenylboronic acid
PVR	phase volume ratio
Pyr	sodium pyruvate/pyruvic acid
OH^-	hydroxide ion
Q^+	trioctylmethylammonium cation
STY	space–time yield (mol/(l min))
t_1	start time of extraction (in ISPR processes)
TOMAC	trioctylmethylammonium chloride
U	stirring speed (1/min)
V	volume (l)

Indices

0	initial
aq	aqueous phase
f	feed
org	organic phase

processes where the product was removed by gas stripping [17,18], pervaporation [19], electrodialysis [20] or extraction [21–24]. An overview over recent developments was given by Schügerl and Hubbuch [13]. Reactive extraction combined with fermentation has, amongst others, been simulated for the production of lactic acid [21], phenyl acetic acid [25] and phenylalanine [26]. The model published by Yabannavar and Wang in 1991 for lactic acid is based on mass balances for fermenter and extractor and does not contain kinetics. In 2002, Gaidhani et al. [25] published a model for the extraction of phenyl acetic acid that included thermodynamics, kinetics and diffusion in the immobilised enzyme beads. The detailed kinetic model published by Takors in 2004 [26] for the production of L-phenylalanine was furthermore used to determine rate-limiting steps and to identify further optimisation steps. All these models do not include coextraction of substrates. This is normally not necessary in whole cell biocatalysis as the accordance of simulations and measurements in the cited articles shows.

The application of integrated product removal is more difficult if the reaction is catalysed by purified enzymes instead of whole cells. In whole cell processes the desired product is produced by the cell metabolism from structurally very different nutrients. In reactions catalysed by isolated enzymes only a single reaction step (and therefore chemical modification) takes place, which means that the substrates are chemically very similar to the product. Therefore it is likely that the substrates are removed by the integrated separation method as well. The selectivity of the product removal is crucial for successful integration of reaction and product removal. A detailed model for the enzyme catalysed production of isomaltose with integrated adsorption has been published by Ergenzinger et al. consisting of hydrodynamics, kinetics of enzymatic synthesis and thermodynamics [27]. The coadsorption of substrate has been incorporated by a modification of the Langmuir isotherm describing the adsorption of the product. In our case the extraction of compound mixtures showed that the coextraction of GlcNAc, ManNAc and Pyr has to be taken into account in case of neuraminic acid production. The development of a process with successful integrated product removal would be desirable because one of the major drawbacks of neuraminic acid synthesis are the unfavourable equilibria of both reaction steps and the high excess of pyruvate applied. Integration of reaction and extraction may result in higher product yields, if the equilibria can be shifted significantly, or simplified downstream processing because of product accumulation in the second phase. There are several reasons to simulate the process before running experiments: The technical equipment for an integrated product removal may be quite complex and the experimental conditions can be optimised much faster using a model. By simulation of the process with varying parameters proper reaction conditions for first experiments can be determined. Another reason to simulate the process in this special case is that reaction and extraction cannot be combined without a complex reactor system because reaction and extraction do not proceed at the same pH value. For the successful integration of extraction not only a favourable extraction equilibrium is necessary, but also advantageous extraction rates. This means that Neu5Ac must not be extracted much slower than the other compounds because this would prevent the accumulation in the organic phase necessary to shift equilibria. Furthermore, the achieved improvement in product

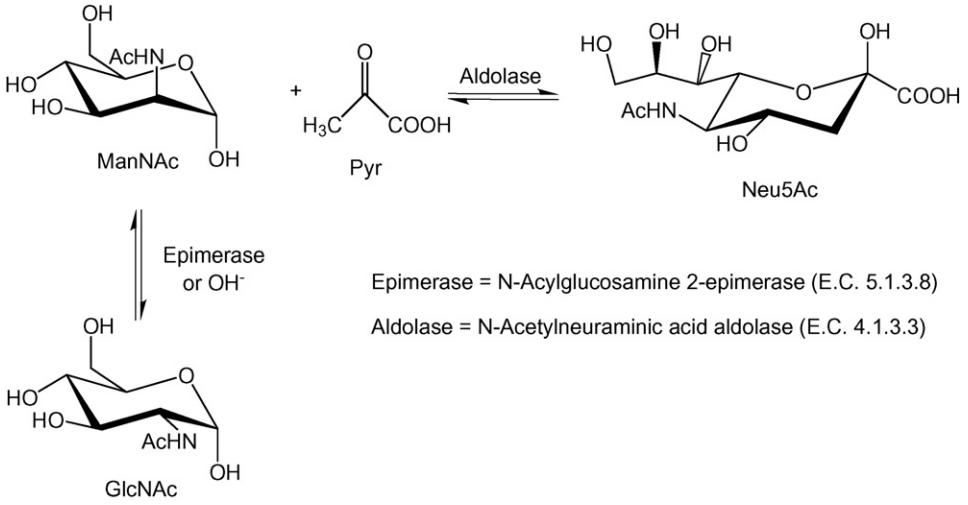

Fig. 1. Synthesis of neuraminic acid from GlcNAc by combination of GlcNAc epimerisation and aldolase catalysed reaction.

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yield or facilitated downstream processing has to be high enough to countervail against the increased equipment costs caused by the integration of a downstream processing step. Generally, an increasing number of compounds involved makes it more difficult to estimate possible advantages and drawbacks. Modelling is a useful tool to gain a better insight into complex systems and to study the influence of various parameters. Therefore we investigated the extraction rates of all compounds and used a kinetic model to simulate the extraction process. The model was combined with a kinetic model describing Neu5Ac synthesis [5] which allows the simulation of integrated product removal under varying process conditions. By simulation of the integrated process with varying initial concentrations and phase volumes it was possible to estimate the usefulness of integration of reaction and extraction.

2. Theory

For the extraction of Neu5Ac and the other compounds (except sodium pyruvate) a two-step mechanism is assumed [11]. In the first reaction step, the compounds react with phenylboronic acid to form esters.

$$A + PBA^- \rightleftharpoons A\text{–}PBA^- + 2H_2O \quad (1)$$

This type of reaction of polyols with boronates has been investigated before [28,29]. In this case, the reaction is probably located in the aqueous bulk phase near the interface, because a great amount of PBA is located in the aqueous phase at basic pH (compare Fig. 8).

The second step is the extraction of the PBA–carbohydrate complex mediated by TOMAC via an ion-exchange mechanism.

$$A\text{–}PBA^- + QCl \rightleftharpoons QA\text{–}PBA + Cl^- \quad (2)$$

This reaction step is supposed to take place at the interface because TOMAC does not dissolve in the aqueous phase. Fig. 2 shows a scheme of the mechanism. The combination of PBA derivatives and TOMAC as carriers was used before for extraction of aliphatic diols and common sugars like glucose and fructose [30,31] and the investigation of sugar transport through liquid membranes [32,33]. The extraction of neuraminic acid was performed with more lipophilic phenylboronic acid derivatives like *tert*-butylphenylboronic acid or 4-biphenylboronic acid as well, but in these systems extraction does not occur to a considerable amount [11].

The description of the kinetics of interfacial mass transfer in reactive extraction of organic compounds, especially amino acids, by ion transfer was subject to several publications since the 1980s [34–40]. The kinetic model used here to describe the reactive two-step extraction of the carbohydrates and pyruvate is based on a model published by Chan and Wang [41] for the reactive extraction of amino acids. The model was modified to describe the two-step mechanism as presented in Section 4 part 1.

3. Material and methods

3.1. Chemicals

Sodium pyruvate was obtained from Fluka (Buchs, Switzerland), *N*-acetyl-D-mannosamine from Sigma–Aldrich (Steinheim, Germany). *N*-Acetyl-D-glucosamine and *N*-acetylneuraminic acid were provided by Julich Chiral Solutions (Jülich, Germany, now part of Codexis). The pH value was adjusted using sodium hydroxide or hydrochloric acid. To minimise the volume error, the phases were saturated with each other before use.

3.2. Determination of extraction kinetics

The Lewis-type cell used in this study is shown in Fig. 3. The cell consists of an glass cylinder, inside diameter 6.05 cm and height 6.06 cm, which was divided into two halves by a PTFE circular disc with an circular whole in the middle and a ring gasket. The interfacial area could be varied by the use of different PTFE discs. The cell was equipped with a thermo jacket to maintain a uniform temperature in the cell during measurements. All measurements were performed at 25 °C. Agitation of the liquids in the upper and lower compartments was performed by conventional stirrers operating in opposite directions. The stirring speed was adjusted to 100 rpm unless specified differently. The volume of each phase was 73.5 ml.

The aqueous phase in which GlcNAc, ManNAc, Neu5Ac or Pyr were dissolved (initial pH value was adjusted with sodium hydroxide to 12.7) was first introduced to the cell, and then the organic solution (*n*-heptane/1-octanol 85:15 with PBA and TOMAC) was placed carefully without disturbing the interface. Samples of 50 μl were taken from the aqueous solution at scheduled time intervals.

3.3. Determination of equilibrium data

The determination extraction equilibrium data for GlcNAc, ManNAc, Neu5Ac and Pyr is described in detail in ref. [11].

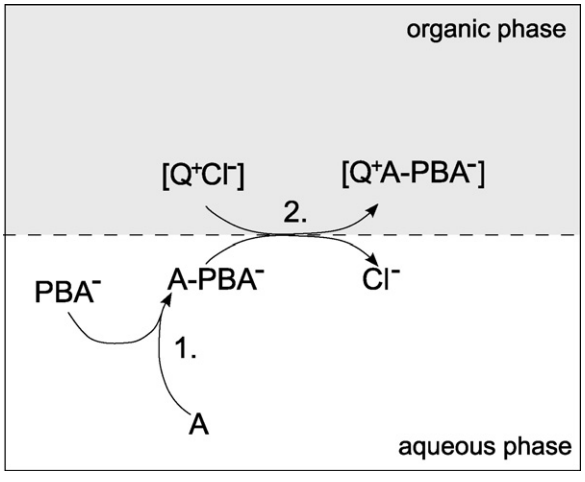

Fig. 2. Scheme of the mechanism assumed for the extraction of GlcNAc, ManNAc and Neu5Ac.

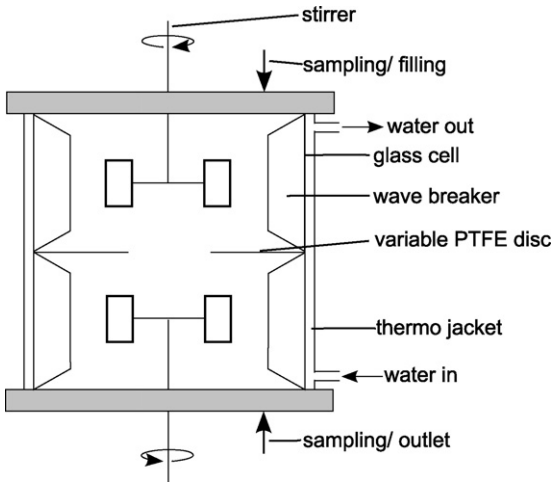

Fig. 3. Experimental apparatus (mass transfer cell).

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The results for various initial concentrations and pH values are presented therein.

3.4. Extraction of compound mixtures

Seven millilitres of a solution were treated with sodium hydroxide to adjust the solution to a pH of 12.7–13.0 and extracted with equal volumes of 0.5 mol/l (mixture 1 (M1)) or 1.0 mol/l (mixtures 2–4 (M2–M4)) carrier solution for 5 min. The pH value of the aqueous phase at equilibrium was 10.9 ± 0.1. The solution was centrifuged for 5 min to accelerate phase separation. After centrifugation the organic phase was removed and aliquots were taken from the aqueous phase.

3.5. HPLC/IC

The concentrations of GlcNAc, ManNAc, Neu5Ac, pyruvate and PBA were determined by HPLC-measurement using a Biorad Aminex HPX-87H analytical column. Samples were eluted at 65 °C with 0.006 mol/l sulphuric acid at 0.8 ml/min, and monitored with refractive index and UV at 203 nm. Chloride concentrations were determined by ion chromatography or calculated via mass balances. Ion chromatography was performed using a Hamilton PRP-X110S column, a Metrohm 828 IC Dual Suppressor and monitored with an Alltech 550 Conductivity Detector at 35 °C. A buffer containing 1.7 mmol/l sodium hydrogen carbonate, 1.8 mmol/l sodium carbonate and 0.1 mmol/l sodium thiocyanate was used as mobile phase.

4. Results and discussion

4.1. Extraction kinetics

According to the well established interfacial reaction model, the rate of a liquid–liquid reactive extraction in a stirred system can be controlled by either film diffusion processes or the kinetics of the chemical reactions taking place in the system. When chemical reactions are controlling the rate of mass transfer, the extraction rate is independent of the stirring speed [42,43]. The influence of the agitation speed on the initial extraction flux is shown in Fig. 4. It is found that the extraction rate is independent of the stirring rate above 70 rpm. This indicates that the kinetics of chemical reactions in the system are rate limiting under these conditions. However the interface becomes instable when the speed is higher than 110 rpm. A stirring speed of 100 rpm was chosen for the experiments to ensure that all measurements are performed in the plateau region

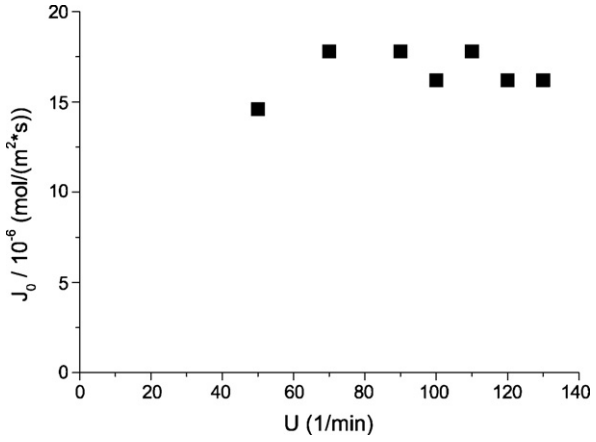

Fig. 4. Initial extraction flux of GlcNAc in dependence of agitation speed (c_0(GlcNAc) = 0.01 mol/l, c_0(PBA) = 0.1 mol/l, c_0(TOMAC) = 0.1 mol/l).

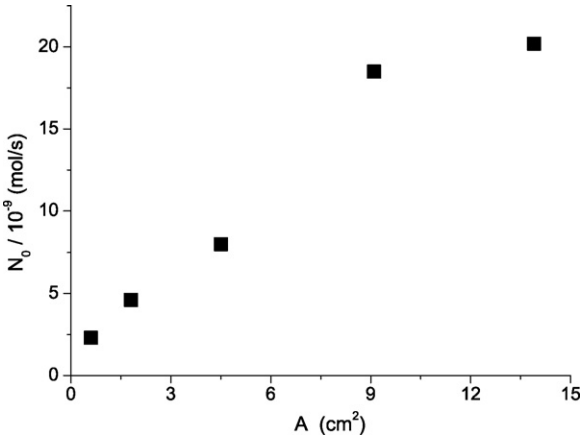

Fig. 5. Dependence of initial extraction rate of GlcNAc on the size of the interfacial area (*Conditions*: c_0(GlcNAc) = 0.01 mol/l, c_0(PBA) = 0.1 mol/l, c_0(TOMAC) = 0.1 mol/l, T = 25 °C, PVR = 1).

and therefore under a chemical reaction controlled regime. The mass transfer rate at a stirring speed of 100 rpm was determined three times to estimate the experimental error and was found to be $17.8 \pm 1.3 \times 10^{-6}$ mol/(m^2 s).

The chemical reaction can take place either at the liquid–liquid interface or in the bulk phase. If the extractant (TOMAC in this case) is insoluble in one phase and the reaction takes place at the interface, the initial mass transfer rate depends on the extractant concentration and the size of the interfacial area. Figs. 5 and 6 show the dependence of the initial extraction rate on the interfacial area and extractant concentration. In case of increasing interfacial area, a clear dependence is found indicating that the area is stable in the examined range. In case of increasing carrier concentration, the initial flux first increases proportionally, but reaches a maximum at 0.06 mol/l initial carrier concentration. This saturation-like curve progression is probably due to adsorption of the carrier at the interface. When all adsorption space at the interface is occupied, the initial flux does not increase with increasing carrier concentration any more.

After determination of the rate-limiting step and its location, the reaction kinetics were determined for all compounds involved in Neu5Ac synthesis. For the description of the time course a model developed by Chan and Wang [41] for the extraction of amino acids was utilised. For the case of interfacial reaction controlled

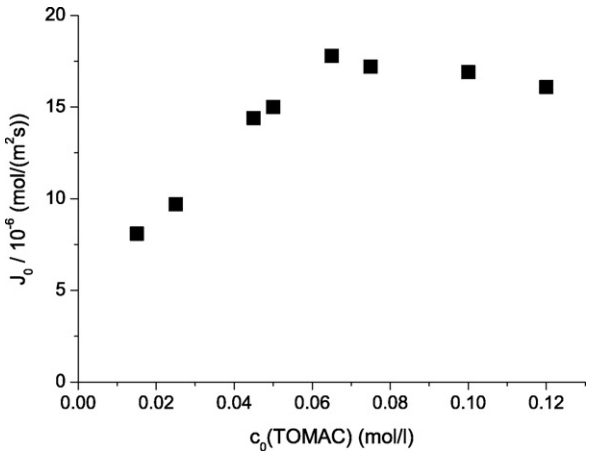

Fig. 6. Dependence of initial extraction flux of GlcNAc on the extractant concentration (*Conditions*: c_0(GlcNAc) = 0.01 mol/l, A = 4.52 cm^2, T = 25 °C, PVR = 1, c_0(PBA) = c_0(TOMAC)).

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Table 1
Determined constants for the applied extraction model (K_3 is not listed because it is not relevant in this case, compare Section 4.1)

	K_{PBA} [l/mol]	k_2 [dm/min]	K_1 [l/mol]	K_3	K_{ex} [l/mol]
GlcNAc	15.5	2.60×10^{-3}	105.9	–	0.125
ManNAc	128.6	2.60×10^{-3}	105.9	–	0.033
Neu5Ac	22.4	6.45×10^{-3}	7.9	–	1.35
Pyr	–	2.77×10^{-3}	28.3	–	0.017

extraction the equation for the interfacial flux J can be simplified and the flux J of every single compound can be described by Eq. (3).

$$J_A = \frac{k_{2,A}K_{1,A}}{K_{ex,A}} \times \frac{((K_{ex,A}/\sigma_A)[A][QCl] - [Cl^-][QA - PBA])}{1 + K_{1,A}[QCl] + K_{3,A}[QA - PBA]} \quad (3)$$

$K_{ex,A}$ is the overall extraction constant. $K_{1,A}$ and $K_{3,A}$ are equilibrium constants for adsorption and desorption at the interface. $k_{2,A}$ is the forward rate constant for the interfacial ion exchange. All constants except $K_{ex,A}$ were fitted according to Chan and Wang and are summarised in Table 1. $K_{1,A}$ and $k_{2,A}$ can be determined from the slope and intercept when plotting $1/J_0$ vs. $1/[QCl]_0$. The plot for Neu5Ac, GlcNAc and Pyr is shown in Fig. 7. A plot for ManNAc is not presented, because under the chosen extraction conditions the epimerisation of ManNAc to GlcNAc is much faster that the extraction. Because ManNAc reacts to GlcNAc, which is in excess due to the thermodynamic equilibrium, and both compounds are extracted concurrently, the interfacial flux of ManNAc cannot be determined. It is assumed that the transport of GlcNAc and ManNAc *via* the interface is the same because of their very similar structure. Therefore the kinetic constants for GlcNAc and ManNAc should be the same. Furthermore, the extraction in the stirred cell cannot be monitored until equilibrium is reached, because, due to the fixed ratio of phase volume and interfacial area, it takes several days and all compounds are not stable under basic conditions for such a long period of time. So the constant describing the equilibrium concentration $K_{ex,A}$ was fitted to detailed equilibrium data obtained for this system before [11]. With known $K_{1,A}$, $k_{2,A}$ and $K_{ex,A}$ the constant for desorption from the interface $K_{3,A}$ should be obtainable by curve fitting of extraction courses. Curve fitting showed that $K_{3,A}$ equals zero for all compounds. From this it can be estimated that the desorption of the TOMAC/A-PBA$^-$ ion pair from the interface is much faster than the extraction step and therefore it does not influence the kinetics of the extraction.

The parameter σ_A in Eq. (3) describes the fraction of active species of the carbohydrate (A) that can react at the interface. Chan and Wang calculated the active ionic species of amino acids via pK_a

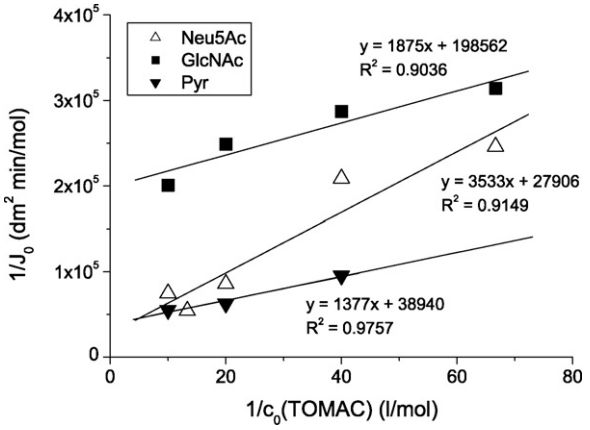

Fig. 7. Plot of $1/J_0$ vs. $1/c_0$(TOMAC) for the determination of $k_{2,A}$ and $K_{1,A}$ (c_0(A) = 0.009 mol/l, c_0(PBA) = c_0(TOMAC), T = 25 °C, PVR = 1, stirring speed 100 rpm).

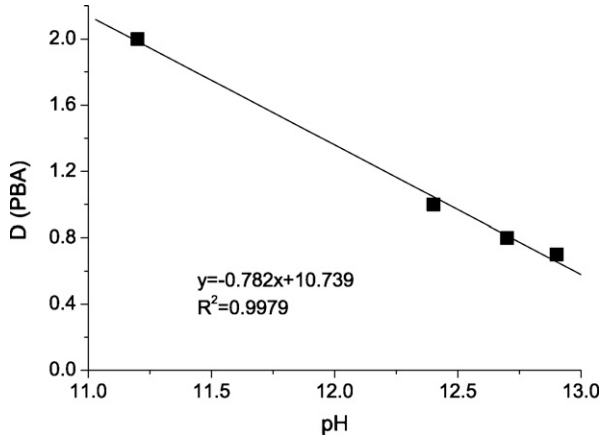

Fig. 8. Distribution coefficient of PBA at different pH values.

and pH of the aqueous phase. In this case, the parameter is used to calculate the amount of A-PBA$^-$ (defined in Eq. (1)). σ_A is now defined as

$$\sigma_A = \frac{1 + K_{PBA,A}[PBA^-]}{K_{PBA,A}[PBA^-]} \quad (4)$$

where $K_{PBA,A}$ is the equilibrium constant for the reaction of carbohydrate with PBA as described in Eq. (1). $K_{PBA,A}$ can be determined by measuring the change in pH when carbohydrate and PBA are mixed [29]. The values for $K_{PBA,A}$ are given in Table 1. [PBA$^-$] is the negative charged boronate of PBA. The distribution of PBA between organic and aqueous phase is shifted towards the aqueous phase with increasing pH value. Fig. 8 shows the dependence of the distribution coefficient D ($D = c_{org}/c_{aq}$) of PBA on the pH value of the aqueous phase. Between a pH value of 11 and 13 the dependence can be described by a linear equation. In the model, this equation is used to calculate the PBA concentration in the aqueous phase from the initial concentration at a given pH. Assuming that the PBA concentration at the interface is at equilibrium during extraction, the concentration of PBA$^-$ at the interface can be calculated via the PBA concentration and the pK_a value of PBA which is 8.8. This assumption may be appropriate because in earlier investigations the ion exchange reaction at the interface has been identified to be the rate-limiting step. Pyruvate does not react with PBA. The pyruvate ion is extracted via ion exchange by TOMAC. σ_{Pyr} can therefore be calculated according to Chan and Wang. The equation can be simplified because pyruvic acid is, in contrast to the zwitterionic amino acids examined by Chan and Wang, a monovalent acid.

$$\sigma_{Pyr} = 1 + \frac{[H^+]}{K_A} \quad (5)$$

The extracted amount does not depend on the pH value of the aqueous phase between a pH value of 10 and 13 significantly, because at basic pH values pyruvate exists in its ionic, extractable form to 100% and σ_{Pyr} is 1.

Fig. 9 presents experimental data and simulated extraction courses for all compounds involved in neuraminic acid synthesis. The dashed lines represent the equilibrium concentrations as obtained from equilibrium experiments (data has been published in ref. [11]). The figure shows that the model is suitable to describe the initial flux over the interface, especially for Neu5Ac and Pyr. The small difference between experimental and simulated equilibrium concentrations is due to the fact that the model has to be valid for different initial and therefore equilibrium concentrations.

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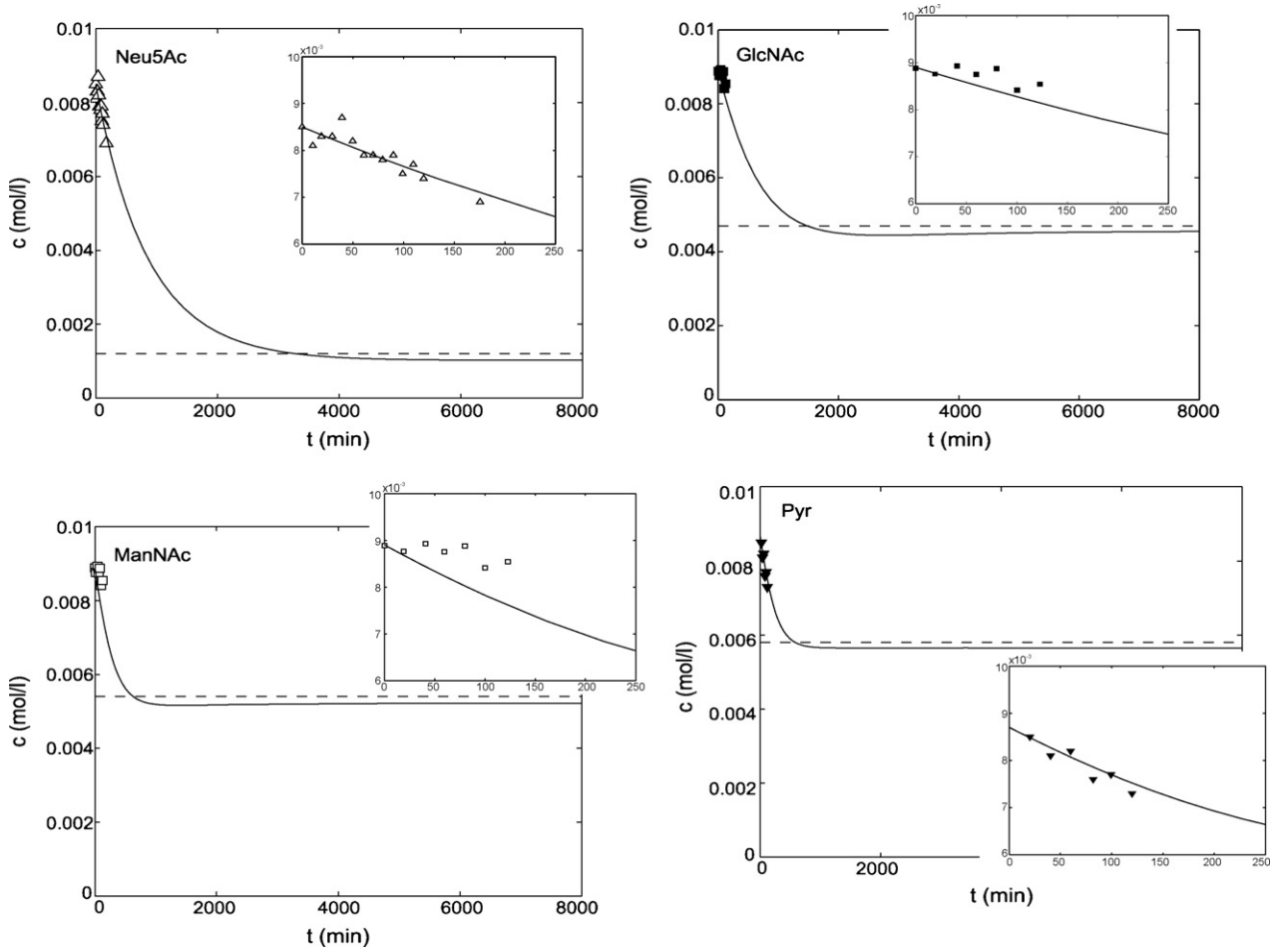

Fig. 9. Simulated extraction courses and experimental points for all compounds involved in neuraminic acid synthesis. The dashed lines show the equilibrium concentration.

4.2. Extraction of reaction mixtures and simulated integrated product removal

When Neu5Ac is produced at large scale, the compound concentrations are usually much higher than in the stirred cell experiments and the solutions are mixtures of all compounds. As we intended to simulate the extraction of reaction solutions the developed model had to be tested for compound mixtures, especially at high concentrations, as well. The simulations indicated that the model is not suitable to describe the equilibrium of mixtures well because the simulated extraction is more effective than observed in experiments (data not shown). Therefore $K_{ex,A}$ had to be fitted for mixtures again. The determined values are 0.68, 0.69, 0.38 and 0.68 for Neu5Ac, GlcNAc, ManNAc and Pyr respectively. Four mixtures (M1–M4) with varying concentrations of the four compounds have been investigated and the extraction was simulated. M1 and M2 are reaction solutions while M3 and M4 were mixed and extracted to investigate solutions with high GlcNAc and Pyr excess. Experimental and simulated aqueous phase equilibrium concentrations are presented in Table 2. Experimental and calculated values show good concordance and the ratios of the compound are reproduced by the model very well. The measurements and the equilibrium constants for compound mixtures show that the selectivity in mixtures is not as high as calculated from single compound measurements. This is probably due to the fact that Chan and Wang's model was derived for single compounds, although the competitive extraction of all compounds by the same extractant is taken into account in our model by the association in the differential equation system.

The kinetic model applied to describe the synthesis of neuraminic acid from GlcNAc was published before [5] and describes the reaction very well in a wide concentration range. The model was combined with the extraction model presented in this work to

Table 2
Extraction of compound mixtures

Identifier	GlcNAc	MaNAc	Neu5Ac	Pyr
M1				
c_0 (mol/l)	0.136	0.026	0.123	0.323
c_{exp} (mol/l)	0.076	0.015	0.058	0.187
c_{sim} (mol/l)	0.088	0.019	0.077	0.186
M2				
c_0 (mol/l)	0.123	0.017	0.142	0.280
c_{exp} (mol/l)	0.051	0.010	0.075	0.139
c_{sim} (mol/l)	0.055	0.010	0.062	0.115
M3				
c_0 (mol/l)	0.319	0.046	0.248	0.204
c_{exp} (mol/l)	0.210	0.029	0.136	0.093
c_{sim} (mol/l)	0.168	0.029	0.128	0.099
M4				
c_0 (mol/l)	0.096	0.014	0.232	0.364
c_{exp} (mol/l)	0.045	0.012	0.108	0.145
c_{sim} (mol/l)	0.048	0.009	0.114	0.168

Comparison of experimental and simulated values. c_0: initial concentration, c_{exp}: experimental value (aqueous phase concentration after extraction), c_{sim}: calculated value. *Conditions*: PVR = 1, $T = 25\,^{\circ}C$, equilibrium pH 11.0 ± 0.2. Mixture 1 (M1): c_0(TOMAC) = 0.5 mol/l, c_0(PBA) = 0.5 mol/l. M2–M4: c_0(TOMAC) = 1.0 mol/l, c_0(PBA) = 1.0 mol/l.

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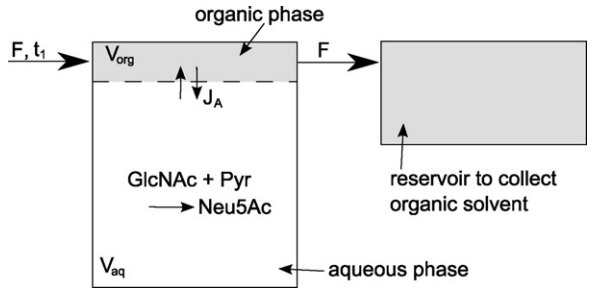

Fig. 10. Scheme of simulated integrated reaction and extraction in a batch reactor with continuous renewal of the organic phase (F, flow rate (l/min); t_1, time when extraction is started; J_A, interfacial flux of compound A).

simulate integrated product removal. Integrated product removal was simulated in a semi-continuous process where the aqueous phase, in which the reaction takes place, is stationary while the organic phase is continuously fed and removed with a flow rate F from a starting time t_1. A scheme of the process is presented in Fig. 10. It is assumed that both phases are ideally mixed, i.e. there are no concentration differences within a phase. The process was simulated with various flow rates, initial GlcNAc, Pyr, TOMAC and PBA concentrations, phase volume ratios (PVR = V_{org}/V_{aq}) and varying interfacial area. The simulations of this ISPR process under various conditions showed that the interfacial area between the phases is the most important parameter. It has to be quite small relative to the aqueous phase volume. Otherwise the coextraction of the substrates would proceed too fast and hence the substrates would not be available for reaction anymore. For illustration an exemplary parameter set is shown in Fig. 11. The figure presents the space–time yield (STY) of an integrated process with fixed conditions of the aqueous phase. The results show that a small change of the interfacial area has a relatively large influence on the space–time yield while big changes in carrier concentration or volume of the organic phase do not influence the space–time yield very much.

Table 3 presents three case studies of the integrated process: One with GlcNAc excess, one with pyruvate excess and one with equal GlcNAc and Pyr initial concentrations. The processes are compared with the corresponding reaction in a batch reactor without concurrent extraction. The conditions of reaction and extraction are the same for these three processes and are given in the caption of Table 3. Integrated product removal does not increase the yield of the process due to concurrent extraction of substrates, but, if GlcNAc or Pyr is applied in excess, the product can be enriched in the organic phase which simplifies further downstream processing. The enrichment is expressed by the ratio of Neu5Ac to GlcNAc and pyruvate in the aqueous and the organic solvent respectively. In this case organic solvent means all organic solvent that has been used in the process and is collected after being removed from the reactor (compare Fig. 10). In all cases presented in Table 3 the space–time yields of the processes with and without integrated extraction are the same, because they proceed to the same conversion in the same time. Table 3 shows that an enrichment of Neu5Ac in the organic phase concurrent to reaction is possible. In the presented processes 57–62 g Neu5Ac were produced. For the first enrichment of Neu5Ac 0.36–0.42 l *n*-heptane, 0.06–0.07 l 1-octanol, 170–198 g TOMAC and 51–60 g PBA were consumed. Of course further purification, e.g. by repeated extraction and/or precipitation is necessary. Usually, the first purification prior to precipitation or crystallisation

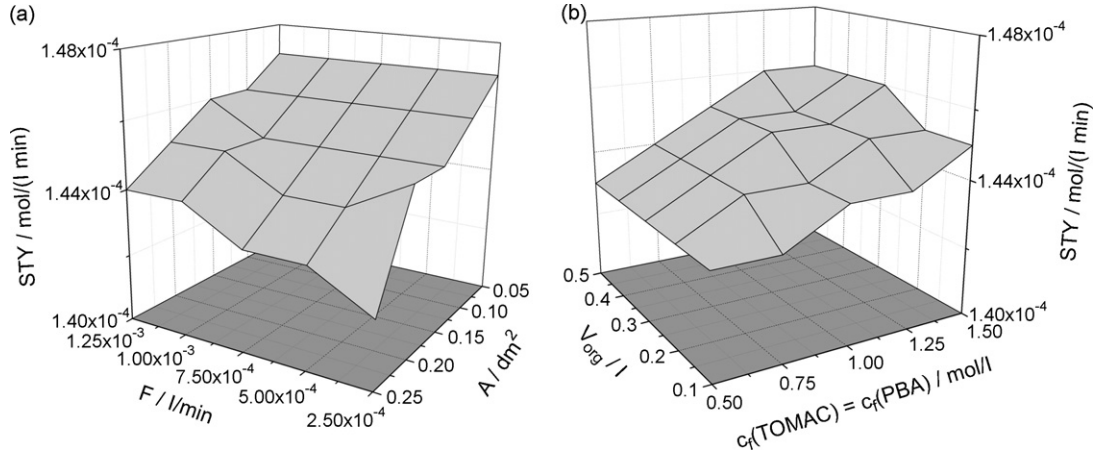

Fig. 11. Influence of interfacial area, carrier concentration, organic phase volume and flow rate on the space–time yield (at 98% of equilibrium conversion) of an exemplary integrated process. *Reaction conditions:* c_0(GlcNAc) = 0.3 mol/l, c_0(Pyr) = 0.5 mol/l, 24,000 U/l aldolase, 1500 U/l epimerase, $V_{0,aq}$ = 1 l, T = 25 °C. (a) V_{org} = 0.1 l, c_f(PBA) = c_f(TOMAC) = 1 mol/l. (b) A = 0.2 dm^2, $F = 5 \times 10^{-4}$ l/min.

Table 3
Comparison of neuraminic acid synthesis without and with integrated product removal

c_0(GlcNAc) (mol/l)	c_0(Pyr) (mol/l)	Without extraction			With extraction				
		STY (mol/(l min))	c(Neu5Ac)/c(GlcNAc)	c(Neu5Ac)/c(Pyr)	STY (mol/(l min))	c(Neu5Ac)$_{aq}$/c(GlcNAc)$_{aq}$	c(Neu5Ac)$_{aq}$/c(Pyr)$_{aq}$	c(Neu5Ac)$_{org}$/c(GlcNAc)$_{org}$	c(Neu5Ac)$_{org}$/c(Pyr)$_{org}$
0.5	0.3	1.20×10^{-4}	0.75	1.65	1.19×10^{-4}	0.63	1.45	1.34	2.39
0.3	0.5	1.47×10^{-4}	2.06	0.60	1.46×10^{-4}	1.80	0.53	3.52	0.91
0.4	0.4	1.35×10^{-4}	1.36	1.08	1.34×10^{-4}	1.11	2.28	0.91	1.54

For the processes without extraction the space–time yield (STY) was calculated at 98% of equilibrium yield. When the reaction is performed with integrated extraction, the product Neu5Ac can be accumulated in the organic phase. *Conditions:* V_{aq} = 1 l, T = 25 °C, 24,000 U/l aldolase, 1500 U/l epimerase. With extraction: V_{org} = 0.1 l, c_0(TOMAC) = 1 mol/l, c_0(PBA) = 1 mol/l, $F = 5 \times 10^{-4}$ l/min, A = 0.1 dm^2, t_1 = 50% of total reaction time.

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is done via chromatographic procedures [10,8]. A detailed description was given by Mahmoudian et al. [8] in 1997. In the described reaction 1020 g Neu5Ac were produced during reaction. The chromatographic purification prior to precipitation consumed more than 35 l of water, 33.4 l 0.5 mol/l sodium metabisulfite solution and an unknown amount of 1 mol/l sodium hydroxide solution. A comparison of the separations per gramme of product shows that even a multi-step extraction is competitive to chromatographic procedures.

5. Conclusions

A kinetic model describing the reactive extraction of *N*-acetyl-D-glucosamine, *N*-acetyl-D-mannosamine, pyruvic acid and *N*-acetylneuraminic acid is presented. The extraction model was combined with another kinetic model, which describes the enzymatic synthesis of Neu5Ac from GlcNAc, to simulate integrated product removal.

Simulations showed that a batch process with continuous renewal of the organic phase is feasible to enrich the product in the organic phase. A crucial parameter is the interfacial area between both phases. As the interfacial area is the major parameter that influences the velocity of extraction it can be concluded that the fine tuning of the kinetics of separation and reaction is essential in the development of processes with integrated product removal, especially when the selectivity of separation is not very good. Therefore kinetic models of reaction and separation are important tools in the development of ISPR processes.

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7. Summary

According to the objectives mapped out in chapter 2 the results of this work can be divided into three parts:

- **Kinetic modelling of neuraminic acid synthesis.** For both reaction steps (enzyme catalysed epimerisation, enzyme catalysed aldol condensation) a kinetic model was developed. The determined constants include substrate and product inhibition constants as well as inhibition constants for the interaction of the two systems. The kinetic model is feasible to describe the neuraminic acid synthesis in a wide range of concentrations and in different reactor types, which was demonstrated by a fed-batch experiment. The model was used to simulate the reaction in different reactor types and optimise reaction parameters like substrate concentrations, feed flow and the ratio of the two biocatalysts. The optimisation led to an improvement in space-time yield ($3.2\ 10^{-3}$ mol l^{-1} h^{-1}) by a factor >2 compared with processes published before.
  Furthermore it was shown by simulation that in fed-batch reactors the composition of the reaction solution that has to be treated in downstream processing can be designed to meet the requirements or rather selectivities of the applied separation method.

- **Development of a new downstream processing method.** Based on the different interactions of carbohydrates with boronates, a reactive extraction with phenylboronic acid (PBA) and trioctylmethylammoniumchloride (TOMAC) was developed for the separation of neuraminic acid from the other compounds involved in its synthesis. For best performance of the extraction system different solvents as organic phase and different PBA derivatives were tested. Phenylboronic acid and a solvent mixture of n-hexane or n-heptane and 1-octanol were

identified to give the best results. This system was characterised with different substrate and carrier concentrations and at different equilibrium pH values. A two-step mechanism for the extraction was proposed based on experimental results. For all compounds (Neu5Ac, GlcNAc, ManNAc, Pyr) the equilibrium constants for both steps and the overall extraction were determined.

- **Kinetic modelling of downstream processing.** The kinetics of the reactive extraction of the compounds involved in Neu5Ac synthesis were determined in a Lewis-type stirred cell. A model developed by Chan and Wang in 1993 for the extraction of amino acids was modified to describe the developed extraction of carbohydrates, including the assumed two-step mechanism. The model was used to simulate the extraction of reaction solutions with concentrations simulated for fed-batch reactors. Furthermore, the integration of reaction and extraction was simulated. Thus a process was developed in which the product can be accumulated in the organic phase concurrent to reaction. In three case studies with different substrate concentrations it was shown that the product can be enriched in the organic phase, that the interfacial area between the phases is a crucial parameter and that the extraction can compete with chromatographic procedures that are used today for purification of Neu5Ac from reaction solutions prior to precipitation.

8. Discussion

The simulations performed for the synthesis of neuraminic acid showed that the model is feasible to describe the reaction under various conditions and in different reactor types. If important parameters of the process like substrate costs, enzyme availability or the applied product separation method change, the model can be used to optimise the reaction conditions according to the new requirements quite fast. One of the parameters that changed during the last years for this process as well as many other biotransformations is the availability of the required enzymes. With the emerging relevance of biotechnology for the production of fine chemicals some companies, e. g. Codexis, have found a new market sector providing enzymes at large scale for conversions that are interesting in industrial and pharmaceutical processes. Trends like this will speed up the development of efficient biocatalytic processes in the coming years.

With the reactive extraction of Neu5Ac, GlcNAc, ManNAc and Pyr a new separation method for this system was developed. The method can be used for the enrichment of neuraminic acid by extraction of reaction solutions. At low carbohydrate concentrations and high carriere excess it could also be utilised for the removal of all compound from aqueous solutions, e. g. waste water streams.
One problem involved in the industrial application of this method is the high solubility of phenylboronic acid in the aqueous phase at basic pH values. On on hand, this is necessary for the first step of the extraction (the reaction of PBA with the carbohydrate), on the other hand the recycling of the carrier is complicated. A screening of more PBA derivatives or perhaps even other boronates could help to identify a compound that is not as soluble in the aqueous phase but reacts with the carbohydrates to a higher exent. Another improvement could be the identification and use of a multifunctional carrier that reacts not only with the polyol groups of the carbohydrates but also with

the carboxy group of Neu5Ac. Such a carrier could lead to better selectivies.

The developed kinetic model for the extraction of carbohydrates with PBA and TOMAC is a useful tool for the simulation of such extraction processes. If other PBA derivatives are screened and found useful as carriers for this reaction, the model can be adapted by the equilibrium data and some kinetic measurements in the Lewis-type stirring cell. In this work the developed model was used to simulate the extraction of reaction solutions and for the development of a neuraminic acid production process with concurrent enrichment of the product in an continously renewed organic phase. Furthermore the model can be used as part of simulations of multi-step extractions or the extraction in devices like extraction columns or mixer-settlers if the extraction is supposed to be applied at industrial scale.

Figure 8.1 shows the state of the art in the optimisation of the neuraminic acid production process before (blue line) and after this work was performed (red line). The parameter evaluation shows that there is low potential for further optimisation in case of the substrate costs because *N*-acetyl-D-glucosamine can be obtained from easily available natural sources (compare page 8). The knowledge about thermodynamics and kinetics of the reactions have been broadened by this work and, including the obtained results, the possibilities to overcome the drawbacks of unfavourable equilibria by means of substrate concentrations, substrate feeding, temperature shifts etc. are

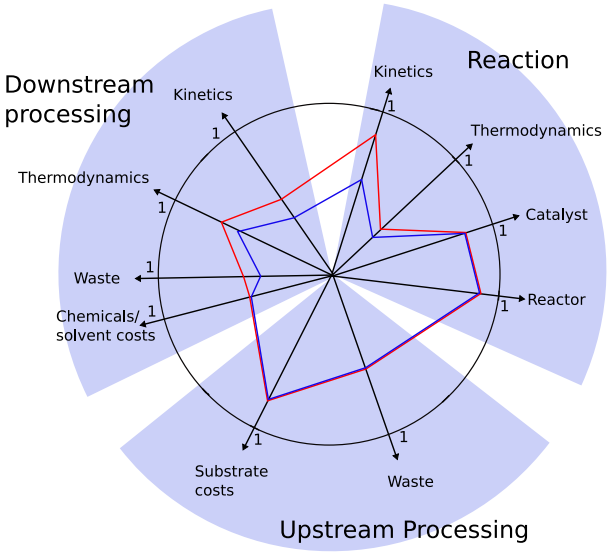

Figure 8.1.: State of the art in the neuraminic acid production process.

Author	Conversion %		Enzyme consumption U/mol(Neu5Ac)		STY mol l^{-1} h^{-1}
	GlcNAc	Pyr	Epimerase	Aldolase	
Blayer 1999[a)]	15.0	31.3	-	$1.1 \cdot 10^7$	$5.2 \cdot 10^{-4}$
Maru 1998[b)]	76.9	37.6	$3.2 \cdot 10^3$	$1.3 \cdot 10^4$	$1.8 \cdot 10^{-3}$
Zimmermann 2007[c)]	41.1	40.7	$3.6 \cdot 10^3$	$5.8 \cdot 10^4$	$3.2 \cdot 10^{-3}$

Table 8.1.: Comparison of the most important Neu5Ac production processes published in the last 10 years. In all cases epimerisation and aldol condensation were carried out in one pot. Process description: a) Alkaline epimerisation and enzyme catalysed condensation, batch, GlcNAc excess. b) Enzyme catalysed epimerisation and condensation, fed-batch, pyruvate excess. c) Enzyme catalysed epimerisation and condensation, fed-batch, equimolar GlcNAc and pyruvate.

almost exploited. This work also contributed to the choice of the reactor and operating mode by simulation of the reaction in fed batch reactors under various conditions. The performed improvement can be illustrated by comparing the developed process with similar processes (where epimerisation and aldol condensation are performed in one pot) that have been published before. Table 8.1 presents such a comparison of the developed fed-batch process with two other processes published in the last ten years. The combination of alkaline epimerisation and enzyme catalysed aldol condensation in one pot, as published by Blayer *et al.* in 1999 has the lowest space-time yield (STY). The prominent reason for this is the degradation of pyruvate and loss of enzyme activity at alkaline pH values, which also leads to a very high enzyme consumption per mole of product. The large scale process (200 l reactor) published by Maru *et al.* in 1998 and the process presented in this work are both fed-batch processes. Our kinetic analysis and optimisation led to an improvement in space-time yield by a factor of almost 2. Compared with Maru's process two major factors can be stressed that led to the improvement in space-time yield: 1. The optimisation of the catalyst ratio. Maru *et al.* did not use enough aldolase compared with the epimerase applied so that the aldol condensation was the limiting reaction step. 2. The constant feed of pyruvate from a certain point of reaction keeps the reaction far from equilibrium. Maru *et al.* let the reaction proceed to equilibrium before further pyruvate was fed which takes a lot of time.

Concerning the optimisation of the catalysts there is still some potential because the

Author	Consumed chemicals
Mahmoudian 1997	34 l water
(chromatographic	33 l 0.5 mol/l sodium metasulfite solution
procedure)	unknown amount 1.0 mol/l sodium hydroxide solution
	unknown amount of chromatographic bed materials
Zimmermann 2007	6.6 l n-heptane
(integrated	1.1 l 1-octanol
extraction)	0.93 kg PBA
	3.0 kg TOMAC

Table 8.2.: Amount of chemicals consumed per produced kilogramme of product in the case studies presented in section 6.3 (average) and by a chromatographic procedure.

enzymes have not been subject to directed evolution yet. But as the enzymes are readily available, do not hinder the reaction by inhibitions very much and are not one of the major cost factors of the process, the optimisation of the catalysts would probably not be reasonable from a economical point of view at the moment.

Still, the most potential for further optimisation of the process lies in the part of downstream processing. The established methods (chromatography or precipitation with glacial acetic acid) produce a lot of waste and are one of the major cost factors. An improvement of downstream processing was possible by development, simulation and optimisation of an extraction method for neuraminic acid. The developed process is suitable to enrich Neu5Ac in an organic phase concurrent to reaction. This purification step was performed chromatographically before, as described by Mahmoudian *et al.* in 1997. Table 8.2 compares the amount of chemicals consumed per produced kilogramme of product in the extraction case studies presented in section 6.3 (average) and by the chromatographic procedure published by Mahmoudian *et al.* [Mahmoudian et al. 1997].
Nevertheless, further optimisation of the process should deal with downstream processing and the further development of the reactive extraction presented in this work might be an opportunity.

A major enhancement of the process optimisation performed here was the developement of kinetic modells for reaction and downstream processing which enabled the optimisation of both process parts separately as well as the simulation of concurrent

reaction and extraction. An optimisation of Neu5Ac extraction was only possible cosidering the reaction kinetics and led to the conclusion that the size of the interfacial area between the aqueous and the organic phase, and therefore the velocity of phase transfer, is a crucial parameter in the process.

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A. Appendix

A.1. Neuraminic acid synthesis

A.1.1. Characterisation of enzymes

Effect of cofactors on the epimerase

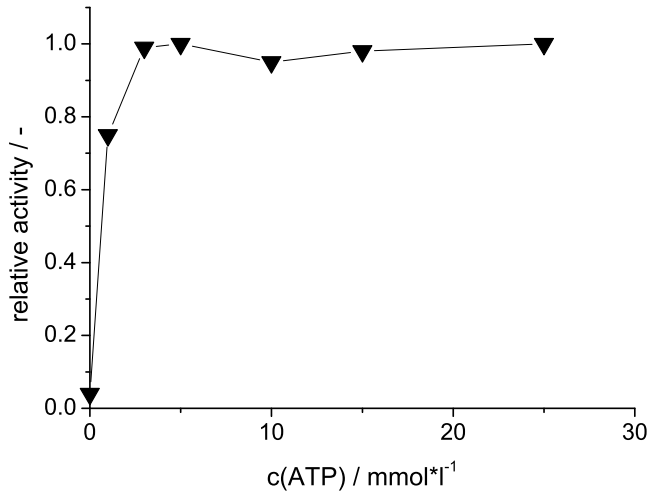

Figure A.1.: Relative activity of the employed N-acylglucosamine-2-epimerase CDX390 at different adenosine triphosphate (ATP) concetrations. Assays were performed as described on page 599 in section 6.1.

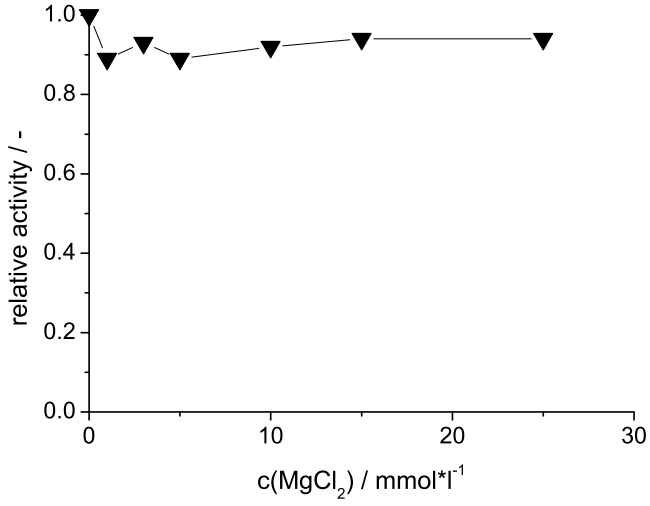

Figure A.2.: Relative activity of the employed N-acylglucosamine-2-epimerase CDX390 at different $MgCl_2$ concetrations. Assays were performed as described on page 599 in section 6.1.

pH-Optima

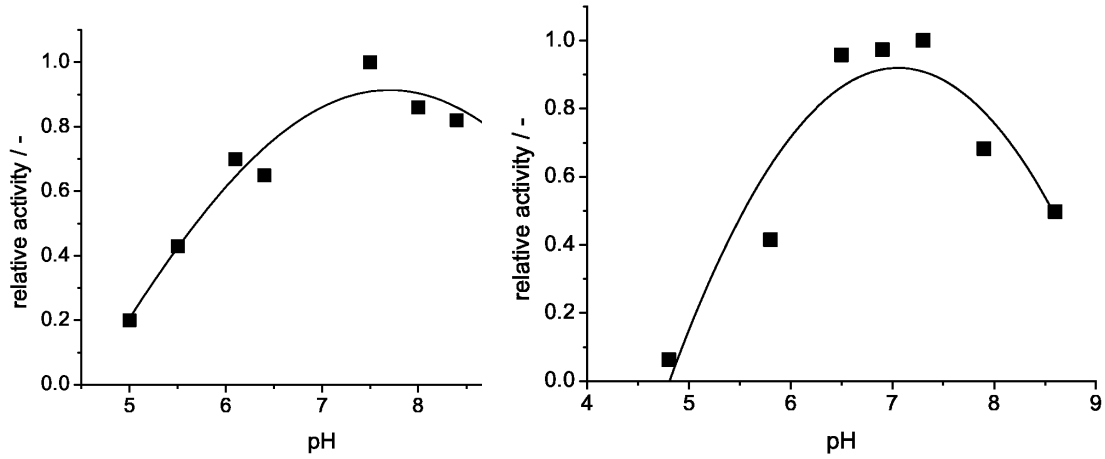

Figure A.3.: Relative activity of the employed enzymes at different pH values (left figure: epimerase, right figure: aldolase). Assays were performed as described on page 599 in section 6.1.

A.1.2. Simulation

Validation of simulation by experiments

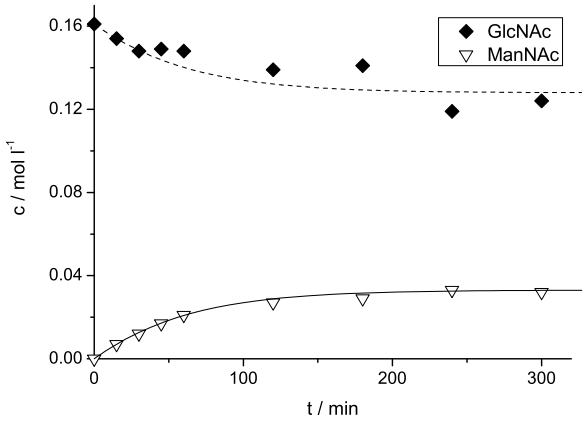

Figure A.4.: Example of an epimerisation of GlcNAc to ManNAc: Comparision of experimental values (markers) and simulated reaction course (lines). Conditions: c_0(GlcNAc) = 0.16 mol/l, c(epimerase) = 1800 U/l, pH = 7.5, T = 25 °C, c(ATP) = 2 mmol/l, c($MgCl_2$) = 2 mmol/l.

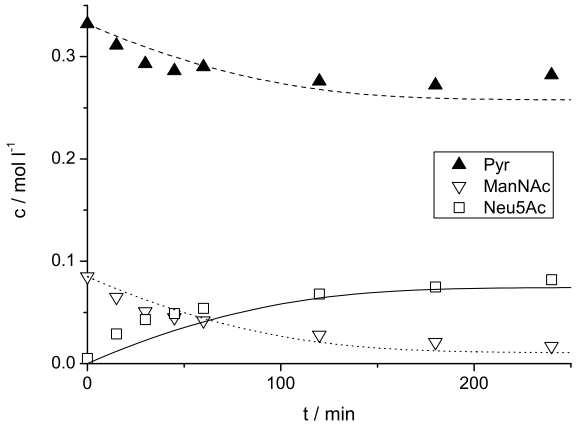

Figure A.5.: Experimental values (markers) and simulated reaction course of an exemplary aldol condensation (lines). Conditions: c_0(Pyr) = 0.33 mol/l, c_0(ManNAc) = 0.085 mol/l, c(aldolase) = 21100 U/l, pH = 7.5, T = 25 °C.

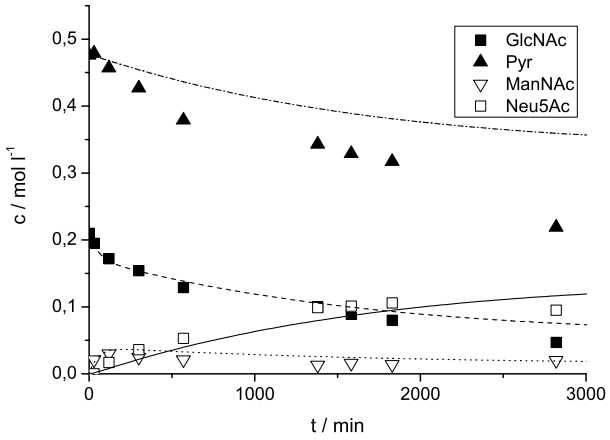

Figure A.6.: Experimental points (markers) and simulated reaction course (lines) of an exemplary enzymatic Neu5Ac synthesis from GlcNAc. Conditions: c_0(GlcNAc) = 0.21 mol/l, c_0(Pyr) = 0.48 mol/l, c(epimerase) = 1200 U/l, c(aldolase) = 35600 U/l, pH = 7.5, T = 25 °C, c(ATP) = 2 mmol/l, c($MgCl_2$) = 2 mmol/l.

A.2. Reactive extraction

Solvent	extracted	Solvent	extracted
Diethylether	44.7 ± 3.4 %	Toluene	59.5 ± 7.8 %
Diisopropylether	38.0 ± 0.9 %	Dichloromethane	50.1 ± 18.9 %
MTBE	32.5 ± 6.6 %	n-Hexane	45.3 ± 6.8 %
p-Xylene	21.8 ± 0.2 %	n-Hex./1-Oct. (85:15)	88.1 ± 1.4 %
o-Xylene	47.1 ± 5.6 %	n-Hept./1-Oct. (85:15)	87.1 ± 2.6 %
1-Octanol	32.0 ± 3.1 %		

Table A.1.: The amount of neuraminic acid extracted from the aqueous phase at equilibrium with different solvents used as organic phase and PBA/TOMAC used as carrier. Conditions: c_0(Neu5Ac) = 0.01 mol $\cdot$ l^{-1}, c_0(PBA/TOMAC) = 0.1 mol $\cdot$ l^{-1}, T = 25 °C, PVR = 1.

pH	GlcNAc	ManNAc	Neu5Ac	Pyr
10	15.5 ± 3.0 %	26.5 ± 8.6 %	64.8 ± 1.4 %	49.5 ± 5.3 %
11	47.0 ± 2.4 %	39.6 ± 0.9 %	85.6 ± 2.4 %	32.9 ± 2.3 %
12	54.0 ± 1.9 %	37.8 ± 2.6 %	84.6 ± 1.3 %	34.1 ± 0.6 %
13	23.7 ± 0.4 %	26.4 ± 31.9 %	67.7 ± 2.7 %	46.0 ± 1.5 %

Table A.2.: Amount of carbohydrates and sodium pyruvate extracted at different pH values. Conditions: $c_0(A) = 0.01$ mol $\cdot$ l^{-1}, c_0(PBA/TOMAC) = 0.1 mol $\cdot$ l^{-1}, T = 25 °C, organic phase n-heptane/1-octanol (85/15), PVR = 1.

c(A)/c(Carrier)	GlcNAc	ManNAc	Neu5Ac	Pyr
0.01/0.02	7.6 ± 3.0 %	16.0 ± 7.3 %	4.3 ± 1.7 %	9.6 ± 1.3 %
0.01/0.05	30.3 ± 1.9 %	22.1 ± 6.9 %	11.3 ± 1.6 %	31.8 ± 7.6 %
0.01/0.1	59.7 ± 8.6 %	39.2 ± 2.2 %	87.1 ± 2.6 %	32.1 ± 5.3 %
0.05/0.5	57.0 ± 3.7 %	47.3 ± 5.6 %	81.7 ± 4.2 %	51.7 ± 4.0 %
0.1/0.5	47.5 ± 0.8 %	48.7 ± 4.6 %	67.2 ± 2.8 %	51.4 ± 1.8 %

Table A.3.: Amount of carbohydrates and sodium pyruvate extracted at varying substrate and carrier (PBA/TOMAC) concentrations. Conditions: T = 25 °C, organic phase n-heptane/1-octanol (85/15).

GlcNAc		**ManNAc**	
pH	% reextracted	pH	% reextracted
1.1 ± 0.29	44.72 ± 2.97	1.45 ± 0.01	10.58 ± 1.27
1.9 ± 0.05	43.95 ± 1.27	2.15 ± 0.01	6.64 ± 5.8
2.71 ± 0.77	47.9 ± 1.98	4.01 ± 0.4	11.77 ± 1.49
5.67 ± 0.21	41.96 ± 15.85	6.65 ± 0.47	9.66 ± 1.68
6.89 ± 0.61	47.21 ± 6.73		
Neu5Ac		**Pyr**	
pH	% reextracted	pH	% reextracted
1.1 ± 0.02	71.59 ± 0.47	1.17 ± 0.25	23.49 ± 3.42
1.78 ± 0.03	59.77 ± 1.79	1.93 ± 0.11	12.34 ± 1.16
2.7 ± 0.04	17.21 ± 0.12	2.57 ± 0.07	5.89 ± 2.78
4.89 ± 0.01	2.94 ± 0.63	5.11 ± 0.32	3.29 ± 0.21
5.66 ± 0.22	3.1 ± 1.4	6.82 ± 0.02	3.87 ± 0.56
3.63 ± 0.3	5.73 ± 1.11		

Table A.4.: Amount of carbohydrates and pyruvate recovered after extraction with PBA/TOMAC in n-heptane/1-octanol and subsequent reextraction of the organic phase with acidic aqueous solution. Conditions: $c_0(A) = 0.01$ mol $\cdot$ l^{-1}, c_0(PBA/TOMAC) = 0.1 mol $\cdot$ l^{-1}, T = 25 °C, organic phase n-heptane/1-octanol (85/15), PVR = 1.

Erklärung

Hiermit versichere ich, dass ich die vorliegende Arbeit selbständig angefertigt und ohne fremde Hilfe verfasst habe und keine außer den von mir angegebenen Hilfsmitteln und Quellen dazu verwendet habe. Die den Werken inhaltlich und wörtlich entnommen Stellen sind als solche kenntlich gemacht.

Rostock, April 2008 .....

Vera Zimmermann