Benefits of Enzyme Kinetics Modelling*

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Mathematical models, especially when coupled with modern computer techniques, prove to be very effective in searching for optimal operating conditions and creating an optimal microenvironment for the biocatalyst in order to optimise productivity. Therefore, the study of the theoretical model for the enzyme reaction system is of interest for the industrial application of the biocatalyst. Theoretical modelling of the enzyme kinetics and the reactors can be used to find optimal operation points and to increase our knowledge about the process. The model usually contains information on the particular biocatalytic reactions. It also includes the mass balance equations in the reactor. The model has to be effective for a wide range of variations of the internal process variables.

The benefits of enzyme kinetics modelling are demonstrated in this review with some examples. These are: continuous (R)-mandelic acid production in the enzyme membrane reactor, enzymatic dipeptide synthesis, the synthesis of L-*tert*-leucine, the synthesis of CMP-Neu5Ac, the synthesis of L-erythrulose, the resolution of racemic alcohol and the synthesis of trisaccharide catalysed by a multi-enzyme system.

Keywords:
Enzyme kinetics, modelling of enzyme reactions, enzyme membrane reactor.

Introduction

During last two decades the continuous operation of enzyme reactors has been realised in practice,[1] so that the design of such reactors is becoming increasingly important. In order to design a reactor for the isolated enzyme catalytic system, it is important to examine the kinetic properties of the enzymes in some detail. Also, knowledge of the enzyme reaction kinetics is essential for understanding how enzyme reactors work. To assemble a complete picture of an enzyme reactor operation, it is necessary to integrate the enzyme reaction kinetics and the mass balance in the reactor. Consequently our major task in this work is to blend the enzyme kinetic measurement and the modelling of the enzyme kinetics to obtain a coherent overall strategy and an analysis of the enzyme reactors. Development of a satisfactory model for the enzyme kinetics is, of course, only one element of a strategy for an improved bioprocess design.

A few years ago modelling was not viewed as quite so significant for current industrial practice. It was popularly imagined to be what academics do.[2] But now the situation is rapidly changing. LONZA, a Swiss company, one of the biggest fine chemicals producers in the world, routinely applies modelling to avoid formation of inhibiting by-products of the biotransformation as well as a tool for industrial bioprocess integration. They showed[3] that modelling had helped to find the most cost-effective mode for L-carnitine production, to develop and practice an adequate process control system for 6-OH nicotinic acid as well as methylpyrazine carboxylic acid production. Also, process simulation had helped to find the most cost-effective biotransformation design for nicotinamide production.[3] Lastly, they concluded that modelling methods were adequate tools to save costs.

Modelling as a reaction engineering principle has been finding a role in enzymatic reactions[4,5,6] and this is expected to grow with the use of biocatalysis.

In our consideration of the enzyme kinetics, the complex nature of the modelling was indicated. In that context, we examined the different types of approximations that could be introduced in order to simplify the kinetic description of the enzyme reaction to a practical, workable level while at the same time trying to minimise errors introduced by the approximation. We face similar problems in the enzyme reactor design and analysis.

The approximations we made are: no diffusion occurs in our reaction system and our reactors behave ideally.

In this paper an approach for the development of the enzyme kinetic model is introduced in the following steps: experimental determination of the

*Dedicated to Prof. Christian Wandrey on the occassion of his 60th birthday

initial reaction rate, mathematical formulation of the initial reaction rate, parameter identification, mathematical formulation of an overall reaction rate, experimental verification of the model prediction, combination with the reactor configuration to find a relation between reaction rates and reactant concentration, and finally calculation and prediction on the basis of the developed model.

Experimental determination of initial reaction rate

The most important characteristics of an enzyme are the fundamental data about the kinetics of the catalysed process and knowledge about the factors that affect the kinetic properties. The two characteristics, the Michaelis-Menten constant K_m and the maximal velocity V_m, are especially important data. They describe the nature of the relationship between the initial reaction rate and the substrate concentration. If substrate and/or product inhibitions occur the corresponding K_i values are also important.

The actual form of **the intrinsic rate equation** of an enzymatic reaction depends on the mechanism of the enzymatic transformations, which can be very complex.[7,8] In carrying out the characterising study of an enzyme, the enzyme should be as pure as possible, preferably recrystallised several times, and in any case should be quite free from any other enzyme acting on similar substrates. The substrates should also be as pure as possible and quite free from any other substances on which the enzyme may act. In carrying out the specificity study it is usual to select a reference substrate, which is generally the most readily attacked biological substrate, and to work out the optimum conditions for this substrate.

On the other hand, for the technical application it is not always possible to use the pure enzyme and its natural substrate. The enzymatic kinetic measurements existing in the literature are mainly concerned with the characterisation of biocatalyst and not with design problems. These measurements refer to conditions of pH, temperature and substrate concentration needed to characterise the enzymes. The use of such data for the reactor design can lead to misinterpretation because the investigations are mainly carried out in a concentration range not interesting for technical applications. In industry high concentrations are desirable to avoid too large reactor volumes for a given productivity. Therefore, the kinetic measurements have to be carried out under conditions relevant to large-scale processes.[9]

The influence of all the reaction components has to be determined while there may be complex interaction in the coupled system. Hence, in the reactor design problem we have to use **the formal reaction rate** that can be quite different from the intrinsic reaction rate. The kinetics models including formal reaction rate are, of course, data-driven and empirical. In the case of one substrate reaction it is usually a non-linear Michaelis-Menten equation combined with product and/or substrate as well as some other reaction component inhibition. The inhibition is mostly competitive. In the case of two substrates, the simplified two-substrate Michaelis-Menten equation combined with the inhibitory effect of product and/or substrate is usually used. This equation resulted from a sequentially ordered mechanism such that substrates must bind in a specified order for reaction to occur. Many oxidoreductases have been shown to react following this mechanism that yielded the lowest number of parameters in the kinetic model.

The actual form of the intrinsic rate equation of an enzymatic reaction depends on the chemical mechanism of the enzymatic transformation. In the case of the formal rate equation that is used for the reactor design, the reaction mechanism is no longer so important.

In fact, "unless there are good positive reasons for using the more complicated of two equations, we should always select the simpler of the two if both fit the data equally well".[9a]

Using non-linear regression methods to fit the experimental data performs the determination of the model parameters from the experimental data of the initial rate of the reaction.

Data from the measurement of the initial rate of the forward and reverse reaction at different substrate concentrations and/or product concentrations were fitted to the kinetic equations using a non-linear least-squares regression technique and optimisation algorithms such as Marquardt, Nelder-Mead, Gauss-Newton, Rosenbrock etc. The estimated parameters serve as the values of initial estimates of the parameter determination through the fitting procedure to the overall reaction rate.

Overall reaction rate

Attempts at attaining good enzyme kinetics models are made by simple trial and error. This is because the form of the model may be suggested simply by the result of an empirical curve-fitting procedure. The equipment by which empirical information is obtained is usually the batch reactor. All that has to be determined is the extent of the reaction at various times by following the concentration of a given component. Therefore experience with the enzyme kinetics is very important.

The overall reaction rate models attempt to relate the kinetics to concentrations by the differential system including mostly non-linear functions. The effects of all the reaction components have to be taken into consideration. The reverse reaction has to be integrated into the kinetic model because the same active site of an enzyme catalyses both the forward and the reverse reaction often even under the same conditions. The overall reaction rate is written as a sum of individual initial reaction rate steps including the influences of all reaction components. Usually these kinetics models describe the reaction rate in a given control volume with constant composition and temperature. Models of this type usually simply use ordinary differential equations (ODE-s) that give the compositions as a function of a single independent variable, in this case time.

The optimised parameter values can be easily determined from the experimental data obtained by measuring the substrate and/or product variation with time by using numerical integration coupled with non-linear regression. The number of parameters to be optimised should be as low as possible. Some programs have not been designed to work properly if the number of parameters exceeds six.[6] Computer modelling with package software (Scientist[10], MatLab[11] etc) has made it possible to treat more coupled reactions, which results in more coupled ODE-s.

Combination with the reactor configuration

The next step for further developing the model is to find the relation between the reaction rates and the reactor configuration.

The above-mentioned formal kinetic model for the overall reaction rate and the balance equations for a reactor configuration were used to predict the process behaviour and to give a rule for optimising productivity.

To validate the kinetic model, the data from batch reactor experiments at different initial concentrations of substrate or enzyme concentrations should be in good agreement with simulated data obtained by means of numerical integration.

Optimisation analysis

An optimisation analysis was usually carried out to determine some of those process characteristics: optimum yield conditions, optimum productivity conditions, optimum selectivity conditions, optimum space-time-yield conditions, and/or optimum operating conditions.

The optimisation of the productivity or space-time-yield is still an interesting field of research, especially for continuously operated processes. The enzyme kinetics can substantially contribute here with the aid of mathematical modelling including the kinetics and different modes of reactor operation.

Examples demonstrating the modelling of the enzymatic reactions

Theoretical modelling of the enzyme membrane reactor can be used to exemplify how to find the optimal operation points and how to increase our knowledge about the process. This means the use of as much physical, chemical and biological a priori knowledge as possible. This is especially important if the model has to be valid for a wide range of variations of internal process variables.

Continuous (R)-mandelic acid production in the enzyme membrane reactor

The first example to demonstrate the strategy and the benefits of enzyme kinetic modelling is continuous (R)-mandelic acid production in an enzyme membrane reactor.[12] It was demonstrated that the enzyme D-(-)-mandelic acid dehydrogenase (MaDH) could be employed for the stereo-specific synthesis of (R)-mandelic acid (Ma), a useful raw material for semi-synthetic β-lactam antibiotic production. In the characterising study of the enzyme a K_m-value of 0.22 mM was obtained.[13] The use of this value for the reactor design led to mainly speculative predictions of process behaviour.

A continuous production of (R)-mandelic acid was carried out in an enzyme membrane reactor. The two enzymes: D-(-) mandelic acid as the production enzyme and formate dehydrogenase as the coenzyme-regenerating enzyme, as well as the PEG-bound cofactors, was separated from the product stream by means of an ultrafiltration membrane. Enforced flow across the membrane and complete mixing in the reactor with a magnetic stirrer ensured that the enzyme membrane reactor behaved as a continuously operated stirred tank reactor (CSTR). For such a multi-enzyme system operating in an enzyme membrane reactor it is desirable to formulate a mathematical model for coupled enzyme reactions in order to understand the complex interrelationships between the two reactions and to optimise its performance.

The enzyme kinetics was assumed to be of mixed type comprising Michaelis-Menten-type kinetics together with uncompetitive substrate inhibi-

tion and competitive product inhibition. A detailed kinetic evaluation of the individual reactions was carried out in order to determine the effect of all substrates and products on the activity of the two enzymes involved. The K_m value of MaDH obtained for phenyl-glycofiy acid (PhGly) was significantly higher in comparison with the intrinsic one.[10]

The kinetic investigation showed that in such a multi-enzyme system, the complex interaction would occur between all reaction components. Meanwhile, the overall rate of (R)-mandelic acid production may be written as the sum of the individual reaction steps including the reciprocal influences of all reaction components.

The behaviour of the enzyme reactor was simulated by means of the kinetics and simultaneous calculations of the mass balances of any reactant.

By means of the stationary solution of mass balance, the optimal enzyme ratio was calculated. It was clear that the optimal ratio for the activity of the production enzyme and the regeneration enzyme was not unity and furthermore it depended on the residence time.

Reactions

Enzymatic dipeptide synthesis

$$\underset{A}{\text{Phth-Tyr-OR'}} + \underset{B}{\text{SerOR'}} \xrightarrow{(R_1)} \underset{P}{\text{Phth-Thy-Ser-OR'}} \quad (1)$$

$$\underset{A}{\text{Phth-Tyr-OR'}} + \underset{I}{H_2O} \xrightarrow{(R_2)} \underset{Q}{\text{Phth-Tyr-OH}} + \underset{B}{\text{SerOR'}} \quad (2)$$

$$\underset{P}{\text{Phth-Thy-Ser-OR'}} + \underset{I}{H_2O} \xrightarrow{(R_3)} \underset{Q}{\text{Phth-Tyr-OH}} + \underset{B}{\text{SerOR'}} \quad (3)$$

R' – Et- or NH_2-
(1) – aminolysis
(2) – hydrolysis
(3) – secondary hydrolysis

Fig. 1 – *The kinetically controlled synthesis of dipeptide catalysed by serine protease α-chymotrypsine (α-CT)*

Kinetic models

Two substrate kinetics according Ping-Pong mechanism:

$$-\frac{dc_A}{dt} = \frac{\frac{V_{m,AB} \cdot c_A c_B}{K_{m,B}} + \frac{V_{m,AI} \cdot c_A \cdot c_I}{K_{m,I}}}{\text{denom}} \qquad \frac{dc_P}{dt} = \frac{\frac{V_{m,AB} \cdot c_A c_B}{K_{m,B}} - \frac{V_{m,PI} \cdot K_{m,AI} \cdot c_I \cdot c_P}{K_{m,I} \cdot K_{m,P} \cdot K_{m,BQ}}}{\text{denom}}$$

$$-\frac{dc_B}{dt} = \frac{\frac{V_{m,AB} \cdot c_A c_B}{K_{m,B}} - \frac{V_{m,PI} \cdot K_{m,AI} \cdot c_I \cdot c_P}{K_{m,I} \cdot K_{m,P} \cdot K_{m,BQ}}}{\text{denom}} \qquad \frac{dc_Q}{dt} = \frac{\frac{V_{m,AI} \cdot c_A c_I}{K_{m,I}} - \frac{V_{m,PI} \cdot K_{m,AI} \cdot c_I \cdot c_P}{K_{m,I} \cdot K_{m,P} \cdot K_{m,BQ}}}{\text{denom}}$$

$$\text{denom} = c_A + \frac{K_{m,AI} \cdot c_B}{K_{m,B}} + \frac{K_{m,AB} \cdot c_B \cdot c_P}{K_{m,B} \cdot K_{i,P}} + \frac{K_{m,AI} \cdot c_I}{K_{m,I}} + \frac{c_A \cdot c_B}{K_{m,B}} + \frac{c_A \cdot c_I}{K_{m,I}} + \frac{K_{m,AI} \cdot c_I \cdot c_P}{K_{m,I} \cdot K_{m,Q} \cdot K_{m,PQ}}$$

Michelis-Menten double substrate kinetics:

$$R_1 = \frac{V_{max,A} \cdot c_A}{K_{mA} \cdot \left(1 + \frac{c_P}{K_{iP}}\right) + c_A} \cdot \frac{c_B}{(K_{mB} + c_B)} \qquad \frac{dc_A}{dt} = -R_1 \cdot E - R_2 \cdot E$$

$$\frac{dc_B}{dt} = -R_1 \cdot E + R_2 \cdot E + R_3 \cdot E$$

$$R_2 = \frac{V_{max,Ah} \cdot c_A}{K_{mAh} + c_A} \qquad \frac{dc_P}{dt} = R_1 \cdot E - R_3 \cdot E$$

$$R_3 = \frac{V_{max,P} \cdot c_P}{K_{mP} + c_P} \qquad \frac{dc_Q}{dt} = R_2 \cdot E + R_3 \cdot E$$

Fig. 2 – *Kinetics models for the kinetically controlled synthesis of dipeptide catalysed by serine protease α-chymotrypsine (α-CT)*

Enzymatic dipeptide synthesis

The short-chain peptides are of increasing interest, especially as building blocks for pharmaceuticals, clinical nutrition and chiral auxiliaries therefore requiring large-scale synthesis methods. As high chemical and optical purity of the desired product is essential, the side reactions and the racemisation have to be minimised during synthesis. These requirements are best fulfilled by kinetically controlled enzyme-catalysed peptide synthesis. As is known, enzymatic peptide synthesis can be carried out either in a thermodynamically or kinetically controlled manner.

In the kinetically controlled synthesis a covalent acyl-enzyme as the reactive intermediate (A) can transfer the acyl group either to a second amino acid (aminolysis, B) or to water (hydrolysis, I). In the former case, the dipeptide product P, in the latter case, the first amino acid (not activated, Q) is obtained in this competitive reaction (Figs. 1 and 2). By controlling the reaction conditions using reaction engineering methods, the secondary hydrolysis can be minimised.[14,15]

In our[15] initial attempts to model the enzymatic peptide kinetics, we used the well-known ping-pong mechanism. This mechanism leads in general to rather unwieldy equations. These equations have too many parameters for practical application (Fig. 2). The enzymatic peptide kinetics equation can also be well approximated by the equation for the Michaelis-Menten double substrate kinetics considering a competitive product inhibition (Table 1, Fig. 2). Both models were validated[15] with batch enzymatic dipeptide (Tyr-Ser) synthesis catalysed with serine-protease α-chymotrypsine by comparing the calculated and the experimental concentration-time curves (Fig.3). Obviously, there is a better agreement between the predicted concentration-time curves according to the Michaelis-Menten double substrate kinetic model and the observed experimental data.

The synthesis of L-*tert*-leucine

The synthesis of L-*tert*-leucine is an example of the application of cofactor regeneration in an industrial continuous process.[16,17] The modelling introduced demonstrates that the high total turnover numbers can be achieved both with the "polymer--enlarged" and with the "native" cofactor. The total turnover number specifies how many moles of product are formed per mole of consumed cofactor.

The kinetics of the substrate and the cofactor of this reaction can be described by Michaelis-Menten double substrate kinetics, taking into consideration a competitive product inhibition.

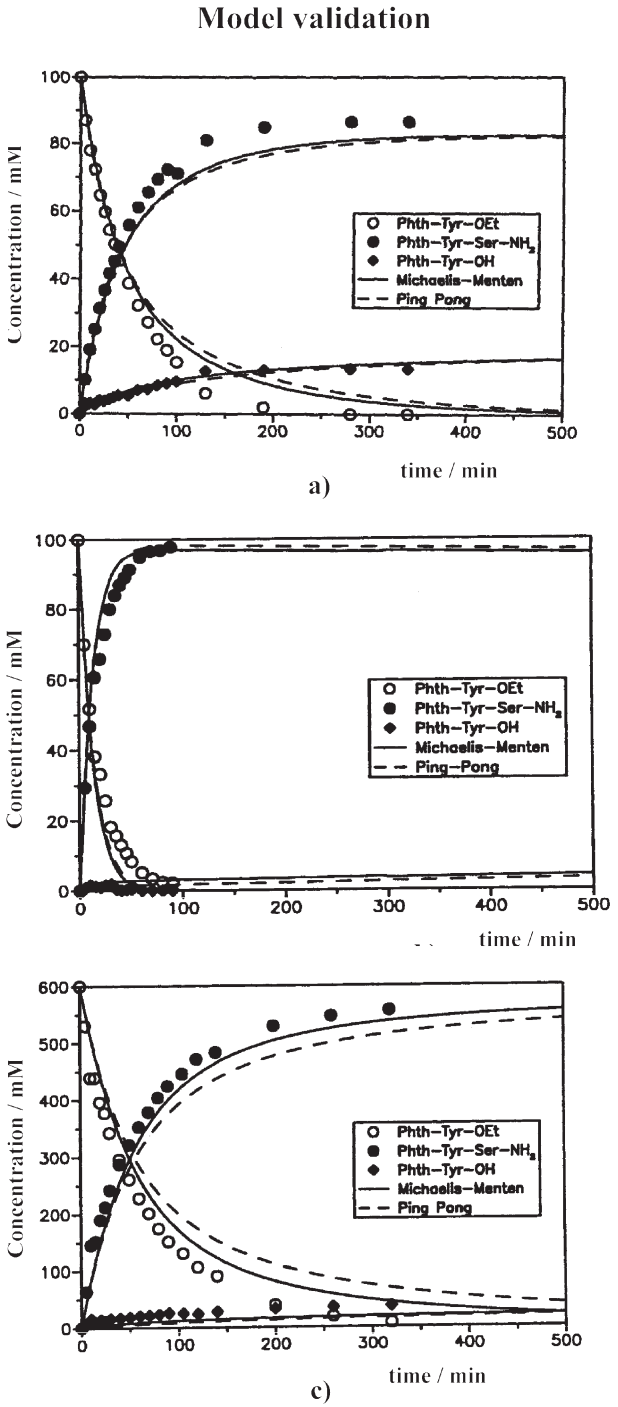


Fig. 3 – *The comparison of two kinetics models fitted on experimental data from the synthesis of Phth-Tyr-Ser.NH$_2$:*
a) – 100 mM Phth-Tyr-Oet, 100 mM Ser-NH$_2$; pH 9.0; 0.01 mg/mL α-CT.
b) – 100 mM Phth-Tyr-Oet, 600 mM Ser-NH$_2$; pH 9.0; 0.01 mg/mL α-CT.
c) – 600 mM Phth-Tyr-Oet, 600 mM Ser-NH$_2$; pH 9.0; 0.01 mg/mL α-CT.

The calculations indicated the way in which the total turnover number depends on the cofactor concentration. The dependence shown is valid for a conversion of 90 % adjusted by the corresponding variation of the residence period. Whereas the

space-time yield rises with an increasing cofactor concentration, the total turnover number achievable simultaneously drops. This behaviour is found both for the polymer-enlarged and for the native cofactor. The space-time yield is somewhat higher for the native cofactor since a higher reaction rate is achieved due to somewhat more favourable kinetic parameters. From the economic point of view, a compromise must be made between space-time yield and total turnover number, which directly determines the cost of the cofactor. Due to the low price now achieved, the native cofactor can be used economically in the continuous process for the smaller achievable total turnover numbers.

The understanding of the enzyme reaction engineering of such systems is useful in determining the optimum reaction conditions.

The overall reaction rate for the formation of L-tert-leucine as a function of the concentration of trimethylpyruvate and the cofactor concentration for 90 % substrate conversion rises with the increasing cofactor concentration whereas it drops with the rising substrate concentration. At the given conversion of 90 %, this drop can be attributed to increasing product inhibition. The activity ratio of the two enzymes applied also influences the achievable space-time yield. Different conversions are achieved at a constant enzyme ratio by varying the residence time. In calculating the enzyme molar fraction, the enzyme activities determined under the initial reaction rate conditions are used. At low conversions the maximum space-time yield must therefore be found at the molar fraction of 0.5. As the conversion increases, the maximum of the achievable space-time yield is shifted towards smaller enzyme molar fractions since the production enzyme and the regeneration enzyme are influenced to a different extent by the concentrations of the reactants, which are changed with increasing conversion. To achieve the same reaction rates for both enzymes under the concentration conditions prevailing in the reactor, the production enzyme must be applied in larger quantities due to the very strong product inhibition.

The CSTR is by far one of the simplest reactor configurations for continuous process realisation and is widely used in many biochemical reactions.

While being advantageous for reactions with substrate inhibitions this reactor is disadvantageous in cases of severe product inhibition as in the synthesis of L-*tert*-leucine from trimethylpyruvic acid. A system of two membrane reactors in series can be proposed as a solution for the reaction in question.

From the economic point of view, a compromise has to be made between the space-time yield, the conversion and the activity of two enzymes that are used. Different conversions at a constant enzyme ratio are achieved by varying the residence time. A comparison among the three types of reactors shows that the maximum space-time yield is found in the batch reactor at the enzyme activity fraction of 0.5. The maximum of the achievable space-time yield in the batch reactor is shifted towards smaller values in the CSTRs in series. The smallest enzyme activity fraction is found for the single CSTR. This can be explained by the fact that the production enzyme (LeuDH) and the regenerating enzyme (FDH) are influenced to a different extent by the concentrations of the reactant and the product. These concentrations are changed according to the different increase of conversion in each reactor type. The main reason responsible is the product inhibition by L-*tert*-leucine. Therefore, with increasing conversion the maximum of the space-time yield is shifted to lower enzyme activity fractions for all three reactors. It is therefore possible to minimise the biocatalyst cost in the reactor by maximising the space-time yield if other destabilising effects are excluded. The minimal biocatalyst cost is achieved in the batch reactor. Even at the highest conversion of 98 % required from the practical point of view, the difference in the biocatalyst cost in the batch and two CSTRs in series is negligible. At that conversion a single-stage CSTR cannot compete with the batch reactor any more.

Therefore by adjusting the proper enzyme activity fraction in the reactor at the highest possible conversion and the maximum space-time yield minimum biocatalyst cost can be achieved.

The synthesis of CMP-Neu5Ac

CMP-Neu5Ac has been evaluated as a potential drug for the treatment of disorders in neuronal function. The synthesis of CMP-Neu5Ac is catalysed by the enzyme[18,19,20] CMP-Neu5Ac synthetase from *Escherichia coli* K-235 (Fig.4).

The kinetics of the synthesis of CMP-Neu5Ac can be adequately described[18] by multiplying two Michaelis-Menten equations together with a competitive inhibition by pyrophosphate and a substrate-surplus inhibition by CTP. For the chemical hydrolysis of CMP-Neu5Ac, a first-order rate equation was used. The set of equations together with the estimated kinetic parameters are given in Fig. 5. To validate the model, the simulation of various syntheses in a batch-operated reactor has been verified experimentally. An example is shown in Fig.6.

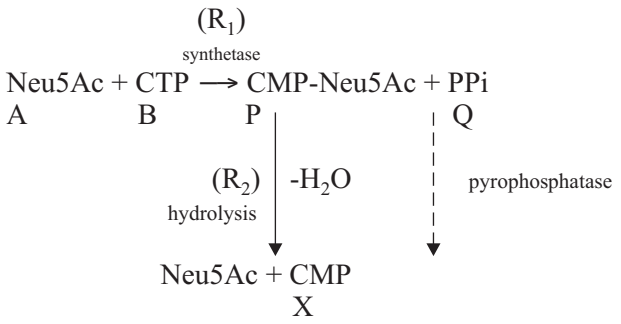
There is good agreement between the predicted and observed concentration time curve.

Fig. 7 shows a comparison between selectivity with and without the addition of an enzyme

Reactions

Enzymatic synthesis of CMP-Neu5Ac

(Cytidine-monophospho-N-Acetyl-neuraminic-acid)



Neu5Ac – N-Acetyl-neuraminic acid
CTP – Cytidine-5'-triphosphate
CMP-Neu5Ac – Cytidine-monophospho-N-acetyl-neuraminic acid
PPi – Pyrophosphate
CMP – Cytidine-5'-monophosphate

Fig. 4 – *The synthesis of N-acetyl-neuraminic acid catalysed by CMP-Neu5Ac synthetase from* Escherichia coli *K-235.*

Kinetic model

$$R_1 = \frac{V_{max} \cdot c_A}{K_{m,A} \cdot \left(1 + \frac{c_Q}{K_i Q}\right) + c_A + \frac{c_A^2}{K_{iA}}} \cdot \frac{c_B}{(K_{mB} + c_B)}$$

$$R_2 = k \cdot c_P$$

$$\frac{dc_A}{dt} = -R_1 \cdot E + R_2 \qquad \frac{dc_Q}{dt} = R_1 \cdot E$$

$$\frac{dc_B}{dt} = -R_1 \cdot E \qquad \frac{dc_X}{dt} = R_2$$

$$\frac{dc_P}{dt} = R_1 \cdot E - R_2$$

Fig. 5 – *Kinetics models for the synthesis of N-acetyl-neuraminic acid catalysed by CMP – Neu5Ac synthetase from* Escherichia coli *K-235.*

(pyrophosphatase) catalysing the destruction of inhibited product (pp_i) in the batch reactor as well as a comparison between the batch reactor and the CSTR for the synthesis of CMP-Neu5Ac. Obviously, product inhibition is still much more dominated, favouring the batchwise production of CMP-Neu5Ac. In this case, the precipitated phosphate only influences the recovery of the enzymes.

Considering the results discussed above, a large-scale synthesis has been performed in the fed-batch reactor in order to compensate for the substrate-surplus inhibition by CTP. To overcome product hydrolysis, short reaction times (less than 8 hours) were employed.

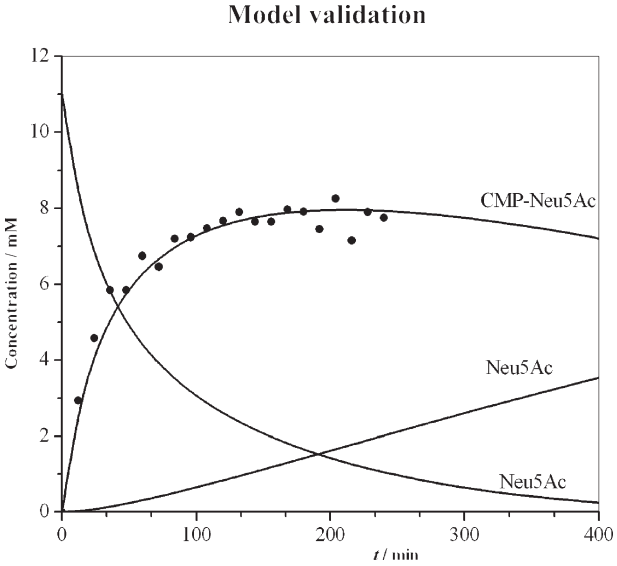


Fig. 6 – *Model validation. 11 mM Neu5Ac, 11 mM CTP; T = 30 °C, pH 8.0*

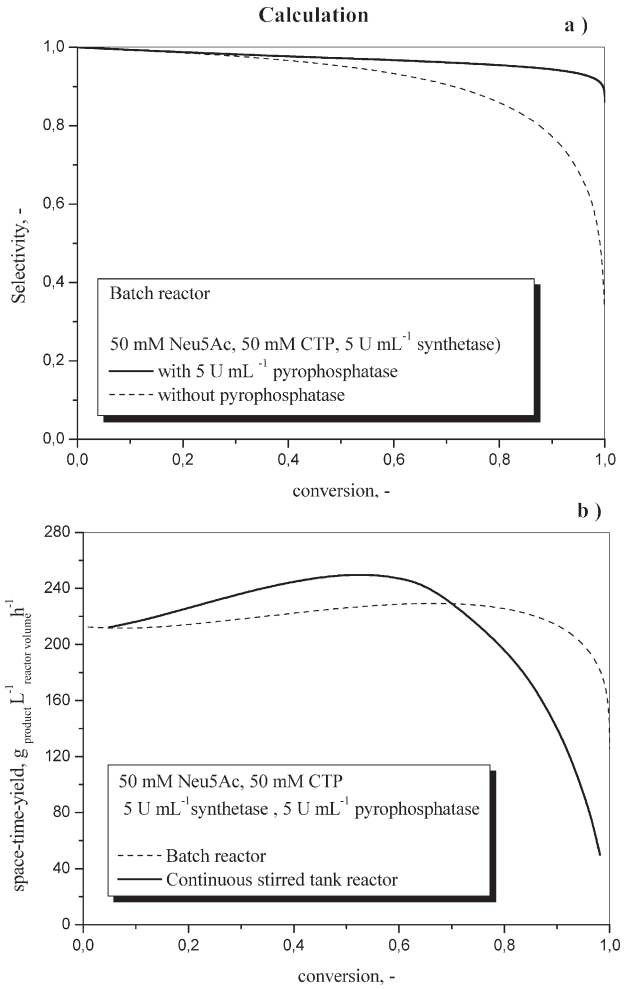


Fig. 7 – *a) – Comparison between selectivity with and without addition of the enzyme pyrophosphatase according to the model depicted in Fig. 5*
b) – Comparison between productivity in the batch and continuous stirred tank reactor (CSTR) according to the model depicted in Fig. 5

The synthesis of L-erythrulose

Transketolase can be used for the synthesis of chiral intermediates and carbohydrates. However, the enzyme is strongly deactivated by the educt.[21,22,23]

When β-hydroxypyruvate (HPA) is used as the donor substrate the reaction becomes irreversible. Transketolase catalyzes the formation of L-erythrulose the formation of L-erythrulose by interconversion of HPA and glycolaldehyde. This reaction system can be described by the Michaelis-Menten double substrate kinetics, considering a competitive product inhibition. For the spontaneous hydrolysis of both starting material, the first-order rate equations were used. The rate of deactivation for transketolase caused by the reactive α-hydroxy aldehyde can be described by the first order rate equation in which the deactivation constant shows the Michaelis-Menten-type dependence on the limiting starting materials.

The CSTR can be a favourable reactor configuration in view of enzyme deactivation, while operating at a high conversion, the substrate concentration is constant at a low level. Consequently, the rate of deactivation should be very small.

While being of advantageous for enzyme deactivation, this reactor is of disadvantageous in the case of strong product inhibition, as in the synthesis of L-erythrulose. Also in the case of transketolase deactivation the steady state can be reached only at a very high enzyme activity (Fig. 8.)

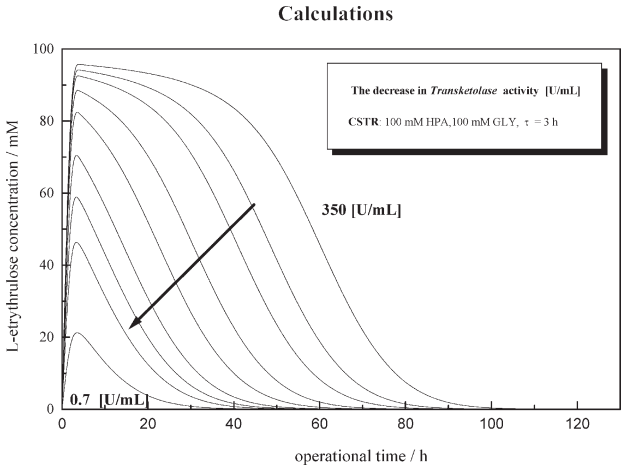


Fig. 8 – *The simulation of CSTR for the synthesis of L-erythrulose catalysed by transketolase according to the published model*[21]. *Product concentration in reactor as a function of the transketolase activity.*

A cross-flow system consisting of two or three equally sized continuously operated stirred tank reactors with distributed (split) feed of the second reactant (called "cross-flow reactor") can be proposed as a solution for the reaction in question.

The cross-flow reactor is a model reactor that can be taken into consideration for the choice of a reactor type for a very strong enzyme deactivation caused by one of the starting materials and to overcome a strong product inhibition as in the batch reactor.

We assumed that the total feed stream of HPA was supplied to the reactor system at one point only and that the product stream left the system at one outlet point. A distributed feed of the enzyme-deactivating starting materials (GLY) along the reactor system might be one of the possibilities. Such applications may be profitable in particular cases, i.e. to suppress undesired enzyme deactivation.

For the cross-flow system consisting of the CSTRs, the mass balance over each reactor is essentially given by equation:

$$\frac{\mathrm{d}C_\mathrm{j}}{\mathrm{d}t} = \frac{C_\mathrm{j,o} - C_\mathrm{j}}{\tau} + v_\mathrm{j} E$$

provided the possibility of more than one feed stream is taken into account. Such a gradual distribution of part of the feed could be realised in practice, but as an extreme model it may serve to investigate problems of reactor optimisation.

We compare the solutions of the CSTR, the cascade of CSTRs with the result for the cross-flow reactor. It can be seen that the CSTR yields higher enzyme consumption than the cross-flow reactor at the same conversion of 90 % and the space-time yield of 85 g L^{-1} D^{-1}.

With the use of the continuous stirred tank reactor or the properly operated cross-flow reactor, the concentration of reactive GLY is intentionally kept relatively low so that the desired product inhibited reaction is favoured and enzyme deactivation is suppressed.

The resolution of racemic alcohol

The necessity of obtaining enantiomerically pure compounds for the pharmaceutical industry is no longer a matter of convenience but a matter of legislation. However, the production of optically active compounds is one of the most difficult problems in the field of speciality chemicals. It was found that the hydrolytic enzymes are particularly well suited because they require no coenzyme and can accept a broad structural range of unnatural esters as substrate.[1,24] The use of oxidoreductases for the production of various chiral compounds, such as hydroxy acids, amino acids or alcohols from prochiral precursors, is a subject of current interest,[25] since coenzyme cost must not be economically limiting.[16,26] Because of the commercial inter-

est in these compounds and their production on a large scale, the kinetic properties of oxidoreductase-catalysed optical resolution need to be studied in detail. Knowledge of the kinetic model is an essential step for the design and optimisation of a continuous process for the production of chiral compounds.

Hence, the resolution of racemic alcohol has been examined in relation to the development of the continuous process based on the use of the enzyme membrane reactor.[27] A model for predicting and interpreting the stereoselectivity of alcohol-dehydrogenase catalysed oxidation and reduction has been formulated based on kinetic data. The model takes into consideration the simple double substrate Michaelis-Menten kinetics. Its application is illustrated by the analysis of representative examples such as the yeast–alcohol-dehydrogenase catalysed resolution of (R, S)-2-butanol and asymmetric synthesis of (S)-2-butanol.

In the biocatalytic systems where the enzyme shows no absolute enantioselectivity, the optimisation of the process requires knowledge of the complete kinetics of the enzymatic reactions. The purity of the remaining enantiomer in such enzymatic resolution depends on the enzyme selectivity as well as on the reactor configuration. (Fig. 9).

The kinetic expressions relating to the progress of the reaction versus the appropriate dependent variable time are developed for the batch reactor and the continuous stirred tank reactor (CSTR). It is found that the maximum ee depends on the reactor configuration and to a certain extent on the kinetic model. To achieve the same ee value in the CSTR as in the batch reactor at a given conversion requires an enzyme with higher enantioselectivity represented in the parameter E, whereas E is defined as

$$E = \frac{v_R}{v_S}.$$

In this case the particular initial reaction rate can be defined as:

$$v_S = \frac{V_{m,S} \cdot \frac{c_S}{K_{m,S}}}{1 + \frac{c_S}{K_{m,S}} + \frac{c_R}{K_{m,R}}} \qquad v_R = \frac{V_{m,R} \cdot \frac{c_R}{K_{m,R}}}{1 + \frac{c_S}{K_{m,S}} + \frac{c_R}{K_{m,R}}}$$

when the both enantiomers compete for the same enzyme site.

Probably, the ee-value is lower at the same degree of overall conversion in the CSTR because the reaction rate of the better-converted substrate decreases more slowly than in the batch reactor with

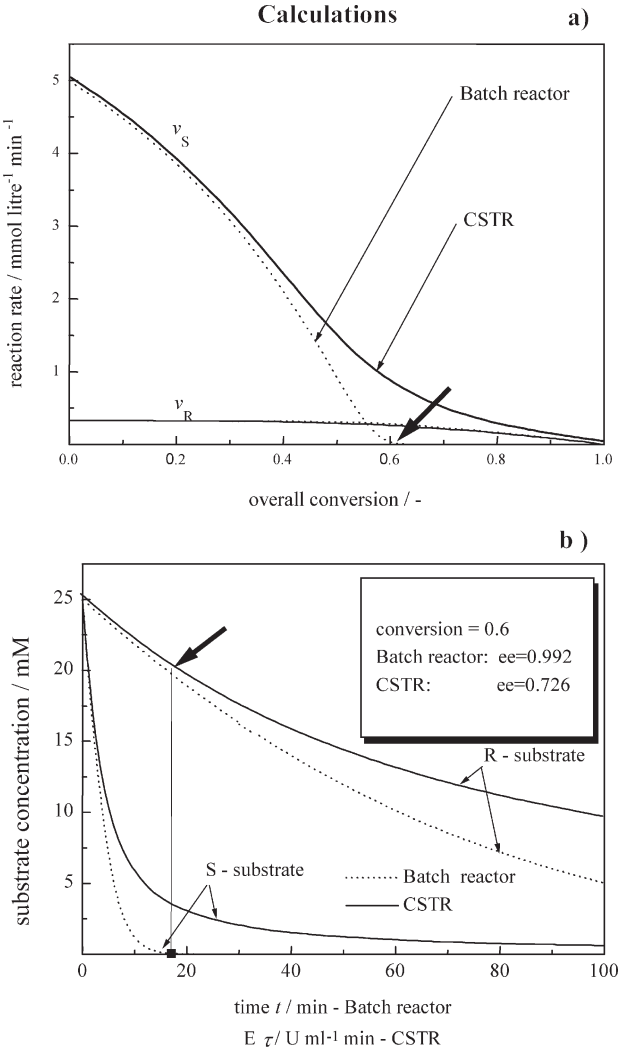


Fig. 9 – *a) – Calculated reaction rate as a function of the overall conversion in the two types of reactors for the enzymatic resolution of the alcohol*[25]
b) – Simulations of the substrate time course in two types of reactors for the enzymatic resolution of the alcohol[25]

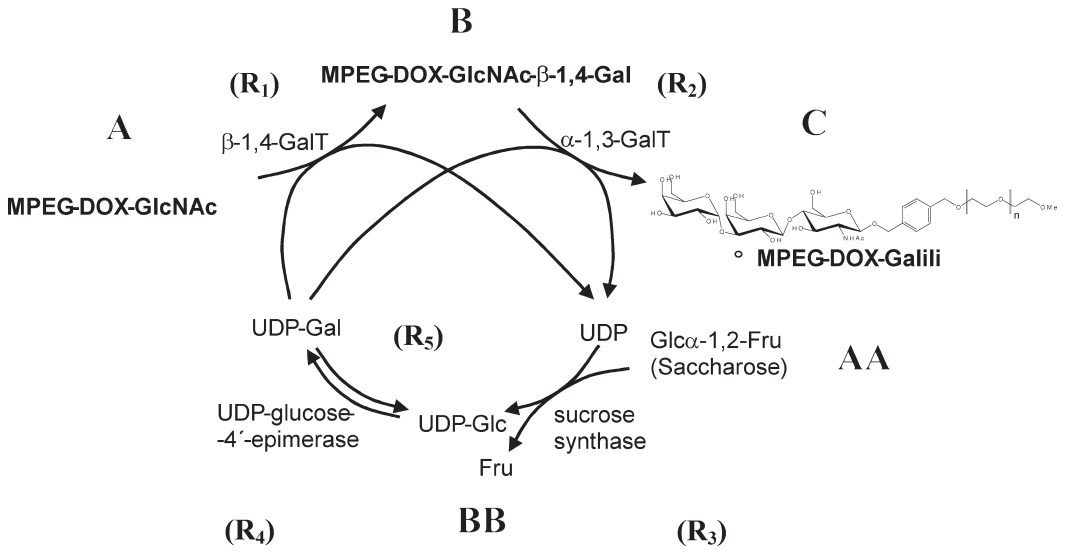
increasing overall conversion due to the convection term and the residence time (Fig. 9b). Hence, the decrease in the concentration of the better-converted substrate over time is quite different in the batch reactor and in the CSTR.

The synthesis of the α-Gal trisaccharide

Polymer-bound α-Gal trisaccharide may be a useful compound to prevent hyper acute xenograft rejection[28]. For synthesis of the desired trisaccharide a multienzyme system (Fig. 10.) was used.[28,29]

The system consists of recombinant β-1,4-galactosyltransferase (β-1,4-GalT), recombinant murine α-1,3-galactosyltransferase (α-1,3-GalT) recombinant sucrose synthase from potato and commercially available recombinant UDP-glucose-4-epime-

Reactions



Lit.: C. H. Hokke, A. Zervosen, L. Elling, D. H Joziasse, D. H. van den Einjnden (1996). Glycoconjugate Journal **13**(4): 687-692.

MPEG 10000 = ω-methoxypoly(ethylene glycol), MW 10000
MPEG-DOX-GlcNAc – polymer-supported *N*-acetyl-glucosamine
MPEG-DOX-GlcNAc-β-1,4-Gal – polymer-supported *N*-acetyl-lactosamine
MPEG-DOX-Galili – polymer-supported α-Gal trisaccharide
UDP – uridine 5'-diphosphate
UDP-Gal – uridine 5'-diphosphogalactose
Glcα-1,2-Fru – saccharose
Fru – fructose

Fig. 10 – *The synthesis of trisaccharide catalysed by a multi-enzyme system*

Kinetic model

$$R_1 = V_{m,1} \cdot \frac{c_A}{K_{m,A} + c_A + \frac{c_A^2}{K_{i,A}}} \cdot \frac{c_{UDP-Gal}}{K_{m,UDP-Gal4} + c_{UDP-Gal}}$$

$$R_2 = V_{m,2} \cdot \frac{c_B}{K_{m,B} + c_B} \cdot \frac{c_{UDP-Gal}}{K_{m,UDP-Gal3} + c_{UDP-Gal}}$$

$$R_3 = V_{m,3} \cdot \frac{c_{AA}}{K_{m,AA} + c_{AA}} \cdot \frac{c_{UDP}}{K_{m,UDP} + c_{UDP}}$$

$$R_4 = V_{m,4} \cdot \frac{c_{UDP-Glc}}{K_{m,UDP-Glc} + c_{UDP-Glc}}$$

$$R_5 = V_{m,5} \cdot \frac{c_{UDP-Gal}}{K_{m,UDP-Gal} + c_{UDP-Gal}}$$

$$\frac{dc_A}{dt} = -R_1 \cdot E_1$$

$$\frac{dc_B}{dt} = R_1 \cdot E_1 - R_2 \cdot E_2$$

$$\frac{dc_C}{dt} = R_2 \cdot E_2$$

$$\frac{dc_{UDP-Gal}}{dt} = -R_1 \cdot E_1 - R_2 \cdot E_2 - R_4 \cdot E_4 - R_5 \cdot E_4$$

$$\frac{dc_{UDP}}{dt} = R_1 \cdot E_1 + R_2 \cdot E_2 - R_3 \cdot E_3$$

$$\frac{dc_{UDP-Glc}}{dt} = R_3 \cdot E_3 - R_4 \cdot E_4 + R_5 \cdot E_4$$

Fig. 11 – *Kinetics models for the synthesis of trisaccharide catalysed by a multi-enzyme system*

rase from *Streptococcus thermophilus*. In the system UDP-Gal was (re) generated *in situ* by the combined action of sucrose synthase and UDP-glucose-4-epimerase. As the intermediate product, polymer-supported *N*-acetyl-lactosamine was formed. The developed model (Fig.11) was validated by fitting the model to the set of experimental data (Fig.12).

A good agreement for this very complex system was obtained. By means of the model, the selectivity and yield were calculated as a function of conversion (Fig.13). The results show that the intermediate in the system could be completely transformed into final product yielding high selectivity at the end of the reaction.

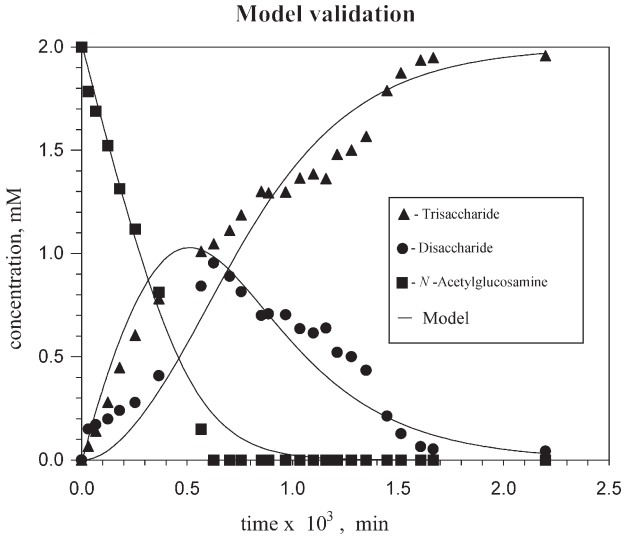


Fig. 12 – *Model validation. 2 mM MPEG-DOX-GlcNAc, 0.4 mM UDP-Glc, 200 mM Saccharose; V = 40 mL; T = 25 °C; pH 7.2; 20 mM HEPES-buffer; 25 mM KCl; 1 mM DTT.*

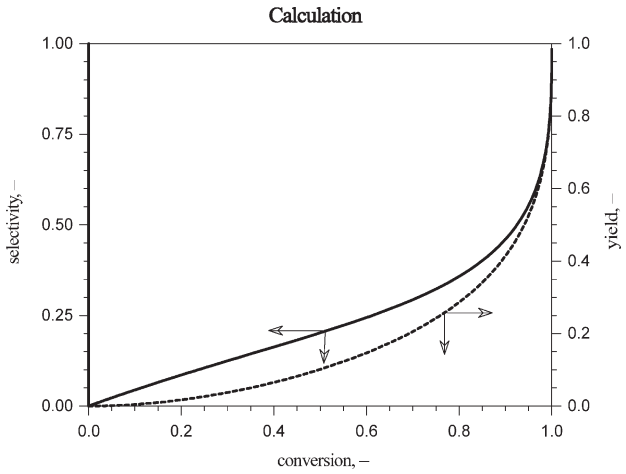


Fig. 13– *Selectivity and yield as functions of conversion in the synthesis of trisaccharide catalysed by a multi-enzyme system.*

Conclusion

It can be concluded that mathematical models, especially when coupled with modern computer techniques, prove to be effective in searching for optimal conditions and creating an optimal micro-environment for the biocatalyst in order to optimise productivity.

Therefore, a study of the theoretical model for the enzyme reaction systems in the reactor is of interest for industrial application of the biocatalyst.

Table I – *Values of parameters in the synthesis of dipeptides*

Parameters	Value
V_{max} [U mg^{-1}]	4.50
K_{mA} [mmol L^{-1}]	22.10
K_{mB} [mmol L^{-1}]	4.85
K_{iA} [mmol L^{-1}]	6.50
K_{iQ} [mmol L^{-1}]	1.77
k [min]	0.0012
E [mg ml^{-1}]	0.507

Table II – *Values of parameters in the synthesis of trisaccharides.*

Parameters	Value
$V_{m,1}$ [U mg^{-1}]	0.13
$V_{m,2}$ [U mg^{-1}]	0.41
$V_{m,3}$ [U mg^{-1}]	0.37
$V_{m,4}$ [U mg^{-1}]	0.59
$V_{m,5}$ [U mg^{-1}]	1.97
$K_{m,A}$ [mmol L^{-1}]	7.71
$K_{i,A}$ [mmol L^{-1}]	7.71
$K_{m,B}$ [mmol L^{-1}]	19.45
$K_{m,UDP\text{-}Gal4}$ [mmol L^{-1}]	2.02
$K_{m,UDP\text{-}Gal3}$ [mmol L^{-1}]	2.22
$K_{m,UDP}$ [mmol L^{-1}]	0.55
$K_{m,UDP\text{-}Glc}$ [mmol L^{-1}]	3.96
$K_{m,UDP\text{-}Gal}$ [mmol L^{-1}]	9.90

References:

1. *Liese, A., Seelbach, K., Wandrey, C.* Industrial biotransformations, Wiley-VCH, Weinheim, pp.93–392.
2. *Beck, M. B, Young, P. C.*, An introduction to system identification, parameter and state estimation, Proc. 4th Int.Conf. Comp. Appl. Ferm. Technol. Cambridge 1988, Computer Applications in Fermentation Technology: Modelling and Control of Biotechnological Processes, Elsevier Sci., Pub., London 1989, pp. 130.
3. *Brass, J. M., Hoeks, F. W. J. M. M., Rohner, M.* Application of modelling techniques for the improvement of industrial bioprocesses, J. Biotechnol. **59** (1997) 63–72.
4. *Wandrey, C.*, Enzyme reaction engineering, **47** (1993) 96–99.
5. *Hogan, M. C.,Woodley, J. M.*, Modelling of two enzyme reactions in a linked cofactor recycle system for chiral lactones synthesis, Chem. Eng. Sci., 55 (2000) 2001–2008.
6. *Straathof, A. J. J.*, Development of a computer program for analysis of enzyme kinetics by progress curve fitting, J. Mol. Catalysis B: Enzymatic **11** (2001) 991–998.
7. *Dixon, M., Webb, E. C.*, Enzymes, 3rd ed. Longman, London, 1979.
8. *Segel, I. H.*, Enzyme kinetics: behaviour and analysis of rapid equilibrium and steady state enzyme systems, Wiley, New York, 1975.

9. *Wandrey, C., Flaschel, E., Schügerl, K.*, Problems in extrapolation of enzymatic kinetic measurements to reactor design using hog kidney acylase as an example, Biotechnol. Bioeng., **21** (1979) 1649–1670

9a. *Levenspiel, O.*, Chemical reaction engineering, 3rd ed. J. Wiley, New York, 1999, pp 380.

10. Handbook SCIENTIST, Micro Math Scientific Software, Salt Lake City, Utah, 1995.

11. *Löwe, A.*, Chemische Reaktionstechnik mit MATLAB und SIMULINK, Wiley-VCH, Weinheim, 2001.

12. *Vasic-Racki, D., Jonas, M., Wandrey, C., Hummel, W., Kula, M-R.*, Continuous (R)-mandelic acid production in an enzyme membrane reactor, Appl. Microb. Biotechnol., **31** (1989) 215–222.

13. *Hummel, W, Schütte, H., Kula, M-R.*, D-(-)-Mandelic acid dehydrogenase from *Lactobacillus curvatus*, Appl. Microbiol. Biotechnol., **28** (1988) 433–439.

14. *Fischer, A., Bommarius, A. S., Drauz, K., Wandrey, C.*, A novel approach to enzymatic peptide synthesis using highly solubilizing N^a-protecting groups of amino acids, Biocatalysis **8** (1994) 289–307.

15. *Fischer, A.*, Zum Einsatz solubilisierender N-terminaler Schutzgruppen von Aminosäuren in der enzymatischen Peptidsynthese, PhD Thesis, University of Bonn (1994).

16. *Kragl, U., Vasic-Racki, D., Wandrey, C.*, Continuous-processes with soluble enzymes, Chem.Ing. Tech., 64 (1992) 499–509. Indian J. Chem. Sect. B **32** (1993) 103–117

17. *Kragl, U., Vasic-Racki, D., Wandrey, C.*, Continuous production of L-tert-leucine in series of two enzyme membrane reactors, Bioprocess Eng., **14** (1996) 291–297.

18. *Klein, T.*, Enzymkatalysierte Herstellung von Cytidinmonophosphat N-Acetylneuraminsäure mit einer Synthetase aus *Escherichia coli* (K 235/CS1), PhD Thesis, University of Bonn (1994).

19. *Kittelman, M., Klein, T., Kragl, U., Wandrey, C., Ghisalba, O.*, CMP-N-acetyl neuraminic-acid synthetase from *Escherichia coli*: Fermentative production and application for the preparative synthesis of CMP-neuraminic acid, Appl. Microb. Biotechnol., **44** (1995) 59–67.

20. *Kragl, U., Klein, T., Vasic-Racki, D., Kittelmann, M., Ghisalba, O., Wandrey, C.*, Reaction engineering Aspects of activated sugar production, Ann. N.Y. Acad. Sci., **799** (1996) 577–583.

21. *Bongs, J.*, Reaktionstechnische Optimierung Transketolase – katalysierter Synthesen, PhD Thesis, University of Bonn (1997).

22. *Bongs, J., Hahn, D., Schörken, U., Sprenger, G. A., Kragl,, U., Wandrey, C.*, Continuous production of erythrulose using transketolase in a membrane reactor, Biotechnology Lett., **19** (1997) 213–215.

23. *Vasic-Racki, D., Schörken, U., Sprenger, A., Liese, A.*, Modeling of reaction kinetics for reactor selection in the case of L-erythrulose synthesis, Bioprocess Biosys. Eng. **25** (2003) 285–290.

24. *Roberts, S. M., Turner, N. J.*, Some recent developments in the use of enzyme catalysed reactions in organic synthesis, J. Biotechnol., **22** (1992) 227–244.

25. *Hummel, W., Kula, M-R.*, Dehydrogenases for the synthesis of chiral compounds, Eur. J. Biochem., **184** (1989) 1–13.

26. *Kragl, U., Kruse, W., Hummel, W., Wandrey, C.*, Enzyme engineering aspects of biocatalysis: cofactor regeneration as example, Biotechnol. Bioeng., **52** (1996) 309–319.

27. *Vasic-Racki, D., Kragl, U., Conrad, D., Wandrey, C.*, Modelling of yeast alcohol dehydrogenase catalysed production of chiral alcohols, Chem. Biochem. Eng. Q., **12** (1998) 87–95.

28. *Brinkmann, N., Malissard, M., Ramuz, M., Römer, U., Schumacher, T., Berger, E. G., Elling, L., Wandrey, C., Liese, A.*, Chemo-enzymatic synthesis of the Galili epitope Galα(1→3)Galβ(1→4)GlcNAc on a homogeneously soluble PEG polymer by a multi-enzyme system, Bioorg. Med. Chem. Lett. **11** (2001) 2503–2506.

29. *Brinkmann, N.*, Polymerunterstützte Synthese von Oligosacchariden in homogener flüssiger Phase, PhD Thesis, University of Bonn (2002).