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MINI-REVIEW

Modelling as a tool of enzyme reaction engineering for enzyme reactor development

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Abstract Strategy of the development of model for enzyme reactor at laboratory scale with respect to the modelling of kinetics is presented. The recent literature on the mathematic modelling on enzyme reaction rate is emphasized.

Keywords Modelling of enzyme kinetics · Enzyme kinetics · Development of biocatalytic reaction · Enzyme reactor development

Introduction

Although industrial biotechnology, which is based on biocatalysis, is one of the oldest technologies (Vasić-Rački 2006), recently a special interest in biocatalytic processes is driven by the sustainable use of limited resources (Wohlgemuth 2009; Ghisalba et al. 2010). Biocatalysis can be broadly defined as the use of enzymes or whole cells as biocatalysts for industrial synthetic chemistry. In comparison to the whole cells, isolated enzymes offer several advantages, including simpler devices, higher process productivity due to higher concentration of catalyst and easier purification of products (Johannes et al. 2006). Thanks to genetic engineering techniques introduced in 1973, enzymes from all kinds of biological sources can now be re-cloned and over-expressed in easily cultivatable micro-organism or cell cultures. With this DNA technology, even enzymes that are rare to this day can be produced in large quantities and at affordable costs (Ghisalba et al. 2010).

Biocatalytic processes are similar to conventional chemical processes in many ways. The most significant difference is that the biocatalytic processes must take into account the enzymatic kinetics and operational stability of enzymes (Johannes et al. 2006). It follows that the biocatalytic processes are increasingly penetrating in the chemical and more particularly the pharmaceutical industry (Bommarius and Riebel 2004). More than 100 industrial biocatalytic processes were analysed until now (Liese et al. 2006). The results of these analyses show that the most used enzymes in industry were hydrolases (44%) and then oxido-reductases (30%). An average of 78% yield, final product concentration of 108 g/L and volume productivity of 372 gL^{-1} day^{-1} were achieved in the production of specialty chemicals. Product concentrations comparable to chemical processes are at least 50–100 g/L (Pollard and Woodley 2007). Because of its enantio-selectivity, biocatalysts are widely used in the pharmaceutical industry. Pharmaceutical companies should develop processes quickly to compensate for high costs of investment in pharmaceutical development and clinical trials and reduce the pressure to develop environmentally friendly processes (Woodley 2008). A prerequisite for effective scale-up of pharmaceutical process is the speed of development, which can be achieved by the use of miniaturized experimentation or mathematical modelling (Pollard and Woodley 2007). Several years ago, modelling was not viewed as so important for current industrial practice. It was popularly imagined to be what academics do. But now, with demand for fast development of biocatalytic processes, the situation is changing rapidly (Brass et al. 1997; Vasić-Rački et al. 2003a). For example, Lonza, a Swiss company, one of the largest fine chemical manufacturers in the world, routinely applied modelling to avoid the formation of by-products,

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which act as inhibitors in biocatalytic process, as well as tools for industrial bioprocess integration. They showed (Brass et al. 1997) that modelling has helped to find the most economical way to L-carnitine production, development and application of appropriate process control system for the 6-OH of nicotinic acid and methyl-pyrazine carboxylic acid production. Also, the simulation process has helped to find the most economical solution for biotransformation design in the production of nicotinamide. In the end, they concluded that the modelling methods are appropriate tools to reduce costs.

Modelling as a principle of reaction engineering certainly has a role in the development of biocatalytic processes and is expected to grow with the use of biocatalysis in industry (Vasić-Rački et al. 2003a; Pollard and Woodley 2007; Gernaey et al. 2010) although modelling techniques are not widely used for biocatalytic processes (Pollard and Woodley 2007).

Sin, Woodley and Gernaey have classified mathematical models in the field of biocatalysis in the following four areas (Sin et al. 2009):

- Biocatalyst: models describing catalysis at a molecular level (Braiuca et al. 2006; Dalby 2007; Lee et al. 2010).
- Reaction: kinetics models describing mechanism and reaction rate.
- Reactor: models describing observed reaction kinetics incorporating mass balances as well as hydrodynamic conditions in reactor.
- Process: models including the interaction between different unit operations involved in a process flow-sheet.

The aim of this paper is to present the strategy of mathematical modelling of the enzyme reactor with the reaction catalysed by isolated soluble enzymes. The usefulness of the mathematical modelling will be illustrated with several examples. Particular, recent literature on the enzyme kinetic modelling will be highlighted.

Modelling of enzyme kinetics

There are lots of papers in the literature, which describe the modelling of enzyme kinetics based on the simplest enzyme reaction mechanism consisting of a binding and a catalytic step. The proposed models are Michaelis–Menten kinetic models for one or two substrates. The purpose of such research, especially when the enzyme was only recently discovered, is to acquire knowledge of the enzyme reaction mechanism and reaction rate and to estimate the kinetic parameters (Bas et al. 2007). Particularly, for kinetics with two substrates, these are very complex mechanistic models as for example ordered Bi–Bi, ping-pong, etc., based on reaction mechanism relating to initial reaction rate. This means that the actual form of equations of the intrinsic initial reaction rate depends on the mechanism of enzymatic transformations, which can be very complex (Vasić-Rački et al. 2003a). These kinetic models contain a significant number of parameters and large experimental and computational efforts are required for a proper prediction of the reaction rate (Straathof 2001).

However, within the enzyme reaction engineering for biocatalytic process development, enzyme reaction rate must be found under the conditions that apply to a potential large-scale process in question (Wandrey 1993; Vasić-Rački et al. 2003a; Bommarius and Riebel 2004; Schmidt et al. 2010). Effect of all compounds in the reaction medium on the so-called formal reaction rate should be taken into account, where high concentrations of substrate and product at high conversions may be particularly strong influence on that rate. Biocatalyst lifetime under operating conditions also plays an important role in determining the formal reaction rate. The kinetic models including the formal reaction rate are data driven and empirical. In these models, a simplified kinetic approach is used where the maxim (Levenspiel 1999) "… 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…" could be applied. Also, simplified kinetic approach is the necessity of complex multi-enzyme cascade reaction system (Findrik and Vasić-Rački 2007; Zimmermann et al. 2007) or complex enzyme reaction system (Hildebrand et al. 2007). However, the literature review will demonstrate that the development of biocatalytic process is using complex mechanistic models as well as simplified data-driven and empirical models so that the mechanistic and empirical models complement each other.

Data-driven model was used for the synthesis of *N*-acetylneuraminic acid through epimerization and the aldol condensation step using epimerase and aldolase (Zimmermann et al. 2007). The authors have developed model describing the complete enzyme-catalysed synthesis of *N*-acetylneuraminic acid from *N*-acetyl-D-mannoseamine. The model was a tool for the prediction of reaction rates at different initial concentrations of substrates, and thus calculation of the reaction time and yield. They showed that the model building is useful for reactor design and optimization.

Sener et al. (2006) investigated the effect of ultrasonication as an alternative method on the kinetics of milk lactose hydrolysis process catalysed by β-galactosidase. The authors have proposed mathematical models to predict the residual lactose concentration and the residual enzyme activity at various process conditions.

Peri et al. (2007) have modelled intrinsic kinetics of enzymatic cellulose hydrolysis. Their investigation coupling experimental methods with mathematical modelling and simulation analysis highlights the changing reaction dynamics of batch cellulose hydrolysis, which is influenced by substrate binding of enzyme and non-competitive product inhibition.

Some authors have developed their kinetic model based on reaction networks and mass action differential equations (Chuang et al. 2004; Leksawasdi et al. 2005; Carvalho et al. 2006).

Chuang et al. (2004) have determined the capacity of computational multiple steady states in a family of enzymatically catalysed oxidation of monophenols by tyrosinase in an isothermal continuous-flow stirred tank reactor (CSTR) by using the deficiency one algorithm and the subnetwork analysis. Their model consists of ten coupled non-linear equations.

Leksawasdi et al. (2005) proposed reaction mechanism of enzymatic conversion of benzaldehyde and pyruvate to (R)-phenylacetylcarbinol, which consists of 20 composite reactions. Based on the reaction mechanism and using the method of King and Altman for deriving the rate equations, they developed mathematical model for the kinetics of their complex enzyme-catalysed reaction.

Kokova et al. (2009) have determined the kinetic parameters and microscopic rate constants using a mechanistic kinetic model, thus identifying the rate limiting steps of the benzoin formation catalysed by benzaldehyde lyase and benzoylformate decarboxylase.

Kurniawati and Nicell (2005) developed a kinetic model of laccase-catalysed reactions on the assumption that the rates of reactions are proportional to the product of the concentrations of reactants raised to the power of the number of molecules reacting. Husson et al. (2010), after two attempts to construct kinetic model for lipase-catalysed acylation based on the mechanisms mentioned in the literature, used data-driven model based on modified sequential ping-pong Bi–Bi mechanism with integrated enzyme activity loss. Other authors of the lipase-catalysed ethanolysis (Torres et al. 2008), the lipase-catalysed esterification of oleic acid (Kraai et al. 2008), the lipase-catalysed resolution remote citalopram intermediates (Wang et al. 2009), the lipase-catalysed synthesis of geranyl butyrate (Varma and Madras 2010), have developed a kinetic models based on the ping-pong Bi–Bi mechanism. It is obviously a popular mechanism for the two substrate reaction, which was also used as a base for kinetic model development of aminotriol catalysed synthesis with integrated use of transketolase and ω-aminotransferase (Chen et al. 2006), transketolase-mediated synthesis of L-erythrulose (Sayar et al. 2009b; Chen et al. 2009) and synthesis of fructosyl-stevioside using β-fructofuranosidase (Suzuki et al. 2002). Sayar et al. (2009a) have extended their kinetic model of transketolase catalysed carbon–carbon bond formation reaction condensing β-hydroxypyruvate and glycolaldehyde to synthesise L-erythrulose to describe various modes of operation as an alternative to a batch process. The alternative continuous and fed-batch operations have been analysed.

Using experiments that were carried out in batch stirred reactor, de Barros et al. 2010 have made a kinetic study of the enzymatic esterification of caproic acid with ethyl alcohol catalysed by *Fusarium solani pisi* cutinase. Different acid and alcohol concentrations (whilst also varying the acid/alcohol molar ratio) were tested, and the results were used to identify the best reaction scheme to describe the results obtained over an extended range of conditions. An ordered ping-pong Bi–Bi mechanism with different modifications is used to ensure that the kinetic model is applicable to a range of experimental conditions that were covered.

Berendsen et al. (2006) developed the detailed model, which extends existing reversible ping-pong Bi–Bi models by taking into account both reversibility and competitive inhibition by both enantiomers as well as substrate inhibition by an acyl donor and an acyl acceptor in reversible transesterification of (R/S)-1-methoxy-2-propanol with ethylacetate catalysed by *Candida antartica* lipase B. For deriving kinetic model they have used King–Altman method.

Other mechanisms for the two-substrate reactions are also used, so for fomate oxidation catalysed by formate dehydrogenase, an enzyme, which needs coenzyme NAD as a second substrate, mechanistic model based on the ordered Bi–Bi mechanism has been developed (Schmidt et al. 2010). The ordered Bi–Bi mechanism was used to analyse the first step in the oxidation of acetyl-CoA in the citric acid cycle catalysed by citrate synthase (Beard et al. 2008).

Strategy of development of model for enzyme reactors

The model development of the enzyme reactor with the reaction catalysed by isolated soluble enzymes consists of:

- Enzyme kinetic modelling
- Reactor modelling, which connects enzyme kinetics with the mass balance for enzyme reactors (batch, CSTR, CSTR cascades, cross-flow, plug flow, etc.), to predict the performance of the reactor and makes it easier to identify the most efficient reactor mode.

Chen at al. (2009) described an efficient approach to development of bioconversion kinetic model that is very similar to our strategy, which is shown in Fig. 1.

First step: characterization of biocatalysts in dependence on pH, T and unnatural substrate

The first step in the strategy of development of model for enzyme reactors is the characterization of biocatalysts in dependence on pH (Bonowski et al. 2010), temperature (Carvalho et al. 2006; Gogou et al. 2010; Grubecki 2010) and unnatural substrate at the concentrations appropriate for scale-up of process (Bommarius and Riebel 2008). Especially when more enzyme systems will be used, it is necessary to find a compromise pH and temperature for more biocatalysts

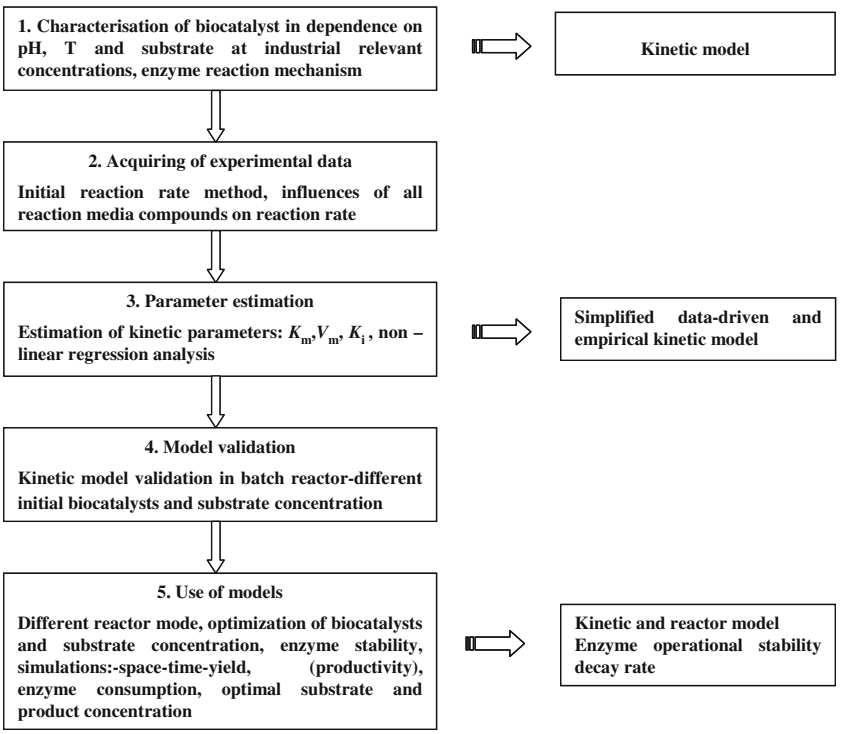

Fig. 1 Strategy of development of model for enzyme reactors

(Findrik and Vasić-Rački 2007). Although the optimum pH was not a problem in enzyme cascade system in which the L-enantiomer of amino acids is produced from the D-enantiomer using four enzymes, it can be a big obstacle in the multi-enzyme reactions in general. Compromise pH value chosen for the four-enzyme systems may be far from optimal and physiological value, and can significantly lower enzyme activity and stability.

Second step: acquiring of experimental data

To obtain useful kinetic model for the development of enzyme reactor, it is necessary to estimate all kinetic parameters from the experimental data. In the second step of the strategy (Fig. 1.), experimental data should be obtained using the initial reaction rate (Jurado et al. 2008) or the integral (progress curve) method (Straathof 2001; Yeow et al. 2004;

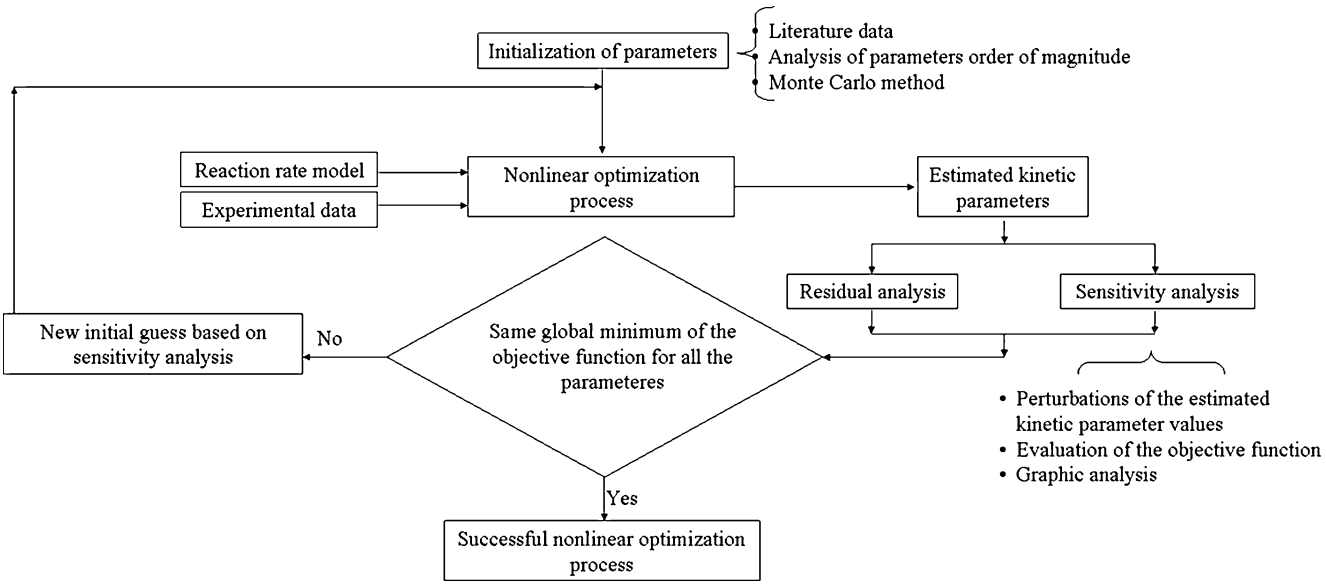

Fig. 2 Methodology for parameter estimation (Alcazar and Ancheyta 2007)

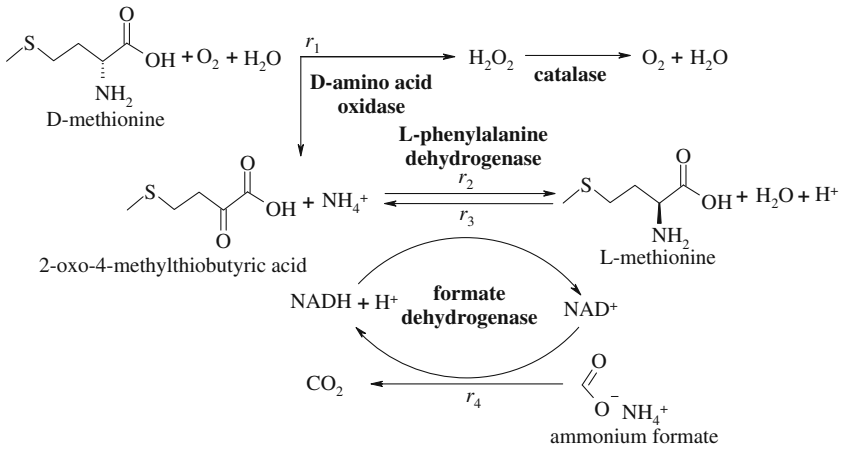

Fig. 3 Reaction scheme for the conversion of D-methionine to L-methionine

Liao et al. 2005; Zavrel et al. 2010a). In particular, for very complex enzymatic reaction systems such as the regeneration of coenzyme, the effect of all compounds in the reaction medium (reagents, products, co-substrates and co-products), on the reaction rates must be examined (Leksawasdi et al. 2005; Findrik et al. 2005a, b; Berendsen et al. 2006; Findrik et al. 2008; Wang et al. 2009).

Third step: parameter estimation

Parameter estimation is carried out in the third step (Fig. 1.). It is a step-by-step procedure (Alcazar and Ancheyta 2007) shown in detail in Fig. 2 and is generally formulated as an optimization problem that minimizes an objective function which represents the fitness of the model with respect to a set of experimental data. The estimation of kinetic parameters in the kinetic models with high parameter accuracy is essential for successful model validation. The model parameters can be estimated by non-linear regression analysis and optimized using an optimization algorithm such as Nelder–Mead algorithm (Nelder and Mead 1965), Brent's algorithm, Levenberger–Marquardt algorithm or others (Yildirim et al. 2003). The numerical values of the parameters are evaluated by fitting the model to the experimental data. The model equations are solved numerically by the fourth-order Runge–Kutta algorithm or similar. The calculated data are compared with the experimental data, recalculated in the optimization routine and fed again to the integration step until minimal errors between experimental and integrated values is achieved. The residual sum of squares is defined as the sum of the squares of the differences between experimental and calculated data.

There are some other approaches for optimal estimation of parameters such as the experimental design (Lindner and Hitzmann 2006; Zavrel et al. 2010b), statistical learning and linear regression model (Borger et al. 2006) or combination of spline theory with linear and non-linear programming (Zhan and Yeung 2011).

To place error bounds on the parameter estimations and to determine which parameters exert the most influence on model results, it is good to perform sensitivity analysis (Hamby 1994; Alcazar and Ancheyta 2007). Woodley and co-workers (Sayar et al. 2009b) have demonstrated the sensitivity analysis procedure on transketolase catalysed reaction between β-hydroxypyruvate and glycolaldehyde to synthesize L-erythrulose. The sensitivities of six-model

Table 1 Mathematical model for the consecutive conversion of D-methionine in L-methionine (Findrik and Vasić-Rački 2007)

Kinetic equations

$$r_1 = \frac{V_{m1}\cdot c_{\mathrm{D-met}}\cdot\gamma_{\mathrm{DAAO}}}{\left(K_m^{\mathrm{D-met}}\cdot\left(1+\frac{c_{2-\mathrm{oxo}}}{K_i^{2-\mathrm{oxo}}}+\frac{c_{\mathrm{NADH}}}{K_i^{\mathrm{NADH}}}\right)+c_{\mathrm{D-met}}\right)}$$

$$r_2 = \frac{V_{m2}\cdot c_{2-\mathrm{oxo}}\cdot c_{\mathrm{NADH}}\cdot c_{\mathrm{NH_4^+}}\cdot\gamma_{\mathrm{L-PheDH}}}{\left(K_m^{2-\mathrm{oxo}}+c_{2-\mathrm{oxo}}\right)\cdot\left(K_m^{\mathrm{NADH}}+c_{\mathrm{NADH}}\right)\cdot\left(K_m^{\mathrm{NH_4^+}}+c_{\mathrm{NH_4^+}}\right)}$$

$$r_3 = \frac{V_{m3}\cdot c_{\mathrm{L-met}}\cdot c_{\mathrm{NAD^+}}\cdot\gamma_{\mathrm{L-PheDH}}}{\left(K_m^{\mathrm{NAD^+}}\cdot\left(1+\frac{c_{\mathrm{NADH}}}{K_{i2}^{\mathrm{NADH}}}\right)+c_{\mathrm{NAD^+}}\right)\cdot\left(K_m^{\mathrm{L-met}}+c_{\mathrm{L-met}}\right)}$$

$$r_4 = \frac{V_{m4}\cdot c_{\mathrm{F}}\cdot c_{\mathrm{NAD^+}}\cdot\gamma_{\mathrm{FDH}}}{\left(K_{m2}^{\mathrm{NAD^+}}\cdot\left(1+\frac{c_{\mathrm{NADH}}}{K_{i3}^{\mathrm{NADH}}}\right)+c_{\mathrm{NAD^+}}\right)\cdot\left(K_m^{\mathrm{F}}+c_{\mathrm{F}}\right)}$$

Mass balance equations in the batch reactor

$$\frac{\mathrm{d}c_{\mathrm{L-met}}}{\mathrm{d}t} = r_2 - r_3 \qquad \frac{dc_{2-\mathrm{oxo}}}{dt} = r_1 - r_2 + r_3 \qquad \frac{\mathrm{d}c_{\mathrm{NAD^+}}}{\mathrm{d}t} = r_2 - r_3 - r_4$$

$$\frac{\mathrm{d}c_{\mathrm{NADH}}}{\mathrm{d}t} = -r_2 + r_3 + r_4 \qquad \frac{dc_{\mathrm{F}}}{dt} = -r_4 \qquad \frac{dc_{\mathrm{NH_4^+}}}{dt} = r_1 - r_2 + r_3$$

D-AAO D-amino acid oxidase, *D-met* D-methionine, *FDH* formate dehydrogenase, *F* formate, *L-PheDH* L-phenylalanine dehydrogenase, *2-oxo* 2-oxo-4-methylthiobutyric acid, γ mass concentration, r reaction rate, t reaction time

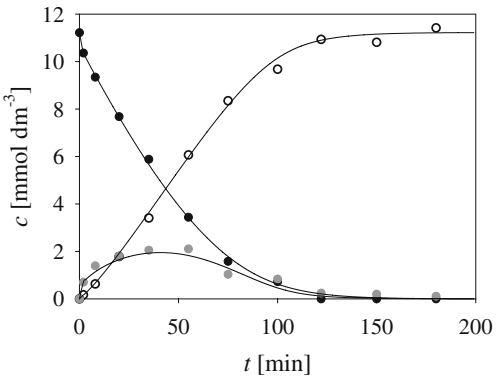

Fig. 4 Conversion of D-methionine to L-methionine in coupled system (30°C, 0.2 mol dm^{-3} phosphate buffer, pH 8.0, γ_{DAAO}=0.0057 mg cm^{-3}, $\gamma_{L\text{-}PheDH}$=0.0038 mg cm^{-3}, γ_{FDH}=1.30 mg cm^{-3}, $\gamma_{catalase}$=0.2 mg cm^{-3}, $c_{D\text{-}methionine}$=11.22 mmol dm^{-3}, c_{NAD^+} = 0.362 mmol dm^{-3}, c_{NH_4F} = 1,000 mmol dm^{-3}). D-methionine (*black circles*), 2-oxo-4-methylthiobutyric acid (*grey circles*), L-methionine (*empty circles*), mathematical model (*black line*)) (Findrik and Vasić-Rački 2007)

parameters were examined and their influence on productivity was shown. It was shown that three parameters of the model could be omitted from further modeling due to their insignificant effect on the productivity. The remaining three parameters had a positive effect on the performance of the reaction and were crucial for further model development.

Peri et al. (2007) used the sensitivity analysis to determine the controlling factors on the enzymatic hydrolysis of cellulose. By knowing the controlling factor, the authors could conclude if some enzyme modifications are required and can be made. They found that the reaction yield could be increased if surfactants are added to the medium which would make the enyzme more inaccessible for glucose. In this case, sensitivity analysis could bring great insight and help in the problem of glucose desorption on the β-glucosidase.

Horenko et al. (2005) have analysed the problem of non-linear sensitivity analysis for models of reaction kinetics. They analysed the effect of non-linearity on the overall dynamics by comparing the fully non-linear system to the linearized system.

Zavrel et al. studied the coupling of 3,5-dimetoxy-benzaldehyde molecules catalysed by benzaldehyde lyase. The mechanistic kinetic model was tested and the kinetic parameters were estimated. Since standard deviation of some kinetic parameters was very high, they used a dynamic sensitivity analysis and correlated their results with the uncertainty level of the estimated kinetic parameters (Zavrel et al. 2008). The sensitivity analysis was followed by model revision. The number of model parameters was lowered and their standard deviation was greatly improved.

Srividhya et al. (2010) investigated the sensitivity of the Michaelis–Menten reaction for different initial substrate-enzyme concentration ratios, number of time courses and number of data point. They demonstrate the importance of experimental design for time course assays and the possibilities of sensitivity analysis in that manner.

Fourth step: model validation

The set of estimated parameters with high parameter accuracy is used for model validation performing in the fourth step of strategy shown in Fig. 1. The developed kinetic model is associated with the mass balance equations for the batch reactor. This extended model must be experimentally confirmed in a batch reactor at different substrate, co-substrate and/or enzyme concentration. The validation of the model is carried out by comparing experimental data with simulations under identical conditions. It is important to validate a model by using data other than those applied to identify parameters. Simulation data obtained from the extended model using computer and experimental data from batch reactor must match each other with an acceptable accuracy (Vrsalović Presečki et al. 2007; de Barros et al. 2010).

Fifth step: use of models

Finally, the last step in the strategy of development of biocatalytic processes with isolated soluble enzymes is the use of models (Zimmermann et al. 2007; Sayar et al. 2009a). Once a meaningful and accurate kinetic-reactor model exists, the computer simulations should enable optimization of the biocatalyst and substrate concentrations at different reactor mode (Kragl et al. 1996; Vasić-Rački et

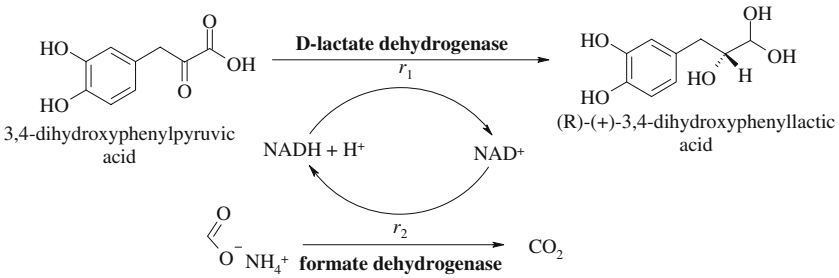

Fig. 5 Reaction scheme for the 3,4-dihydroxyphenylpyruvic acid reduction catalysed by L-lactate dehydrogenase

al. 2003b). Then the performance of biocatalytic processes for example in the so-called repetitive reactor under optimal biocatalyst concentration will contribute to assessing the enzyme operational stability decay rate, which will extend the model (Findrik et al. 2010). These models include the kinetic model, the mass balances equation for reactor and enzyme operational stability decay rate and enable the prediction of the space-time yield, consumption of enzyme, coenzyme consumption expressed as total turn-over number of enzyme reaction with coenzyme regeneration (Wichmann and Vasić-Rački 2005) for batch and/or continuous process.

Examples that demonstrate the usefulness of mathematical modelling in enzyme reaction engineering

The above described strategy of enzyme reactor development will be explained in the following examples.

Particularly interesting is the process of biocatalytic enzyme cascade system in which the L-enantiomer amino acids is produced from the D-enantiomer (Findrik and Vasić-Rački 2007) using four enzymes shown in Fig. 3. Considering that there are four enzymes present in the reaction system, compromise pH should have been chosen, which was suitable for all of the enzymes (D-amino acid oxidase, catalase, L-phenylalanine dehydrogenase and formate dehydrogenase). Although the optimum pH was not a problem in this case, it can be a big obstacle in the multi-enzyme reactions in general. Compromise pH value chosen for the four-enzyme systems may be far from optimal and physiological value, and can be significantly lower enzyme activity and stability. Except pH, there were other problems to be solved. The number of possible interactions between the compound and the enzyme is multiplied if there are a lot of enzymes and compounds present in the reactor (Kragl et al. 1993). These interactions affect the reaction rate, which can hardly be described with the known mechanistic kinetic model. It is, therefore, appropriate to use data-driven models. They have less kinetic constants, which can be evaluated independently and have a defined physical meaning. Such a mathematical model is presented for the biotransformation of D-methionine into L-

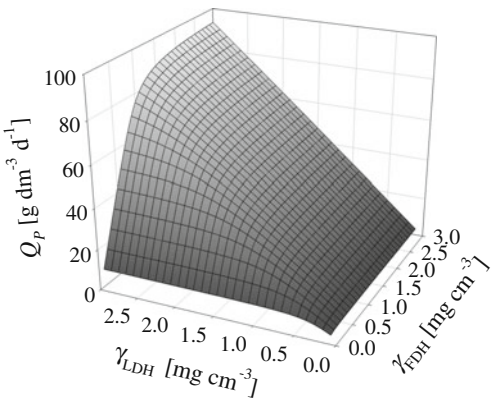

Fig. 6 Dependence of volumetric productivity on the concentration of enzymes lactate dehydrogenase (*LDH*) and formate dehydrogenase (*FDH*; $c_{\text{dihydroxyphenylpyruvate}}$=4.69 mmol dm^{-3}, c_F=36.85 mmol dm^{-3}, c_{NAD^+}=4.95 mmol dm^{-3}, t=8.5 min) (Findrik et al. 2005a)

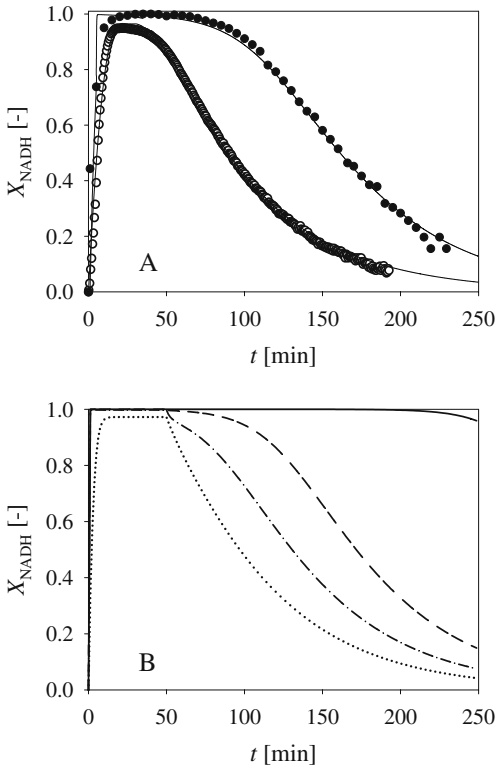

Fig. 7 NADH oxidation catalysed by NADH oxidase in the enzyme membrane reactor in two different buffers (pH 9) at 30°C. **a** τ=60 min (experimental results (*circles*): Tris-HCl buffer (*black*), glycine-sodium pyrophosphate buffer (*white*), model simulations (*line*)). **b** Model simulations at different enzyme concentration (c_{NADH0}=0.2 mmol dm^{-3}, $t_{\text{deactivation start}}$=50 min, τ=60 min, k_d=0.056 min^{-1} and kinetic parameters from Tris-HCl buffer). *Dotted line*, $\gamma_{\text{NADH oxidase}}$=0.045 mg cm^{-3}; *dash-dotted line*, $\gamma_{\text{NADH oxidase}}$=0.5 mg cm^{-3}; *long dashed line*, $\gamma_{\text{NADH oxidase}}$=5.0 mg cm^{-3}; *black line*, $\gamma_{\text{NADH oxidase}}$=30.0 mg cm^{-3}) (Findrik et al. 2007)

Table 2 Mathematical model of NADH oxidation catalysed by NADH oxidase in the continuously operated enzyme membrane reactor (Findrik et al. 2007)

Kinetic equations	Enzyme deactivation
$r = \frac{V_{m3} \cdot c_{NADH} \cdot \gamma_{NOX}}{K_{m2}^{NADH} + c_{NADH} \cdot \left(1 + \frac{c_{NAD^+}}{K_{i2}^{NAD^+}}\right)}$	$V_m = V_{m0} \cdot e^{-k_d \cdot t}$
Mass balance equations in the continuously operated enzyme membrane reactor	
$\frac{dc_{NAD^+}}{dt} = \frac{c_{NAD^+0} - c_{NAD^+}}{\tau} + r$	$\frac{dc_{NADH}}{dt} = \frac{c_{NADH0} - c_{NADH}}{\tau} - r$

k_d operational stability decay rate constant, τ residence time, *NOX* NADH oxidase

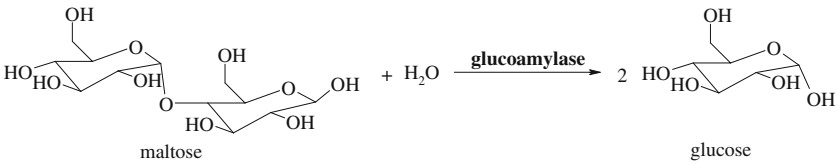

Fig. 8 Reaction scheme for the hydrolysis of maltose catalysed by dextrozyme

methionine in Table 1. It consists of kinetic equations (r_1-r_4) and the mass balances for the batch reactor. The impact of each component of reaction mixture on the initial reaction rate was examined for each enzyme in system in particular. The reverse reaction and the potential inhibition by products and substrates were investigated as well. Kinetic constants were estimated from experimental data using non-linear regression. Process model was confirmed by experiments in a batch reactor and was used to find the optimal initial conditions of reaction. It was possible to predict the concentrations (Fig. 4) of all compounds in the investigated reaction system, and calculate and/or optimize the enzyme concentration to achieve the desired substrate conversion in the shortest possible time. In cascade enzyme systems it is very important to choose the right concentration of substrates and enzymes required to start the reaction(s), which was done using the developed model.

Kinetic model, incorporated into the model of the process, may be useful for process optimization. Such optimization was done for the α-keto acid reduction to α-hydroxy acid catalysed by L-lactate dehydrogenase (Findrik et al. 2005a, b). The reaction scheme was shown in Fig. 5. Coenzyme regeneration was carried out using formate dehydrogenase. Influence of catalytic and regenerating enzyme on process productivity is investigated using the process model. The results shown in Fig. 6 show that the concentration of LDH and FDH affect the volumetric productivity. Volumetric productivity in this process is more influenced by the concentration of catalytic enzyme that is far more expensive. The concentration of formate dehydrogenase is important up to 1.5 mg cm^{-3}, and at concentrations higher than this has no effect.

It is well known that the enzyme activity and operational stability are key factors in industrial bioprocess. As the enzyme activity inevitably lowers during the process, it is very important to predict the outcome of the process taking into account this decay. Special form of concentration vs. time curve, which was obtained experimentally, and also as computer simulations using the model of the process, shows decay of the operational stability of enzymes in a continuous process. (Vasić-Rački et al. 2003b; Findrik et al. 2005a, b; Findrik et al. 2007). By using the process model with the first-order kinetic model for enzyme stability decay, product concentration can be predicted and enzyme can be added to prevent the effect of the activity drop on the productivity. Figure 7a presents the data and simulation of NADH concentration during the experiment carried out with NADH oxidase in the continuously operated enzyme membrane reactor, which clearly indicates to enzyme operational stability decay. Process model shown in Table 2 was not able to simulate the experimental results, but the model that includes the operational stability decay, which is described by first-order kinetics, describes the data correctly, as shown in Fig. 7a. Also, by increasing the enzyme concentration in the reactor enzyme operation stability decay becomes less visible, high product concentration can be achieved and steady state in reactor can be maintained (Fig. 7b). In the case of NADH oxidase, the concentration required to achieve a stable steady state in a continuous reactor was too high (up to 30 gcm^{-3}) (Findrik et al. 2007).

A good example of interaction between process modeling and operational stability of enzymes has been researching operational stability of a commercial enzyme named dextrozyme, which catalyses hydrolysis of maltose (Findrik et al. 2010) presented in Fig. 8. Experiments were performed in different types of reactors, where the decline in enzyme activity was observed during the reaction. This was particularly evident in the continuously operated enzyme membrane reactor, because it was not possible to achieve a stable steady state. Conversion-time curve had a shape similar to that shown in Fig. 7. The first-order constant of enzyme operational stability decay rate was determined from experiment in the batch reactor, where the same amount of enzyme was used in three sequential trials (Fig. 9). The developed process model that integrates kinetic model, the

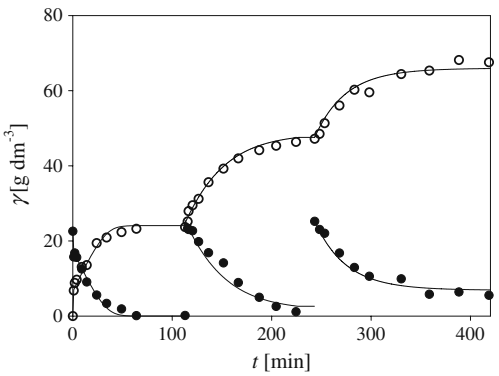

Fig. 9 Maltose hydrolysis in the repetitive batch reactor (0.1 mol dm^{-3} phosphate buffer, pH 5.5, $V_{reactor}$=0.025 dm^3). $c_{maltose,0}$=22.57 gdm^{-3}, $c_{maltose,1}$=23.33 gdm^{-3}, $c_{maltose,2}$=25.21 gdm^{-3}, $\varphi_{enzyme}=5.09\cdot10^{-5}$, 65°C (maltose (*black circles*), glucose (*white circles*), mathematical model (*black line*)) (Findrik et al. 2010)

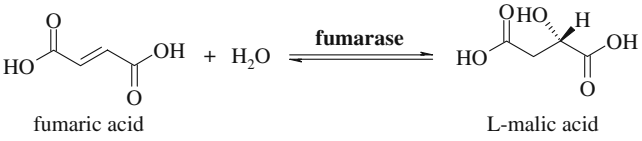

Fig. 10 Reaction scheme for the dehydration of fumaric acid L-malic acid catalysed by fumarase

rate of enzyme operational stability decay and the mass balance equation of the reactor, described sequential experiments in a batch reactor reasonably well. The model was used to optimize a continuous process.

In most practical cases the rate of decay of the enzyme operational stability can be described by first-order kinetics (Bailey and Ollis 1986). There are several mechanisms of the enzyme operational stability decay, which are discussed in the literature, but they are very difficult to prove. Kinetic models that come out of such mechanisms are more complex (Vrabel et al. 1997; Gibbs et al. 2005; Polakovic and Bryjak 2002) and have more than one kinetic constants. Different data-driven kinetic model for the decay of the operational stability of enzyme is proposed for the synthesis of erytrulose catalysed by transketolase (Vasić-Rački et al. 2003b; Sayar et al. 2009b). It was found experimentally that one of the substrates significantly affects the operational stability of the enzyme.

Generally, to determine the operational stability of enzymes, enzyme activity can be monitored during the experiment using independent test of enzyme activity (if any), or using a series of independent experiments in the batch reactor.

The following example shows the use of process model to calculate the amount of biocatalyst for the process in question. To calculate the required concentration of yeast, which could give the same result of conversion and

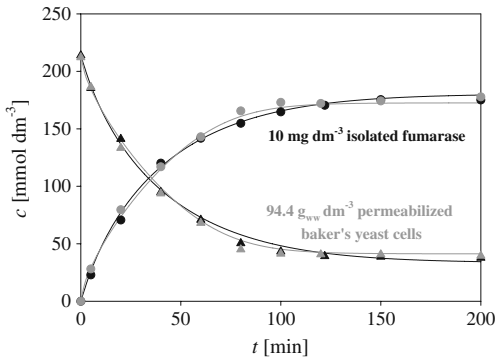

Fig. 11 Comparison of L-malic acid production in the batch model (0.1 mmol dm^{-3} phosphate buffer, 30°C, $V_{reactor}$=100 cm^3) by isolated fumarase ($\gamma_{fumarase}$=10 mg dm^{-3}) and permeabilized yeast cells (γ_{yeast}=94.4 $g_{ww}dm^{-3}$), L-malic acid (*circles*), fumaric acid (*triangles*), isolated fumarase (*black symbols*) and permeabilized yeast cells (*grey symbols*) (Vrsalović Presečki et al. 2007)

Table 3 Biocatalyst concentration, biocatalyst productivity (Q_{BP} product produced daily per amount of enzyme used) and biocatalyst consumption (BC) in the continuous production of L-malic acid at τ=900 min and $c_{0,fumaric\ acid}$=250 mM and $X_{fumaric\ acid}$=0.7 (Vrsalović Presečki et al. 2007)

Parameters	Isolated fumarase	*Saccharomyces bayanus*	Baker's yeast
γ			
mg dm^{-3}	2.63		
$g_{ww}dm^{-3}$		180	315
Q_{BP}			
mmol day^{-1} mg^{-1}	106.49		
mmol $day^{-1}g_{ww}^{-1}$		1.56	0.89
BC			
mg $mmol^{-1}$ day^{-1}	0.0094		
g_{ww} $mmol^{-1}$ day^{-1}		0.64	1.12

productivity at the same time as 10 mg dm^{-3} isolated fumarase, a model process for the dehydration of fumaric acid, which is shown in Fig. 10, was used (Fig. 11) (Vrsalović Presečki et al. 2007). It was found that with 94.4 g of baker's yeast, it is possible to get the same productivity as well as with 10 mg isolated fumarase. Since the natural microbial enzyme concentration is relatively low, the concentration of yeast necessary to duplicate the results of the experiment was so high.

Using the same process model, the volumetric productivity of process (Q_P; daily produced product concentration) and

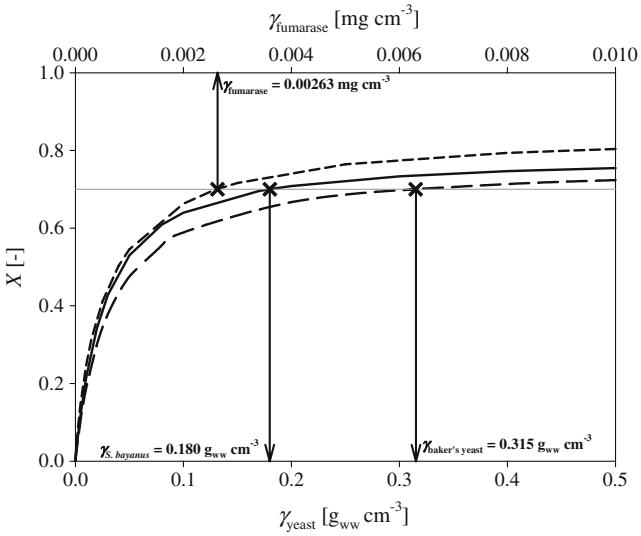

Fig. 12 The dependence of steady-state conversion on initial biocatalyst concentration in the L-malic continuous production at τ=900 min and $c_{0,fumaric\ acid}$=250 mmol dm^{-3}—simulated by model (isolated fumarase (*short dashed line*), *Saccharomyces bayanus* cells (*solid line*) and baker's yeast cells (*long dashed line*)—*Saccharomyces* sp.) (Vrsalović Presečki et al. 2009)

biocatalyst consumption (BC; the amount of enzyme used for daily production of 1 mmol of L-malic acid) was calculated with the estimated amount of biocatalysts. The data are presented in Table 3. This model was also used to optimize the continuous process. Figure 12 shows the dependence of the steady-state conversion on biocatalyst concentrations. It was found that 1 mg of purified fumarase in continuous production of L-malic acid corresponds to the use of enzyme in 68 g (wet weight) cells of *Saccharomyces bayanus* or in 120 g (wet weight) cells of baker's yeast.

Conclusions

Mathematical modeling is very useful in the development of biocatalytic reaction and reactor to develop a process model that enables prediction of operational stability of enzymes. Using the developed model, process optimization containing the conversion of the substrate, the yield of product, productivity of process and biocatalyst consumption is possible from the experiment in the laboratory scale. Although the biocatalytic process modeling is not used very often, the literature shows that there is more and more interest in this topic.

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