

## THE KINETIC MODELS OF THE BIOPROCESS WITH FREE AND IMMOBILIZED CELLS

*The kinetic models are fundamental knowledge of biochemical and microbial processes. The aim of the work is the kinetic model research based on biochemical and microbial mechanisms of the cell growth, the substrate consumption and the product formation. The engineering goal is to obtain ethanol as a fuel from renewable resources by a biosynthesis process. The engineering goal requires the inclusion of the inhibitory effect of a high substrate and ethanol concentration in the kinetic models. The interactions of the ethanol synthesis with the growth biomass mechanism were researched. The models of the ethanol forming mechanisms were incorporated in the complex structure of the kinetic models. The researches of the kinetic models were realized by computer simulation and experimental verification at the ethanol biosynthesis process from glucose with free and immobilized cells *Saccharomyces cerevisiae*. The model parameters and the limits were estimated by experimental validation. The base of the verified deterministic and semi-empiric kinetic models was created. The model base may be onward used for the process simulation models generating.*

*Key words: Ethanol biosynthesis, free and immobilized cells, kinetic model structured and validation.*

Mathematical models based on physical and chemical laws (e.g., mass and energy balances, thermodynamics and chemical reaction kinetics) are frequently employed in the process description. These models are conceptually attractive because a general model for any system size can be developed, even before the system is constructed. On the other hand, an empirical model can be devised, which simply correlates input–output data without any physicochemical analysis of the process.

A very important kinetic equation according to Monod, gives an expression for the growth rate of an organism when the growth is limited by one substrate. The Monod equation is analogous to the Briggs–Haldane solution of the Michaelis–Menten model for the kinetics of a single enzyme. If the growth is considered to be the result of a sequence of enzymatic reactions, in which one reaction is much slower than all others, the Michaelis–Menten equation can be a good fit, which the Monod equation often gives in growth modeling. This reasoning is, however, by no means unique. An equation of the form of the basic Monod equation is obtained starting from other various modeling assumptions [1].

Although the growth of a microorganism is an unusually complex phenomenon, it is often possible to represent this growth by relatively simple laws. J. Von Liebig's pioneering studies are basic for the idea of the substrate–limiting cell growth. He formulated the Law of the Minimum, which described the effect of essential nutrients on the cell growth. It states that the growth is

controlled not by the total of the resources available, but by the scarcest resource. This concept was originally applied to plants. The growth of an organism may be dependent on a number of different factors. The availability of these may vary so that at any given time one is more limiting than the others. Liebig's Law states that growth only occurs at the rate permitted by the most limiting. Liebig's Law has been extended to the description of the Michaelis–Menten's rates of enzymatic reactions and Monod's growth biological populations.

Some authors [2] discarded the conclusion that Monod proposed the equation on a theoretical base, indicating that the major difference between the Monod equation for the microbial growth and the Michaelis–Menten equation for the enzymatic reaction lies on the process state, i.e. equilibrium for the enzymatic reaction and non–equilibrium for a microbial growth process. A number of authors have pointed out that microorganisms, from thermodynamics point of view are to be considered as open systems, which is not in the state of thermodynamic equilibrium [3], but in the dynamic equilibrium. The dynamic equilibrium is a steady state in which there are a continuous reactant input and transformations of the reaction product.

The production of fuel ethanol from renewable resources has initiated the research of the kinetic model based on the biochemical and microbial mechanisms of the cell growth, the substrate consumption and the product formation. Most studies seek to improve the efficiency by increasing the rate of the ethanol production. Allain's generated model of a system has suggested that a cell–free process should be capable of producing ethanol much more efficiently than the microbial based process [4]. Najafpur et al. developed a model, which can be used for designing a large scale immobilized cell reactor column for a continuous production of high ethanol concentration [5]. The model

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generated by using the experimental design method, confirms that zinc, sodium and potassium ions have significant main effects on the ethanol production by *S. cerevisiae* but no interactive effects on the ions [6]. The global perspectives for the development of ethanol obtained from cellulose, as the most widespread renewable resource, are analyzed in the papers [7].

In this paper, the live growing cells were an open system, which is characterized by the dynamic equilibrium. The steady state concentrations in the dynamic equilibrium are different from the thermodynamic steady state concentrations. Therefore, the reaction progress is permanent according to equilibrium. A microorganism for the maintenance and growth consumes the energy, which is simultaneously generated.

## MATERIALS AND METHODS

The anaerobic ethanol fermentation process with free suspended and immobilized *S. cerevisiae* cells was researched. The nutritive medium content, (g/l): extract yeast 10.0, NaCl 1.0, CaCl<sub>2</sub>·2H<sub>2</sub>O 0.2, KH<sub>2</sub>PO<sub>4</sub> 2.0, FeCl<sub>3</sub>·6H<sub>2</sub>O 0.01, MgSO<sub>4</sub>·7H<sub>2</sub>O 1.7, NH<sub>4</sub>Cl 2.0, C<sub>6</sub>H<sub>12</sub>O<sub>6</sub> 20.0. The nutrient medium was prepared and sterilized in a citrate buffer solution in pH 5. The nutritive medium content was optimized in an object function of the maximum rate of cells growth. The kinetic measurements were realized at the temperature of 32°C.

For free suspended cells, the kinetic experimental data are gained in the batch bioprocess with the initial substrate concentration  $c_{S0} = 100\text{--}250 \text{ g/dm}^3$  and the inoculum  $c_{X0} = 0.75 \text{ g/dm}^3$ , in a well-mixed process vessel by the initial rate method.

For immobilized cells in the Ca-alginate gel bead, the experimental data are gained in the batch process with a good mixing at the initial substrate concentration  $c_{S0} = 100\text{--}250 \text{ g/dm}^3$  and a very low immobilized cells concentration in the free volume of the process vessel  $c_{X0} = 3.50 \text{ g/dm}^3$ . In order to eliminate the restricting effect on the bioprocess kinetics, the phenomena of the convection, inner and interphase transfer of the glucose substrate mass and the ethanol and CO<sub>2</sub> production are researched experimentally by increasing the recirculation-mixing rate. The bioprocess effectiveness was determined by the independent kinetic and diffusion experiment. The effectiveness of the immobilized biocatalyst is determined by the effective diffusion coefficient, which is modeled and confirmed on the experimental data [8].

The process model is verified on the experimental data obtained on the flow chemostat and the column with the bed immobilized biocatalyst. The column parameters are porosity  $\varepsilon = 0.51$ , immobilized cells concentration in void volume of the column  $c_X = 163.33 \text{ g/dm}^3$  and the recirculation flow that enables a well-mixed system.

*Immobilization procedure* – For immobilized preparations the sodium alginate type Texamid-578

Henkel (Germany) was used. The immobilization was performed in a two-needle system shown elsewhere [9]. The experiments described in this paper were performed with alginate beads obtained from 2% alginate solution entrapping 20% yeast cells, hardened in 0.1 mol/L CaCl<sub>2</sub> bath for 2 h. The fermentation was carried out with standard beads 3 mm in diameter without the protective layer, and two-layer beads 3 mm in diameter and 0.2 mm thin external cell-free alginate layer.

*Analysis* – The concentration of entrapped cells was determined by dissolving a given mass of gel (3 beads) in 5 ml of 0.28 mol/L K<sub>2</sub>HPO<sub>4</sub>, for 15 min. Following a suitable dilution, the samples were spectrophotometrically assayed at 540 nm [10]. The residual glucose in the medium was estimated using the 3,5-dinitrosalicylic acid method [11]. Ethanol was assayed by alcohol dehydrogenase [12].

## MODELS DEVELOPEMENT

The mathematical expressions describing the rates of the biomass and the product formation and the rates of the substrate utilization are based on Monod's model and its derivated forms. The aim is to describe both free and immobilized cells ethanol fermentation with a simple model. The Monod's model has been modified to include terms representing the maintenance coefficient, as well as the effects of the product and the substrate inhibition.

The maintenance processes need to provide the energy that is to maintain the system, even if the growth is absent. The maintenance requirement is assumed to be a constant independent of the growth rate of the cells.

The kinetics of the microbial populations can be configured so as to include the inhibition phenomena, the growth associated and the non-growth associated patterns of the product formation. The system can be structured to contain either one or two different microbial populations or the diauxic utilization of multiple substrates as different carbon-energy sources. The two populations can interact through competition, commensalisms, cross-inhibition or predation or through the combination of these relationships.

### Free suspended and immobilized cells

Microbiological transformation processes are the natural state of live and growing free-suspended cells. At batch bioprocesses, they are limited by a low cell concentration and a short life half time. The multiple uses required the additional separation subsystem. Continual systems are limited by cell washing from the reactors at the rate higher than a specific growth rate. A fed batch technique partially solves the problem of the low cell concentration. The processes with recirculation do that as well. In all bioprocesses with free cells, a volumetric productivity is relatively small because of the

inhibitory effect of the high concentration of both substrate and the product to the cell growth.

Immobilized systems provide a high cell concentration in the process, high productivity, a better control and incomparably higher operational stability. There are two approaches in the development of the immobilized cells.

The first one is the immobilized biocatalyst design without or with minimum cell washing. The goal is the high starting cell concentration which is maintained constant throughout the process by the effect of the matrix, two-layer and lowering nutrient concentration which is limiting for the growth, and is not a substrate e.g. nitrogen. A very high operational stability is achieved that way. The technique of immobilization in two-layer particles additionally decreases cell washing and increases the operational biosystem stability.

The immobilized biocatalyst designed with cell washing, allows a limited growth of the cells that are washed from the matrix. This technique is used at the production of the starter cultures. While, at the same time, the immobilized and washed cells, which function as free, increase the total effect on the productivity. However, washed cells and non-controlled growth in the matrix lead to matrix destruction with the exploitation time, thus decreasing the operational biosystem stability.

#### The structure of the kinetic model for bioethanol synthesis

The structure of the kinetic model of the isotherm anaerobic bioprocess consists of the model of biomass growth kinetics and of the models of the substrate consumption and the product forming kinetics. The models of the biomass growth generated on the basis of the mechanism and stoichiometrics are analyzed. At the same time, the models on the basis of the substrate consumption and the product forming pattern are analyzed. For each generated model of the biomass growth kinetics, the determined number of structures of bioprocess kinetic models is formed including models of different mechanisms of the patterns of the substrate-to-product conversion.

#### The models of biomass growth kinetics

In this paper eight kinetic models of the biomass growth were generated. The four models represent deterministic models generated from mechanisms and stoichiometric. These models include different inhibitory effects of a high substrate and the product concentration and effects of the maintenance to the cell growth (MODEL 1–4).

Mixed models (MODEL 5–8) represent semi-empirical models. A part of these models is deterministic, multiplied by empirical members for the growth inhibition by a high substrate and product concentration. This approach allows mixed semi-empirical models generation and they include

boundary values of the substrate, the product or metabolite concentration that stop the cell growth or biosynthesis. It is also possible to describe a biological switching, like a pattern changing at the second product forming, diauxic process or processes with two different microbial population.

MODEL 1. Monod's growth biomass model for a single-substrate limitation,

$$r_X = \frac{dc_X}{dt} = c_X \cdot \frac{\mu_m \cdot c_S}{K_M + c_S} \quad (1)$$

MODEL 2. Monod's biomass growth model, which includes the maintenance process,

$$r_X = \frac{dc_X}{dt} = c_X \cdot \frac{\mu_m \cdot c_S}{K_M + c_S} - m \cdot c_X \quad (2)$$

MODEL 3. The biomass growth model, which includes a non-competitive substrate and a product inhibition,

$$r_X = \frac{dc_X}{dt} = c_X \cdot \frac{\mu_m \cdot c_S}{K_M + c_S + \frac{c_S^2}{K_{IS}} + \frac{c_S \cdot c_P}{K_{IP}}} - m \cdot c_X \quad (3)$$

MODEL 4. The biomass growth model, which includes a non-competitive substrate and a competitive product inhibition,

$$r_X = \frac{dc_X}{dt} = c_X \cdot \frac{\mu_m \cdot c_S}{K_M + c_S + K_M \frac{c_P}{K_{IP}} + \frac{c_S^2}{K_{IS}}} - m \cdot c_X \quad (4)$$

MODEL 5. Mixed semi-empirical biomass growth model, which includes a modified non-competitive substrate inhibition and a linear product inhibition,

$$r_X = \frac{dc_X}{dt} = c_X \cdot \frac{\mu_m \cdot c_S}{K_M + c_S + \frac{c_S^2}{K_{IS}}} \cdot \left(1 - \frac{c_P}{P_m}\right) - m \cdot c_X \quad (5)$$

MODEL 6. Mixed semi-empirical biomass growth model, which includes a modified non-competitive substrate and a product inhibition and a linear product inhibition,

$$r_X = \frac{dc_X}{dt} = c_X \cdot \frac{\mu_m \cdot c_S}{K_M + c_S + \frac{c_S^2}{K_{IS}}} \cdot \frac{c_P}{K_P + c_S + \frac{c_P^2}{K_{IP}}} \cdot \left(1 - \frac{c_P}{P_m}\right) - m \cdot c_X \quad (6)$$

MODEL 7. Mixed semi-empirical biomass growth model, which includes a multiplied of modified non-competitive substrate and a product inhibition,

$$r_X = \frac{dc_X}{dt} = c_X \cdot \frac{\mu_m \cdot c_S}{K_M + c_S + \frac{c_S^2}{K_{IS}}} \cdot \frac{c_P}{K_P + c_S + \frac{c_P^2}{K_{IP}}} - m \cdot c_X \quad (7)$$

MODEL 8. Modification of the Levenspiel's semi-empirical model which includes basic Monod's model extended by the substrate and the product inhibition,

$$r_X = \frac{dc_X}{dt} = c_X \cdot \frac{\mu_m \cdot c_S}{K_M + c_S} \cdot \left(1 - \frac{c_P}{P_m}\right)^{n_P} \cdot \left(1 - \frac{c_S}{S_m}\right)^{n_S} - m \cdot c_X \quad (8)$$

In the bioprocess with alive and growing cells, the molecular mass of a cell may not exactly be defined and determined. The partial knowledge of the biomass average composition may not link with the effective molar concentration. Also, due to the cell growth and death with time, the biomass concentration changes.

By using the analogy with an enzymatic catalysis theory that is based in a dynamic balance, the microbial process with the live and growing cell may be described by a modified Monod's growth model, by transforming Eq. (1),

$$\mu = \frac{r_X}{c_X} = \frac{1}{c_X} \frac{dc_X}{dt} = \frac{\mu_m \cdot c_S}{K_M + c_S + \frac{c_S^2}{K_{IS}} + \frac{c_S \cdot c_P}{K_{IP}}} - m \quad (9)$$

If molar concentration  $c_S$  and  $c_P$ , Michaelis' constant  $K_M$  and inhibitory constants and are multiplied with the adequate molar mass of the substrate  $M_S$ , the product  $M_P$ , and the average molar mass of the cells  $M_X$ , the dimensional correct Monod's equation with the specific growth rate  $\mu$  in units of the specific mass rate (gx/gxh) is obtained.

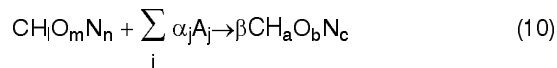
Regardless the complexity of a biological mechanism in the growing cell, the last instance is the transformation processes of the substrate into the product which are taking place on the enzyme. Assuming that the part of the enzyme in growing cells was constant, then the concentration of the cell biomass in the bioprocess would follow the appropriate change of the enzyme concentration in the mol and mass units. This approach is necessary, considering the fact that the experimental data are gained for the cell biomass in the units of the mass concentration during the microbe process model validation.

The non-structured model, that does not take the inner microorganism structure, is used at the bioprocess system modeling. The partial structuring is related to the enzyme in the cell biomass content. The model is also distributed because the condition of the culture is characterized by the total biomass concentration  $c_X$  ( $g_{SM}/dm^3$ ). If the structure of the cell biomass is supposed to be constant on average and independent from the cell age, in that case the Monod's non-structured and distributed model may be considered theoretical.

### Kinetic models of the substrate consumption

The substrate requirements for the increase in biomass can be considered to be composed by two contributions: the substrate requirements for the synthesis of the precursors for biomass, which can be calculated from the atomic conservation principle, and the substrate requirements for the biomass synthesis from precursors.

The organisms take up substrates, such as a carbohydrate, oxygen, nitrogen source etc., and convert them to cellular mass, products (for example ethanol) and metabolites (water, carbon dioxide etc.). From the principle of conservation of atomic species a generalized stoichiometric equation can be derived, which could take the form:



where  $CH_1O_mN_n$  is the composition formula of the main energy supplying substrate,  $A_j$  are other substrate metabolite and products ( $O_2$ ,  $CO_2$ , nitrogen source),  $\alpha_j$ ,  $\beta$  are stoichiometric coefficients, and  $CH_aO_bN_c$  is the composite formula of the micro-organisms.

On average, 8% of the biomass is composed of the elements other than C, H, O and N, so that, in order to calculate the actual requirements, 8% has to be deducted from the values calculated here. In anaerobic condition, the oxygen source is a glucose substrate. This decreases the biomass yield and increases the ethanol yield. If the substrate is glucose, which is nitrogen non-content, then it is necessary to add the nitrogen source as another substrate (term  $A_j$  in Eq.10).

Many authors researched the initial nitrogen concentration effect on a maximal specific growth rate and a maximal production rate of ethanol [13, 14]. These researches confirmed the basic Monod's model of the substrate-nitrate limiting cell growth.

$$r_X = \mu \cdot c_X \quad (11)$$

where

$$r_X = r_N \cdot Y_{X/N} \quad \text{and} \quad \mu = \frac{\mu_m c_N}{K_N + c_N} \quad (12)$$

and parameters values:  $\mu_m=0.294$ ,  $K_N=0.1$  and  $Y_{X/N}=6.84$ .

The initial nitrogen concentration influence was investigated. The model validity is confirmed and the maximal specific rate was gained at  $0.2 \text{ g}_N/dm^3$ . Their conclusion is in good agreement with that of Saita and Slaughter [15], who found that nitrogen has a stimulating effect on the fermentation rate through its function as a substrate for protein synthesis, and not by an activation of glycolytic enzymes by ammonium ions.

The nitrogen itself influences the cell growth, especially for the synthesis of the precursor for the biomass, and it may be used for the control of the undesirable biomass growth at immobilized cells.

If the substrate is a simultaneous carbon source for the cell growth, the reactant which is converted to ethanol, the energy source for a biotransformation and the source for the maintenance energy, then a balance of the substrate consumption is as following,

$$\begin{aligned} \left(\frac{dc_S}{dt}\right) &= \left(\frac{dc_S}{dt}\right)_{\text{biomass}} + \left(\frac{dc_S}{dt}\right)_{\text{product}} + \\ &+ \left(\frac{dc_S}{dt}\right)_{\text{growth energy}} + \left(\frac{dc_S}{dt}\right)_{\text{maintenance energy}} \end{aligned} \quad (13)$$

In the bioprocess synthesis with immobilized cells, the balance (Eq. 13) was reduced,

$$\left(\frac{dc_S}{dt}\right) = \left(\frac{dc_S}{dt}\right)_{\text{product}} + \left(\frac{dc_S}{dt}\right)_{\text{maintenance energy}} \quad (14)$$

Under the restrictive condition, e.g. a drastically growth limitation with glucose or nitrogen, the minimum energy produced is consumed primarily as the maintenance energy and then as the cell growth energy.

### Kinetic models of the product synthesis

Bioprocesses are, in general, an integrated biochemical, physiological and physicochemical component. The simultaneous solving of the biomass growth model, with the models of the substrate consumption and the product forming, enables a valid simulation of the real bioprocess dynamics.

The additional analyses are directed to the interaction of a biochemical component with a biological one. The enzymatic bioprocesses are different than the bioprocesses in growing cells in the dynamics of the enzymatic complex quantity increase at growing cells. This is why the additional phenomenon, which should be analyzed and modeled, is considered to be the phenomenon of the biotransformation interaction with the cell growth. The three basic mechanisms of the product forming are chosen for the analysis and modeling i.e. the growth-associated, non-growth-associated or metabolically non-coupling product forming and the non-growth associated forming of the secondary metabolite.

The modeling of these mechanisms leads to the transformation in the kinetic models of the product forming and the substrate consumption. For this reason the kinetic model of the biomass growth stays unchanged.

Biochemical kinetic models are developed and they include a modified Monod's biomass growth model for the processes inhibited by the high substrate and product concentration, in mechanistic and empiric terms. The product forming and substrate consumption models are generated by the three elected and supposed product-forming mechanisms.

For the growth associated product formation (Eqs. 15–17), the rates of the product formation and the rate of the substrate utilization are specified as stoichiometrically proportional to (i.e. associated with) the biomass growth rate.

Non-growth associated product formation, as a result of metabolic uncoupling, is simulated by specifying that only the biomass growth rate is the subject to substrate and product inhibition (Eq. 22). The product formation is linked to the substrate utilization and ceases when the substrate runs out. The product formation is thus not directly associated with the biomass growth and the substrate utilization is not coupled with the growth (Eqs. 23–24). In the absence of inhibition, the product formation pattern is growth associated. The model is thus consistent with experimental observations of metabolic uncoupling.

When non-growth associated product formation is modelled as a phenomenon associated with the secondary metabolite formation, the rate of the product formation is linked to the endogenous rate of the cellular degradation (i.e. endogenous metabolism). The product formation is thus a process that is secondary to the biomass growth. In addition, the product formation but not growth is inhibited by the concentration of the substrate. This inhibition pattern simulates that observed with the production of a wide variety of secondary metabolites. This approach is in many ways superior to the mathematical model, which has most often been used to describe the growth and non-growth associated product formation by the "mixed-growth-associated" model of Leudeking and Piret [16].

Unlike Leudeking and Piret's model, the models generated by this approach recognize the fact that there are many reasons behind the non-growth associated patterns of the product formation; e.g. metabolic uncoupling and secondary metabolite formation. In contrast, Leudeking and Piret's model is a curve fitting relationship which does not differentiate between the different mechanisms behind the non-growth associated product formation. In addition, in these models, the product and biomass formation are stoichiometrically balanced with the substrate utilization. This is not the case with Leudeking and Piret's model which is unable to predict a cessation of the product formation.

The modeling methodology of microbial kinetics is represented in detail on the models structured according to the biomass growth model with the non-competitive substrate and the product inhibition (MODEL 3), coupled with the models of the substrate consumption and models of growth and non-growth associated product formation.

### Growth associated model product formation

In the growth-associated model product forming (MODEL 3.1), which includes the growth biomass model with a non-competitive substrate and the product inhibition Eqs. (15–17), the technique formed of the kinetic model structure for the bioprocess with free cells (MODEL 3.1.1) and the bioprocess with immobilized cells (MODEL 3.1.2) is presented.

$$r_X = \frac{dc_X}{dt} = c_X \cdot \frac{\mu_m \cdot c_S}{K_M + c_S + \frac{c_S^2}{K_{IS}} + \frac{c_S \cdot c_P}{K_{IP}}} - m \cdot c_X \quad (15)$$

$$-r_S = -\frac{dc_S}{dt} = c_X \cdot \frac{v_{ms} \cdot c_S}{K_M + c_S + \frac{c_S^2}{K_{IS}} + \frac{c_S \cdot c_P}{K_{IP}}} \quad (16)$$

$$r_P = \frac{dc_P}{dt} = c_X \cdot \frac{v_{mp} \cdot c_S}{K_M + c_S + \frac{c_S^2}{K_{IS}} + \frac{c_S \cdot c_P}{K_{IP}}} \quad (17)$$

For the bioprocess kinetic model (MODEL 3.1.2) with the immobilized cell Eqs. (18–20), which includes the growth-associated model product forming, the

model Eqs. (15–17) was accomplished on the presumption that cells concentration is constant,

$$r_X = \frac{dc_X}{dt} = 0 \quad (18)$$

$$-r_S = -\frac{dc_S}{dt} = c_X \cdot \frac{v_{ms} \cdot c_S}{K_M + c_S + \frac{c_S^2}{K_{IS}} + \frac{c_S \cdot c_P}{K_{IP}}} \quad (19)$$

$$r_P = \frac{dc_P}{dt} = c_X \cdot \frac{v_{mp} \cdot c_S}{K_M + c_S + \frac{c_S^2}{K_{IS}} + \frac{c_S \cdot c_P}{K_{IP}}} \quad (20)$$

The basic postulate of the immobilized system Eq. (21), as a bioprocess system in which the specific biomass growth rate  $\mu$  is equal to the maintenance coefficient  $m$ , follows from the condition Eq. (18),

$$c_X \cdot \frac{\mu_m \cdot c_S}{K_M + c_S + \frac{c_S^2}{K_{IS}} + \frac{c_S \cdot c_P}{K_{IP}}} = m \cdot c_X \quad (21)$$

The model of a live non-growth cell may approximate this system ( $\mu = m$ ). In Eq. (21) the maintenance coefficient  $m$  represents the energy demands just to maintain the activity in the biomass. This condition may be got by the control of the nitrogen concentration in nutrient medium, following Eq. (12). In other approach the specific growth rate may be expressed as a function of two limiting nutrients, the main substrate glucose and the growth limiting nutrient nitrogen.

### Non-Growth associated model product formation

The non-growth-associated model product forming (MODEL 3.2), which is metabolic uncoupling and the including model biomass growth with the non-competitive substrate and the product inhibition, is generated in the form of Eqs. (22–24) for free suspended cells (MODEL 3.2.1), by analogy with the model Eqs. (15–17)

$$r_X = \frac{dc_X}{dt} = c_X \cdot \frac{\mu_m \cdot c_S}{K_M + c_S + \frac{c_S^2}{K_{IS}} + \frac{c_S \cdot c_P}{K_{IP}}} - m \cdot c_X \quad (22)$$

$$-r_S = -\frac{dc_S}{dt} = c_X \cdot \frac{v_{ms} \cdot c_S}{K_M + c_S} \quad (23)$$

$$r_P = \frac{dc_P}{dt} = c_X \cdot \frac{v_{mp} \cdot c_S}{K_M + c_S} \quad (24)$$

The kinetic model (MODEL 3.2.2) of the bioprocess with the immobilized cell for a non-growth-associated mechanism product forming Eqs. (25–27) from model Eqs. (22–24) and on the presumption that cells concentration is constant, was accomplished.

$$r_X = \frac{dc_X}{dt} = 0 \quad (25)$$

$$-r_S = -\frac{dc_S}{dt} = c_X \cdot \frac{v_{ms} \cdot c_S}{K_M + c_S} \quad (26)$$

$$r_P = \frac{dc_P}{dt} = c_X \cdot \frac{v_{mp} \cdot c_S}{K_M + c_S} \quad (27)$$

### Parameter estimation and model validation

The experimental data from a kinetic experiment with free and immobilized cells were used for the kinetic parameter estimation.

ŽAABODY- = The kinetic model of free cells Eqs. (15–17) including the growth-associated and the model Eqs. (22–24) including the non-growth-associated product forming mechanism are fitted to the experimental data from the bioprocess with free suspended cells.

The kinetic model of immobilized cells Eqs. (18–20) including the growth-associated and the model Eqs. (25–27) including the non-growth-associated product forming mechanism, are fitted to the experimental data from the bioprocess with immobilized cells.

For identification on the model parameters, the average values of different process variables (biomass, glucose and ethanol) for three repeated experiments, at different time points, were taken. The model parameters were calculated based on an ordinary least squared (LS) method and assisted by a computer program, which minimized the deviations between the model prediction and the experimental results.

In this paper the two methods were tested. The first method is based on the LS method, which minimized the deviation between the differential equation form model and the numerical differentiation of the experimental data. The Levenberg-Marquardt iterative fitting method is used for the parameter estimation [17].

In the second method, for calculating the model predictions, the system of differential equations was solved using an integration program based on the Runge-Kutta method of the fourth order. The optimization program for the direct search of the minimum of the multivariable function was based on the method of Rosenbrock [18].

The minimization criteria in parameter optimization computer program were:

$$\text{MINSS} = \sum_i^n \sum_j^k \Delta_{ij}^2 W_j^2 \quad (28)$$

where MINSS represents the sum of the weighed residues square,  $i$  and  $j$  are the number of experimental data points and the number of variables, respectively,  $W_j$  is a statistical weight of each variable ( $W = 1/\sigma$ ), where  $\sigma$  is the variance of repeat experiments, and  $\Delta_{ij}$  is difference between the model and the experimental value.

The model validity is statistically estimated by Fisher's test. Fisher's numerical value  $F_{\text{calc}}$  is a measure of deviation of the simulated data in the model to the experimental data from the process. It is numerically determined as a relation of the variance nonadequacy of the model to the experimental data and the variance of experimental data.

$$F_{\text{calc}} = \frac{\sigma_{\text{ad}}^2}{\sigma^2(Y_{\text{exp}})} = \frac{\sum_i (Y_{\text{mod}_i} - Y_{\text{exp}_i})^2}{\sum_i (Y_{\text{mod}_i} - \bar{Y}_{\text{exp}})^2} \quad (29)$$

where,  $Y_{\text{exp}}$  and  $Y_{\text{mod}}$  are the experimental and simulation data,  $n$  and  $k$  are the number of the experimental data and model parameters, and  $\bar{Y}_{\text{exp}}$  is the mean value of experimental data.

The Fisher's test was used for a comparative analysis of more valid models and the adoption of the adequate.  $F_{\text{calc}}^{\text{tot}}$  is determined for the total model and  $F_{\text{calc}}^{\text{X}}$ ,  $F_{\text{calc}}^{\text{S}}$  and  $F_{\text{calc}}^{\text{P}}$  for all the components partially by using a variance additive principle. For a preliminary comparative analysis and preliminary model validity estimation, the correlation coefficient was used.

## RESULTS AND DISCUSSION

### Growth associated model product formation

The Model 3.1 for free suspended and immobilized cell is fitted to the experimental data. The parameters are estimated for different initial substrate

concentrations. The results of the estimation for free system are presented in Table 1, and for the immobilized system in Table 2.

### Non-Growth associated model product formation

Model 3.2 for free suspended and immobilized cells is fitted to the experimental data. The parameters are estimated for different initial substrate concentrations. The results of the estimation for free system are presented in Table 3, and for the immobilized system in Table 4.

### Comparative analysis of the growth and non-growth associated model product formation

The comparative analysis of free-suspended cell kinetic models with the growth and non-growth associated product forming, show the dominant influence of the inhibition by high glucose and ethanol concentration on the cell growth. This is especially illustrated in Figure 1, comparing Figures a2 and b2 i.e. a3 and b3. The same conclusion is drawn by a comparative analysis of the values of a partial and total Fischer's test in Table 1 for the growth associated model and Table 3 for the non-growth associated model.

Table 1. The parameter estimation of free-system kinetic Model 3.1.1, that includes the growth-associated mechanism of product forming ( $c_{\text{SO}} = 50-250 \text{ g/dm}^3$ ,  $c_{\text{XO}} = 0.75 \text{ g/dm}^3$ ,  $m = 0.02$ )

$c_{\text{SO}}$	$\mu_m$	$v_{\text{ms}}$	$v_{\text{mp}}$	$K_M$	$K_{\text{IS}}$	$K_{\text{IP}}$	$F_{\text{calc}}^{\text{X}}$	$F_{\text{calc}}^{\text{S}}$	$F_{\text{calc}}^{\text{P}}$	$F_{\text{calc}}^{\text{tot}}$	$F_{\text{tab}}$	$r_{\text{cor}}$
50	0.362	3.220	1.514	2.276	140.0	37.55	0.160	0.042	0.024	0.226	2.484	1.000
100	0.266	2.933	1.361	1.551	357.0	33.15	0.167	0.088	0.051	0.306	2.084	0.999
150	0.250	2.826	1.241	9.676	606.1	34.87	0.931	0.671	0.315	1.918	1.841	0.997
200	0.205	2.348	0.993	50.740	944.0	30.67	1.560	0.124	0.360	2.044	1.592	0.999
250	0.280	2.101	0.819	435.40	9977.0	206.10	10.34	4.540	0.761	15.64	1.385	0.999

Table 2. The parameters estimation of the immobilized system kinetic Model 3.1.2, that includes a growth-associated mechanism of product forming ( $c_{\text{XO}} = 3.5 \text{ g/dm}^3$ )

$c_{\text{SO}}$	$v_{\text{ms}}$	$v_{\text{mp}}$	$K_M$	$K_{\text{IS}}$	$K_{\text{IP}}$	$F_{\text{calc}}^{\text{S}}$	$F_{\text{calc}}^{\text{P}}$	$F_{\text{calc}}^{\text{tot}}$	$F_{\text{tab}}$	$r_{\text{cor}}$
50	2.813	1.265	105.9	44.19	5.43	0.017	0.019	0.036	1.955	1.000
100	4.718	2.255	106.0	54.78	6.26	0.181	0.171	0.353	1.757	0.998
150	8.336	3.900	509.3	73.88	11.7	0.123	0.073	0.195	1.624	1.000
200	6.525	2.847	1417.0	4453.00	262.50	0.050	0.028	0.078	1.757	1.000
250	4.422	1.733	1652.0	12710.00	444.00	0.110	0.203	0.313	1.642	0.999

Table 3. The parameter estimation of free-system kinetic Model 3.2.1, that includes the non-growth-associated mechanism of product forming ( $c_{\text{SO}} = 50-250 \text{ g/dm}^3$ ,  $c_{\text{XO}} = 0.75 \text{ g/dm}^3$ ,  $m = 0.02$ )

$c_{\text{SO}}$	$\mu_m$	$v_{\text{ms}}$	$v_{\text{mp}}$	$K_M$	$K_{\text{IS}}$	$K_{\text{IP}}$	$F_{\text{calc}}^{\text{X}}$	$F_{\text{calc}}^{\text{S}}$	$F_{\text{calc}}^{\text{P}}$	$F_{\text{calc}}^{\text{tot}}$	$F_{\text{tab}}$	$r_{\text{cor}}$
50	0.352	2.170	1.020	1.716	112.6	43.85	0.022	0.008	0.015	0.045	2.484	1.000
100	0.231	1.799	0.834	5.382	1269.0	110.50	0.114	0.023	0.053	0.190	2.084	0.999
150	0.225	1.641	0.721	16.270	420.3	65.58	0.030	0.142	0.257	0.429	1.841	0.998
200	0.170	1.010	0.411	40.070	4019.0	37.48	0.570	0.206	0.322	1.097	1.592	0.998
250	0.235	2.101	0.819	435.400	10000.0	206.10	0.567	0.349	0.413	1.330	1.385	0.999

Table 4. The parameter estimation of the immobilized system kinetic Model 3.2.2, that includes the non-growth-associated mechanism of product forming ( $c_{X0}=3.5 \text{ g/dm}^3$ )

CSO	$v_{ms}$	$v_{mp}$	$K_M$	$F_{calc}^S$	$F_{calc}^P$	$F_{calc}^{tot}$	$F_{tab}$	$r_{cor}$
50	1.620	0.727	81.73	0.002	0.001	0.003	1.955	1.000
100	2.769	1.297	155.6	0.001	0.001	0.001	1.757	1.000
150	4.486	2.093	383.2	0.011	0.002	0.014	1.624	1.000
200	6.318	2.754	1384	0.049	0.028	0.077	1.757	1.000
250	4.834	1.892	1815	0.110	0.175	0.285	1.642	0.999

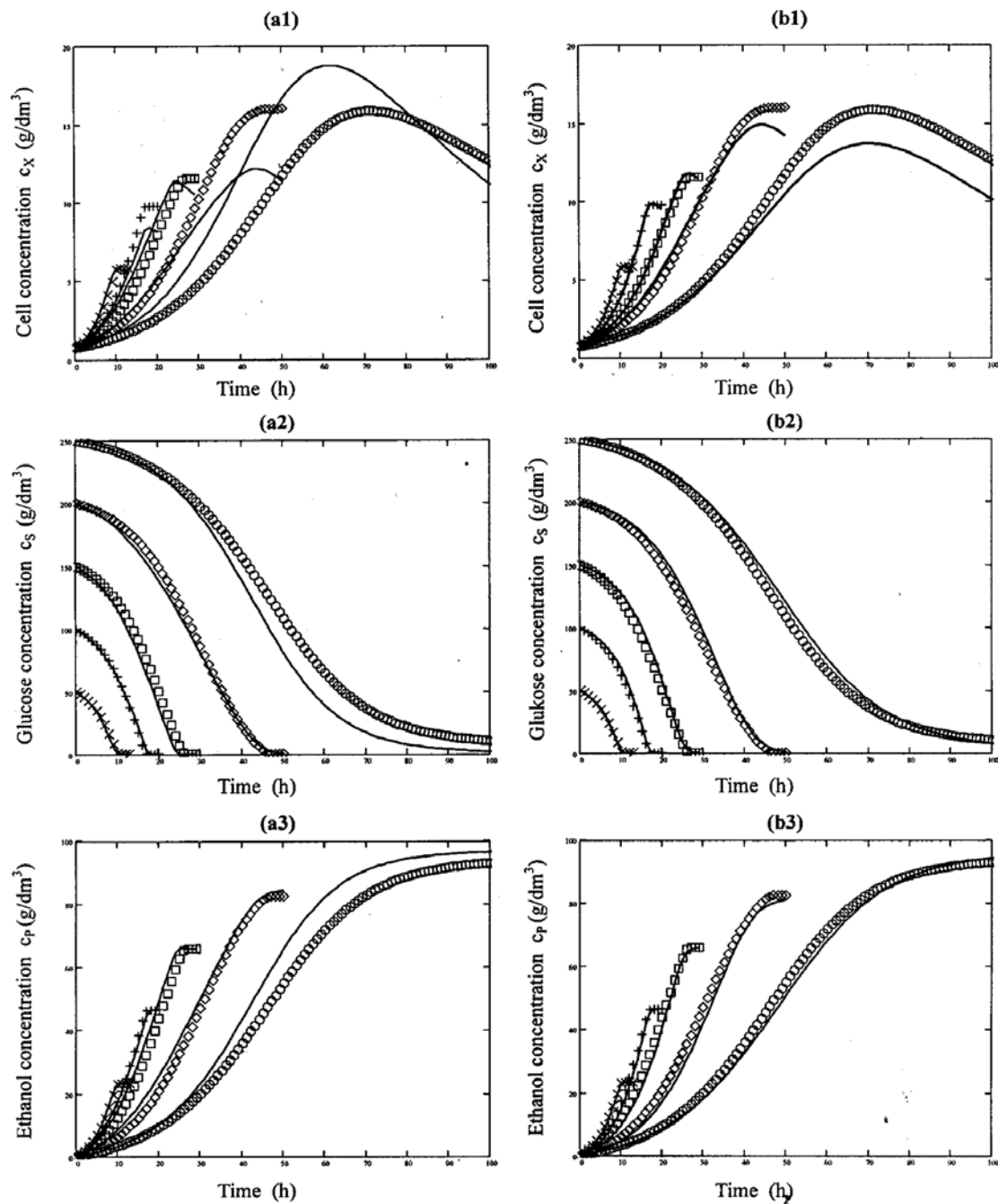


Figure 1. Growth cells, substrate consumption and product formation kinetic curves for free suspended cells, with growth associated (a) and non-growth associated (b) product forming patterns. The experimental data at different initial glucose concentration 50, 100, 150, 200 and  $250 \text{ g/dm}^3$  (x, +, □, ◇, o) and corresponding curves simulation (—) in the MODEL 3.1.1 and MODEL 3.2.1, with parameter values from Table 1 and Table 3.

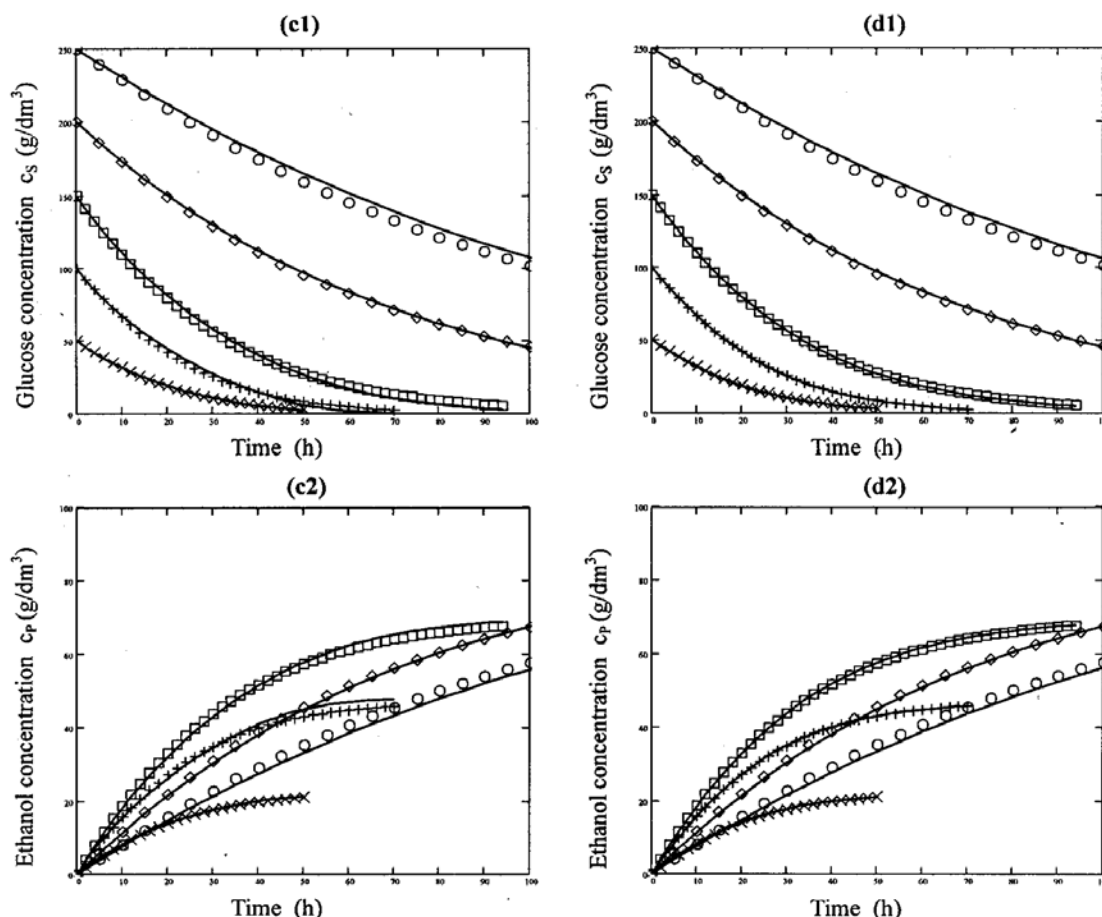


Figure 2. Substrate consumption (c1,d1) and product formation (c2,d2) kinetic curves for immobilized cells, with growth associated (c) and non-growth associated (d) product forming patterns. The experimental data at different initial glucose concentration 50, 100, 150, 200 and 250 g/dm<sup>3</sup> (x, +, □, ◇, ○) and corresponding curves simulation (–) in the MODEL 3.1.2 and MODEL 3.2.2, with parameter values from Table 2 and Table 4.

The Fischer's test (Table 2 and 4) and a corresponding comparative analysis of the progress curves for the substrate consumption and product forming (Figure 2), show better comparison of the experimental and simulated data on MODEL 3.2.2 as well as for the non-growth associated product forming with immobilized cells.

The kinetic models gained from semi-empirical growth model (MODEL 5–8) are also tested for growth associated and non-growth associated pattern product forming. These models show a good comparison with experimental data.

#### Analysis of the biological switchig

The biological switching effect was illustrated by the analysis of the semi-empirical MODEL 5.1 (Eqs. 30–32), which generated from the growth model (Eq. 5).

$$r_X = \frac{dc_X}{dt} = c_X \cdot \frac{\mu_m \cdot c_S}{K_M + c_S + \frac{c_S^2}{K_S}} \cdot \left(1 - \frac{c_P}{P_m}\right) - m \cdot c_X \quad (30)$$

$$-r_S = \frac{dc_S}{dt} = c_X \cdot \frac{v_{ms} \cdot c_S}{K_M + c_S + \frac{c_S^2}{K_S}} \cdot \left(1 - \frac{c_P}{P_{mm}}\right) \quad (31)$$

$$r_P = \frac{dc_P}{dt} = c_X \cdot \frac{v_{mp} \cdot c_S}{K_M + c_S + \frac{c_S^2}{K_S}} \cdot \left(1 - \frac{c_P}{P_{mm}}\right) \quad (32)$$

The model parameters  $P_m$  and  $P_{mm}$  represent the corresponding ethanol concentration which stopped the cell growth and ethanol synthesis respectively. They are determined in the independent experiment.

In Figure 3a, the growth cells kinetic simulated in Model 5.1 when the parameter values are  $P_m = P_{mm} = 84$  g/dm<sup>3</sup> was presented. The process simulation with different parameters values  $P_m = 84$  g/dm<sup>3</sup> and  $P_{mm} = 95$  g/dm<sup>3</sup> was presented in Figure 3b.

Figure 3 and a corresponding Fischer's test show that the models with different parameter model values are more valid, especially in the domain of high ethanol concentration. Therefore, at kinetic simulations by deterministic models, the biologically limiting was included in the mathematical model.

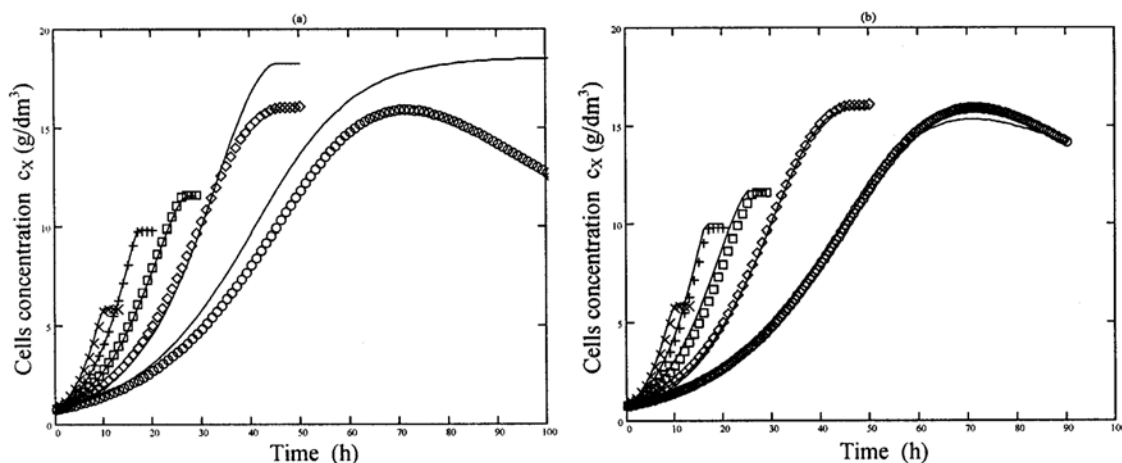


Figure 3. Cell growth kinetic simulated in the MODEL 5.1 with parameter values  $P_m = P_{mm} = 84 \text{ g/dm}^3$  (a) and with different parameter values  $P_m = 84 \text{ g/dm}^3$  and  $P_{mm} = 95 \text{ g/dm}^3$  (b). The experimental data at different initial glucose concentration 50, 100, 150, 200 and 250  $\text{g/dm}^3$  (x, +, □, ◇, ○) and corresponding simulation curves (-).

## CONCLUSION

In this paper, the base of the kinetic model for free and immobilized cells was generated. The structuring of the kinetic model in the form of a differential equation system, which is simultaneously solved at simulation, enables the bioprocess kinetic analysis and modeling including different mechanisms of the product synthesis. The kinetic model structure consists of the model of biomass kinetic growth and kinetic models of the substrate consumption and product synthesis.

Four models are mechanistically based on the mass and energy balances, dynamic equilibrium, a biochemical reaction rate, conservation of atomic species and stoichiometric of the reaction. Other kinetic models are mixed semi-empirical, derived by modification with empirical terms that were determined in independent experiments.

The interaction of biological and biochemical processes is analyzed for growth-associated and non-growth-associated mechanisms of ethanol biosynthesis from glucose. It is confirmed that the method of the model structure modification with the appropriate validation, can be successfully applied for confirmation of the bioprocess mechanism, the choice of the convenient or the elimination of the inadequate.

From generic kinetic models, the biomass growth model, taking into account non-competitive substrate and product inhibition, and non-growth pattern product forming, was confirmed. The validations of the semi-empirical model were confirmed, but many of their terms are not generated based on physical and chemical laws or their biological switch, and are necessary to be researched.

## NOMENCLATURE

$c_x$	$\text{gX dm}^{-3}$	– concentration of the cells (dry weight)
$c_s$	$\text{gs dm}^{-3}$	– concentration of the substrate
$c_p$	$\text{gP dm}^{-3}$	– concentration of the product

$F_{\text{calc}}^{\text{tot}}$	–	– Fisher's distribution for a validation test
$F_{\text{calc}}^X, F_{\text{calc}}^S, F_{\text{calc}}^P$	–	– Fisher's distribution for a partial validation test
$K_M$	$\text{gs dm}^{-3}$	– Monod's constant of cells growth rate
$K_N$	$\text{gs dm}^{-3}$	– Monod's constant of cells growth rate related to nitrogen
$K_{IP}$	$\text{gP dm}^{-3}$	– inhibition constant for a competitive inhibition by product
$K_{IS}$	$\text{gs dm}^{-3}$	– inhibition constant for a noncompetitive inhibition by substrate
$m$	$\text{gx gx}^{-1} \text{h}^{-1}$	– maintenance coefficient for cells growth
$P_m$	$\text{gs dm}^{-3}$	– critical product concentration above reaction cannot proceed
$S_m$	$\text{gs dm}^{-3}$	– critical substrate concentration above reaction cannot proceed
$r_X$	$\text{gx dm}^{-3} \text{h}^{-1}$	– rate of growth (production) of the cells related to glucose
$r_S$	$\text{gs dm}^{-3} \text{h}^{-1}$	– rate of the substrate consumption
$r_P$	$\text{gP dm}^{-3} \text{h}^{-1}$	– rate of the product formation
$r_N$	$\text{gx dm}^{-3} \text{h}^{-1}$	– rate of growth (production) of cells related to nitrogen
$r_{\text{cor}}$	–	– correlation coefficient
$Y_{X/N}$	$\text{gx gn}^{-1}$	– coefficients of the cells yield related to nitrogen

## Greek symbols

$\mu$	$\text{gx gx}^{-1} \text{h}^{-1}$	– specific growth rate
$\mu_m$	$\text{gx gx}^{-1} \text{h}^{-1}$	– maximum specific growth rate
$v_s$	$\text{gs gx}^{-1} \text{h}^{-1}$	– specific rate of the substrate consumption
$v_p$	$\text{gP gx}^{-1} \text{h}^{-1}$	– specific rate of the product formation
$v_{mp}$	$\text{gP gx}^{-1} \text{h}^{-1}$	– maximum specific rate of the product formation
$v_{ms}$	$\text{gs gx}^{-1} \text{h}^{-1}$	– maximum specific rate of the substrate consumption
$\sigma_{\text{ad}}^2$	–	– nonadequacy variance
$\sigma^2(Y_{\text{exp}})$	–	– variance of the experimental data

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