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Review

Review-Development of Models of Ethanol Synthesis and Production by Using Principles of the Theory of System Analysis. Personal Experience.

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Abstract

The objective of this review article was to highlight the authors' more than 30 years' experience in the field of model development procedure of ethanol biosynthesis. During all these years a powerful system analysis theory and its decomposition principles were applied not only for studying and description of microbial kinetics but for the needs of bioprocesses and bioreactors/photobioreactors optimization and scale-up. Special attention was given to the description of phenomena and techniques involved in ethanol synthesis on single and two substrates utilization processes by wild and genetically modified strains. The method of simultaneous saccharification and fermentation of starch to ethanol (SSFSE) by genetically modified microbial *Saccharomyces cerevisiae* YPB-G strain is presented to fully illustrate the strength of the applied theory. Another modern method for extractive fermentation and ethanol production is presented as well. Diauxic growth phenomena in ethanol synthesis is the example showing a modeling approach by connecting knowledge from molecular and population hierarchic levels. This modeling work helped tremendously to find new insights on the control of internal metabolic mechanisms of the cells and how the processes can be guided to an optimal trajectory. A parameter identification procedure was performed by using the latest achievements in global search methods. The applied strategy of model development was extremely successful in obtaining new knowledge about the microbial systems' behavior as well as for fast and robust bioprocesses development. The review article can be very useful for modelers and young scientists working in the fields of biotechnology, bioengineering, metabolic and chemical engineering.
Key words-modeling, system analysis, ethanol, kinetics, parameters identification, genetic algorithm.

Резюме

Целта на тази обзорна статия е да се представи тридесетгодишния научен опит на авторите в областта на моделиране на кинетиката на етанолния биосинтез. През всичките тези години се е прилагала теорията на системния анализ и нейните принципи на декомпозиция като мощен инструмент не само за изучаване и описание на микробната кинетика, но така също и за нуждите на оптимизация и мащабиране на биопроцеси и биореактори/фотобиореактори. Специално внимание бе отделено на описание на механизмите и техниките при синтеза на етанол на базата на усвояване на един или два субстрата от диви и генетично модифицирани щамове. Методът на едновременно озахаряване и ферментация на нишесте до етанол от генетично модифициран микробен шам *Saccharomyces cerevisiae* YPB-G е представен за да илюстрира изцяло стабилността на приложената теория. Друг съвременен метод за производство на етанол като екстракционна ферментация също е представен. Статията за «Diauxic» растеж в синтеза на етанол е пример, показващ моделиране, чрез свързване на знанията от молекулно и популационно йерархични нива. Тази работа по моделиране помогна изключително много, за да се намерят нови идеи за контрол на вътрешните метаболитни механизми на клетките и как тези процеси могат да бъдат оптимално провеждани. Процедурата на параметрична идентификация се извършва чрез използване на най-новите достижения в областта на методите за търсене на глобален екстремум. Приложената стратегия за разработване на кинетични

модели беше изключително успешна за получаване на нови знания за поведението на микробните системи, както и за бързо и стабилно разработване на биопроцесите. Статията може да бъде много полезна за моделатори и млади учени, работещи в областта на биотехнологиите, биоинженерство, метаболитната и инженерната химия.

Introduction

The quantitative description of bioreaction processes must first pass through their modeling and simulation. By applying mass and energy balances, the engineer is able to evaluate almost any bioreactor system. Having the knowledge about microbial kinetics and microbial physiology, the process engineer can build a complex model of the bioprocess and bioreactor system and to solve optimization and scale-up tasks. During the modeling procedure scientists and engineers make an attempt to study and analyze a real, most often complex, situation, where many parallel processes take place in the bioreactor vessels. This usually requires simplification of the situation in an understandable physical, chemical, and biochemical analog. In order to successfully solve the task, the mathematical description must have physical sense, formulated in complex mathematical models. The contribution made to biotechnology by a biochemical engineer's modeling approach is an undeniable fact. However, to build a robust and working model it is not enough to use only basic fundamental principles, such as material balances.

There is more than that. The system analysis theory and its principles provide the strategy for building a working model of any chemical or biological sophisticated system (Kaffarov, 1985; Kafaroff *et al.*, 1979; Kafaroff *et al.*, 1985). The authors have used the knowledge of this powerful tool for more than 30 years in Russia, Bulgaria, Brazil and the USA in order to describe the dynamic behavior of many **biotechnological** (Kroumova and Wagner, 1995; Kroumov *et al.*, 2005c; Wenzel *et al.*, 2006a; Wenzel *et al.*, 2006b], **wastewater treatment processes** (Fiorentin *et al.*, 2015; Borba *et al.*, 2014; Módenes *et al.*, 2015; Kroumov *et al.*, 2005b; Trigueros *et al.*, 2010a; Trigueros *et al.*, 2010b; Trigueros *et al.*, 2007; Fagundes-Klen *et al.*, 2007) **containig heavy metals** (Borba *et al.*, 2008), **bioreactors** (Kroumov *et al.*, 1986; Kroumov *et al.*, 1987; Kroumov *et al.*, 1990; Kroumov *et al.*, 1991; Kroumov and Gimenes, 2002a; Kroumov and Gimenes, 2002b; Kroumov and Gimenes, 2002c), **photobioreactors** (Kroumov, 2013a; Kroumov, 2013b; Kroumov, 2014; Kroumov, 2015; Kroumov *et al.*, 2015a; Kroumov *et al.*, 2015b), and **algae kinetics** (Kroumov *et. al.*, 2013; Kroumov *et. al.*, 2015; Crofcheck *et al.*, 2000a; Crofcheck *et al.*, 2009b; Crofcheck *et al.*, 2010).

The review paper was intended to cast light on ethanol production processes and their mathematical descriptions (Kroumov, 1999; Kroumov, 2002e; Kroumov *et al.*, 2002d; Kroumov *et al.*, 2006; Tait *et al.*, 2006; Tait *et al.*, 2005a; Tait *et al.*, 2005b; Tait *et al.*, Tait *et al.*, 2005c; Tait *et al.*, 2005d; Tait *et al.*, 2004a; Tait *et al.*, 2004b; Kroumov *et al.*, 2005a). The presented examples and all cited works of the kinetic models will convince the reader that the application of the theory of system analysis may save time and money during the overall bioprocess development procedure.

Materials and Methods

Model development procedure based on the system analysis theory

System analysis theory and its principles

The main stages of the system analysis theory (Kaffarov, 1985; Kaffarov *et al.*, 1979; Kaffarov *et al.*, 1985) applied to the development of a bioprocess kinetic model can be presented as follows: (1) Determination of the task of the modeling, choice of the criterion of optimality; (2) Analysis of the bioprocess – qualitative analysis of the structure of the biosystem, decomposition of the biosystem in sub-systems; building of the models of the sub-systems; parametric identification of the models of the elements of the sub-systems; (3) Synthesis of the system – development of the complex models of the sub-systems and their relationships; simplification and combination of all the models into a complex bioprocess kinetic model; search for optimal working conditions of the bioprocess based on the model simulations; experimental verification of the developed simple and complex models; (4) Solving the modeling task – determination of the optimal structure of the kinetic model on the basis of the chosen criterion of effectiveness; solving the bioprocess scale-up problems on the basis of the developed complex kinetic model.

Examples

Simultaneous Saccharification and Fermentation of Starch to Ethanol (SSFSE) - applications of the principles of system analysis theory to SSFSE sys-

tem

A non-structural model describing a SSFSE process by a recombinant strain of *S. cerevisiae* YPB-G (Altintas *et al.*, 2002) was developed, published (Kroumov *et al.*, 2006; Kroumov *et al.*, 2004) and presented elsewhere (Maicon *et al.*, 2005). The scheme of the model development algorithm is shown below. In Figure 1 the mechanisms of the SSFSE processes were formalized in two hierarchic levels of knowledge. The first one considers processes of enzymatic hydrolysis of starch, and the second one combines microbial physiology processes. The dynamics of enzyme excretion and plasmid stability are not taken into consideration.

First hierarchic level

The first hierarchic level (see Table1) considers enzymatic hydrolysis of starch by glucoamylase (this enzyme in our case is secreted by the recombinant strain). A mathematical description of the hydrolysis process of starch as two substrate fractions was developed and published in details by Polakovic & Bryjak (2004). This knowledge was adapted and applied to starch hydrolysis by the recombinant

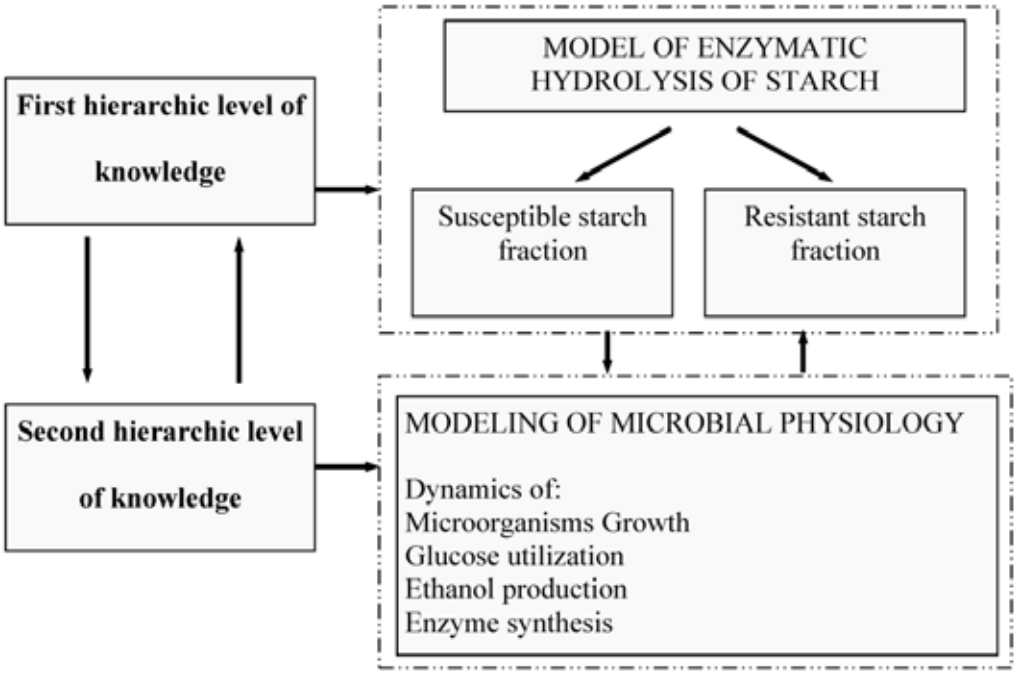


Fig. 1. A scheme of model development algorithm.

strain of *S. cerevisiae* YPB-G.

Second hierarchic level

The second hierarchic level (Table. 1, see Eq. 4-7) involves knowledge about the microbial kinetics of the population. Several physiological phenomena of the recombinant strain *S. cerevisiae YPB-G* have been described with simple unstructured mathematical formulas taken from our kinetic models catalogue database (Birol *et al.*, 1998). The microbial kinetic model is flexible, and equation (4) (glucose balance) is a key equation, which unites the first and second hierarchic levels of knowledge. The enzyme synthesis rate (see eq.7) is represented as a function of the specific growth rate and total starch concentration. The model of SSFSE is completed and it describes enzyme hydrolysis of starch by glucoamylase secreted by the recombinant strain *S. cerevisiae* YPB-G. It also describes microbial growth, glucose synthesis and utilization, and ethanol accumulation in the medium. More details about the identification procedure, the applied hybrid genetic algorithm (GA) and the new decomposition approach can be found elsewhere (Maicon *et al*, 2005, Kroumov *et al.*, 2006]. The values of kinetic and stoichiometric parameters were estimated using the experimental data of Altintas *et al.*, 2002. Several sets of batch data of recombinant strain cultivation on starch were examined. Here only a set called SG30 was used with the above initial conditions. It must be noted that the identification of the 14 model parameters is not an easy task. The straightforward use of the search method is always going to fail. We have developed and applied a new decomposition approach of the objective function in order robustly to find its global extreme. The details can be found elsewhere (Maicon *et al.*, 2005, Kroumov *et al.*, 2006). Here the experimental data of Altintas *et al.*, 2002 are presented.

Table 1. SSFSE system - two hierarchic levels of the model

First hierarchic level	Second hierarchic level
Susceptible starch fraction balance-Eq.(1) $\frac{dS_{sus}(t)}{dt} = -\frac{k_{sus} * Enz(t) * S_{sus}(t)}{K_m * \left(1+\frac{Glu(t)}{K_{glu}}\right) + \frac{S_{sus}(t)^2}{K_{starch}} + S_{sus}(t) + S_{res}(t)}$	Glucose balance -Eq. (4) $\frac{dGlu(t)}{dt} = -1.111.\frac{dS_{total}(t)}{dt} - \left(\frac{1}{Y_{x/s}}.\frac{dX(t)}{dt} + \frac{1}{Y_{p/s}}.\frac{dEt(t)}{dt}\right)$
Resistant starch fraction balance-Eq. (2) $\frac{dS_{res}(t)}{dt} = -\frac{k_{res} * Enz(t) * S_{res}(t)}{K_m * \left(1+\frac{Glu(t)}{K_{glu}}\right) + \frac{S_{res}(t)^2}{K_{starch}} + S_{sus}(t) + S_{res}(t)}$	Ethanol balance -Eq. (5) $\frac{dEt(t)}{dt} = \frac{q_{p\max}.Glu(t).Et(t).\left(1-\frac{Et(t)}{Et_{max}}\right)}{(K_{sl}+Glu(t))\left(K_{psl}+Et(t)+\frac{Et(t)^2}{K_{pi}}\right)}X(t)$
	Biomass balance- eq. (6) $\frac{dX(t)}{dt} = \frac{\mu_{max}.Glu(t)\left(\frac{S_{total}(t)}{S_0}\right)}{K_s + Glu(t)}X(t)$
Total starch balance-Eq.(3) $\frac{dS_{total}(t)}{dt} = \frac{dS_{sus}(t)}{dt} + \frac{dS_{res}(t)}{dt}$	Enzyme balance-Eq.(7) $\frac{dEnz(t)}{dt} = \frac{(\mu_{max}+\beta).Enz_{max}.S_{total}(t)}{K_{enz}+S_{total}(t)} - \frac{\left(\frac{dX(t)}{dt}\right)Enz(t)}{X(t)} - \beta.Enz(t)$
where Enz-Enzyme concentration (U/m^3); Glu-Glucose concentration (kg/m^3); S-Starch concentration (kg/m^3); Et-Ethanol concentration (kg/m^3); X-Biomass concentration (kg/m^3); R-starch utilization rate (kg/m^3/h); $R_{Glformation}$-glucose formation rate (kg/m^3/h); $R_{Glutilization}$-glucose utilization rate (kg/m^3/h); R_{enz}-enzyme synthesis rate (U/m^3/h); krate constant (1/h); K-inhibition and saturation constants (kg/m^3); K_m-Michaelis' constant (kg/m^3); q_p-specific ethanol production rate (1/h); $Y_{x/s}$-yield coefficient of cell growth (kg/kg); $Y_{p/s}$-yield coefficient of product (kg/kg); per-percent of susceptible starch (-); t-time (h); Greek symbols - μSpecific growth rate (1/h); enzyme dilution rate (1/h); ***Subscripts*** - res-resistant starch; sus-susceptible starch; 0-initial; total-total; max-maximum. **Note:** One unit of enzyme (U) represents an enzyme necessary quantity to produce from starch 1 kg of glucose per hour. Initial conditions for all task and fixed parameters of the model: Glu_0=3.5 kg/m^3; S_0=30 kg/m^3; X_0= kg/m^3; Et_0=0.5 kg/m^3; Enz_0=0.001 U/m^3; ß=0.05l/h; K_{enz}=1 kg/m^3; Enz_{max}=6.05U/m^3; K_m=0.406 kg/m^3;	

The analysis of the results presented in Fig. 2 evidence the predictive power of the new developed kinetic model of SSFSE and the new decomposition method applied for the parameter identification. As can be seen in Fig 2, the simulated dynamic profiles of starch decomposition and utilization (Fig 2a) show how the resistant and susceptible starch fractions were degraded through the SSFSE process. The enzyme profile shown offered new knowledge about the overall SSFE process when limitation, inhibition and effects take place on two hierarchic levels of the system. The experimental data and model simulation of the total starch degradation (Fig 2b) and glucose formation and utilization (Fig2c) are almost perfectly predicted by the model for the given initial conditions. This very well corresponds with the profiles given in Fig. 2a. Understandably, the experimental dynamic profiles of biomass (Fig. 2d) and ethanol (Fig. 2e) showed significant deviations from the simulated ones. In biotechnology this is a well-known fact and can be explained by the nature of the population growth, as well as by the measurement errors of the analytical procedure used.

Simultaneous fermentation and ethanol extraction (SFEE)

This example shows the application of a new method of fermentation and ethanol synthesis when ethanol is a strong inhibitor of its own production. The objective of this work was to model the continuous mode of simultaneous fermentation and extraction of ethanol (SFEE) in order to obtain maximum productivity. The model considers a solvent distribution coefficient value into the confidence range. Different kinetic models were applied. Two kinetic models representing the significant effect of the substrate and product inhibition were chosen. The response surface analysis (RSA) methodology was applied to study the influences of the control variables on the SFEE system. The simulation results have shown that the solvent phase dilution rate is influenced mainly by the product inhibition, while glucose inhibition limits its own feeding con-

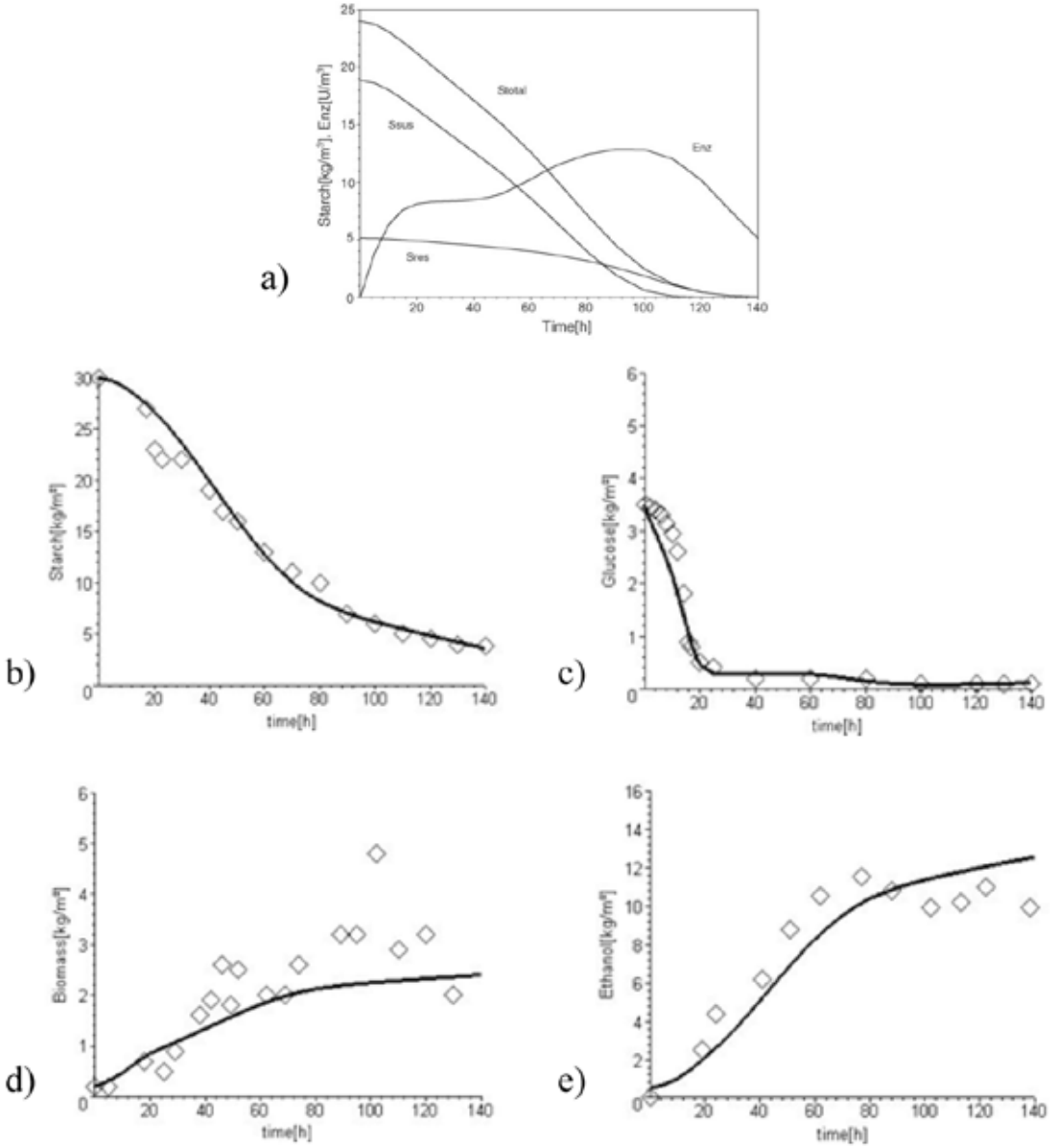


Fig. 2. Search for minimum of least square objective function by using hybrid GA and a new developed decomposition approach: a) Simulation-Dynamic profiles of the state parameters of the new developed SSFSE kinetic model (Kroumov *et al.*, 2006); b) Starch; c) Glucose; d) Biomass; e) Ethanol.

centration. The main control variable of the system was the watery phase dilution rate. A hybrid genetic algorithm (GA) with particular codification of the crossovers, mutation and elitism technique was used for the optimization of ethanol productivity. The optimized control variable values were obtained for watery and solvent phase dilution rates and for feeding glucose concentration, respectively. The value of the overall productivity of 21.84 kg/m³ is in accordance with the optimization results published in the literature.

Note: In SFEE, the appropriate solvent identification can be considered to be a crucial step. Kollerup and Daugulis (1985, 1986) presented studies with excellent final results of solvent identification. Recently, Pistikopoulos and Stefanis (1998) have analyzed identification methods and a promising molecular modeling methodology. However, if satisfactory solvent characteristics cannot be reached during the procedure of solvent identification, genetic engineering can be used as a tool for development of solvent resistant modified strains. The mathematical modeling of the batch process was considered by Honda et al. (1987) and was successfully applied for a mixture of solvents by Shi et al. (1990).

The SFEE model

A general mathematical model for SFEE based on the system analysis approach (see Fig.3) was developed.

The model considers continuous operation in a well-mixed reactor. The balance equations of the model are presented in Table.2.

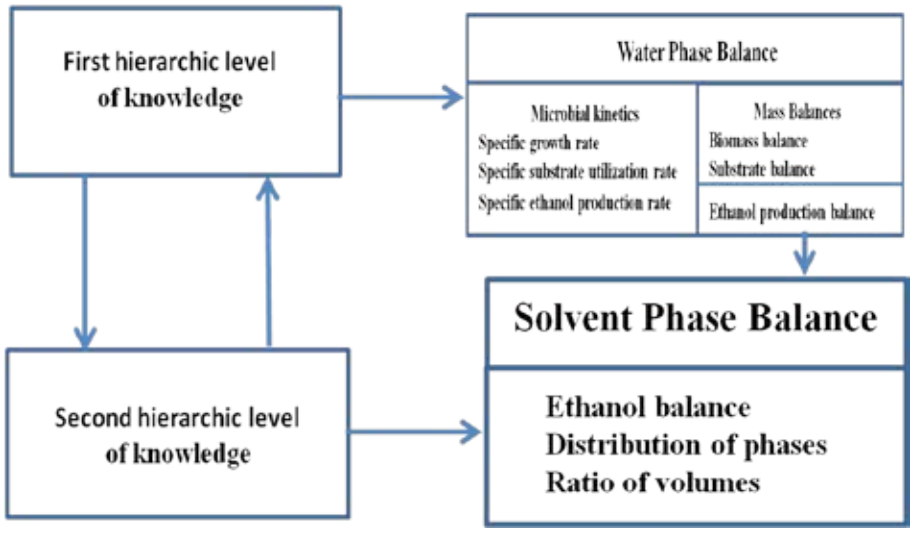


Fig.3. Scheme of the algorithm of SFEE model development.

Table.2 SFEE system -Two hierarchic levels of the model

First hierarchic level	Second hierarchic level	
Biomass balance-Eq.(8) $\frac{dX(t)}{dt} = \mu X - D_w X$	Ethanol balance in the solvent phase-Eq. (11) $\frac{dEt_s(t)}{dt} = \frac{m}{(1+m\eta)}\left(\nu_p X - D_w Et_w - \eta D_s Et_s\right)$	
Glucose balance-Eq. (9) $\frac{dGl(t)}{dt} = \nu_s X + D_w (Gl_{in} - Gl)$	Distribution coefficient-Eq. (12) $m = \frac{Et_s}{Et_w}$	Ratio of volumes Eq. (13) $\eta = \frac{V_s}{V_w}$
Ethanol balance in the water phase-Eq.(10)	$\frac{dEt_w(t)}{dt} = \frac{1}{(1+m\eta)}\left(\nu_p X - D_w Et_w - \eta D_s Et_s\right)$	
Nomenclature: X – biomass concentration, (kg/m³); Gl – glucose concentration, (kg/m³); Et_w – ethanol concentration in the broth liquid phase, (kg/m³); Et_s – ethanol concentration in solvent liquid phase, (kg/m³); Dw – dilution rate of broth liquid phase, (1/h); Ds – dilution rate of solvent liquid phase, (1/h) ; m – distribution coefficient, (-); η - relation between solvent phase volume and aquatic phase volume, (-); μ - specific growth rate, (1/h); ν_s - specific substrate utilization rate, (1/h); ν_p - specific ethanol production rate , (1/h); μ_{max} - maximum specific growth rate, (1/h); ν_{max} -maximum ethanol production rate, (1/h); K_{ss} , K_{is} , K_{sp}, K_{ip}, K_p - saturation and inhibition constants, (kg/m³); $Et_{max,s}$, $Et_{max,p}$ - maximum ethanol concentration in solvent and watery liquid phase, (kg/m³); $Y_{p/s}$ - yield coefficient , (kg product / kg substrate); $Y_{x/s}$ - yield coefficient, (kg biomass/ kg substrate).		
First hierarchic level-Microbial kinetics modeling		
Specific growth rate-Eq.(14) $\mu = \mu_{max} \cdot \frac{Gl}{K_{ss} + Gl + \frac{Gl^2}{K_{is}}} \cdot \left(1 - \frac{Et_w}{Et_{max,s}}\right)$	Specific ethanol production rate-Eq.(15) $\nu_p = \nu_{max} \cdot \frac{Gl}{K_{sp} + Gl + \frac{Gl^2}{K_{ip}}} \cdot \left(1 - \frac{Et_w}{Et_{max,p}}\right)$	
Specific substrate utilization rate-Eq. (16) $\nu_s = -\frac{\nu_p}{Y_{p/s}}$		
Evaluated values of kinetic parameters for the proposed model μ_{max}=0.4 h^{-1}; K_{ss}=0.48 kg/m³; K_{is}= 205.2 kg/m³; $Et_{max,s}$=87 kg/m³; $Y_{p/s}$= 0.48 kg/kg; υ_{max}=1.4 h^{-1}; K_{sp}=0.666 kg/m³; K_{ip}= 303.0 kg/m³; $Et_{max,p}$=114 kg/m³; strain-*Saccharomyces cerevisiae* NRRL Y132.		

The factor η is defined as the ratio between the solvent and water phase volumes. The partition (distribution) coefficient (m) is defined as the ratio between the ethanol concentration in the broth phase and the ethanol in the solvent phase. For convenience, some scientists fixed the value of (m) (Honda *et al.*, 1987).

Different kinetic hypotheses can be applied for the model development of SFEE process. In this work, Ghose and Thyagi's model was analyzed and applied (see Table.2, Eq. 14-16). The model was verified on the basis of experimental data presented by Dourado *et al.*, 1987.

Optimization procedure

An analysis of sensitivity of the optimization variables can be performed initially using the RSA method. The method shows the objective function (productivity) in relation to the control variables of optimization. When analyzing the results, some of the variables during the optimization process can be discarded. The RSA analysis (Silva *et al.*, 1999) also assists in the identification of the superior and inferior limits of the search space of each control variable, minimizing the computational work with genetic algorithm (GA).

The three control variables of optimization are as follows: water phase dilution rate, solvent phase dilution rate and glucose concentration in the feeding flow. For analysis purposes, a three-dimensional representation of the relation between productivity and two control variables was chosen. The third variable was fixed in quasi-optimal value. The system response as a function of the control variables is shown in Figures 4-6. The overall ethanol productivity was considered zero near the critical conditions of wash-out point and can be written as follows:

The range of control variables was determined

$$\mathrm{Pr}oductivity = \frac{\left(D_w Et_w + \eta . D_s Et_s\right)}{\left(1+\eta\right)} \qquad (17)$$

on the bases of their microbiological and physical meaning. To perform the optimization search, a hybrid genetic algorithm (GA) was used as a global optimizer. The search for a global solution by GA was carried out applying a selection method of parents (tournament method). We have used the performance of hybrid GA with the following parameter values: crossover=0.6 and mutations=0.2. As a rule of thumb, the crossover probability is generally in the range of 0.5-0.85. The best solution from every generation is preserved in the next one. This is in case the GA does not find a better solution during the search. A higher value of this elitism operator typically leads to premature convergence identified by the GA (Katare *et al.*, 2004). Re-initialization of chromosomes corresponds to 20% of a new generation. More detail about the hybrid GA can be found in Singh *et al.*, 2005; Katare *et al.*, 2004). If during the search GA reaches the i^{th} lower or upper parameter bound, a penalty on objective function is applied. A population size of 40 has been determined and the GA was run for 300 generations.

Results and Discussion

The Ghose and Thyagi's model considers a hypothesis that specific utilization and ethanol formation rate can be modeled as a function of a specific growth rate. This assumption has the convenience and advantage to show that in microbial physiology the specific growth rate of microbial population has key importance. Taking into account this fact, the system can be formalized by building it from three dependent sub-systems, and the biomass balance can be considered a key factor, where the specific growth rate controls the other cell functions. Substrate limitation and inhibition effects are presented in the Andrews type model and ethanol inhibition is considered a function of the critical inhibitory concentration. The system can be provisionally divided into two hierarchic levels. The first one includes the knowledge about microbial physiology and overproduction of ethanol by fermentation. On this level, the system behavior is studied for the two chosen kinetic models. The second hierarchic level contains knowledge about the physical process of ethanol extraction by organic solvent. The thermodynamics of liquid-liquid extraction can be represented in the simplest possible way by two coefficients. It is assumed that the solvent does not inhibit microbial growth and ethanol production, i.e. the solvent is biocompatible.

For preliminary studies, the relation between the volumes of the solvent and broth phases was fixed η=0.25, and the distribution coefficient has a value of m=2. The value of these control variables depends on the balance between the phases and limits of solubility of the solvent in the water phase. The RSA of the Ghose and Thyagi's kinetic model has shown the following system responses (see Figures 4-6).

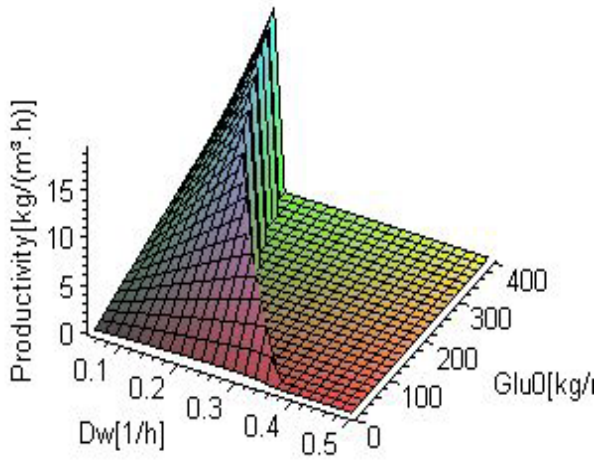


Fig. 4. RSA of Dw versus Glu_0 using Ghose and Thiagy's kinetic model–Ds=3(h^{-1}).

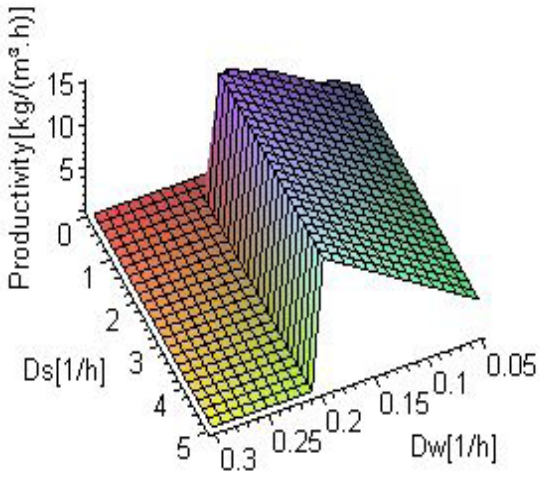


Fig. 5. Surface analysis of Dw versus Ds for Ghose and Thiagy's model–Glu_0=200 kg/m³.

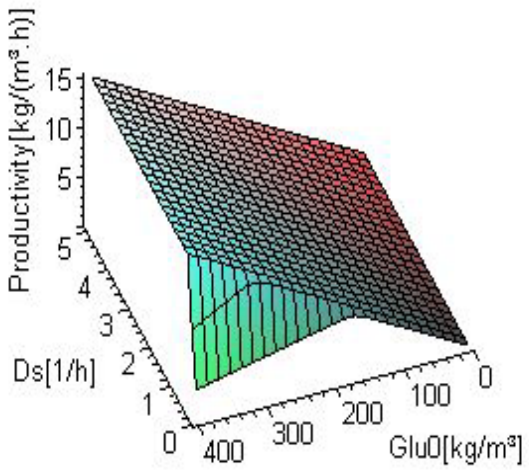


Fig. 6. RSA of Ds versus Glu_0 using Ghose and Thiagy's model–Dw=0.2 (h^{-1}).

Analyzing the graphs of Figures 4, a „wash-out" phenomenon can be observed, which supposes a penalty on the objective function when the search procedure reaches the region near this point. On the other hand, the productivity is zero on the right-hand side of the "wash-out" point and its maximum is near the left-hand side of this point. The D_w variable has exerted bigger influence on the system in terms of "wash-out" phenomenon. Figure 4 shows that the D_w-Glu gives crucial information about the maximum ethanol productivity. Based on this information, different operational conditions can be selected to perform an optimal ethanol production process. Figure 5 indicates that the search for an optimal D_w value is a crucial step of ethanol productivity maximization. For the chosen kinetic models, the D_s-D_w correlation is insignificant. The D_s influence on the system can be neglected since the ethanol inhibition effect on cells is very low. In Figure 6, the analyzed „wash-out" phenomenon shown in Figure 4 is not observed. The maximum productivity is reached with the increase of both control variables, D_s and Glu_0 (D_w is fixed). The system behavior was analyzed in detail by using Ghose and Thyagi's kinetics. Response surface analysis (RSA) showed the key role of D_w control during the optimization procedure. The D_w values near 0.37 should be taken with caution. The applied hybrid GA performed excellently for the chosen system and optimal solutions were found within a short computational time. The main difficulty of the optimization procedure was to find the optimal value of GA parameters. The RSA can be very helpful for localization of space search for system optimal solutions, minimizing the computational time. The optimal control variables and productivity values for Ghose and Thyagi's model are presented in Table 3.

The productivity presented in Table 3 corresponds to the optimal values of Glu_0, D_w and D_s. The results obtained during the search by GA have shown process productivity of 23 kg/(m³.h). The productivity simulated with the chosen kinetic models and solvent extraction is about three times higher than the productivity obtained by the conventional ethanol production process.

Conclusions

The process of extractive fermentation for ethanol production was modeled applying two different kinetics hypotheses. The model considers the thermodynamics of liquid–liquid extraction as simply as possible, utilizing the distribution coefficient

Table 3. Optimal parameter values estimated by GA for Ghose and Thyagi's model

Optimal parameter values estimated by GA for Ghose and Thyagi's model				
Variable	Glu_0	D_w	D_s	Productivity kg/(m³.h)
Model	337	0.169	1.11	21.84

as a constant. The microbial kinetics of the Ghose and Thyagi's model can be considered practically useful. The RSA of the system showed that the chosen control variables Glu_0, D_w and D_s had a significant effect on the SFEE process. The correlation between the control variables was analyzed in accordance with the microbial physiology theory and liquid-liquid extraction thermodynamics. The RSA methodology is a very helpful tool for localization of the space of optimal solutions. The adapted hybrid GA for the optimization of the SFEE process performed excellently. An optimal productivity value of 21.84 kg/m³ was obtained and compared to the results of others. The SFEE process optimization results showed a three times higher productivity than that obtained by the conventional ethanol production process. Finally, the SFEE process is very challenging for industrial applications because of the high reductions of the production cost.

Modeling of Ruminucoccus albus bacterial growth on cellobiose–xylose mixture

A model was developed for mixed substrates (cellobiose and xylose) utilization by *Ruminococcus albus* B199.

Provisionally, the system was divided into two sub-systems (see Fig. 7), where phenomena taking place on molecular and population levels were analyzed and modeled. On molecular level, the model describes regulatory mechanisms of induction – repression processes, taking into consideration the available knowledge of catabolic pathways and changes of the key enzyme activity, which is considered to be a rate-controlling step for the utilization of the non-preferable substrate (xylose). On population level, the specific growth rate on mixed substrates was established as an additive function of growth rates on single substrates. The product formation rate of *Ruminococcus albus* B199 could be described as partially linked with the specific growth rate or, more precisely, linked with the substrate utilization rate. By using material balances, the kinetics of mixed substrates utilization, growth and product formation in batch processes were investigated. The developed model was a useful tool for microbial kinetic experimental design and biochemical study of key enzymes involved in the metabolic regulation of *Ruminucoccus albus* B199. An understanding of the internal regulatory processes is important for the ruminant animal sciences (Russell et al., 1979; Strobel, 1993; Thurston *et al.*, 1993, 1994), fermentation using cellulose and hemicellulose hydrolysates.

Many theoretical models have been developed to describe microbial growth on multiple substrates. The simple unstructured models developed by Lee *et al.* (1974), Tsao & Hanson (1975) and Yoon *et al.* (1977) are inadequate to explain the complex cellular regulatory process. Further contributions in the modeling of this system are the structural models of Domach *et al.* (1984), Joshi & Palsson (1988), Nikolajsen *et al.* (1991) and Palsson and Joshi, (1987). Such models incorporate too many parameters to be used for experimental design and optimization of fermentation processes. The most widely accepted models of Bajpai & Ghose (1978), Imanaka & Aiba (1977) and Van Dedem & Moo-Young (1977) were based on the lac operon theory of Jacob and Monod (1961) and included detailed mechanisms of repression and induction.

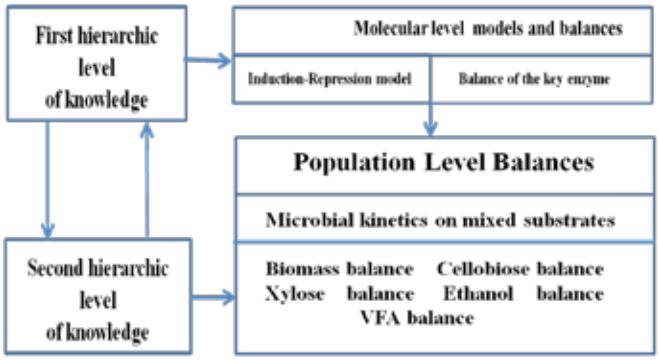
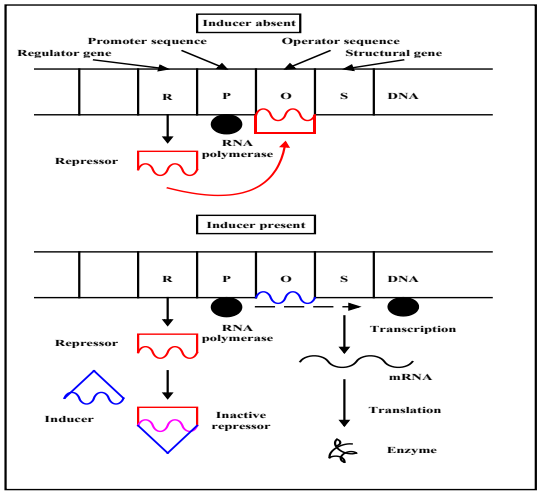


Fig. 7. A scheme of the system of mixed substrate utilization.

Recently, a cybernetic approach (Baloo & Ramkrishna, 1991a; Baloo & Ramkrishna, 1991b; Kompala, 1982; Kompala *et al.*, 1984; Kompala *et al.*, 1986; Ramkrishna, 1982; Ramkrishna & Ramkrishna, 1996; Straight & Ramkrishna, 1994; Yoon *et al.*, 1977) was applied for modeling microbial growth on multiple substrates. On the other hand, to explain complex regulatory processes, modeling should be based on the knowledge of the biochemical pathways of a particular microorganism. The lac operon theory of Jacob and Monod (1961) was adapted in order to develop the induction-repression model of cellobiose-xylose utilization by *Ruminococcus albus B199*. Briefly about the theory, the "xylose" operon is assumed to consist of four major regions. The "R" gene codes for an inactive repressor molecule. This molecule in the absence of an inducer, such as xylose, is able to bind to the operator region "O" of the operon. This binding of the active repressor to the operator blocks the transcription process by mRNA polymerase, which begins at the promoter region "P" of the operon. In this repressed situation, the "S" gene (DNA encoding the xylose key enzyme) is not transcribed and the required key enzyme is not formed. When there is an inducer present to bind with the inactive repressor, then operator region is free and mRNA polymerase travels from the promoter region throughout the operator and transcribes the "S" gene to form therequired key enzyme. The mathematical description of this phenomenon is based on the assumption that a chemical equilibrium exists between the repressor molecule, inducer and the operator region. An induction-repression mechanism was successfully used for description of yeast growth on glucose-cellobiose mixture by Bajpai & Ghose (1978). Applying this to cellobiose-xylose utilization by *R. albus B199*, the relationship between the inactive repressor molecule (R), xylose and the operator can be written as follows:



$$R + nXyl \underset{\leftarrow}{\overset{k_1}{\rightarrow}} R.nXyl \tag{18}$$

$$O + R.nXyl \underset{\longleftarrow}{\overset{k_2}{\longrightarrow}} O.R.nXyl \tag{19}$$

where R- is the cytoplasmic repressor; O- is the operator; Xyl- is inducer; n-is the number of ligand binding sites for inducer on the repressor molecule R.

The equilibrium constants K_1 and K_2 are defined by:

$$K_1 = \frac{[R.nXyl]}{[R][Xyl]} \tag{20}$$

$$K_2 = \frac{[O.R.nXyl]}{[O][R.nXyl]} \tag{21}$$

The material balances for the operator and total repressor concentrations are:

$$[O] = [O] + [O.R.nXyl] \tag{22}$$

by assuming [R] >> [O] then

$$[R] = [R] + [R.nXyl] \tag{23}$$

where R.n.Xyl- is the repressor-inducer complex, which prevents R from binding to an operator gene where it would block the transcription of the structural gene;

O.R- is the operator-repressor complex whose formation blocks mRNA polymerase formation.

Using the low of mass action the fraction of free operator genes can be written:

$$\frac{O}{O_t} = \frac{(1 + K_1 . Xyl^{\,n})}{(1 + K_1 . Xyl^{\,n} + k_1 R_{t1})} \tag{24}$$

The fraction of the gene promoter occupied by the catabolic repressor is expressed Imanaka & Aiba (1977).

$$\frac{R}{R_t} = \frac{(1 + K_2 . CB^{\,m})}{[1 + (1 + k_2 R_{t2}) . K_2 . CB^{\,m})]} \tag{25}$$

The rate of enzyme synthesis can be assumed to be proportional to the mRNA polymerase concentration in the cell, which itself is proportional to the fraction of free operator genes. Hence,

$$\frac{d\left(\frac{E}{E_o}\right)}{dt} = \alpha . \left(\frac{O}{O_t}\right) \left(\frac{R}{R_t}\right) - \beta \left(\frac{E}{E_o}\right) \tag{26}$$

where E- is the induced key enzyme (xylose isomerase) concentration in repressed state; E_0- is the key

enzyme concentration under completely de-repressed state; E/E_0- depends upon the fraction of free operons made available by the inductive action of xylose and upon the extent of catabolic repression of cellobiose; α- is the key enzyme synthesis rate constant; β- is the enzyme decay rate constant

Thus, the induction-repression model on the molecular level is completed and is based on the following assumption: -the interaction between the inducer and the cytoplasmic repressor can be treated as a chemical equilibrium; -the equilibrium is established instantaneously; -the number of cytoplasmic repressor molecules is large compared to the amount of operators and it is small compared to the inducer molecules.

The E/E_0 ratio represents the degree of catabolic repression occurring in the cell on the basis of O/O_t, R/R_t interaction. When E/E_0 is equal to 1, the key enzyme is in fully de-repressed state. For values of E/E_0 less than 1, the system is proportionally repressed. It is assumed that there is only one enzyme that is induced, or if there is more than one, only one is produced at a slow rate (limiting step) as a result of induction and synthesis of the same enzyme is catabolically repressed. Hence, the E/E_0 ratio can be considered to be a function of two control parameters (xylose and cellobiose concentrations). For development of a growth kinetics model, it is assumed that the rate of xylose utilization is directly proportional to the key enzyme concentration (xylose isomerase).

It is important to note that measurements of absolute key enzyme activity (concentration) not required, which is very important for practic applications. The model requires only the initi... values of the relative enzyme level (relative to the maximum one during balanced exponential growth on xylose). Such initial value can be used to characterize the state of the inoculum and can be determined from a single substrate batch experiment. The specific growth rate of *R. albus* B199 on xylose can be written as follows:

$$\mu_{xyl}m = \mu^{\circ}_{xyl}m.\left(E/E_{\circ}\right) \tag{27}$$

Therefore, E/E_0 could be rewritten as the ratio of ($\mu_{xyl}m/\mu^{o}_{xyl}m$), where $\mu_{xyl}m$ is the maximum growth rate on xylose in the presence of cellobiose and $\mu^{o}_{xyl}m$ is the maximum growth rate on pure xylose.

$$\left(E/E_{\circ}\right) = \mu_{xyl}m / \mu^{\circ}_{xyl}m \tag{28}$$

Microbial growth on multiple substrates can be represented by the following equation:

$$B + S_i \ldots \xrightarrow{E_i} (1 + Y_i).B + \ldots \tag{29}$$

The utilization of the *i*th substrate (S_i) by the biomass (B) is assumed to be catalyzed by a key enzyme (E_i) representing the whole set of enzymes catalyzing the metabolic pathways of growth on (S_i). The key enzyme required for utilization of a given substrate (cellobiose or xylose) must be synthesized before growth can occur on the substrate. Hence, growth of *R. albus* B199 on cellobiose-xylose can be considered to be made up of contributions from growth on cellobiose and xylose involving catabolic repression mechanism. Therefore,

$$\mu_{cb} = \mu_{CB}m \frac{CB}{(K_{CB} + CB)} \tag{30}$$

$$\mu_{xyl} = \mu_{xyl}m \cdot \frac{Xyl}{(K_{xyl} + Xyl)} = \mu^{\circ}_{xyl}m \cdot \left(E/E_{\circ}\right) \frac{Xyl}{(K_{xyl} + Xyl)} \tag{31}$$

Modeling of batch growth

Using the above kinetic models and the mass balances, mathematical expressions describing batch process of biomass accumulation and substrate utilization can be written as follows:

Biomass

$$\frac{dX}{dt} = \sum_i \mu_i.X = (\mu_{CB} + \mu_{xyl}).X \tag{32}$$

Cellobiose

$$\frac{dS_{CB}}{dt} = -1/Y_{CB}.\mu_{CB}.X \tag{33}$$

Xylose

$$\frac{dS_{xyl}}{dt} = -1/Y_{xyl}.\mu_{xyl}.X \tag{34}$$

VFA - volatile fatty acids

$$\frac{dVFA}{dt} = 1/Y_{vfa}.q_s^{CB}.X + 1/Y_{vfa}.q_s^{xyl}.X \tag{35}$$

Ethanol

$$\frac{dEtOH}{dt} = 1/Y_{EtOH}.q_s^{CB}.X + 1/Y_{EtOH}.q_s^{xyl}.X \tag{36}$$

The process of mixed substrate utilization is fully described by equations 26, 30-36. The most

important information of this study is the information about bacterial diauxic growth as a result of two substrate utilization processes controlled by a catabolic repression mechanism. For this reason, the information from equations 35 and 36, describing product formation, is not under consideration in the present work.

Materials and methods are published elsewhere. More details about biochemical pathways and assays may be found elsewhere (Russell *et al.*, 1979; Thurston *et al.,* 1993; Thurston *et al.*, 1994).

Results and Discussion

Series of batch experiments with different cellobiose-xylose ratio were performed to evaluate the catabolic repression hypothesis describing *Ruminococcus albus* B199 growth. Experimental design details can be found elsewhere (Kroumov, 1999).

Experiments in tubes with different cellobiose-xylose ratio were performed. In all cases, the total substrate concentration of 1 kg/m^3 and xylose inoculum was chosen. Figure 8 shows the growth curves of 9 tubes processes and substrate ratio changing from pure xylose via 15:1, 10:1, 5:1, 1:1, 1:5, 1:10, 1:15 to pure cellobiose. Each curve represents the average value of three parallel tests. *R. albus B199* utilized cellobiose first and when its concentration fell to the low level, the utilization of xylose took place. A maximum of the growth rate was observed on pure cellobiose. Diauxic growth appeared in ratios “D” and “E” where the concentration of cellobiose was lower than that of xylose. In “B” and “C” ratios there was co-utilization of xylose. By changing the ratio the diauxic lag became progressively suppressed. These observations were in agreement with the results of others (Bajpai & Ghose, 1978; Kompala *et al.*, 1986) on multiple substrates.

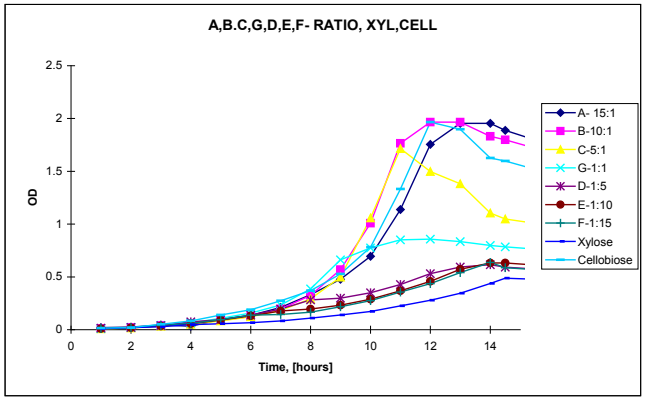


Fig. 8. Growth of *R. albus* B199 on cellobiose-xylose mixture.

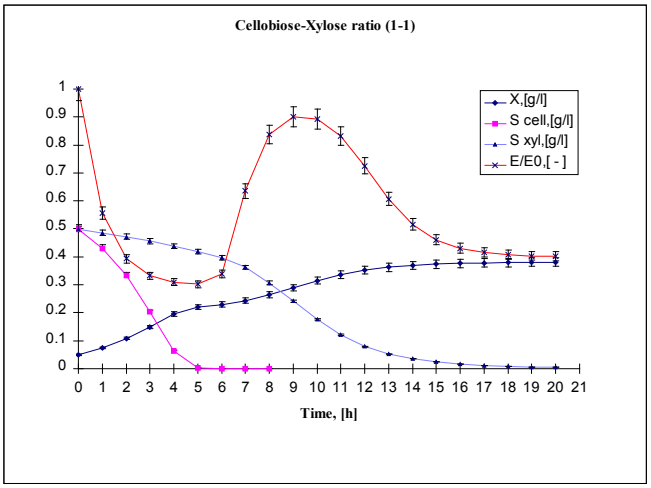


Fig. 9. Batch growth of *Ruminuccocus albus* B199 on CB: Xylose ratio (1:1).

Parametrical identification and simulations

Experimental results show that the maximum specific growth rate of *R. albus* on cellobiose is greater than on xylose substrate. In mixed substrates experiments, the decrease in μ_{xyl} depends on the decrease in E/E_0. On the other hand, the relative enzyme activity of the key enzyme (E/E_0) is a function of the repressor-inducer ratio, and for the *R. albus* B199 strain the most dramatic repression effect is observed in the cellobiose-xylose ratio (1:5; 1:10) Fig. 10 and Fig. 11. The detailed description of the identification procedure can be found elsewhere (Kroumov, 1999). The final values of constants used in the simulations: Kinetic parameters-$\mu_{xyl}m$=0.358 1/h; $\mu_{CB}m$=0.18 1/h; K_{CB}=0.07 kg/m^3; K_{xyl}=0.2 kg/m^3; Y_{CB}=0.28 kg/kg; Y_{xyl}=0.386 kg/kg; Induction repression parameters- K_1=438 [-]; K_2=14723 [-]; m=1; n=2.3; k_1R_{t1}=1.5 [-]; k_2R_{t2}= 2.3 [-]; α=1 [-]; β=1 [-].

The initial conditions for the experiments and integration of the model equations were determined as follows: X_0=0.05 kg/m^3; E/E_0=1. The initial concentrations of cellobiose and xylose were determined by the substrate ratio for the total amount of carbohydrates equal to 1 kg/m^3.

Figure 9 shows the simulation and experimental results of diauxic consumption of cellobiose and xylose for the substrate ratio 1:1 (or concentration of 0.5 kg/m^3 for each sugar). Cellobiose is consumed first and because of the strong catabolic repression, the relative enzyme activity reaches the minimum. It starts increasing when cellobiose in the reactor is exhausted and then reaches the maximum, which is about 90% of its initial value. Xylose consumption rate increases as the key enzyme concentration builds up. The enzyme activity falls

again as xylose concentration drops, which results in a reduced consumption rate. In the growth curve, there is a clear indication of a second lag phase for about 3 hours, where the overall specific growth rate is close to zero. For *R. albus* B199, the duration of this lag phase is determined from the time taken for complete de-repression of operators by xylose, i. e. from the values of the constants in equation 26.

Figure 10 presents the results from batch experiments for the substrate ratio of 1:5 (cellobiose concentration – 0.167 kg/m^3; xylose concentration –0.833 kg/m^3). In the growth curve, the duration of the second lag phase is about 3 – 4 hours. The minimum of the relative enzyme activity (E/E_0) is slightly higher than in the substrate ratio 1:1. After the cellobiose consumption, the relative enzyme activity reaches the maximum of about 98% of the fully de-repressed state. This fact is obvious because xylose concentration is higher compared to that in the substrate ratio 1:1. The appearance of a second lag phase in all three chosen ratios is a manifestation of repression of xylose-consuming key enzyme synthesis. The evidence that the key enzyme is induced by the presence of xylose is indicated by very long lag periods (5-7 hours, even more!) observed when actively growing cells of inoculum are cultivated on the cellobiose medium.

Figure 11 presents data about *R. albus* B199 growth based on the cellobiose-xylose substrate ratio 1:10 (cellobiose concentration–0.09 kg/m^3, and xylose concentration- 0.9 kg/m^3). In this experiment, the catabolic repression effect is similar to the one observed at 1:5 ratio. The growth curve shows a very long second lag phase. The recovery of the relative enzyme activity is about 98.6% of the fully de-repressed state of the key enzyme. The simulation results (dash lines) and experimental data (dotted line) for the three substrate ratios presented in Fig. 9, 10, and 11, respectively, are a very good manifestation of the chosen parametrical identification procedure. The error between the model and experimental data was in the range of 3% - 12%.

Finally, all biochemical and molecular level studies were performed on the basis of simulation results of the developed model. The analysis of sensitivity applied to equation 26 showed that the power prediction of the model was high for the different operational conditions.

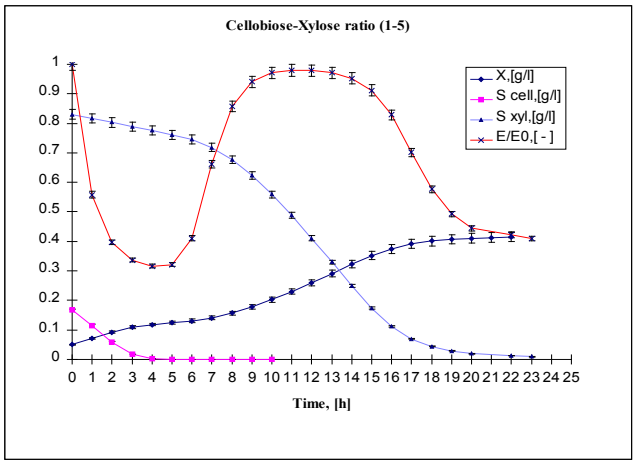


Fig. 10. Batch growth of *R. albus* B199 on CB:Xylose ratio (1:5).

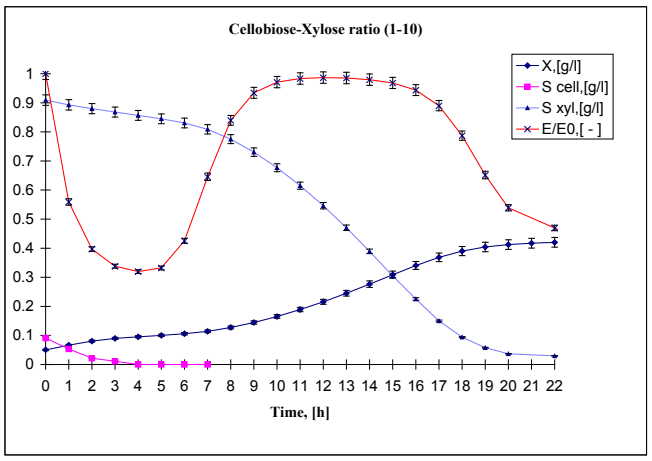


Fig. 11. Batch growth of *R. albus* B199 on CB:Xylose ratio (1:10).

Conclusions

In this work, it was shown that anaerobic growth of ruminal bacterium *Ruminococcus albus* B199 on cellobiose-xylose mixture can be described successfully by an induction-repression model. The developed model performs well with different cellobiose-xylose ratios and it is a powerful tool for different practical applications. Based on the model simulations, the experimental design for biochemical and strain modification studies was performed with unexpected great success.

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