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MODEL BASED BIOMASS YIELD OPTIMIZATION AND CONTROL FOR *E. coli* BL21 (DE3) HEPATITIS B CORE ANTIGEN (HBcAg) PRODUCER FED-BATCH FERMENTATION PROCESS

Summary of Doctoral Thesis

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DECLARATION OF ACADEMIC INTEGRITY

I hereby declare that the Doctoral Thesis submitted for the review to Riga Technical University for the promotion to the scientific degree of Doctor of Engineering Sciences is my own and does not contain any unacknowledged material from any source. I confirm that this Thesis has not been submitted to any other university for the promotion to other scientific degree.

Oskars Grīgs .....
Date: .....

The Doctoral Thesis has been written in the Latvian language, it contains Introduction, Review of Literature, Methods, Discussion of Experiments, Conclusions, Bibliography with 148 reference sources. It has been illustrated by 24 figures and 16 tables. The volume of the Thesis is 114 pages, not including 1 appendix.

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CONTENTS

GENERAL OVERVIEW OF THE THESIS

Introduction

In the fed-batch fermentation processes of microorganisms, which are realized in the bioreactors, unique products, which are of significant importance and demanded in the world consumption, are produced. In such processes a precise substrate feeding rate (feeding profile) control has a significant role, which directly influences the biomass growth rate and the biomass yield at the end of the process. The fact mentioned above plays a critical role in the biomass synthesized target product production with a maximal yield and quality.

The Latvian Biomedical Research and Study Center has developed *E. coli* BL21 (DE3) pBR327 hepatitis B core-antigen (HBcAg) producer. HBcAg is being intensively studied for vaccine development/production against hepatitis B virus, as well as a tool for hepatitis B virus diagnostics, and core-antigen structure as a various poly-/olynucleotide gene and immune system stimulating gene and immune system stimulating sequence carrier. Particular producer cultivation process transfer from flask (0.5 l) to laboratory scale bioreactor (5 l) is essential/relevant. Laboratory scale bioreactor is an intermediate stage for process scale up till bioreactors of 5–10 m^3. One of the most significant scale-up tasks is appropriate feeding rate profile evaluation and its control in the process to maintain controlled and optimal biomass growth.

The challenges for the control of the feeding rate in the fermentation process are, in fact, that notably varying specific substrate consumption rate, which in *E. coli* glucose consumption case is approximately within 0.5–1.0 g-substrate/g-biomass/h, which influences biomass physiological state, physicochemical and technological parameters, feed substrate concentration in the fermentation medium should be kept continuously within narrow, low concentration ranges, which for typical *E. coli* glucose feed processes are in the order of 10^{-2}–10^{-4} g/l.

It is possible to provide control according to the substrate feeding rate by using model based feeding rate calculation and combining it with advanced feeding rate control methods. For this purpose, precise fermentation model is required, which provides the possibility for modeling process state variables – biomass (x), substrate (s) and acetate (a) concentrations. Model predictive (MPC) feeding rate control can be applied by using a simplified process model without acetate influence on modeling. Advanced fed-batch control is appropriately applicable according to the on-line pH or pO_2 sensor readings.

The Aim and Tasks

Using model-based fed-batch process calculation and advanced feeding rate control, to evaluate and control substrate feeding profile for *E. coli* BL21 (DE3) pBR327 fermentation process, for maximal and controlled biomass yield obtainment at the end of the process.

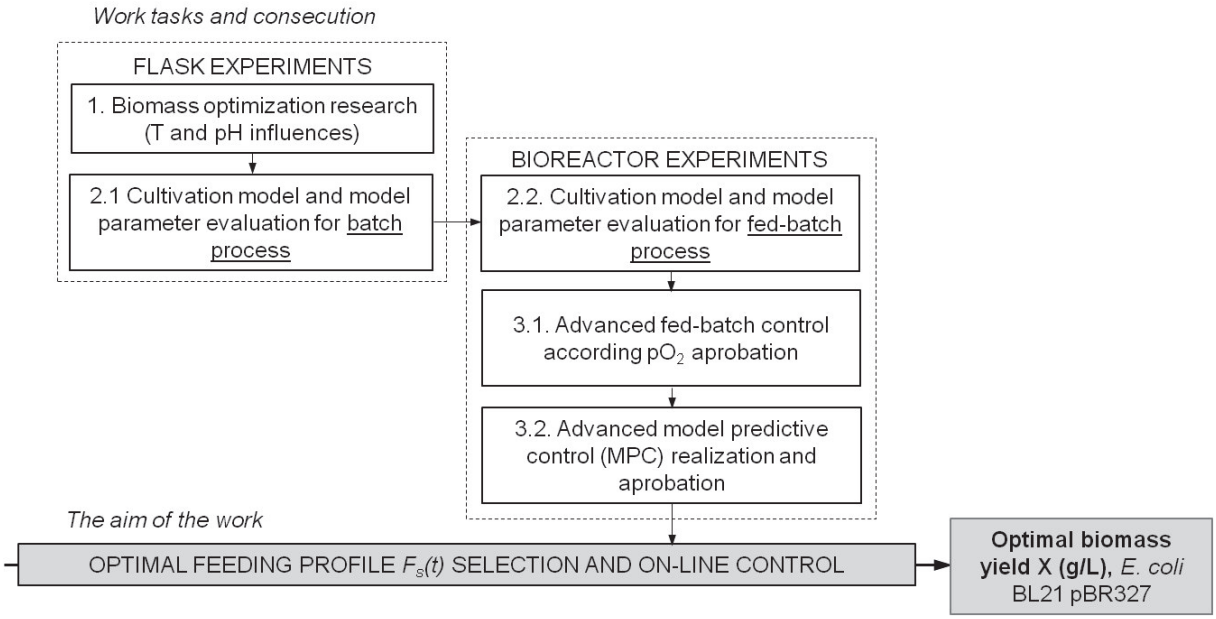

Fig. 1. The connection of the task and sub-tasks solved in the Thesis.

Scientific Novelty and Main Results

The new and unique recombinant *E. coli* BL21 (DE3) pBR327 hepatitis B core-antigen producer (Latvian Biomedical Research and Study Centre) model-based biomass production fermentation process optimization. Desirable feeding rate ($F_s(t)$) and biomass ($x(t)$) profiles and their advanced on-line control have been suggested and tested for biomass production process.

E. coli BL21 (DE3) pBR327 fermentation process state variables – biomass (x), substrate (s), and acetate (a) – have been analyzed, mathematical model precision has been described and the respective specific parameters of the model have been assessed/defined.

Application

The evaluated optimal feeding and biomass growth profiles in combination with approbated advanced feeding rate control methods can be used for recombinant *E. coli* BL21 (DE3) pBR327 hepatitis B core-antigen production fermentation process scale-up and control for pilot/production processes.

The described model and model parameter evaluation methodology can be used for the evaluation of similar fermentation process model and model parameters.

The advanced feeding rate control methods have been implemented and approbated in the control systems of JSC «Biotehniskais Centrs» for commercial bioreactors.

Approbation of the Research Results

The scientific achievements and main results of the research have been presented in 11 international conferences, summarized in 5 full text scientific manuscripts and 3 peer-reviewed conference proceeding abstracts.

REVIEW OF THE LITERATURE

In the bioreactor accomplished microorganism fermentation processes play a significant role in obtaining many products consumed by society. These products are used in medicine, food processing, dairy consumption and also in other industries [1, 2], and one of their characteristics is that their production using conventional technologies, such as chemical engineering, is practically non-realizable or not economically feasible. In majority of the industrial fermentation processes, particularly the ones used to obtain recombinant proteins and other medicines used in health care, continuous, substrate limited microorganism feeding is used [3, 4]. In this way it is possible to achieve a higher cell biomass and increased product yields compared to batch processes, where no additional substrate is added when initial amounts from batch medium are consumed. Fermentation process modeling is used for calculation of substrate feeding profile $Fs(t)$ before the process, as well as for its control within the process [5, 6]. Fermentation process development usualy starts in the laboratory flask or bioreactor scales, where scale-up step usualy is 1:5 or 1:10. Besides the traditional stainless stell bioreactor application, within the last decade, the single-use technology has become actual [7].

Significant research, including activities in Latvia, is conducted in the field of obtaining recombinant hepatitis B core-antigen (HBcAg) virus-like particles [8–10]. Interest on hepatitis B virus (HBV) gene cloning and expression generates perspective in *E. coli* fermentation process to synthesize a HBV vaccine component and tools for diagnosis of various hepatitis B disease stages, as well as using core-antigen structure as various immunologic epitope and poli-/olinucleotide gene and carrier of immune system stimulating sequences. HBcAg and its derivative obtainment from *E. coli*, *S. cerevisiae* un *P. pastoris* microorganism cultivation processes are being intensively studied. Obtainable biomass amount in this kind of *E. coli* high cell density fermentation processes, without application of diafiltration, is up to 150 g of dry cells per liter [3]. Recombinant protein yield in this type of *E. coli* processes usually is about 0.1–50 g/l. For HBcAg expression using *E. coli*, usually limited glucose fed process is used, where glucose concentration in the culture should be kept within narrow ranges of 0.005–0.5 g/l to provide the possibility to control the biomass growth rate and to avoid accumulation of un-desirable by-product like acetate. Acetate accumulation above 1.5–2 g/l negatively influences biomass growth and product formation [11]. It is known that acetate formation occurs due to unbalanced substrate metabolism, when part of the consumed substrate is converted to acetate and it is excreted in culture media. This unbalanced metabolism occurs under increased glucose concentration conditions, which increase the glucose consumption rate. Exceeding the critical glucose consumption rate, which usually decreases within the process from 1.2 to 0.5 (glucose)/g (biomass)/h, the acetate accumulation begins in culture medium [5, 12].

Kinetic and bioreactor model combination gives a complete mathematical description of the fermentation process, and this model can be used for fermentation process simulation. Before model application, it is important to choose the most appropriate or available model parameters. A few of these parameters, such as a mass flow to and from the reactor, are working parameters dependent on process control.

However some parameters, such as biomass specific growth rate (μ) and biomass yield from substrate (*Yxs*), are related to the physiology of the cell [13]. Due to biotechnological process variability, process model and model parameters should be identified for each particular fermentation process of microorganisms. Process models applied in manufacturing process control should also be validated [14].

Advanced feeding rate control in substrate limited processes intends for application of analytic and instrumental tools [15]. To determine substrate and biomass amounts in culture medium, feedback can be either direct, using direct substrate and biomass on-line or off-line measurements, or in-direct, for example, using parameters, such as pO_2, pH, uptake/dispense of O_2/CO_2 etc. Direct substrate determination from culture medium with on-line sensors is limited because of the lack of the appropriate sensors/technology. The disadvantages of direct substrate determination methods are some short time delay for measurement reception. For determination of process variables, like biomass specific growth and substrate consumption rates, model based methods and tools such as program or soft sensors are also applied.

For provision of fed-batch process repeatability, model predictive control (MPC) is used [6]. MPC advantage is a possibility of relatively simple model application for biomass growth prediction and appropriate substrate feeding profile calculation taking into account time-varying process conditions.

EXPERIMENTAL PART

In Thesis highlighted the main problem, e.g., optimal (maximal) *E. coli* BL21 (DE3) pBR327 biomass yield obtaining by application of optimal feeding profile; the diagram of solved tasks is shown in Fig. 1.

E. coli BL21 (DE3) (ATCC, American Tupe Culture Collection) recombinant strain saturate pBR327 plasmid for hepatitis B core-antigen expression (Latvian Biomedical Research and Study Centre). Culture media and feeding solution composition were taken from R. Baipaj research [16].

Devices and Equipment

Cultivation in the flasks. For bioreactor inoculum preparation, as well as for flask experiments, shaker/incubator ES-20 (Biosan, Riga, Latvia) was used. In biomass yield optimization experiments standard 250 ml Erlenmeyer flasks with initial working volume of 50 ml were used. In other flask cultivations standard 500 ml Erlenmeyer flasks with initial working volume of 100 ml were used.

Cultivation in the bioreactor. Fermentation processes were conducted in 5.4 l laboratory bioreactor EDF-5.4/BIO-4 (Riga, Latvia) (see Fig. 2). The demonstrative experiment was conducted in the single-use 5.7 l bioreactor CerCell 5.7 (Holte, Denmark).

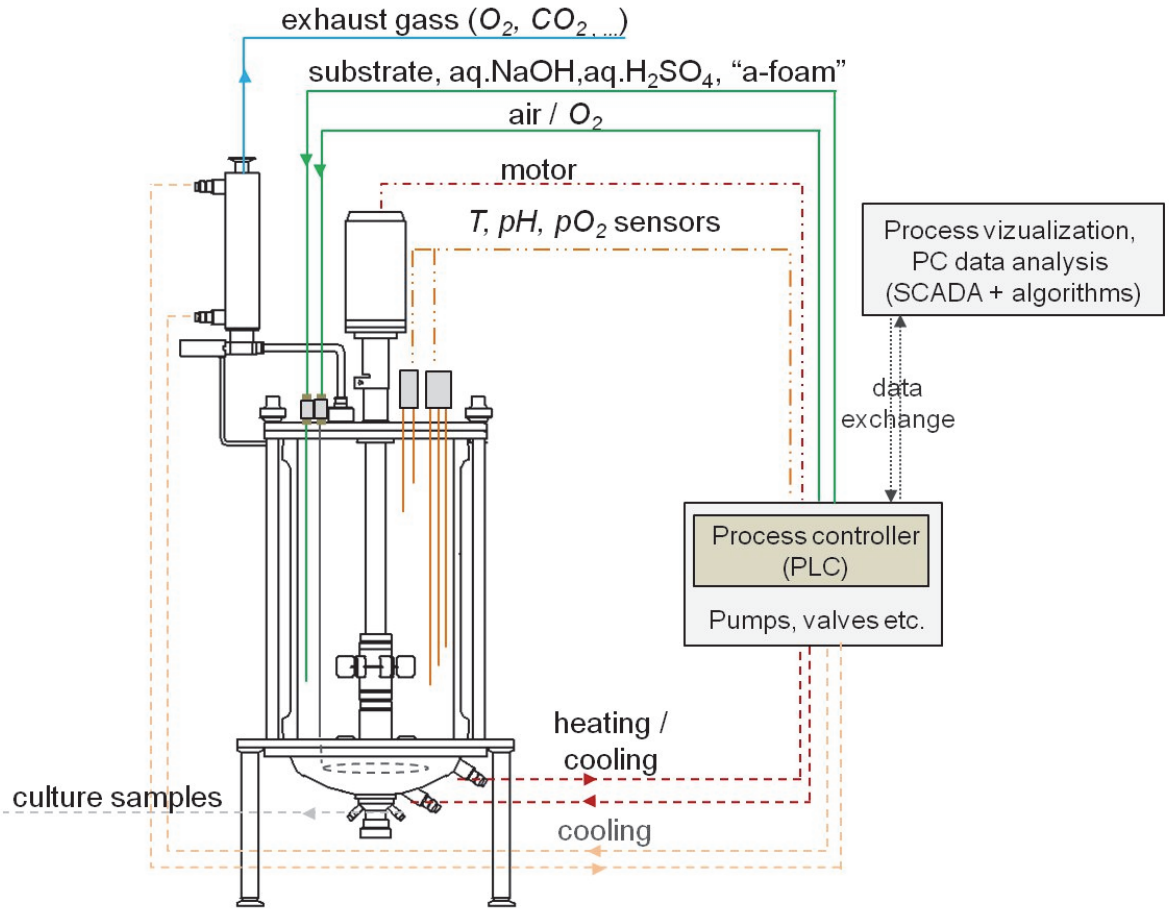

Fig. 2. EDF-5.4 principle scheme of the bioreactor.

Analysis and Control

E. coli BL21 (DE3) pBR327 biomass growth during the process was monitored by spectrophotometric optical density (OD, 560 nm) measurements (Jenway, 6300, Essex, England). Biomass concentration was calculated using previously determined biomass and OD correlation coefficient of 0.45. Glucose was measured enzymatically (AccuChek ACTIVE, Roche, Basel, Switzerland). Acetate concentration was determined using HPLC (Agilent Technologies, Inc., Santa Clara, CA, USA, Agilent 1100, stationary phases: Aminex HPX).

In different experiments the feeding solution was added with peristaltic pumps periodically (peristaltic pump included in EDF-5.4 standard package) or continuously (Longer-Pump, BT100–2J, Baoding, Hebei, China). The following sensors were used in the bioreactor control: pH (Ingold, Toledo 405-DPAS SC K85/120), pO_2 (Ingold, Toledo InPro 6800), O_2 and CO_2 concentration analysis in exhaust gass (Bluesens, BlueInOneFerm, CO_2: 0–25 Vol.%, O_2: 0.1–25 Vol.%), biomass (Optek, ASD19-EB-01).

EDF-5.4/BIO-4 standard T, pH un pO_2 control algorithms were developed by the engineers of JSC «Biotehniskais Centrs». Advanced model predictive fed-batch control and data processing algorithms in *Matlab* environment were developed by Professor Dr. Vytautas Galvanauskas (Kaunas University of Technology, Department of Automation). The author of the Thesis configured the above-mentioned algorithm control parameters specifically adapting them to *E. coli* BL21 (DE3) pBR327 cultivation process.

Pre-culture of 100 ml, grown in batch medium (14–16 h, OD_{560} = 4.0–4.5), was prepared for fermenter inoculum. pH value of 7.0 ± 0.2 was controlled using 30 % NaOH and 20 % H_2SO_4 solutions, temperature and pO_2 was selected at 37.0±0.2 ˚C and 40±5 %, respectively. Achieving selected lower pO_2 point, then pO_2 initially was controlled by manipulating stirrer speed until allowed maximum, and then by manipulating inlet air oxygen enrichment impulse length. In several experiments, pO_2 control was implemented with 3rd cascade – substrate feeding pump. Oxygen enrichment pulse length was explicit in percent from period T=20 s. Air flow at the process was 1.7 l/min, meanwhile oxygen flow rate at the time of oxygen valve pulse was 0.33 l/min. Outlet gas condenser was used to reduce evaporated culture volume loss. Foam level was controlled by adding anti-foam agent A (Sigma).

Substrate feeding profile for glucose limited and controlled biomass growth was calculated as follows:

$$F_s = \frac{1}{s_f}\left(\frac{\mu_s}{Y_{xs}} + m_s\right) \cdot V_0 \cdot x_0 \cdot e^{\mu_{(set)}(t-t_0)}, \qquad (1.)$$

where F_s – substrate feeding rate, ml/min; x_0 – biomass at the beginning of fed-batch, g/l; V_0 – volume at the beginning of fed-batch; $\mu_{(set)}$ – selected specific biomass growth rate, 1/h; s_f – substrate concentration in feeding solution, g/l; Y_{xs} – biomass yield from substrate, g/g; m – biomass maintenance energy, g/g/h; t – process time, h; t_0 – process time at the beginning of fed-batch.

Feeding profile control in several bioreactor experiments differed, depending on a particular experiment phase and the available technical solutions and equipment.

Modeling

Fermentation process modeling, described in the Doctoral Thesis, was performed using mechanistic mathematical models, which are available in the literature [12, 17. 18]. Average parameter values selected from literature available data [3, 5, 12, 16, 19–21, 23–25] were used as initial parameters for modeling of flask and bioreactor experiments. Manual parameter tuning/identification, on the basis of model fitting to experimental data, was performed to improve modeling quality. Identified parameter values are summarized in the chapter "Results".

Model precision was calculated as a sum of root mean square deviations referred to the mean value of experimental data. As a result, the relative deviation of the model from the experimental data (in percent) was gained.

$$x_s = \sqrt{\frac{\sum\left(x_{i,exp} - x_{i,mod}\right)^2}{n}} \tag{2.}$$

$$\bar{x}_{exp} = \frac{\sum x_{i,exp}}{n} \tag{3.}$$

$$r_x = \frac{x_s}{\bar{x}_{exp}} \cdot 100\ \%, \tag{4.}$$

where x_s – modelled component x standard deviation from x; $x_{i,exp}$ – experimentally evaluated x in the point i; $x_{i,mod}$ – modelled x in the point i; n – number of experimental measurements; $\bar{x}_{exp}$ – experimental measurement mean value; r_x – modelled x model relative deviation from experimental data in %.

Equation 4 was used for biomass (r_x), substrate (r_s) and acetate (r_a) modeling precision calculations.

EXPERIMENTAL RESULTS AND DISCUSSION

1.Biomass Optimization Research for *E. coli* BL21 (DE3) pBR327 Cultivation in Flask Scale

E. coli BL21 (DE3) pBR327 biomass optimization research was started in the flask scale to faster become familiar with the system, therefore avoiding unnecessary and lengthy bioreactor experiment conductance. Experiment planning using design of experiments (DOE) was applied to reduce the number of experiments, to achieve the maximal accuracy of the measurements from a limited number of experiments and to find out the optimal conditions of the research process in the flask scale. At the beginning of the optimization, the plan was selected in the way to obtain as much information as possible about the main factors influencing biomass yield. The concentration of the biomass at the end of fermentation process (x_{end}) was selected as an optimization criterion, whose value was maximized (see Fig. 3).

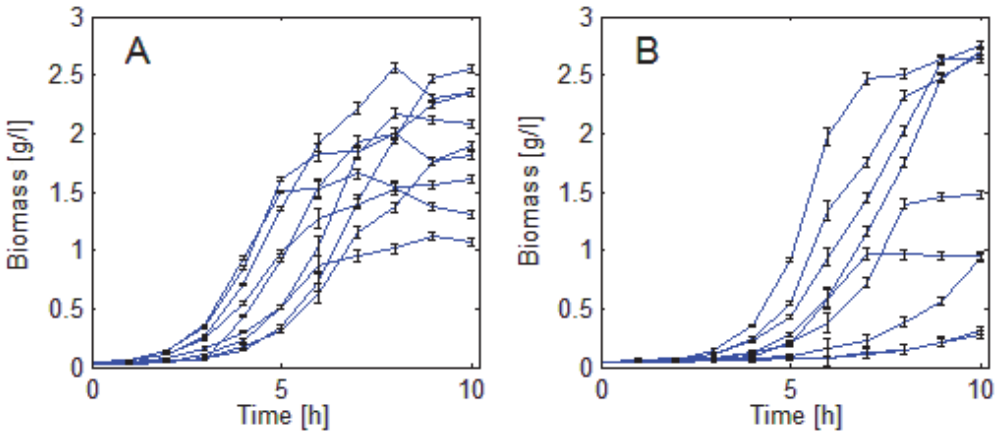

Fig. 3. The dispersion of the biomass outcome during the optimization procedure.
(A) Experimental results from the 1st optimization,
(B) experimental results from the 2nd optimization.

After temperature and pH optimization, the robustness was examined, during which the influence of manipulable parameter deviation on the x_{end} was analyzed. Such uncontrollable parameters as ambient temperature, pressure, humidity, merging of the different raw material batches can potentially influence the result precision. The final task of the experiment series was to mechanistically model the biomass yield depending on the set temperature and pH. The results obtained within optimization procedure are expressed with 2D response surface plot (see Fig. 4). After the performance of the experiment series, the model was obtained and the main factors influencing the model were defined.

After carrying out the 2nd optimization experiment series, the model/equation no. 5 with correlation R^2=0.93 and forecast Q^2=0.39 was obtained. The statistic and measurement experimental error of 0.09 g/l was calculated based on results gained from four experiments (3rd experiment – the central point/spot experiment) at the point T=30 °C, pH=8. However, if regression equation were simplified by excluding the least relevant factors from equation no. 6, the model correlation quality decreased – R^2=0.86, but the forecast quality increased – Q^2=0.67.

$$x = -275.3 - 0.0522T^2 - 3.328pH^2 + 4.1384T + \ \ldots$$
$$\ldots + 54.616pH - 0.0968pH \cdot T \qquad (5.)$$

$$x = -49.215 + 4.0224T - 1.511pH - 0.06328T^2 \qquad (6.)$$

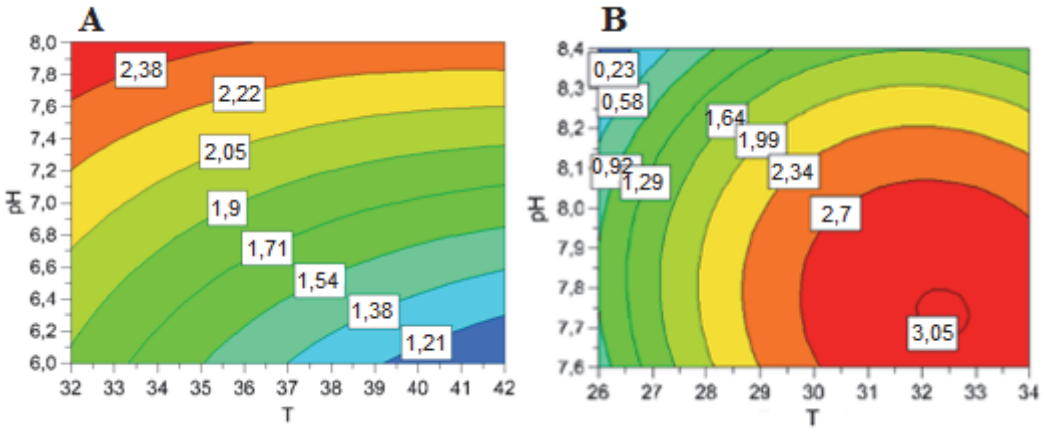

Fig. 4. The results of the biomass optimization shown as a 2D surface response plots. (A) Experimental results from 1st optimization, (B) experimental results from 2nd optimization taking into consideration all regression equation coefficients.

In case if all regression coefficients (equation no. 5) were taken into consideration to obtain regression equation, the maximal forecast of biomass was established – 3.06±0.09 g/l at T = 32.4 °C, pH=7.7. Experimentally at this point, the detected value was 2.92±0.09 g/l, which within error limits corresponds to the modelled value. In the case of simplified regression model (equation no. 6) by excluding the least relevant factors, the maximal forecast of biomass was established – 3.22±0.09 g/l at T=31.6 °C and pH=7.6. In this case, the value gained experimentally was 2.98±0.09 g/l, and within error limits it differed from the predicted value by 1.9 %, but this result was found to be the highest (optimal) biomass yield. If the differences between predicted and practically achieved x_{end} values are compared in percentage, conclusion can be made that the predictability is higher when in the regression model all factors are taken into account. Thereby an increase in forecast coefficient Q^2 did not improve the correlation quality between predicted and experimentally achieved biomass.

2. Cultivation Model and Model Parameter Identification for *E. coli* BL21 (DE3) pBR327 Fed-Batch Process in the Laboratory Flasks

For initial assessment of *E. coli* BL21 (DE3) pBR327 growth parameters and the structure of the mathematical model, which potentially in the best way would describe the biomass and substrate dynamics, separately planned experiments were carried out. The selected mechanical model consisted of four mass balance/equilibrium equations and separate kinetic expressions/equations, which describe the uptake/appearance speed of the most relevant cultivation environment components depending on other component concentration. Selected mass balance equations describe biomass (x), glucose (s), acetate (a) and oxygen (o_2) concentration dynamics as well as the volume (V) change in time.

The structure of the model and determination of its parameters were performed based on information on the modeling of similar processes available in the literature [5]. Four model cases of different complexity were chosen: (a) only the concentration of the substrate (Mono kinetics) in the substrate consumption rate was taken into account [22]; in the second case (b) additionally the effect of synthesized acetate influence on substrate and acetate consumption rate and biomass formation [12]; in the third case (c) the case "b" was supplemented with the influence of the oxygen limit as a competitive substrate on glucose uptake rate [13]; in the fourth case (d) the case "a" was supplemented with the influence of the oxygen limit as a competitive substrate on glucose uptake rate.

Table 1

The Nomenclature of Models Used in the Thesis Depending on the Use of Different Complexity Substrate Uptake Models

Model description	**Substrate consumption models**		
	q_s, K_s	q_{ar}, q_{au}, $K_{i,a}$	q_{O2}, K_{O2}
a	*S* consumption	–	–
b	*S* consumption; *A* formation, inhibition and consumption		–
c	*S* consumption; *A* formation, inhibition and consumption; O_2 limit influence on S		
d	*S* consumption	–	O_2 limit influence on S

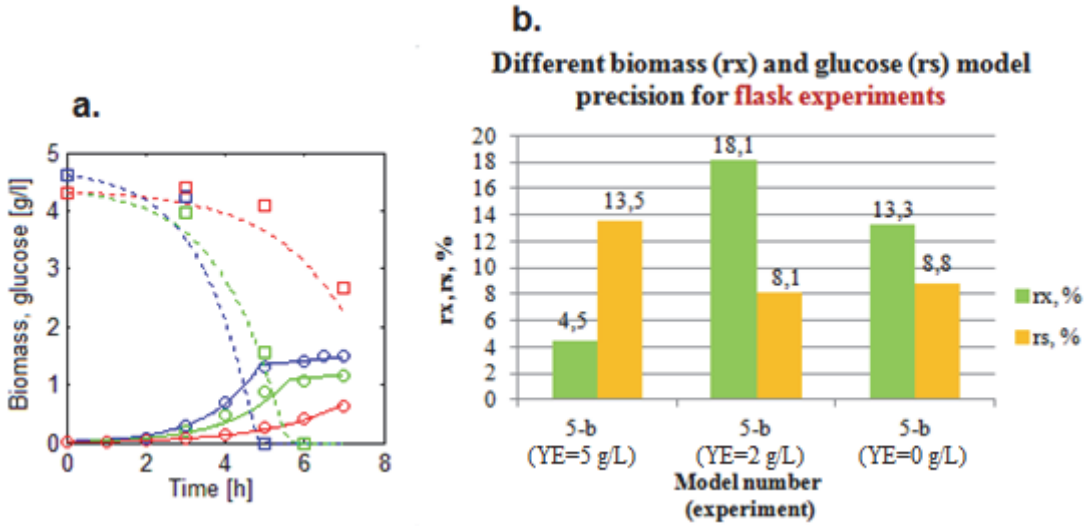

Fig. 5. **Plot a**: the *E. coli* BL21 (DE3) pBR327 biomass growth character under different yeast extract initial concentration conditions; red color denotes the experiment with the yeast extract initial value of 0 g/l, green – 2 g/l, blue – 5 g/l. Experimentally measured parameters: the concentration of biomass (circles) and glucose (squares). Lines correspond to the modelled trajectories. **Plot b**: precision of *E. coli* BL21 (DE3) pBR327 cultivation model 5-b for flask experiments with yeast extract concentration (YE) at the beginning of the process – 5, 2 and 0 g/l.

For investigation of the influence of the yeast extracts on Y_{xs} and q_{sMax}, two additional experiments were performed, where in one of the experiments, the yeast extract was added in a concentration of 2 g/l, and in the other experiment the extract was not added at all. In Fig. 5, the modeling results of these experiments are summarized. The modeling was made with some adjustment of previously identified parameters – Y_{xs}, Y_{xa} and q_{sMax}.

Table 2

Identified Parameter Values for *E. coli* BL21 (DE3) pBR327 Flask Experiments with Different Yeast Extract Initial Concentration, the Comparison with Data Available in Literature (In Bold Indicated Tuned Values)

Param.	Units	References [3, 5, 12, 16, 19–21, 23–25]	YE 5 g/l	YE 2 g/l	YE 0 g/l
Y_{xs}	g/g	0.50	**0.67**	**0.58**	**0.49**
Y_{xa}	g/g	0.41	**0.507**	**0.507**	**0.507**
Y_{xar}	g/g	0.25	0.25	0.25	0.25
Y_{as}	g/g	0.832	0.832	0.832	0.832
q_{sMax}	g/g/h	1.69	**2.29**	**2.29**	**1.49**
q_{aMax}	g/g/h	0.153	0.153	0.153	0.153
q_{sKr}	g/g/h	1.13	1.13	1.13	1.13
K_s	g/kg	0.081	0.081	0.081	0.081
K_a	g/kg	0.05	0.05	0.05	0.05
K_{O2}	g/kg	$3.2*10^{-6}$	$3.2*10^{-6}$	$3.2*10^{-6}$	$3.2*10^{-6}$
$K_{i,a}$	g/kg	10.82	10.82	10.82	10.82
m	g/g/h	0.024	0.024	0.024	0.024

The present research showed that the yeast extract had a significant influence on parameters Y_{xa} and q_{sMax}, which should be taken into account for precise process modeling. During the selection of parameters, the value of Y_{xa} was increased. The total precision of biomass and substrate modeling for the described flask experiments (YE 5.2 and 0 g/l) was in the range of 4.5–18.1 % and 8.1–13.5 %, respectively. Identified model parameter values are summarized in Table 2.

3. Cultivation Model and Model Parameter Identification for *E. coli* BL21 (DE3) pBR327 Fed-Batch Process in the Bioreactor

For the calculation of fed-batch profile, as well as for model predictive feeding profile control in the bioreactor process, the mathematical model identification is necessary. For the identification of bioreactor fermentation model, initially identified model and model parameters from flask experiments were used. Further model and model parameter re-identification were made to improve the comprehensive acetate influence counting model. Further, the mathematical model of a simplified and more universal fermentation process was identified to be applied for model predictive fed-batch control (MPC).

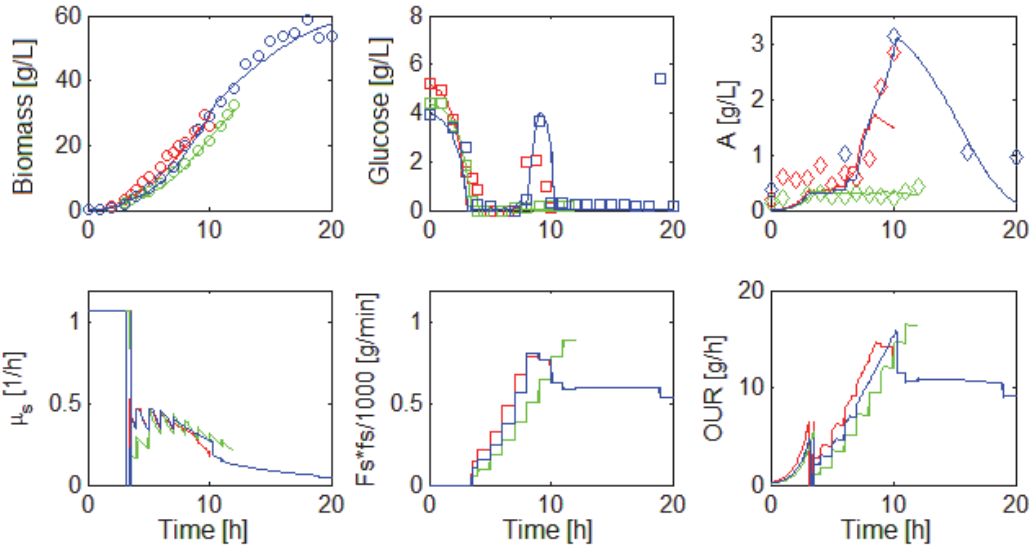

Fig. 6. *E. coli* fed-batch process and modeling results taking into account acetate formation and re-consumption. Exp. a-1 (red), exp. a-2 (green), exp. a-3 (green). Experimental measurements (circles, squares, rhombs), modelled parameters (lines).

For feeding rate profile calculation for the first "a" series experiments, the necessary parameters Y_{xs} and m_s were identified from previously made flask experiments; rest of the parameter values used in modeling are shown in Table 3. The modeling of culture growth with calculated feeding profile initially was performed with model "a" (see Table 1) obtained from the flask experiments due to its highest precision. This model was supplemented with the yeast extract influence on Y_{xs}, as well as with the glucose concentration inhibiting influence on glucose consumption. Due to pO_2 control around 40 % from the maximal O_2 saturation, the pO_2 influence on glucose and acetate oxidative uptake was restricted to the minimum.

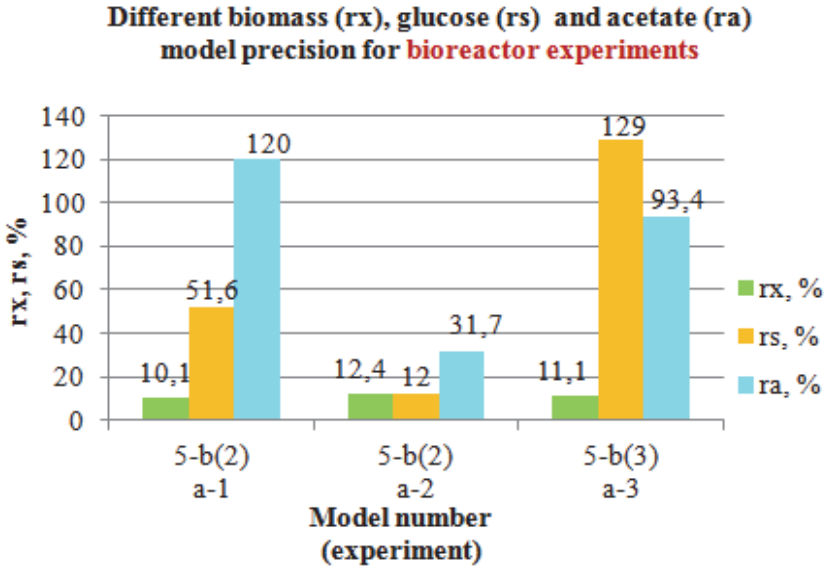

Fig. 7. Comprehensive *E. coli* BL21 (DE3) pBR327 cultivation model improvement results from fed-batch bioreactor "a" series processes.

The process a-1 was performed with μ = 0.5 l/h to examine the possible synthesis of the acetate. Using this kind of fed-batch profile, the accumulation of the acetate

occurred till 2.9 g/l. It was examined that in the experiments a-2 and a-3, after 8 h (x = 14.5 g/l, OUR~9 g/h) and 6 h (x = 10.0 g/l, OUR~9 g/h), respectively, of reaching the maximal stirrer speed (800 rpm), the oxygen transfer rate (OTR) limit was reached.

The obtained similar experimental results by performing control of similar processes a-1 and a-3 show good process and analytic measurement repeatability (see Fig. 6). From the experiment a-3, it is evident that after start of the automatic F_s control according to pO_2 signal (~10^{th} hour of the process), which more limits the amount of available substrate in the culture, the culture begins to uptake the synthesized acetate. The maximal biomass concentration of 58.6 g/l in such a process was obtained. The maximal acetate concentration of 3.13 g/l was observed. The identified value parameters are summarized in Table 3. Identified model precision for experiment a-3 was 11.1 % for biomass, 129.0 % for glucose and 93.4 % for acetate (see Fig. 7).

Table 3

Values of the Identified Comprehensive Model Parameters for Bioreactor Experiments; Comparison with Available Parameters from the Literature (In Bold Indicated Tuned Values)

Parameter	Units	Ref. (see Table 1)	Identified param. for. *E. coli* BL21 (DE3) pBR327 bioreactor process
Y_{xs}	g/g	0.50	**0.42**
Y_{xa}	g/g	0.41	0.41
Y_{xar}	g/g	0.25	**0.1**
Y_{as}	g/g	0.832	**0.667**
q_{sMax}	g/g/h	1.69	**1.49**
q_{aMax}	g/g/h	0.153	**0.140**
q_{sKr}	g/g/h	1.13	**1.19**
K_s	g/kg	0.081	**0.005**
K_a	g/kg	0.05	**3.41**
$K_{i,a}$	g/kg	10.82	**3.45**
m	g/g/h	0.024	**0.037**
Additional parameters for the bioreactor model			
Y_{xRE}	g/g	–	**0.504**
Y_{bx}	g/g	–	**0.001**
t_{RE}	h	–	**3.5**
$K_{x,au}$	g/kg	–	**27.21**
$K_{i,s}$	g/kg	55.15	**60**
$K_{r,a}$	g/kg	3.6	**0.001**
q_{sKr1}	g/g/h	–	**1.52**

Simplified and more universal fermentation process model for application in the model predictive controller (MPC) can be applied [6]. In such a model actual biomass growth and substrate uptake behavior in the running process should be monitored and re-identification of the parameters Y_{xs} and q_{sMax} should be made.

Further experiments of "b" series were carried out, where the previously modelled and selected reference feeding profile model predictive control depending on the manual biomass and glucose analysis was made. Modeling of these series experiments was performed with the simplified model, which did not take into account the formation and re-consumption of acetate and its influence on the biomass growth; it was assumed that the substrate uptake was only influenced by concentration of glucose (Mono kinetics) and inhibition effect of glucose. The obtained experimental and modeling results are shown in Figs. 8 and 9. From the experimental data it can be seen that the modeling quality for biomass is rather high in exponential growth phase. After the exponential growth phase, the precision of the model is considerably lower. The mean modeling precision of the biomass (r_x) for "b" series experiments is 29.1 %. The mean modeling precision of the glucose concentration in a complete range (r_s) is 100.1 %, which can be evaluated as satisfactory, as a similar result was obtained with fermentation process model described in the previous chapter. The identified fermentation model parameters for "b" series experiments are summarized in Table 4

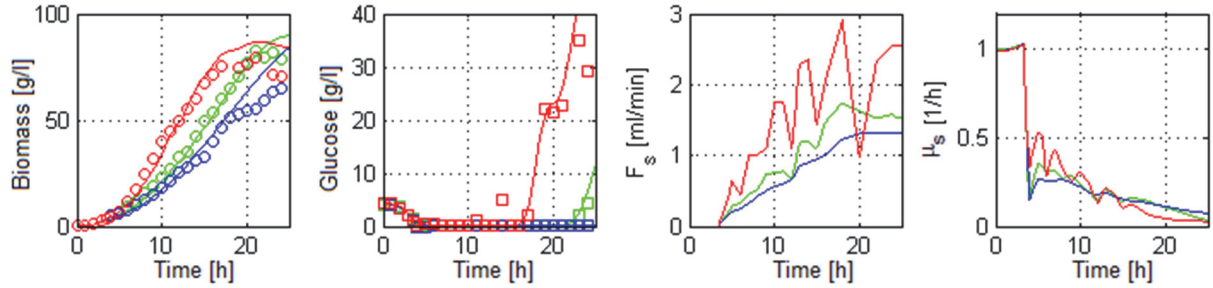

Fig. 8. *E. coli* BL21 (DE3) pBR327 "b" series fed-batch process and modeling results, where a simplified fermentation process model was used. Exp. b-1 (blue), exp. b-2 (green), b-3 (red). Experimental measurements (circles, squares), modelled parameters (lines).

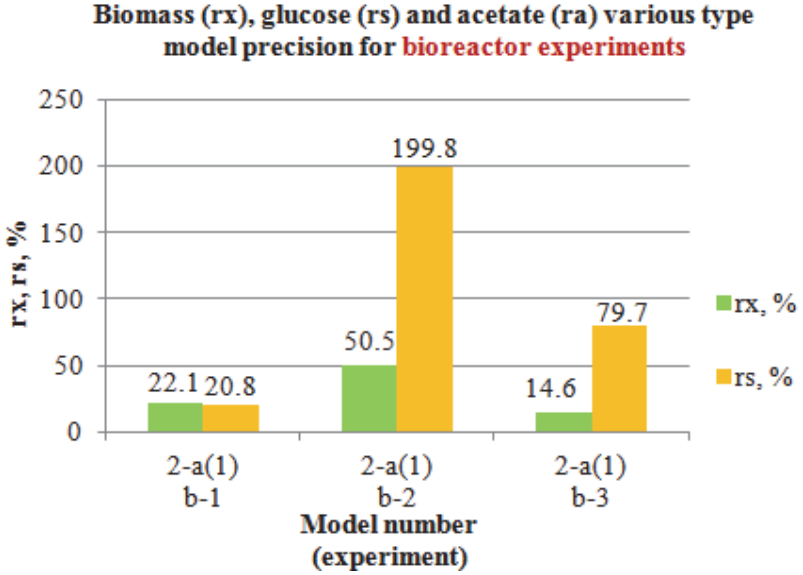

Fig. 9. Simplified *E. coli* BL21 (DE3) pBR327 cultivation model improvement results from fed-batch bioreactor "b" series processes.

Table 4

Values of the Identified Simplified Model Parameters for Bioreactor Experiments; Comparison with Available Parameters from the Literature (In Bold Indicated Tuned Values)

Parameter	Units	Ref. (see Table 1)	Identified param. for. *E. coli* BL21 (DE3) pBR327 bioreactor process
Y_{xs}	g/g	0.50	**0.55**
q_{sMax}	g/g/h	1.69	**1.15**
K_s	g/kg	0.081	**0.005**
Additional parameters for the bioreactor model			
Y_{xYE}	g/g	–	**0.45**
Y_{bx}	g/g	–	**0.001**
t_{YE}	h	–	**35**
$K_{i,s}$	g/kg	55.15	**30**
K_{xMax}	g/kg	86	**97.5**

4. Advanced Feeding Rate Control according to pO_2 Signal for *E. coli* BL21 (DE3) pBR327 Process

After more detailed (acetate) model and model parameter identification, pO_2 cascade control PID parameter tuning from preliminary experiments, calculation of desirable feeding profile for desirable biomass growth (X), substrate (S) and acetate (A) trajectory achievement, and test experiment were performed (see Fig. 10). The aim of the test experiment was to control the second part of the process as much as possible closer to the pre-defined X, S, A and pO_2 conditions using feeding rate control according to the pO_2 signal. The substrate feeding rate control according to pO_2 signal was selected to verify this control principle, which was being widely used industrially due to the achievable lower O_2 mass transfer rates in comparison with laboratory or pilot scale bioreactors.

The online measurements represented as lines.

The particular method is convenient for proving a limited substrate fed-batch process in the second phase, when a precise calculation of the substrate feeding rate is complicated due to the explicit complexity of the process conditions in the process second phase. It should be noted that using this principle, the specific growth rate of microorganisms is limited (reduced) in the phase of automatic feeding rate control according to pO_2 signal, as the substrate feeding rate is reduced according to the culture available O_2.

In the controllable range of the complete process (3–19 h), pO_2 control quality of 39±8 % was achieved, which was close to the set value 40±5 %.

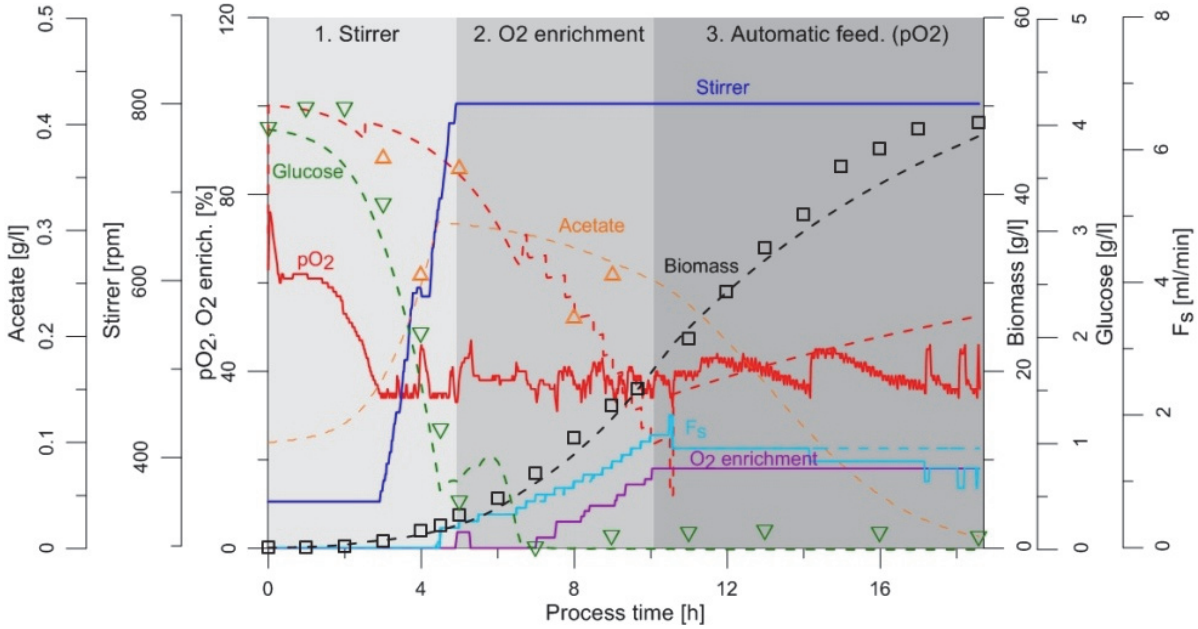

Fig. 10. *E. coli* BL21 (DE3) pBR327 fed-batch test experiment of pO_2 cascade control assessment. Analytic measurements for biomass, glucose and acetate represented as symbols (□, ▽ and △, respectively); modeling results represented as dashed lines.

The optimized control strategy gave a stabile biomass growth without considerable accumulation of acetate in fed-batch phase. At the end of the process, the biomass concentration of 48.15 g/l was achieved and the concentration of acetate during the complete process did not exceed 0.37 g/l. Dissolved oxygen partial pressure (pO_2) cascade control functioned well, and pO_2 was controlled very close to the preset range. Automatic fed-batch phase ensured robust pO_2 control and minimal optimized feeding rate profile reduction, by ensuring the predicted parameter of process productivity (amount of the biomass) in technologically defined range.

5. Advanced Model Predictive Feeding Rate Control for *E. coli* BL21 (DE3) pBR327 Process

The mathematical model identified in the 3rd chapter was further used for model based feeding rate control (MPC). For this purpose, the experiment series "c" was carried out. In the present research it was shown methodologically, how a particular principle can be applied for desirable reference feeding/biomass growth profile evaluation. Due to accumulation of the experimental data, it is possible to more precisely identify model parameters. After starting the processes with MPC, automatic correction of selected initial feeding profile takes place in order to follow the process biomass XV_{proc} trajectory as it was calculated (modeled) for the reference XV_{ref}.

Due to slightly varying process initial biomass (X_0) and glucose (S_0) concentrations and sub-optimal process control, culture growth profile in the process batch phase (before fed-batch start) will vary even when identical feeding rate profile is applied. This means that in such a process time will vary in which glucose available from batch solution will be consumed and when fed-batch should be started. In this case in order not to limit or overfeed the culture, one should shift pre-set fed-batch start time to earlier or later time.

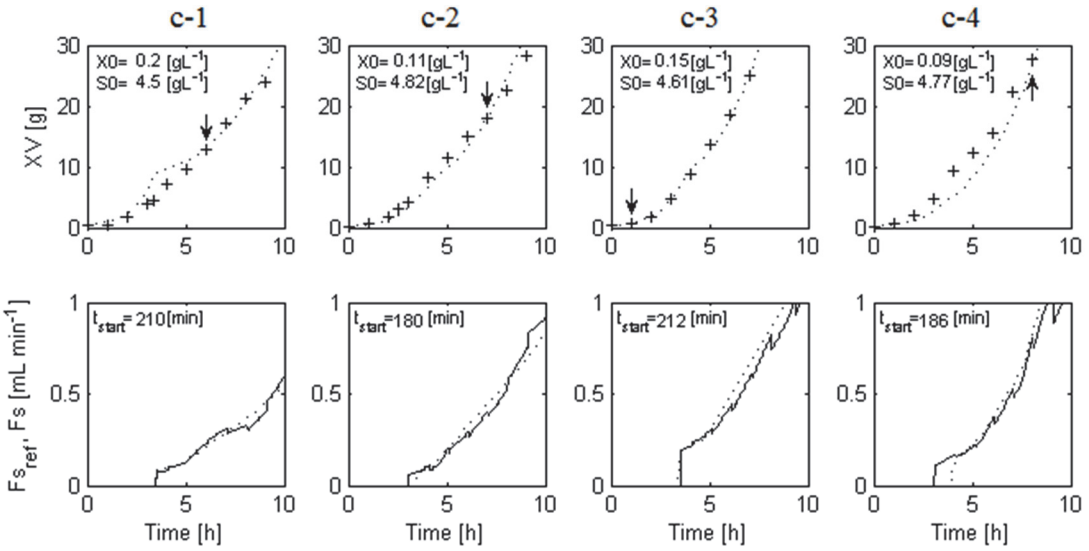

Fig. 11. Process initial conditions (X_0 and S_0) and feeding start times (t_{start}) (below) for processes EXP-1 – EXP-4. (– –) set (modeled) reference profiles, (+) estimated, (–) applied feeding rate, (↓) indication of time moments when biomass reached set references.

Similar X_0 and S_0 parameters for c-1 and c-3 (see Fig. 11) resulted in the same fed-batch start at the process time of 210–212 min. Similar initial conditions were obtained for c-2 and c-4. In c-2 and c-4, initial X_0 was lower and S_0 was higher compared to c-1 and c-3. Nevertheless, the faster biomass growth led to earlier fed-batch mode initiation at the process time of 180–186 min. Time points when the process biomass has reached the set reference values are indicated with arrows in Fig. 11.

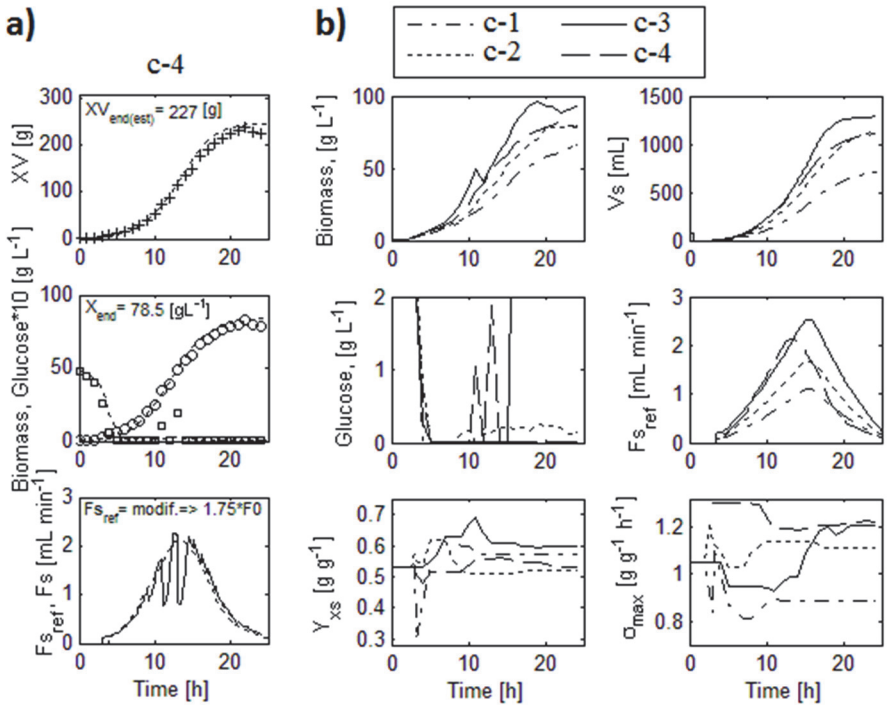

Fig. 12. Biomass amount control results (c-4): summarized overview of parameter value evolution in experiments c-1–c-4. a) (– –) Set (modeled) reference profiles, (+) estimated on-line, (▫) off-line glucose, (○) off-line biomass, (–) applied feeding rate. b) Comparison of experiments c-1 – c-4. Lines for biomass, glucose, Y_{xs} and σ_{max} are drawn and interpolated between off-line analytical measurements made every 1–2 h.

For „c” experiment series feeding profiles yielding biomass exponecial growth in range of 0.15–0.50 1/h, were selected. Figures 12 and 13 show “c” experiment series results. Estimated biomass amounts and concentrations in the process end for the experiments c-1 – c-4 were 157 g (65.8 g/l), 225 g (79.8 g/l), 290 g (93.2 g/l), and 227 g (78.5 g/l), respectively. Estimated biomass ($XV_{end(est)}$) and experimentally measured ($XV_{end(exp)}$) differences ($d_{end(est)}$, $d_{end(exp)}$) from the reference profiles (XV_{ref}) in the process end (24 h) were 4.6 and 3.8 %, respectively. Smaller XV_{est} deviations from the reference profiles were achieved at 20 h of the process where the mean difference was 3.0 %. This can be explained by the fact that the model has not precisely modeled the late biomass stationary growth phases. To model this stage with higher precision, a more complex process model is required taking into account potential (by) product (acetate, protein, etc.) [17, 26], formation and biomass physiological state (cell starvation, ratio of viable cells, etc.) [27]. Obviously, precision of the selected (modeled) culture volume reference profile has an impact on $d_{end(est)}$. Higher numerical value of $d_{end(est)}$ compared to $d_{end(exp)}$ indicates that biomass estimation quality could be improved by more precise volume on-line estimation. Additionally, it can be seen from the results of c-4 that model parameters used for reference profile selection were not optimal for the case where excessively increased feeding profile was chosen for exponential growth phase. Taking into account the initial conditions (X_0 and S_0) of the c-4, model does not reflect the observed significant glucose accumulation at 11 process hour.

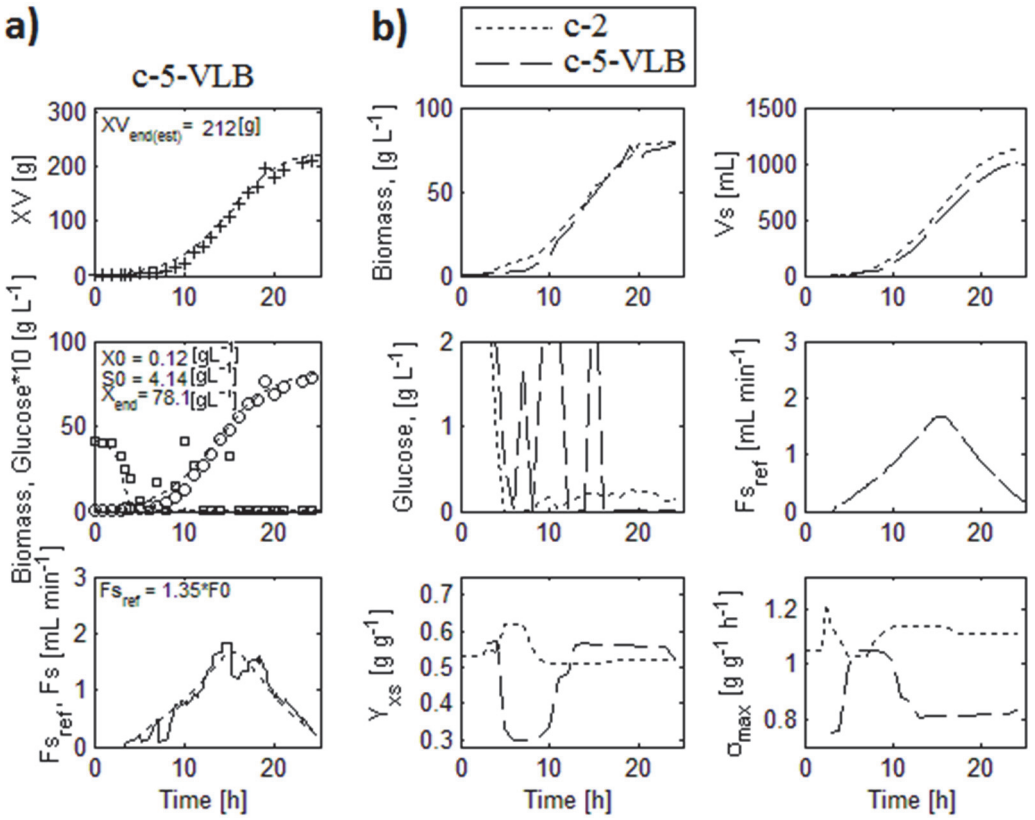

Fig. 13. Biomass amount control results (c-5-SUB) in a single-use reactor and its comparison with c-2. a) (– –) Set (modeled) reference profiles, (+) estimated on-line, (▫) off-line glucose, (○) off-line biomass, (–) applied feeding rate. b) Comparison of experiments c-5-SUB and c-2. Lines for biomass, glucose, Y_{xs} and σ_{max} are drawn and interpolated between off-line analytical measurements made every 1–2 h.

From Y_{xs} and σ_{max} adaptation results, additional useful information may be extracted. If a brief overview of biomass production process is made, biomass yield on substrate could become an indicator, how efficient the main carbon source is converted into biomass. Higher yield potentially indicates that smaller amount of feed is wasted for undesirable by-product synthesis. Starting from 8–9 hour of the process, some correlation could be observed between the estimated Y_{xs} trends for explorative experiments (see Fig. 12, b). It is interesting that the highest yield at the end of the process was observed in the experiments with the slowest and fastest feed rate profiles, an average of 0.57 g/g for EXP-1 and 0.60 g/g for EXP-3. Relatively small difference of the yield between EXP-2 and EXP-4 was observed, 0.52 g/g and 0.54 g/g, respectively. It correlates well with the biomass at the end of the process (231 g and 247 g) taking into account that substrate feed volume (V_s) was practically the same for these experiments at the end of the process. The estimation results for σ_{max} show that the values have fluctuated within the range of 1.05±0.2 g/g/h. The variations may be related to different substrate limitation conditions within the performed experiments.

For the MPC control approach comparison on the reactors of different configuration, test run in a single-use lab bioreactor was carried out (see Fig. 13, a un b). For this experiment, the same reference profiles as in c-2 were chosen to maintain culture growth without critical glucose accumulation and at the same time to achieve comparatively good biomass yield. Initial biomass and sugar concentrations were similar to c-2. Nevertheless, possibly due to different temperature controller PID parameter tuning (till the 6 process hour) in order to compensate differences between two reactors (mainly because of different material and specific area of heat exchange element), frequent temperature oscillations of 36.8±0.9 ºC occurred at the initial phase of the process. This most probably led to longer batch lag phase (by 3–4 hours) and much lower process biomass at this stage. Despite this fact, the culture was able to recover and closely reached reference biomass at 17 h (see Fig. 13, a). Feeding phase started at 210 min. Biomass of 212 g (78.1 g/l) was reached at the end of the process. Biomass estimate ($d_{end(est)}$) deviation from pre-set reference at the end of the process was 4.3 %. Glucose concentration did not exceed 4.1 g/l.

Model-based evolution of feeding profile and model-predictive control were demonstrated on recombinant *E. coli BL21* biomass cultivation processes in conventional and single-use bioreactors. Best match to the reference profiles was observed in the processes without significant glucose accumulation and in all processes until the beginning of biomass stationary growth phase around 20 h of the process. Estimated biomass mean deviation from the pre-set references was 4.6 % at the end of the processes. It was close to the experimentally measured (3.8 %). By means of sequentially performed experiments, it was possible to test the feeding profiles of different scale and shape, maintaining exponential growth with specific growth rates of 0.2, 0.3 and 0.45 ±0.05 h^{-1}. In this way, by performing the feeding profile modification, the so-called desirable “golden batch” feeding profile may be achieved and selected as a reference for the particular process of interest. Successful test experiment in the single-use laboratory bioreactor was performed. Biomass estimate ($d_{end(est)}$) deviation from the pre-set reference was 4.3 % at the end of the process for this experiment.

The implemented process control and its performance may be additionally improved. More specific process model adapted for *E. coli*, for example, taking into account by-product (protein, acetate, etc.) synthesis/consumption and information about biomass physiological state (cell starvation, ratio of viable cells, etc.), may enhance precision of the process modeling. Improved accuracy of volume estimation by means of direct weighing of solution bottles or reactor may also improve the precision of biomass estimation and its subsequent control. The proposed approach has been implemented in a commercially available bioreactor system (JSC «Biotehniskais Centrs») and is intended for a wide range of potential users.

CONCLUSIONS

1. Research on the feeding rate control in fermentation processes has shown that for ensuring this procedure with high biomass yield obtainment, it is required to use fermentation process modeling and advanced feeding rate control method, where the determination of the biomass is still limited because of the availability and the lack of appropriate analytical and instrumental tools.
2. From the flask experiments it is possible in relatively short time to evaluate the nature of *E. coli* BL21 (DE3) pBR327 growth, the biomass measurement precision and the fermentation process initial model, which further has successfully been used to calculate the feeding rate for experiments in the bioreactor. In the chosen cultivation medium, the maximal achievable biomass is 3.5 % from the optimized biomass yield from the bioreactor.
3. The maximal biomass yield of 93.2 g/l has been obtained in the bioreactor fermentation process with the accumulation of a certain amount of substrate in the culture. In this process, the specific biomass growth rate (μ) does not exceed 0.45 1/h. The evaluated desirable biomass concentration till 80 g/l for hepatitis B core-antigen (HBcAg) obtainment with biomass growth rate is not higher than 0.40 1/h.
4. Different complexity mathematical models and model parameters have been identified for biomass, substrate and acetate concentration modeling for *E. coli* BL21 (DE3) pBR327 fermentation process. Research has shown that a more complex mathematical model gives similar substrate and 2.6 times better biomass modeling quality compared to a simplified model.
5. At the end of the process, with controlling feeding rate according to pO_2, in the whole control region, stable pO_2 and substrate control has been achieved. In such a process, the achieved biomass is 0.63 times less than the maximally achieved one. Applying a particular feeding strategy, it is possible to provide safe, un-overfed *E. coli* BL21 (DE3) pBR327 cultivation process. However, the influence of reduced biomass yield on HBcAg at the end of the process should be taken into account.
6. Model based predictive feeding rate control (MPC) system has been developed and approbated for *E. coli* BL21 (DE3) pBR327 reference feeding and biomass profile selection, as well as for automatic feeding start, feeding rate and biomass growth rate control in the process. Advanced feeding rate control methods have been implemented and approbated in the commercially available bioreactor systems (JSC "Biotehniskais Centrs").

LIST OF PUBLICATIONS

Publications in Scientific Journals

1. Grigs O., Galvanauskas V., Dubencovs K., Vanags J., Suleiko A., Berzins T., Kunga L. Model predictive feeding rate control in conventional and single-use lab-scale bioreactors: a study on practical application // Chem. Biochem. Eng. Q. 2016, Vol. 31(1): 75–88.
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