Control Strategies of a Fed-Batch Production of Sterols in Yeasts

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ABSTRACT: Sterols form a large group of organic compounds occurring in plants, in fungi and in animal cells. Main sterol in yeast cells is ergosterol. The aim of our research was 1) to study conditions and factors affecting ergosterol and accompanying sterols formation in fed-batch culture of industrial strain *Saccharomyces cerevisiae* deposited as D7 in culture collection of DFCB-ICTP, 2) on the basis of knowledge analysis to create and to verify a simple mathematical model with the objective of optimising feeding of nutrients, 3) to apply and to verify physiological control based on knowledge of current physiological state of the culture. Experiments were carried out in the laboratory bioreactor unit New MBR equipped with the knowledge-based control system BIOGENES© created at the Department of Computing and Control Engineering. We have tried and experimented with two different approaches: The first approach was based on unstructured, unsegregated mathematical model of the process. For the control of the whole cultivation run the CO_2 profile in exit gas was computed from the prespecified desired specific growth rate with the aid of this mathematical model. Results of these experiments were repeatedly used as a knowledge base for creation of an unstructured mathematical model. Model consists of 14 equations describing rate equations of main components. From simulation procedures several control strategies were derived mainly for biphasic system (growth and stationary phase) varying concentration of ethanol, specific growth rate (controlled by CO_2). The second approach was based on so called physiological control in which the current physiological situation (mainly metabolic state) of the culture is inferred on-line and an appropriate control strategy is selected and a new value of the manipulated or set-point variable is computed. Results of both two different control approaches are similar, but the physiological approach could be more robust than that using the mathematical model. From many conclusions derived from our investigation there are few which can be used for optimisation of ergosterol production and minimisation of unwanted sterols. Industrial verification is in progress.

KEYWORDS: Fed-batch cultivation, *Saccharomyces cerevisiae,* Ergosterol biosynthesis, Mathematical model of, Knowledge-based control of .

I. INTRODUCTION

Sterols occur in membranes of eucaryotic cells (e.g. fungi, plant and animal cells). The most important sterol is ergosterol, the formation of which is dependent on many external and internal factors. Baker´s yeasts belonging to yeast genera *Saccharomyces cerevisiae* can under certain cultivation conditions increase the content of intracelularly formed-sterols in comparison with other yeast genera. Concentration of yeast sterols changes

significantly when changing extracellular or intracellular conditions which lead to physiological stress. Sterols bound in membranes affect membrane fluidity, permeability, activity of membrane enzymes and growth rate [Arnezeder and Hampel ,1990]. Full description of sterol biosynthesis, sterol mutual changes and their kinetics in yeasts have not been published so far. Cultivation of sterol can be controlled with respect to above mentioned facts. Generally it could be any type of cultivation which favours ergosterol enrichment. Our study is based on fed-batch cultivation technique which is from practical point of view easy to run and control. Most of the control methods for fed-batch process already published are based on controlling the main values of micro-organism state. Parameters such as dissolved oxygen and ethanol concentrations, respiration quotient (RQ) are mostly maintained constant [Arnezeder and Hampel ,1990] , another variable - carbon dioxide concentration in gas phase must form an increasing function due to increasing biomass concentration. Control systems well known for baker´s yeast production and some new concepts were subject of our presentation on CHISA´96 [Rychtera et al. 1996]. Strain of yeast *Saccharomyces cerevisiae* producing higher amount of intracellular sterols was provided by yeast factory and was marked as strain D7. It belongs similarly as baker´s yeasts, from which it was constructed, to facultative anaerobic micro-organisms which possess a typical character of response to lack of oxygen and to excess of carbohydrate under conditions of optimal aeration. The former phenomenon is known as an alcoholic fermentation and the latter as Crabtree effect [DeDeken, 1966]. Under the condition of Crabtree effect (characterised by a critical glucose concentration) there appears an accumulation of ethanol. This phenomenon has been studied and described by many authors since its first disclosure in 1929 [Crabtree,1929]. From various controlled variables we selected those which are directly or indirectly linked with accumulation of sterols [Rychtera et al., 1998]. Manipulated variable was always medium flow rate. External controlled variables were: ethanol concentration, dissolved oxygen concentration, carbon dioxide concentration in exit gas phase and respiration quotient (RQ). Internal controlled variable was specific growth rate. Keulers [1993] already stressed importance of the specific growth rate for the control strategy of yeast growth but to overcome the problem of this non-measurable variable he suggested a simple observer developed on the basis of on-line measurement. This observer is able to estimate the specific growth rate and the cell concentration. In our experiments we used a model approach and its simulation. Several initial laboratory experiments and number of industrial production runs served for the first model identification. Simulation gave us feeding profile calculated from the simulated specific growth rate values. Real controlled variable in this case was, however, ethanol or CO_2 concentrations. Influence of medium composition was also tested especially for the sake of significant impact of the C/N ratio. In our paper presented on the CHISA´96 [Rychtera et al., 1996] and CHISA´98 [Rychtera et al., 1998] conferences we described comparison of several control strategies based on ethanol, dissolved oxygen , carbon dioxide concentrations and on specific growth rate (controlled variables) for the simple growth of yeasts where these parameters were either set constant or variable.

II. EXPERIMENTAL

II. 1 BIOREACTOR, MONITORING AND CONTROL

The laboratory fermentation unit used for experiments consists of a laboratory bioreactor with a working volume of 5 l (produced by new MBR Switzerland) equipped with an analogue control unit IMCS 2000 and a Pentium 166 MHz-based computer for control purposes. The analogue control unit is used to stabilize the environmental conditions: bioreactor temperature, pH of the medium, frequency of stirrer revolutions, the flow of air and the level of foam. The unit accepts set-points for the first four variables from the computer. The dissolved oxygen tension is also measured by an oxygen probe from Metler Toledo. For supplying cultivation medium to the bioreactor a DP 200 peristaltic pump from New Brunswick Scientific is used. SERVOMEX Type 1100A and 1400B analyzers with back pressure compensation and regulation and a Baldwin gas sample conditioner are used for measurement of the oxygen and carbon dioxide concentrations, respectively, in the outlet gas. The ethanol concentration in the outlet gas is measured continuously by an METREX instrument (constructed by ICT Prague) and biomass concentration is measured on-line every 5 minutes using CELLEX analyzer (constructed by ICT Prague, Dept. of Computing and Control Engineering). For connecting the computer to all these instruments Advantech data acquisition and control cards were used as an input/output system.

The knowledge-based control system named BIOGENES© was created from the commercial control package Genesis for Windows 3.0 (GFW) from Iconics and expert system CLIPS 6.04 for the PC-Windows platform. Linking both systems, we made use of the Visual Basic compatible scripting language provided with GFW and integrated the 16 bit CLIPS DLL via the CLIPS VB interface DLL into GFW as a periodic application script (1 min period).

Standard process control tasks (data acquisition, data logging, alarming, visualization, direct digital control, etc.) of BIOGENES© are implemented by the individual GFW modules:

- Application Control Strategy executed by the GFW Real Time Server (data acquisition, calculation of physiological variables, direct digital control)
- GraphWorX (visualization, MMI)
- AlarmWorX (alarming)
- TrendWorX (trending, data logging)
- GFW scripting (GFW-CLIPS interface, supporting tasks)

II.2 MICRO-ORGANISM

Yeasts *Saccharomyces cerevisiae D7* are industrial strain prepared as an UV mutant for ergosterol biosynthesis.

II.3 MEDIUM

In order to standardise all experimental conditions at institutions involved in project solution original molasses medium was replaced by optimised synthetic medium consisting of glucose, yeast extract (DIFCO), ammonium sulphate, potassium dihydrogen phosphate, magnesium sulphate, calcium chloride and tap water.

II.4 CULTIVATION STRATEGY

Cultivation is characterised by controlled feeding. Two cultivations, each using a different control approach, will be presented in more detail.

Case A: The whole 24 hour cultivation was divided into two parts differing from each other by values of the specific growth rate (μ). The first part is characterised by constant and higher value of μ (0.05 - 0.15 h^{-1}), in our model case μ = 0.075 h^{-1}) and the second part by lower constant or linearly decreasing value of μ (changing from the upper value to zero). In the first 24-hour experiment the cultivation was controlled by a digital PID controller to track a CO_2 profile in exit gas calculated from a desired specific growth rate profile using an unstructured and unsegregated mathematical process model. After the 1-hour start-up phase where the substrate flow rate was constant the CO_2 PID control was applied to manipulate the substrate supply. The CO_2 PID controller closely tracked the CO_2 set-point profile divided into 12 linear segments except for the initial 3 hours when oscillating (low biomass concentration being the probable cause) and the final 3 hours when the process behaviour diverged from the simulation results due to limited validity of the mathematical model (Fig. 1).

Case B: In the second 24-hour experiment the principle of physiological control has been applied. The cultivation began with an 1-hour start-up phase in which the culture crossed from the initial purely oxidative metabolic state to the border state between the purely oxidative and the oxidative-fermentative metabolism. The substrate flow rate was held constant and increased only once to overcome a stagnation of RQ in the start-up phase. In the subsequent growth phase lasting for 15 hours the substrate feed rate was adjusted by the operator to keep the culture in the slight oxidative-fermentative metabolism characterised by RQ between 1.10 and 1.30 and a low level of ethanol production. The operator based his decisions on the advice and the information about the current metabolic state inferred on-line by the knowledge-based control system. Consequently in the end of sterol production phase the substrate feed rate was kept constant at a low level and stopped in the 21st hour after a total depletion of ethanol. (Fig. 2).

II.5 MATHEMATICAL MODEL

The mathematical model describing the growth, substrate consumption, ethanol and other intracellular component production is based on two papers that were published earlier [Behalová et al., 1986; Sobotka et al., 1982]. Contribution to the bioengineering analysis of the model is based on mass balances of perfectly mixed and aerated fed-batch bioreactor and allows to simulate the time dependence of biomass, sugar, dissolved oxygen, ethanol concentrations in the broth, the time course of intracellular concentration of sterol, protein and polysaccharide fraction, and concentration of carbon dioxide and oxygen in fermentation gases on feeding and aeration rate, including the effect of dose of antifoam agent on aeration capacity of the bioreactor.
As shown in the analysis of the fed-batch fermentation of baker´s yeasts described in the PhD thesis of Paulová [1999], the effect of catabolic repression by the sugar concentration on the growth on ethanol can be for most cases neglected in a fed-batch culture. Several assumptions allow to simplify the original kinetic model to the last version:

The results of the process analysis were summarised by following equations.

I. generation

Specific growth rate on sugar:	μ_1	=	$k_1 S/(k_2 X+ S)$	(1)
Specific growth rate on ethanol:	μ_2	=	0.0	(2)
Specific rate of ethanol formation rate:	r_e	=	$k_3\mu_1$	(3)

II. generation

Specific growth rate on sugar:	μ_1	=	$k_1 S$	(1´)
Specific growth rate on ethanol:	μ_2	=	$k_2 E$	(2´)
Specific rate of ethanol formation rate:	r_e	=	$k_3\mu_1$	(3´)

Applying the mass conservation law in the form of differential mass balances the dynamics of the process can be described as follows:

Balance of ethanol (E):

$$dE/dt = r_e X - Y_{E/X} \mu_2 X - F E/V \quad (4)$$

Balance of biomass concentration (X):

$$dX/dt = \mu_1 X + \mu_2 X - F X/V \quad (5)$$

Balance of sugar (S):

$$dS/dt = -Y_{S/X} \mu_1 X + F (S^o - S)/V \quad (6)$$

Balance of dissolved oxygen concentration (c - mg/L):

$$dc/dt = k_La (y_{O2} P/H - c) - 1000 [Y_{O/X} (\mu_1 + \mu_2) + m_O] X \quad (7)$$

Balance of oxygen in the gas phase as molar fraction (y_{O2}):

$$\varepsilon\, d y_{O2}/dt = P V_G /(RT) [0.21 - y_{O2}]/V - k_La (y_{O2} P/H - c)/32000 \quad (8)$$

Balance of carbon dioxide in the gas phase as molar fraction (y_{CO2}):

$$\varepsilon\, d y_{CO2}/dt = - P V_G /(RT) y_{CO2} /V + [Y_{CO2/X} (\mu_1 + \mu_2) + m_{CO2}] X/44 \quad (9)$$

Balance of fermenter volume (V):

$$dV/dt = F \quad (10)$$

$$k_La = 32000 P V_G (0.21 - y_{O2})/[(RTV)(y_{O2} P/H - c)] \quad (11)$$

This equation can be used for rotation speed 600 (min^{-1}) and airflow rate 3-5 (L/min) with medium volume ranges from 3.5-5 L.

Intracellular balance of egosterol (x_e is expressed as mass fraction of biomass):

$$d\, x_e / dt = k_{1E} - k_{2E}\, x_F - (\mu_1 + \mu_2)\, x_e \qquad (12)$$

II. 6 RESULTS

Results of both different control approaches are shown in Fig. 1 and Fig. 2 and experimental conditions and devices used are described earlier. Use of the mathematical model for the optimal process control synthesis is not described here and will be published later. Based on our previous research we wished to control the physiological state of the culture manipulating the specific growth rate in the case A. In the following figure, there are depicted two diagrams of desired specific growth rate profiles. In our experiment we wished to maintain the second profile during 24 hour fed batch cultivation (Fig. 3 - B).
The figure on the left-hand side represents two phase profile of μ , where the first phase is characterized by specific growth rate μ_1 . In the current time of the cultivation t_c is the growth rate shifted to the second phase characterized by specific growth rate μ_2 . The three unknown values of those parameters can be used for evaluation of optimum of the above formulated criterion.
In the same way as in the case of the profile described in left-hand side figure profile on the right-hand side was formulated. The course of the first phase is same but the second phase is characterized by the slower shift in the specific growth rate μ given by the slope of line, denoted as k.
Based on previous experience both mathematical models describing the control were rewritten into the simulation program using PSI/c language (from TU Delft). Equation (1) in our model is suggested only for the case where concentration of C-source is very low (to prevent from Crabtree effect) but if it increases it should be extended to full Monod type equation.
Our experience when having scaled up model from smaller laboratory bioreactor to larger one shows that the most important for this procedure is investigation of eq. 8 and others which describe impact of oxygen transfer. Values of k_La are very important in this respect.

III DISCUSSION OF RESULTS AND CONCLUSION

Main results of both control strategies verified in case A and B are shown in Fig. 1 and Fig 2. Several important points must be discussed:

<u>*Case A*</u>

- First part (growth phase) is governed by the specific growth rate equal to 0.075 h^{-1}. Growth stopped at 22nd hour of cultivation,
- Glucose concentration is increased rapidly at 14th hour,
- Critical glucose concentration derived from the ethanol-time course is found around 0.1 g/L. Above this concentration a significant increase of ethanol appears
- Maximum of ethanol concentration (1.3 g/L) was reached at 17th hour
- Simultaneous utilisation of glucose and ethanol takes place when glucose concentration is lower than 0.1 g/L.
- Respiration quotient (RQ) equals 1.3 till 13th hour. Final course (from 22nd hour) is caused by assimilation of acidic products (mainly acetic acid)
- This fact is supported also by the course of NaOH and H_2SO_4 consumption.
- Controlled variable - CO_2 fluently increased its value till 14th hour (1.1 % vol.) and the end of the process shows rather strange behaviour given by acid assimilation. The same shows both curves of oxygen (dissolved oxygen concentration, DOC and concentration of oxygen in the gas phase)
- Total volume of medium fed into the reactor (F_{ms}) is very similar in shape to the growth curve
- Concentration of ergosterol shows increasing tendency but decreasing purity, i.e. other sterols - pointed as L1, L2 and L4 increase their concentration. Concentration of L1 and L4 is subject of optimisation. Maximum of ergosterol was reached at 10th hour (2.26 % wt in dry biomass)
- Yield of biomass $Y_{X/S}$ = 0.37 and productivity p_x = 0.185 g/L.h
- Net amount of dry yeast biomass was only 4.9 g/L

Case B

- In comparison with case A the specific growth rate is higher till 4th hour and then linearly decreased,
- Glucose is rapidly increasing from 9th till 13th hour.
- Critical concentration of glucose is also around 0.1 g/L
- Maximum ethanol concentration (2.5 g/L) was reached between 14th and 15th hour
- RQ shows similar type of course
- Similar end of cultivation took place also in case B. On courses of RQ, CO_2, O_2, DOC, F_{as} (consumption of sulphuric acid) and F_{bs} (consumption of sodium hydroxide) utilisation of another substrate (acidic character, metabolisable) is seen
- CO_2 profile is steeper in the first part (till 17th hour), its maximum is 1.9 % vol.
- Shape of the F_{ms} curve is similar to case A, different course in the second half has curve F_m (doses of feed medium in ml/min)
- Higher consumption of medium in case A is given by lower concentration of carbon source in medium
- Concentration of ergosterol and relative concentration of the other sterols are very similar to the those found in case A
- Yield of biomass $Y_{X/S}$ = 0.358 and productivity p_x = 0.326 g/L.h
- Net amount of dry yeast biomass was 8.29 g/L (168 % of the biomass from case A). The secondary reason for this situation is that the volume of medium in case A was lower. This fact is the most remarkable feature of this kind of control. Experiments for its verification must be carried out in the near future.
- For economical production of ergosterol the required quality of yeasts should be as follows: content of ergosterol above 1.0 % DW, dehydroergosterol (L1) less than 8 % of total sterols and dihydroergosterol (L4) with content less than 2.5 % of total sterols. Our experimental results achieved lately were significantly better with content of total sterols above 3 % DW, ergosterol 2.75 % DW, dehydroergosterol less than 8 % total sterols and dihydroergosterol less than 2.6 % total sterols. These values were not reached at the same time but applying optimisation and knowledge-based control system we are able to improve significantly process in large scale.
- An important step towards final knowledge-based control experiments was creation of matrix of physiological stages of yeasts *Saccharomyces cerevisiae* with respect to sterol biosynthesis. Details of this matrix will be published later. All first five metabolic stages (1 - oxidative (growth on glucose only), 2 - border state (between oxidative and fermentative metabolisms), 3 - oxidative-fermentative (growth on glucose accompanied by ethanol production), 4 - oxidative (growth on clucose and ethanol), 5 - oxidative (growth on ethanol only) and 6 - starvation) also exist in our process but from sterol production point of view they are not equivalent. Our studies were mostly directed to stage 1 - 3. In fact stage 1 - 3 (purely oxidative, oxidative/fermentative and stage of ethanol utilisation) do not exist separately . They act together and their participation depends on homogeneity (or heterogeneity) of medium inside the reactor.

References

Arnezeder C., Hampel W.A. 1990, Biotechnol. Lett. 4, 277-282

Behalová, B., Votruba, J. and Beran, K., 1986, Folia Microbiol. 31, 129-137

Crabtree, H.G., 1929, Biochem. J. 23, 536

DeDeken, R.H., 1966, J.Gen Microbial. 44, 149

Keulers M., 1993, PhD. Thesis, Technical University Eindhoven, The Netherlands

Paulová, L., 1995, PhD. Thesis, Institute of Chemical Technology Prague, Czech Republic

Rychtera, M., Votruba, J., Paulová, L., Fiala, J., Melzoch, K. and Náhlík,J., 1996, 12th Int. Congress of Chem. Process Eng. - CHISA ´96, Aug. 25-30, Prague

Rychtera, M., Votruba, J., Melzoch, K., Èermák, J., Fiala, J., Vedlichová, Z., Patáková, P., Kent, C.A., Al-Rubeai, M., Náhlík, J. and Miskiewicz, T., 1998, 13th Int.Congress of Chem.Process Eng. - CHISA ´98, Aug. 23-28, Prague

Sobotka, M., Votruba, J., Krumphanzl, V. a Hradec, V., 1982, Kvasný prùmysl 28, 87-89, (in Czech)

Symbols used

c	concentration of DO in eq. (7)	(mg/L)
DOC	concentration of dissolved oxygen	(mg/L)
DW	dry weight	
E	concentration of ethanol	(g/L)
F	medium flow rate	(L/h)
H	Henry´s constant for oxygen solubility	(Pa.L/mol)
k_1 - k_7	constants in corresponding equations	
k_La	volumetric mass transfer coefficient	(h^{-1})
k_{e1}, k_{e2}, k_{e3}, k_{e4}	coefficients in eq. (16)	
k_{1a}, k_{1b}, k_{1i}	coefficients in eq. (18)	
k_{3E}	coefficient in eq. (19)	
k_{4a}, k_{4b}	coefficients in eq. (20)	
m_e	maintenance coefficient for sterol synthesis	(h^{-1})
m_O	maintenance coefficient based on oxygen	(h^{-1})
P	pressure	(Pa)
RQ	respiration quotient (CO_2 produced/ O_2 consumed)	(-)
r_e	specific rate of ethanol synthesis	(h^{-1})
S	concentration of sugar	(g/L)
t	time	(h)
V	volume of medium in bioreactor	(L)
V_G	air flow rate	(L/h)
X	concentration of yeast dry biomass	(g/L)
x_S, x_e	mass fraction of biomass	(-)
x_f	fraction of free ergosterol in biomass	(-)
$Y_{E/X}$	yield coefficient (ethanol from biomass)	(-)
y_{O2}	molecular fraction of oxygen in air	(-)
y_{CO2}	molecular fraction of carbon dioxide in air	(-)
$Y_{CO2/X}$	yield coefficient (CO_2 from biomass)	(-)
$Y_{O/X}$	yield coefficient (oxygen - biomass)	(-)
μ	specific growth rate = (1/X).dX/dt	(h^{-1})

Symbols in Fig. 1

EtOH	concentration of ethanol	% vol
F_{ms}	total volume of medium fed into the bioreactor at time t	ml
F_m	doses of feed medium	ml/min
F_{bs}	volume of 10% NaOH added into the bioreactor at time t	ml
F_{as}	volume of 10 % sulphuric acid added into the bioreactor at time t	ml
CO_2	content of carbon dioxide in exit gas (% vol.)	
mi	specific growth rate, μ	
L1, L2, L4	sterol contaminants	

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