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Experimental Techniques and Data Analysis to Determine Baker's Yeast Ethanol Dynamics

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Experimental techniques and data analysis to determine baker's yeast ethanol dynamics

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Summary

Dynamical experiments have been done with baker's yeast under feed-rate controlled conditions. The yeast was grown on molasses in an 8 L fed-batch reactor and experiments were done at cell concentrations between 5 and 65 g/L. Small changes in the feed rate was made around a feed rate corresponding to the critical growth rate, where the yeast cell metabolism switches between ethanol consumption and production. A membrane gas sensor was used for on-line measurement of the ethanol concentration in the broth. The measured ethanol signal was used for control and the system was excited through changes in the regulator set-point. Making the experiments in closed loop ensured feed variations to be in the critical range, and thus facilitated reproducible experiments. Data was fitted to a second order difference equation using statistical methods. Results were compared with a theoretically derived model. The process gain could be understood in terms of the underlying stoichiometry by using the "bottle neck" view of yeast glucose metabolism. The process time constant was found longer than what is implied by the simple Monod relation between glucose uptake rate and concentration.

Introduction

The chemostat, and feed-controlled cultivation conditions in general, have proven to be a valuable tool in the research of microbial cell metabolism. In the study of yeast metabolism the chemostat made possible the description of respirative growth on glucose [1]. New on-line measurement techniques provides possibilities for new experiments. The refinement of exhaust gas analysis stimulated research in the role of cell mass formation on the redox balance [2]. The last few years a few groups have focused on transient behaviours in the bioreactor [3]-[6]. This research is highly dependent on rapid on-line measurement of relevant variables in the reactor.

We have developed an experimental set-up for substrate limited cultivation of baker's yeast in a fed-batch reactor [7], which is suitable for dynamical studies. Variations in the broth ethanol concentration is a good indicator of the respiratory state of the yeast culture and it was measured on-line by a membrane gas sensor [8]. The measured ethanol signal was used for control of the feed rate. By introducing test signals through the regulator set-point, dynamical experiments can be done that reveal the character of the switch in glucose metabolism. In this way, by making the experiments in closed loop, reproducible experiments are facilitated.

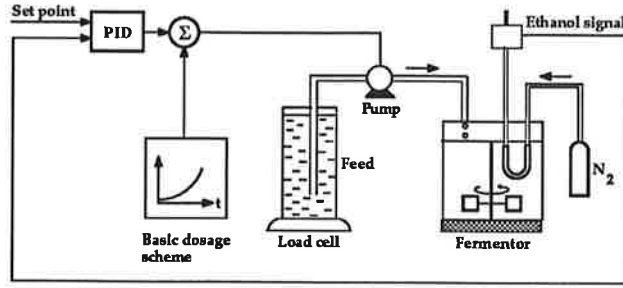


Figure 1. The experimental set-up

In this paper is discussed how such experiments should be valuable to understand metabolism. A mathematical model is derived for yeast ethanol dynamics in the reactor and special attention was paid also to the substrate feeding system and the ethanol sensor. Data from six identification experiments during three cultivations are analysed. Process parameters are obtained by statistical methods and they are interpreted in microbiological terms.

Background

The yeast was grown in a well-mixed fed-batch reactor under substrate limited conditions near the critical growth rate. The oxygen supply was assumed to be sufficient.

Ethanol dynamics in a fed-batch reactor

Mass balance equations are given below. Note that the cell concentration X , as well as the volume of the broth V , increase during a cultivation. Let S denote the substrate and E the ethanol concentration in the broth.

$$\begin{aligned}
 \frac{dV}{dt} &= F \\
 \frac{d(VX)}{dt} &= \mu(S, E) \cdot VX \\
 \frac{d(VS)}{dt} &= -q_S(S) \cdot VX + S_{in} \cdot F \\
 \frac{d(VE)}{dt} &= q_E(S, E) \cdot VX
 \end{aligned} \tag{1}$$

The relation between substrate uptake rates, cell growth and ethanol formation remains to be characterized. Here critical growth rate is assumed and only small variations in the substrate concentrations are considered. Further, the ethanol concentration is not allowed to reach inhibiting concentrations. The following approximations are then appropriate.

$$\begin{aligned}
 \mu(S, E) &= \mu_c \\
 q_S(S) &= q_S^0 + \alpha S \\
 q_E(S, E) &= -q_E^0 + \beta S, \quad E > 0
 \end{aligned} \tag{2}$$

Note that under these assumptions the differential equations become linear.

$$\frac{d}{dt} \begin{pmatrix} VS \\ VE \end{pmatrix} = \begin{pmatrix} -\alpha X & 0 \\ \beta X & 0 \end{pmatrix} \begin{pmatrix} VS \\ VE \end{pmatrix} + \begin{pmatrix} S_{in} \\ 0 \end{pmatrix} F - \begin{pmatrix} q_S^0 \\ q_E^0 \end{pmatrix} VX \tag{3}$$

The critical feed rate $F^0(t)$ to obtain stationarity in the ethanol concentration is proportional to cell mass.

$$S_{in} F^0 = (q_S^0 + \frac{\alpha}{\beta} q_E^0) VX \tag{4}$$

Metabolism operates in the time scale of a few minutes while cell growth is in hours. Over shorter time periods the ethanol dynamics can be approximated with a time invariant model.

$$T \frac{d\Delta S}{dt} + \Delta S = K_1 \Delta F \quad (5a)$$

$$\frac{d\Delta E}{dt} = K_2 \Delta S \quad (5b)$$

The parameters depend on volume and cell concentration as

$$T = \frac{1}{\alpha} \cdot \frac{1}{X} \quad (6a)$$

$$K = K_1 \cdot K_2 = \frac{S_{in}}{\alpha} \frac{1}{VX} \cdot \beta X = \frac{\beta}{\alpha} \cdot \frac{S_{in}}{V} \quad (6b)$$

The ethanol dynamics change during a cultivation according to (6) as cell concentration and volume increase. However there are other factors in the reactor conditions that are not modeled in (2). For instance, the oxygen transfer is often a limiting factor at high cell concentrations. Further, unmetabolizable products of the feed and certain byproducts of cell metabolism, may accumulate and reduce the growth rate.

The experimental task

On-line measurement of the ethanol concentration in the broth is available. First experiments should be done that confirm the simple model (5) of the ethanol dynamics in the reactor. Then it is interesting to determine the process gain K and the time constant T . Experiments at different cell concentrations show if the parameter variation (6) is valid. To determine K_1 and K_2 separately, both ethanol and sugar concentrations must be measured. However, the sugar concentrations are very low and on-line measurement is difficult.

Motivation for doing the experiment in closed loop

The ethanol concentration is a very sensitive indicator of over- and under feeding of the culture. The model (5) shows that the ethanol concentration easily drifts away if the feed rate does not exactly match the substrate demand. In order to determine the process parameters small variations should be done in the feed rate around the nominal value $F^o(t)$ in (5). In order to get accurate parameters several shifts between ethanol production and consumption has to be considered, and the experiment time needed implies that the cell mass increases significantly and thus the feed rate demand. In order to make a reproducible experiment feed-back is valuable. Changes in the feed rate are induced by changing the ethanol set-point to the regulator. In this way changes in the feed rate are guaranteed to be around the nominal feed rate, and the ethanol concentration is kept within certain limits.

Inherent difficulties of the identification problem

In order to simplify the analysis of the data serie, the length of the test period is chosen to be no longer than that the variation in the parameters (6) has a negligible influence on the dynamics. On the other hand, a short test period sets a limit to the maximal time constant that can be distinguished from a pure integrator. Here a test period of about three hours was used.

It is important that the variations in the feed rate are not larger than that the model (5) is valid. This means that the feed rate should not be too low so that the substrate vanishes and the ethanol consumption capacity saturates. Neither should the feed rate be too high so that the substrate concentration increases beyond the valid range of (2).

The dynamics of ethanol production and consumption changes according to (6). The time constant T decreases typically from about 10 min at the start to 0.5 min

at the end of a fed-batch cultivation. In this work the ethanol sensor had a time constant of about 2 min. This fact implies that it will be hard to determine T at high cell concentrations.

Methods

The equipment and cultivation conditions are briefly given below. For details see [7]. The statistical data analysis routines used, are also shortly presented.

Cultivation conditions

Baker's yeast, *Saccharomyces cerevisiae*, was used in this study. Sugar beet molasses with a sugar concentration of 50% (w/v) was used as medium. The feed contained 1.69 kg molasses which was mixed with 1 litre H_2O and the resulting sugar concentration was about 410 g/L. In cultivation 1 and 2 a different batch of molasses was used than in cultivation 3. The yeast strain and the molasses were supplied by Svenska Jästbolaget AB. Cultivations were performed in a fermentor (FLC-B-8 Chemoferm AB, Hägersten, Sweden) with a working volume of 6 litre. The dissolved oxygen concentration was monitored using a galvanic oxygen electrode [9]. The stirrer speed was kept constant during the test periods, except for test period 1 where it was increased in the middle. Temperature was kept at 30° C and pH at 5.0 with $NaOH$, using conventional control.

Ethanol sensor, pump and control system

Ethanol was monitored using a semiconductor gas sensor (TGS 812, Figaro Engineering Inc, Osaka, Japan) in combination with a silicone membrane sampling probe immersed in the cultivation medium [8]. The substrate feed rate was controlled by a peristaltic pump (Ismatec mp-4). The actual feed rate was checked using data from a load cell (Sartorius mp-8) which the substrate vessel was placed on. The balance had a quantization of 0.1 g. A PDP 11/03 microcomputer was used for control, monitoring and data logging. The ethanol signal was prefiltered with a second order Butterworth filter with a time constant of 60 sec. Data was logged on disc every 30 sec. The feed rate was controlled around an exponential basic dosage scheme. Adjustments were made using feedback from the ethanol signal. A PID regulator was used with a sampling interval of 30 sec. See Figure 1.

Statistical data analysis

The test signal to the regulator reference value was a precalculated PRBS sequence with a basic period of 4 min in experiments 1, 2a and 2b, and 6 min in 3a, 3b and 3c. Trends in the data series were removed before further analysis. An exponential trend was usually subtracted from the feed rate signal, corresponding to the critical feed rate $F^o(t)$. However, in data from 2b and 3c, linear trends were more appropriate. Data analysis was done using the interactive program package MATLAB extended with the System Identification Toolbox (MathWorks, Inc). Maximum likelihood methods were used. The data was fitted to black box discrete time ARMAX models with different numbers of parameters. Further, the gain and time constant of equation (5) were determined in the following way. The structure of the integrator (5b) was included in the model by differencing the ethanol signal. The data was then fitted to a first order model, where $y(t) = E(t) - E(t - h)$, $u(t) = \Delta F(t)$, and $e(t)$ is a white noise residual. The sampling interval is denoted by h .

$$y(t) = -a_1 y(t - h) + b_1 u(t - h) + b_2 u(t - 2h) + e(t) + c_1 e(t - h) \quad (7)$$

The gain and time constant of model (5) are here related to (7) by

$$K = \frac{1}{h} \frac{b_1 + b_2}{1 + a_1}, \quad T = -\frac{h}{\ln(-a_1)} \quad (8)$$

provided the sensor time constant is negligible compared to the time constant of the ethanol dynamics. The influence of sensor dynamics is mentioned in the later part of the paper in the discussion.

Results

Special attention was paid to the substrate feeding system and the ethanol sensor, both with respect to calibration and dynamical response.

The ethanol sensor system

The ethanol sensor was calibrated before and after each cultivation. The dynamics of the measurement system can be characterized by a transport delay time T_d and a time constant T_s . Typical values were $T_d = 2$ min and $T_s = 2$ min. In Figure 2, the response of the sensor is compared with the result from such a model.

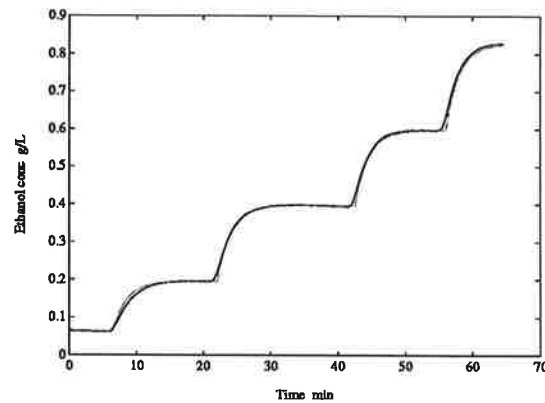


Figure 2. Dynamics of the ethanol sensor during calibration. The solid line is the measured ethanol signal and the dotted line is a first order model with the time constant $T_s = 2$ min. Data was from the calibration after cultivation 3.

The substrate feeding system

Measures were taken to make the feed rate respond within seconds to a change in the pump signal. The feed tubing between substrate vessel, pump and fermentor was kept short, and any air-bubbles in the tubing were eliminated. The feed dropped into the broth from the top of the fermentor and it was easy to see that the drop frequency changed immediately when the pump signal changed. The actual feed rate was monitored by the substrate vessel load cell. In Figure 3, the pump signal is compared with the differenced load cell signal. The high noise level was mainly due to the quantization error of the digital load cell.

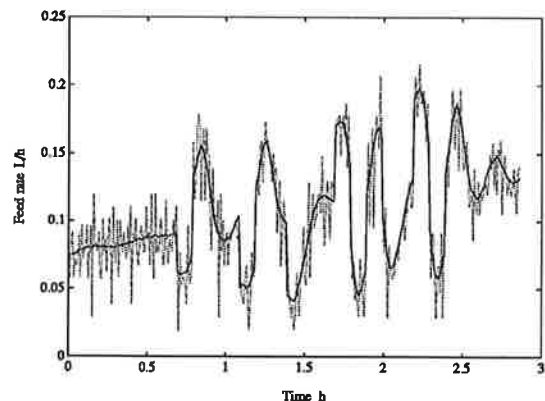


Figure 3. Comparison between the pump signal and the data from the load cell. The solid line is the pump signal and the dotted line is the differenced load cell signal. The data was taken from the start of test period 3b.

Results from identification experiments in closed loop

Several identification experiments were done. In Figure 4. is shown raw data from three test periods during cultivation 3. The measured ethanol signal is compared with the output from the identified model in simulation. Note that the cell concentration increases considerable, see Table 1. During the last test period the regulator was not able to keep the ethanol concentration down. The experiment was terminated before the PRBS sequence was finished.

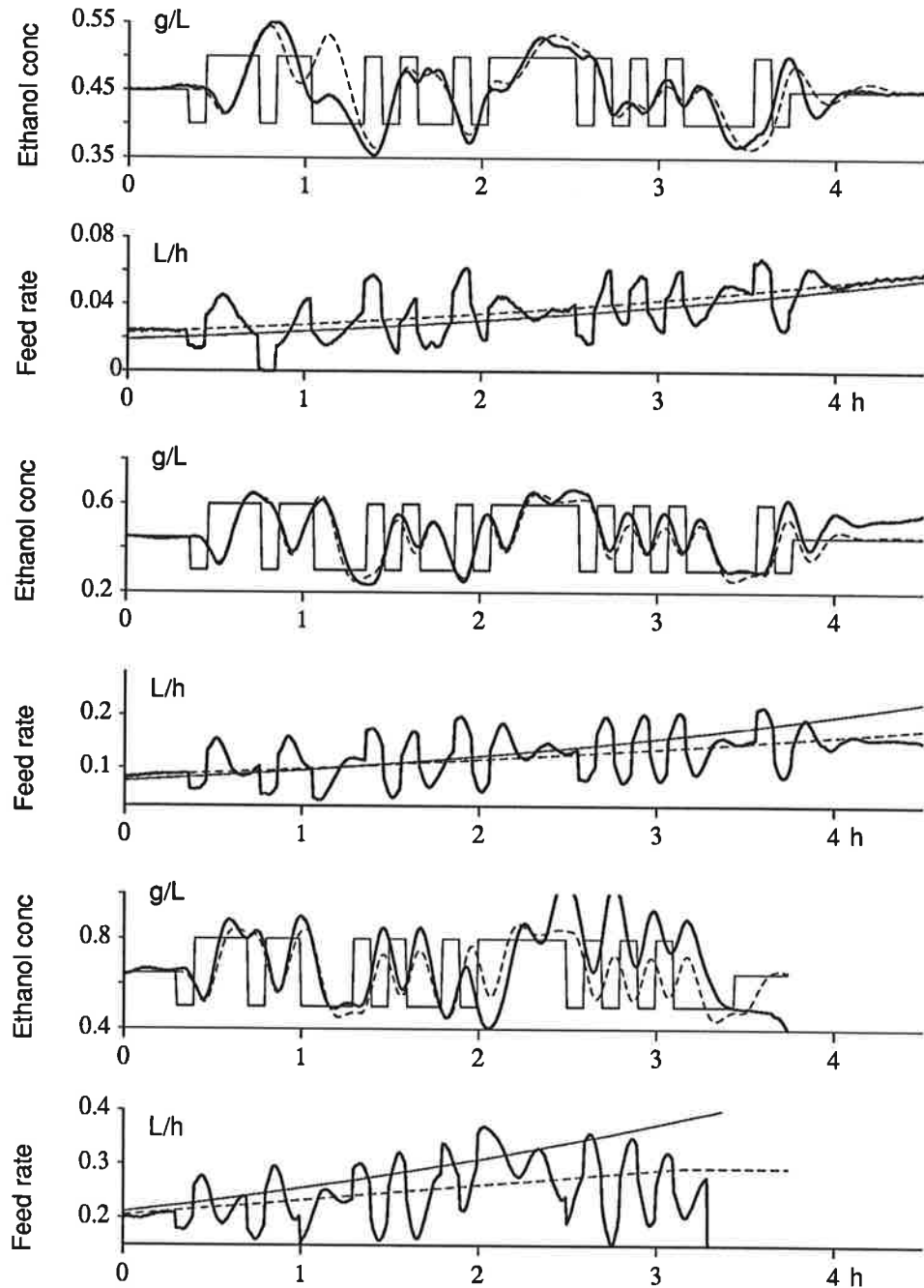


Figure 4. Three identification experiments: a, b and c, during cultivation 3. Thick lines are ethanol concentration and feed rate (upper, lower), thin lines are ethanol set-point and basic dosage scheme (upper, lower), and dashed line is the output from the identified model (upper) and estimated critical feed rate $\hat{F}^c(t)$ (lower).

The identified process gain \hat{K} and time constant \hat{T} for the experiments are summarized in table 1. The identified time delays \hat{T}_d are also given. Dry weight measurements of the cell concentrations were done before and after each experiment except for experiment 1, where the cell concentrations were estimated from the amount of feed consumed. The time period between the two measurements vary and are given in the table.

Table 1. Identified process parameters.

Experiment		1	2a	2b	3a	3b	3c
V	[L]	4.2 - 4.6	4.0 - 4.1	4.8 - 5.4	4.0 - 4.3	4.4 - 5.2	5.8 - 6.3
X	[g/L]	10 - 21	4.6 - 9.3	30 - 48	4.0 - 13	19 - 40	50 - 63
Period	[h]	3.7	3.0	3.0	5.0	4.5	1.5
\hat{K}	[g/L, L]	58	70	88	60	72	74
\hat{T}	[min]	2.2	7.8	6.6	4.2	3.5	2.9
\hat{T}_d	[min]	2.5	4.0	2.0	3.5	2.0	2.0

In order to identify the process parameters \hat{K} and \hat{T} differences of the ethanol signal were fitted to the model (7). One example is given in Figure 5., where data from experiment 3b is used. Note the low noise level. Taking differences of the ethanol signal also reduce the effect of drifts due to poor control, on the identification.

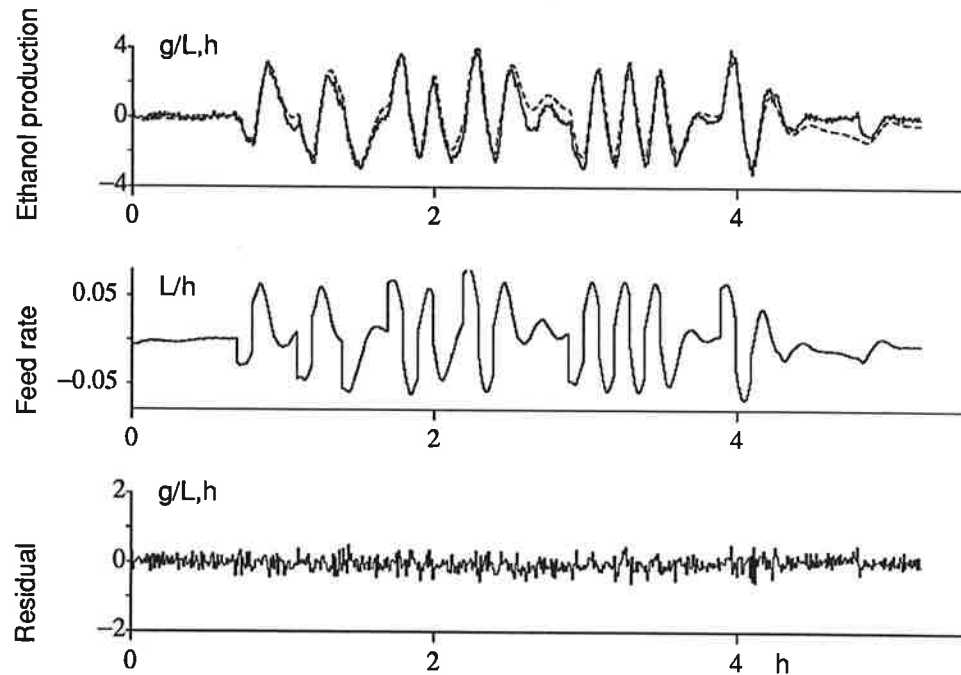


Figure 5. An example of differenced ethanol signal fitted to a first order model (7). The upper diagram shows the differenced ethanol signal divided by the sampling interval, solid line. The dashed line is the output from the model. The variation of the feed rate from the estimated critical feed rate $\hat{F}^o(t)$ is shown in the middle and the lower diagram shows the residual $e(t)$ after identification. Data is from experiment 3b.

Discussion

Comparison of the data with what could be expected

Chemostat studies, e.g. [10], have discussed relations between glucose uptake rate and ethanol formation rate. The data reflect different stationary states of the yeast culture at different dilution rates. The stoichiometry of these states has received much attention. A review is given in [1]. The substrate uptake rate is often described as dependent on the substrate concentration by the Monod relation.

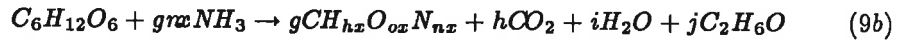
Stoichiometric gain

The stoichiometry of yeast growth can be viewed as a combination of three different paths. A simplifying fact is that the cell composition remains almost constant independent of changes in the metabolism. The chemostat experiments evaluate each path separately.

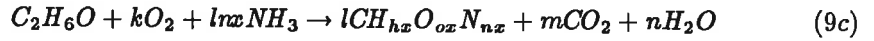
Respiratory growth on glucose



Fermentative growth on glucose, anaerobically



Respiratory growth on ethanol



Elemental balances for C , H , O and N give rise to a system of equations for each reaction. Each system can be solved if one measurement is added, for instance the cell yield coefficients b , h and l . Sensitivity analysis shows a good behaviour.

From the stoichiometric equations (9) a relation between the glucose uptake rate q_G and the ethanol formation rate q_E can be derived, based on the following assumptions. The switch in metabolism between ethanol production and consumption can be viewed as an overflow reaction governed by the respiratory capacity $q_{O_2}^{lim}$ [11]. Further, assume that in the case of a glucose over-feeding, the stoichiometry is a sum of (9a) and (9b). In case of a glucose under-feeding, ethanol is consumed by the remaining respiratory capacity and the stoichiometry is a sum of (9a) and (9c). That is (9c) momentarily absorbs all respirative capacity left over from (9a).

$$q_E = \begin{cases} jq_G^{(b)} = j(q_G - q_G^{(a)}) & = j(q_G - \frac{1}{a}q_{O_2}^{lim}), \quad q_E > 0 \\ -\frac{1}{k}q_{O_2}^{(c)} = \frac{1}{k}(aq_G^{(a)} - q_{O_2}^{lim}) & = \frac{a}{k}(q_G - \frac{1}{a}q_{O_2}^{lim}), \quad q_E < 0 \end{cases} \quad (10)$$

Further (2) and (6b) give

$$K = \frac{\beta}{\alpha} \cdot \frac{G_{in}}{V} = \frac{\partial q_E}{\partial q_G} \cdot \frac{G_{in}}{V} = \kappa \cdot \frac{G_{in}}{V} \quad (11)$$

where using data from [11]

$$\kappa = \begin{cases} j \approx 1.9, \quad q_E > 0 \\ \frac{a}{k} \approx 1.5, \quad q_E < 0 \end{cases} \quad (12)$$

A comparison with the experimentally identified process gains is shown in Table 2. with $\kappa = 1.7$. Note that the classical stoichiometry for glucose- and ethanol-metabolism which do not account for the coupling between metabolism and biomass formation [1], gives $\kappa = 2.0$. From the stoichiometric equations (9) the process gain should vary with $\pm 10\%$ between over- and under-feeding. This could not be distinguished in our data. The gains are in general higher than expected, the difference being most pronounced for high cell densities.

Table 2. Comparison between the experimentally identified process gains \hat{K} and the derived gains from the stoichiometric data, K .

<i>Experiment</i>	1	2a	2b	3a	3b	3c
K [g/L, L]	40	43	35	43	37	30
\hat{K} [g/L, L]	58	70	88	60	72	74

Dynamics of glucose uptake

From stationary chemostat experiments is generally found a Monod relation between the substrate concentration and the uptake rate. Assume that the cells momentarily acquire this uptake rate. Then the expression can be used to get an estimate of the time constant T (6a), in the fed-batch reactor.

$$q_G = q_G^{max} \cdot \frac{G}{G + K_s} \quad (13)$$

$$\alpha = \frac{\partial q_G}{\partial G} = \frac{K_s}{(G + K_s)^2} \cdot q_G^{max}$$

The identified process time constant are compared in Table 3. with what the Monod relation (13) predicts. The parameters q_G^{max} , K_s and G_{crit} are taken from the literature [11].

Table 3. Comparison between the identified process time constants \hat{T} with the time constants T , derived from the Monod model.

<i>Experiment</i>	1	2a	2b	3a	3b	3c
T [min]	0.6	1.2	0.2	0.9	0.3	0.1
\hat{T} [min]	2.2	7.8	6.6	4.2	3.5	2.9

The identified process time constant \hat{T} should be interpreted as a combination of the reactor and sensor time constants. This is illustrated in Figure 6. Here the reactor and sensor time constant were approximated to one time constant and an additional time delay, using a least squares method in the frequency domain [12]. It is clearly seen from the figure that the sensor dynamics gives a bias to the estimate of the reactor time constant and at high cell concentrations the sensor dynamics dominates. However, the bias in \hat{T} is not large enough to account for the differences in Table 3.

The difference between the experimental result and the theoretically derived time constant is not surprising. There are several hypothesis to account for the additional dynamics. First of all the Monod relation is valid only in stationarity and for higher substrate concentrations. The slope α might deviate from the Monod relation at low concentrations. From the underlying switch in the metabolism at the critical glucose

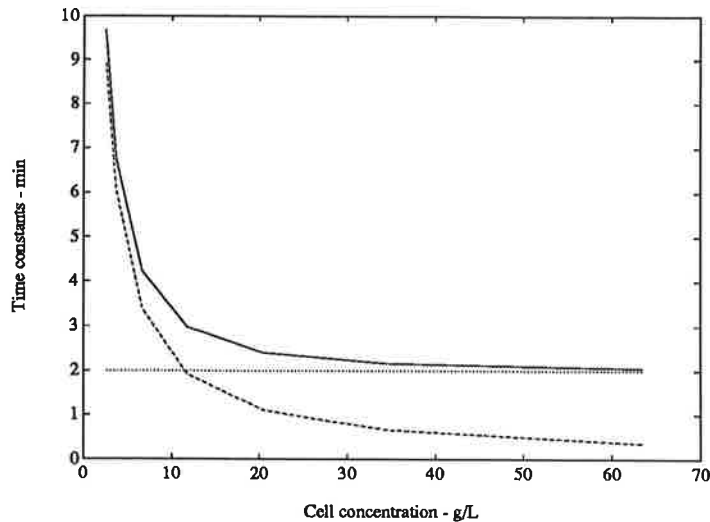


Figure 6. Approximated time constant including ethanol dynamics and sensor. Solid line is the approximated time constant, dashed line is the ethanol dynamics and dotted line is the sensor time constant.

concentration, it is reasonable to suspect two different values for α , as for the stoichiometric gain κ . There are good reasons to believe that the yeast cell increases its substrate uptake gradually with time, and not momentarily, and that introduces dynamics. The more complicated change in metabolism may also be one reason for the high value of the process gain. In [13] experiments with glucose pulses indicate dynamics in the uptake rate. In our experiments molasses were used as substrate and that introduces further dynamics that might not be negligible. Molasses contains a large amount of disaccharides, and they are cleaved extracellularly to monosaccharides. Monosaccharides are then taken up by the cells [14]. Finally, the switch in metabolism in the cells might take some time and thus introduce dynamics. New experiments with equipment for on-line measurements of different sugars at low concentrations, would be most helpful to understand this.

Degree of uncertainty

In order to interpret the data from the dynamical experiments correctly, special attention was paid to the pump and the sensor. Careful calibrations of the equipment are standard routines in a biotechnical laboratory, however test of the dynamical responses are especially important when studying transients in reactors. In this work the pump was found to introduce negligible dynamics as could be seen from the load cell data. However, more important was that the load cell data gave a possibility to check the pump gain and bias after an experiment. The sensor is of course more difficult to check. It was calibrated before and after each cultivation and the zero ethanol level could be checked during a cultivation. Off-line tests are difficult to do. Internal standards during a cultivation might be a possibility.

The statistical analysis of the data gives parameters and also confidence intervals for the parameters. First the data was fitted to black-box models and a second order model was found to be sufficient, even though a third order model is theoretically motivated. Here the process integrator was revealed with an accuracy well within the limitation set by the test period length. Secondly the structure of the integrator was imposed on the model by differencing the measurement data. The filtered data was then fitted to a first order model. In this way the confidence interval of the parameters was substantially reduced. Even better estimates of the physical parameters might be obtained if a tailor made identification algorithm is used. It could exploit the time-varying structure of the equations (3) and also incorporate the knowledge of the sensor dynamics.

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