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Control of substrate feeding in *Escherichia coli* cultures

Stéphane Velut

Department of Automatic Control
Lund Institute of Technology
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Department of Automatic Control
Lund Institute of Technology
Box 118
SE-221 00 Lund Sweden

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Author(s)
Stéphane Velut

Supervisor
Mats Åkesson
Per Hagander

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(Reglering av substratdosering av *Escherichia coli* kulturer)

Abstract

This report investigates feeding strategies for fed-batch cultures of *Escherichia coli*. The DO-stat is a common method that is well suited for handling of limitations in oxygen transfer but may cause over-feeding and overflow metabolism in some situations. The properties of the DO-stat method are studied and some suggestions for tuning are given. Strategies that addresses the problem of overflow metabolism are also described, in particular a probing technique is discussed. A new feeding strategy combining the advantages from the DO-stat method and the probing technique is also suggested.

Key words
Process control, fed-batch cultivation, *Escherichia coli*, glucose feeding, acetate, dissolved oxygen, on-line monitoring, feed-back, recombinant protein production.

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1. Introduction

Today a wide range of chemicals and pharmaceuticals are produced using genetically modified organisms. Recombinant DNA technology makes it possible to insert foreign genes encoding a desired substance into a host organism. New products can be manufactured in this manner by adding genetic information to a suitable cell and growing it in such a way that the desired substance is produced. To provide a suitable environment, parameters such as temperature, pH and dissolved oxygen concentration are monitored and controlled. Automatic control is used to improve the performance and to achieve uniform and reliable results.

A commonly used microorganism for synthesis of proteins is the bacterium *Escherichia coli*. It is a well-characterized organism that can be grown to high cell densities, but it has also drawbacks. Under oxygen-limiting conditions or at high substrate concentrations, the by-product acetate is produced. An accumulation of acetate in the broth results in a decrease of the cell growth and the product yield.

To avoid acetate formation due to over-feeding, many feeding strategies has been developed, see [1]. One of them, using a pulse technique, requires only a minimum of a priori information and can thus be applied to different strains of *E. coli*, see [2] without extensive experimentation.

To maintain aerobic conditions the substrate feed rate must also be chosen such that the corresponding oxygen uptake does not exceed the maximum oxygen transfer capacity of the reactor. A so called DO-stat method that manipulates the feed rate to keep a constant oxygen concentration, permits to achieve a balance between oxygen uptake and oxygen transfer, see [3]. However, this strategy does not guarantee that over-feeding is avoided.

The goal of this work is to study the DO-stat method and to determine critical situations where it yields poor results. Tuning rules will furthermore be presented for this strategy to work well. A new strategy combining the advantages from the pulse strategy and the DO-stat strategy will also be suggested.

2. Background

A fermentation process takes place in a tank called bioreactor or fermenter. The reactor contains the medium, which is a liquid providing the nutrients that cells require for growth and product synthesis.

A microbial culture may be run in three different operating modes: batch, fed-batch or continuous. In a batch process, all the nutrients are initially added into the bioreactor, and the products are removed from the vessel at the end of the cultivation. In a continuous process, nutrients are fed and products are removed continuously. The fed-batch mode is somehow between the two previous: after an initial batch phase, additional substrate is fed, often at a growth-limiting rate, and the products are withdrawn after the cultivation is completed.

The carbon source, that is usually glucose, constitutes the main nutrient in a fermentation, and it is consumed with a concomitant consumption of oxygen. To provide aerobic conditions, air or pure oxygen is sparged into the medium. The oxygen transfer rate is usually regulated by means of a stirrer, which also achieves the mixing.

We will focus on a fed-batch cultivation of *E. coli* bacteria in a small-scale bioreactor. A flow diagram of the process is shown in Figure 1. Two streams enter the system for the supply of glucose and oxygen, whereas exhaust gas leave freely the tank. Ammonia is also provided continuously as nitrogen source as well as for regulation of pH.

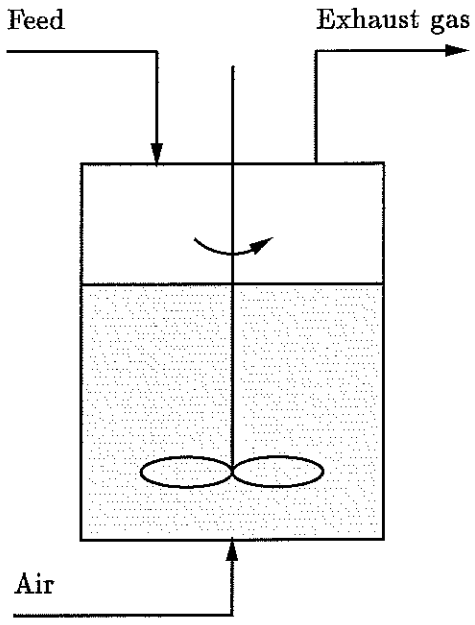

Figure 1 A fed-batch fermenter with mechanical agitation.

2.1 Process model

In order to understand the process dynamics, a model based on mass balances will be derived. The description of acetate formation and consumption is largely based on [4], but the inhibitory effect on growth has not been taken into account. We will assume that the mixing is sufficient to consider temperature and all concentrations uniformly distributed.

Mass balances

- Mass balance for dissolved oxygen gives the following expression:

$$\frac{d(VC_o)}{dt} = K_L a V(C_o^* - C_o) - q_o V X \tag{1}$$

where X, C_o, V are the cell concentration, the dissolved oxygen concentration and the liquid volume, respectively. Further, $K_L a$, q_o, C_o^* denote the volumetric oxygen transfer coefficient, the specific oxygen uptake rate and the liquid-phase concentration of oxygen in equilibrium with the oxygen in gas bubbles. The first term models the oxygen transfer from air bubbles to liquid and the second one describes the oxygen consumption by the cells.

Most sensors measure the partial pressure of dissolved oxygen or the dissolved oxygen tension, not the concentration. Henry's law gives the equilibrium relationship between these parameters for dilute liquid solutions:

$$O = H \cdot C_o \tag{2}$$

where the parameter H depends on the pressure in the fermenter but also on the temperature and the composition of the broth. The oxygen dynamics can be rewritten as:

$$\frac{d(VO)}{dt} = K_L a \cdot V(O^* - O) - q_o \cdot HVX \tag{3}$$

The dynamics of the probe has to be taken into account. It can be formulated as

$$T_p \frac{dO_p}{dt} + O_p(t) = O(t - \tau) \tag{4}$$

with O_p denoting the measured dissolved oxygen tension, τ being the time delay and T_p the time constant introduced by the probe.

- Mass-balance for glucose yields the following expression:

$$\frac{d(VG)}{dt} = FG_{in} - q_g(G) \cdot VX \tag{5}$$

where G, F, G_{in}, q_g are the glucose concentration, the feed rate, the glucose concentration in the feed and the specific glucose uptake rate.

- One can also perform a mass-balance on cell biomass:

$$\frac{d(VX)}{dt} = \mu VX \tag{6}$$

where μ is the specific growth rate.

- The mass balance for acetate can be written as

$$\frac{d(VA)}{dt} = (q_a^p - q_a^c)VA \tag{7}$$

where A, q_a^p, q_a^c denote the acetate concentration, the specific rate of acetate *production* and the specific rate of acetate *consumption*, respectively.

Kinetic expressions The specific glucose uptake rate q_g is modeled to be of Monod type:

$$q_g = q_g^{max} \frac{G}{k_S + G} \tag{8}$$

Part of the consumed substrate is used for maintenance activities and does not give any contribution to the cell biomass. The maintenance is described as

$$q_m = \min(q_{mc}, q_g) \tag{9}$$

where q_{mc} is the maintenance coefficient. As mentioned earlier, oxygen is used to metabolize glucose, and the specific oxygen uptake q_o depends on the specific glucose uptake q_g. It has been observed that the specific oxygen uptake rate reaches an apparent maximum q_o^{max} at the onset of acetate formation. Above the corresponding glucose uptake rate q_g^{crit}, excess glucose is fermented with acetate as main by-product. The specific acetate formation rate can be formulated as

$$q_a^p = \begin{cases} 0, & q_g < q_g^{crit} \\ (q_g - q_g^{crit}) Y_{ag}, & q_g > q_g^{crit} \end{cases} \tag{10}$$

with Y_{ag} being the acetate yield coefficient from glucose.

Acetate may also be consumed, however, this requires oxygen and the consumption of acetate is therefore limited both by the uptake mechanism and by the available respiratory capacity. Hence, no acetate is consumed for glucose uptakes exceeding q_g^{crit}. It is also assumed that glucose is the preferred substrate. The specific rate of acetate consumption q_a^c can be described as

$$q_a^c = \begin{cases} \min\left(\dfrac{q_a^{c,max} A}{k_a + A}, \dfrac{q_o^{max} - (q_g - q_m) Y_{og} - q_m Y_o}{Y_{oa}}\right), & q_g < q_g^{crit} \\ 0, & q_g > q_g^{crit} \end{cases} \tag{11}$$

where it has been assumed that the acetate uptake is of Monod type.

The corresponding oxygen consumption q_o can now be expressed as

$$q_o = \begin{cases} (q_g - q_m) Y_{og} + q_m Y_{om} + q_a^c Y_{oa}, & q_g < q_g^{crit} \\ q_o^{max} = (q_g^{crit} - q_m) Y_{og} + q_m Y_{om}, & q_g > q_g^{crit} \end{cases} \tag{12}$$

where Y_{og} and Y_{om} are the yield coefficients for growth and maintenance respectively, and Y_{oa} denotes the acetate yield coefficient.

Figure 2 shows a graphical description of the relations between specific glucose uptake q_g, specific oxygen uptake q_o, and net specific acetate production $q_a^p - q_a^c$ for zero and high acetate concentrations in the medium. The solid line corresponds to zero acetate concentration and shows that the oxygen uptake saturates and that acetate is produced for q_g above $q_g^{crit} = 0.7$ g/(gh). The dashed line corresponds to the case with a high acetate concentration (A=1 g/l). For low glucose uptake rates the acetate consumption is limited by the acetate uptake while for medium glucose uptakes the limitation lies in

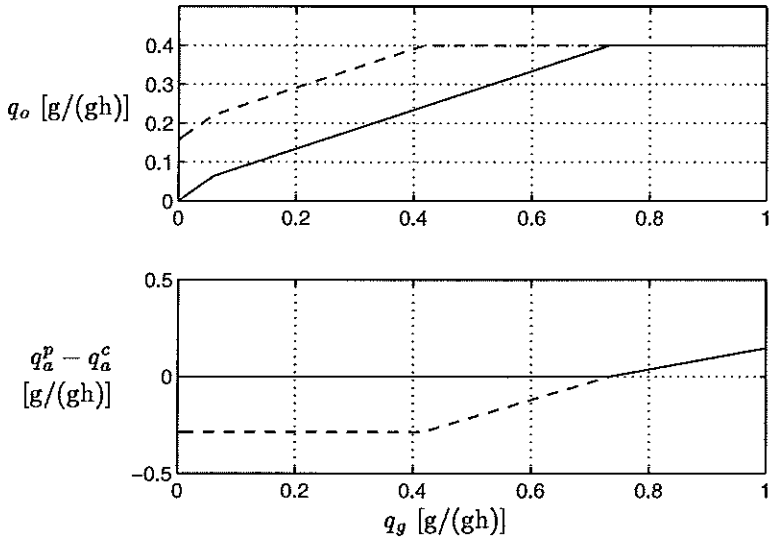

Figure 2 Relations between specific glucose uptake q_g, specific oxygen uptake q_o, and net specific acetate production $q_a^p - q_a^c$ for two different acetate concentrations. A=0 g/l (solid) and A=1 g/l (dashed). The knee in q_o below $q_g = 0.1$ g/(gh) is due to the maintenance description.

the available respiratory capacity. For glucose uptakes above q_g^{crit} acetate is produced as in the case with zero acetate concentration.

The specific growth rate can similarly be expressed as a function of q_g and q_a^c:

$$\mu = \begin{cases} (q_g - q_m)Y_{xg}^{ox} + q_a^c Y_{xa}, & q_g < q_g^{crit} \\ (q_g^{crit} - q_m)Y_{xg}^{ox} + (q_g - q_g^{crit})Y_{xg}^{fe}, & q_g > q_g^{crit} \end{cases} \tag{13}$$

where Y_{xg}^{ox} and Y_{xg}^{fe} denote the biomass yield coefficients from glucose, that is oxidated and fermented, respectively. The biomass yield coefficient from acetate is Y_{xa}.

Oxygen transfer The volumetric oxygen transfer coefficient K_La depends on many parameters such as temperature, viscosity, foaming, air flow rate and stirrer speed. Provided that the air flow rate is constant, K_La can be approximated as an affine function of the stirrer speed

$$K_La = \alpha(N - N_0), \quad N > N_0 \tag{14}$$

2.2 Linearized model

The model previously developed is strongly non-linear. A linearized version can be valuable to get a better insight into the process dynamics. Since we deal with strategies manipulating the feed rate at a constant oxygen concentration, a transfer function from the feed rate to the dissolved oxygen tension will be derived to make the analysis easier.

The glucose concentration in the incoming stream is high enough to neglect volume variations during short time intervals. Conservation of oxygen and

glucose can be rewritten as:

$$\frac{dO}{dt} = K_L a(N) \cdot (O^* - O) - q_o(G) \cdot HX \tag{15}$$

$$\frac{dG}{dt} = \frac{F}{V} G_{in} - q_g(G) \cdot X \tag{16}$$

Since oxygen is poorly soluble, dissolved oxygen present in the broth is rapidly consumed by the cells. Oxygen concentration would drop to zero in few seconds if the aeration rate is not sufficient. In fed-batch cultures where substrate is a limiting factor, the dynamics of glucose concentration is also very fast . Variations in cell biomass and in the volume are conversely slow and they will be neglected when deriving the linearized model.

$$\begin{cases} \dfrac{d\Delta O}{dt} = \dfrac{\partial K_L a}{\partial N}(O^* - O)\Delta N - K_L a \Delta O - \dfrac{\partial q_O}{\partial G} HX \Delta G \\ \dfrac{d\Delta G}{dt} = \dfrac{G_{in}}{V}\Delta F - \dfrac{\partial q_g}{\partial G} X \Delta G \end{cases} \tag{17}$$

$$\begin{cases} T_o \dfrac{d\Delta O}{dt} + \Delta O = K_n \Delta N + K_o \Delta G \\ T_g \dfrac{d\Delta G}{dt} + \Delta G = K_g \Delta F \end{cases} \tag{18}$$

where, assuming that $q_g < q_g^{crit}$

$$\begin{aligned} T_o &= \frac{1}{K_L a} = \frac{1}{\alpha(N - N_0)} \\ T_g &= (\frac{\partial q_g}{\partial G} X)^{-1} \\ K_o &= -\frac{1}{K_L a} HX \frac{\partial q_o}{\partial G} = -Y_{og} H \frac{T_o}{T_g} \\ K_g &= (\frac{\partial q_g}{\partial G} X)^{-1} \frac{G_{in}}{V} = \frac{G_{in}}{V} T_g \\ K_n &= \frac{(O^* - O)}{K_L a} \frac{\partial K_L a}{\partial N} \end{aligned}$$

The transfer function from the feed rate ΔF to the oxygen measurement ΔO_p is

$$G_{of}(p) = \frac{K_o K_g}{(1 + T_o p)(1 + T_g p)(1 + T_p p)} \tag{19}$$

The following parameters values corresponds to a laboratory scale bioreactor and will be used for the simulations in the sequel.

$\alpha = 0.92$ h^{-1}rpm^{-1}	$H = 14000$ %	$N_o = 323$ rpm	$q_g^{max} = 1.0$ g/(gh)
$k_S = 10$ mg/l	$G_{in} = 500$ g/l	$Y_{og} = 0.5$ g/g	$Y_{xg}^{ox} = 0.5$ g/g
$Y_{xg}^{fe} = 0.2$ g/g	$Y_{om} = 1.07$ g/g	$q_g^{crit} = 0.7$ g/(gh)	$T_p = 20$ s
$q_a^{c,max} = 0.3$ g/(gh)	$k_a = 50$ mg/l	$Y_{oa}^{ox} = 0.55$ g/g	$Y_{ag}^{ox} = 0.55$ g/g
$Y_{xa}^{ox} = 0.4$ g/g			

2.3 Cultivation

A fed-batch process can normally be divided into three characteristic phases:

I. First, a batch phase is carried out. The decrease in the dissolved oxygen tension (abbreviation: DO) is owing to the increase in the oxygen uptake by the growing culture. The high glucose concentration results in a growth at a maximum specific rate. As DO has decreased down to the chosen setpoint, the stirrer speed is used as a control signal to keep DO constant.

II. A peak in DO due to the depletion of initial glucose marks the end of the batch phase. From now, many alternatives are available to feed the cells. To avoid acetate formation due to overflow metabolism, the feed rate must however be restricted. Some possibilities are described in chapter 4.

III. As soon as the maximum agitation speed is reached, the nutrient flow rate becomes limited by the oxygen transfer capacity of the reactor. Typically, the feed flow rate is decreasing or constant in order to maintain aerobic conditions.

Figure 3 shows a simulation where a fixed profile in the feed rate has been applied in Phase II whereas the DO-stat strategy is used in Phase III.

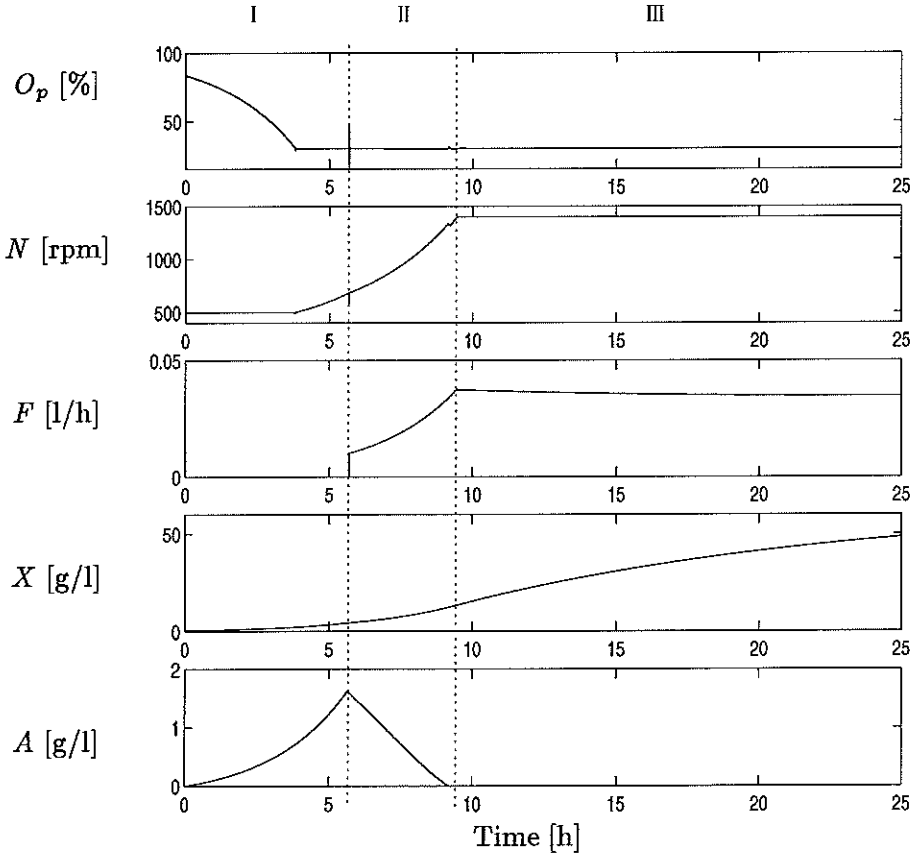

Figure 3 Fed-batch culture with a profile for the feed rate in phase II and the DO-stat strategy in phase III.

3. DO-stat strategy

In fed-batch cultivations, the oxygen uptake rate depends on the feed flow rate while the oxygen transfer rate can be varied for instance by means of the stirrer speed. The principle of the DO-stat method consists in manipulating the substrate flow rate to keep the dissolved oxygen concentration constant. At constant DO there is a balance between the oxygen transfer rate and the oxygen uptake rate. This guarantees that consumption does not exceed the transfer capacity of the reactor and that aerobic conditions are maintained. The strategy is thus well-suited for phase III, where the reactor is operating at the maximum oxygen transfer rate. However, some disturbances in the metabolism may lead to over-feeding and overflow metabolism.

In the DO-stat strategy, the feed rate F is chosen to be the control variable while the stirrer speed N usually remains constant. The structure of the control system is shown in Figure 4.

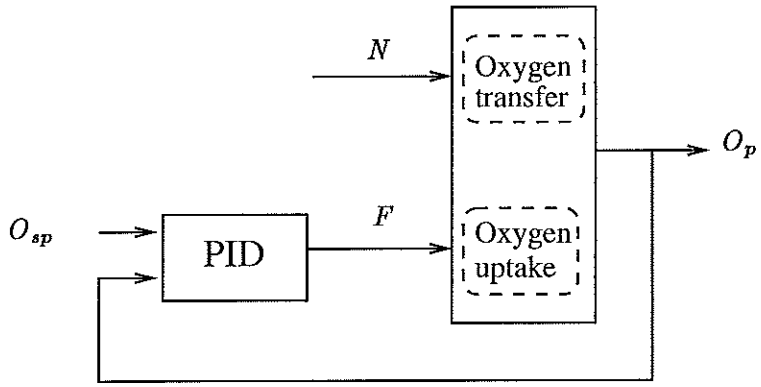

Figure 4 Block diagram of the closed-loop system, using the DO-stat strategy.

The linear model previously derived will serve as a frame to discuss the control problem. One can see that the dynamics of the process depends on many culture variables that are not constant over the cultivation. From a control point of view, this means that adaptive control or gain scheduling may be necessary to overcome problems caused by the process variations. We will thus examine the influence of some culture variables on the process dynamics before designing any controller. We will then point out some critical situations that can be encountered with the DOT-feed strategy before suggesting some practical tuning rules.

3.1 Process variations

We will study the effect of the stirrer speed, the biomass concentration and the glucose concentration on the process behavior. First, changes in one parameter at a time will be considered. The following values will serve as default values:

$$N = 1000 \text{ rpm} \quad X = 10 \text{ g/l} \quad G = 10 \text{ mg/l} \quad V = 2 \text{ l}$$

Then typical changes in the process dynamics during a cultivation will be examined.

Effect of stirrer speed Figure 5 shows the Bode diagrams for the process at three different stirrer speeds:

$$N = 500 \text{ rpm} \quad N = 1000 \text{ rpm} \quad N = 1500 \text{ rpm}$$

One can see that the low frequency gain decreases with an increasing stirrer

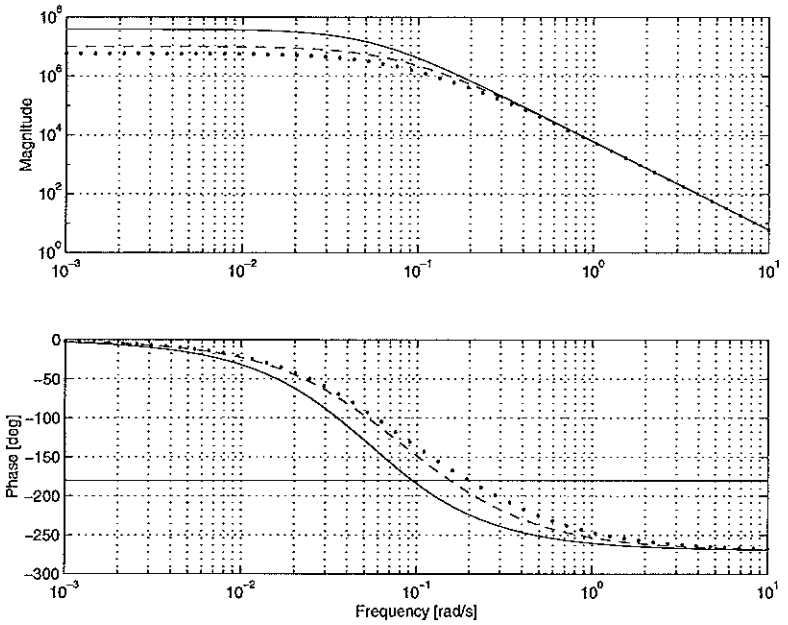

Figure 5 Open-loop Bode diagrams for the process at 500 rpm (solid), 1000 rpm (dashed) and 1500 rpm (points).

speed. As for the phase, one can notice a slight increase. Equation 19 could predict this change. The static gain of the process as well as the time constant T_o are inversely proportional to the agitation speed. One can expect a slower, but more well damped, response of a closed-loop system at high stirrer speeds.

Since the DOT-strategy usually operates at a constant agitation speed, one may often neglect the influence of the stirrer speed while tuning the controller.

Effect of biomass Figure 6 shows the open-loop Bode diagrams for three different cell concentrations:

$$X = 5 \text{ g/l} \quad X = 10 \text{ g/l} \quad X = 40 \text{ g/l}$$

The value of the biomass concentration has a slight influence on the characteristics of the process. One can notice an increase in the gain as well as in the phase at high frequencies due to the decrease of T_g.

Effect of glucose concentration The substrate concentration has a strong influence on the process dynamics. Note that at high glucose concentrations, that is for $q_g > q_g^{crit}$, the linearized model is not valid any more. The specific oxygen uptake rate is then constant and variations in the feed flow rate does not affect the dissolved oxygen concentration.

Figure 7 shows the open-loop Bode diagrams for different glucose concentrations below the critical value (here $G_{crit} = 233$ mg/l):

$$G = 10 \text{ mg/l} \quad G = 100 \text{ mg/l} \quad G = 230 \text{ mg/l}$$

One can see that the phase decreases as well as the gain when the substrate concentration decreases. This can be explained by the saturation in q_g, which results in a smaller T_g.

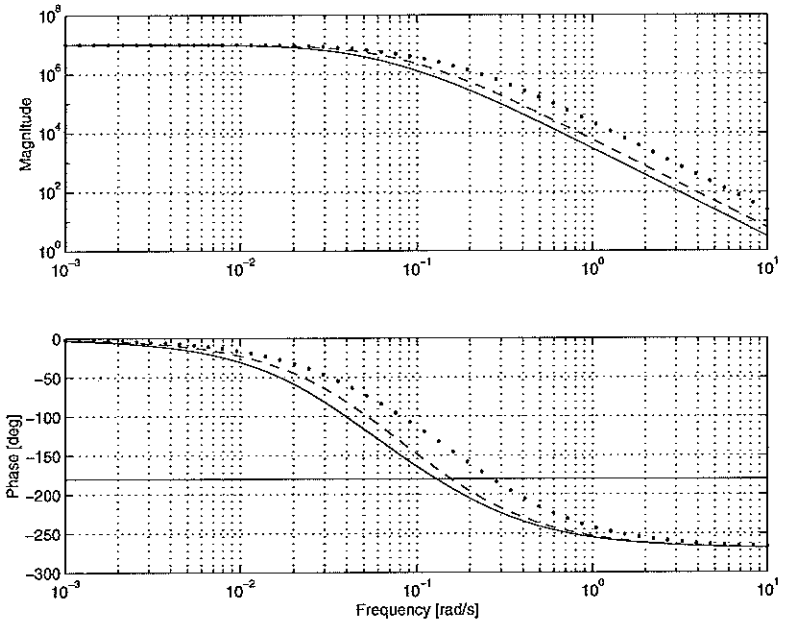

Figure 6 Open-loop Bode diagrams for the process at X = 5 g/l (solid), 10 g/l (dashed) and 40 g/l (points).

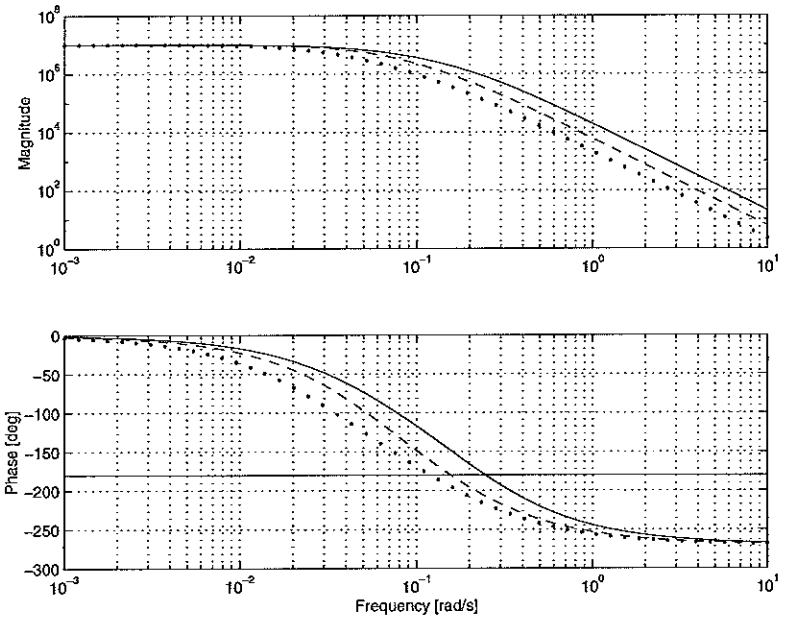

Figure 7 Open-loop Bode diagrams for the process at 10 mg/l (solid), 100 mg/l (dashed) and 230 mg/l (points).

Typical changes Thus far, we have studied the influence of one culture variable at a time, but during a cultivation many parameters change at the same time. It would be relevant to see the typical changes of the process dynamics. Figure 8 indicates the frequency response of the linear model for five operating points given by the simulation shown in Figure 3. The feed rate has been controlled such that glucose was not present at excessive concentrations in the tank. We will later discuss policies to prevent from over-feeding.

The significant changes are the increase in the phase as well as in the gain

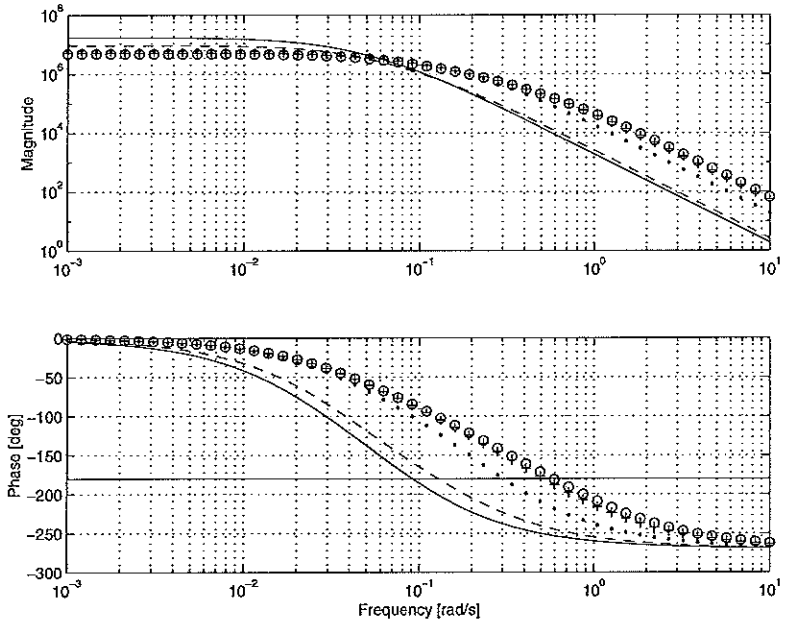

Figure 8 Bode diagrams at time t=6h (solid line), 8h (dashed), 12 (points), 20h (plus), 25h (circles).

at high frequencies. It means that the closed-loop system can be made faster at the end of the cultivation. This is confirmed by the following table where the process gains and time constants were computed for the same instants:

Time [h]	6	8	12	20	25
T_o [s]	10	5.5	3.6	3.6	3.6
T_g [s]	50	33	4.0	1.2	1.0
T_p [s]	20	20	20	20	20
K_oK_g[% l/h]	$4.8 \cdot 10^3$	$2.6 \cdot 10^3$	$1.6 \cdot 10^3$	$1.4 \cdot 10^3$	$1.3 \cdot 10^3$
N [rpm]	720	1040	1400	1400	1400
X [g/l]	4.7	8.6	22	41	48
G [mg/l]	12.3	17.9	6.0	2.1	1.6

The time constant T_g varies with a factor 50 of magnitude, from 50 s to 1s at the end of the cultivation. This can be explained by the relative high glucose concentration in the earlier phase (phase II) in comparison with the later phase (phase III). During the third phase, the oxygen transfer capacity is maximum and the feed rate decreases to maintain aerobic conditions. This results in a lower glucose concentration and faster dynamics. The increasing biomass, which affects T_g, tends also to make the process faster.

As far as the process gain is concerned, the significant decrease from 6h to 8h is due to the increase in the stirrer speed. After the maximum aeration rate is reached, the slight increase in the volume does not affect K_oK_g a lot.

3.2 Control design

Need for adaptive control The previous analysis has indicated significant changes in dynamics between the two fed-batch phases. If DO is controlled by

the feed rate in both phases, it seems hard to use a fixed controller. One would probably need some kind of adjustment of the controller parameters to ensure stability and acceptable performances. During phase III there are less variations in the process dynamics and the need for parameter adaptation does not seem so strong. One can examine the behavior of fixed controllers when the process dynamics change. Two PID controllers were designed to work well at the beginning and at the end of phase III respectively. Figures 9 and 10 show simulations of the closed-loop system for these two operating points. Responses to a disturbance in $K_L a$ are examined.

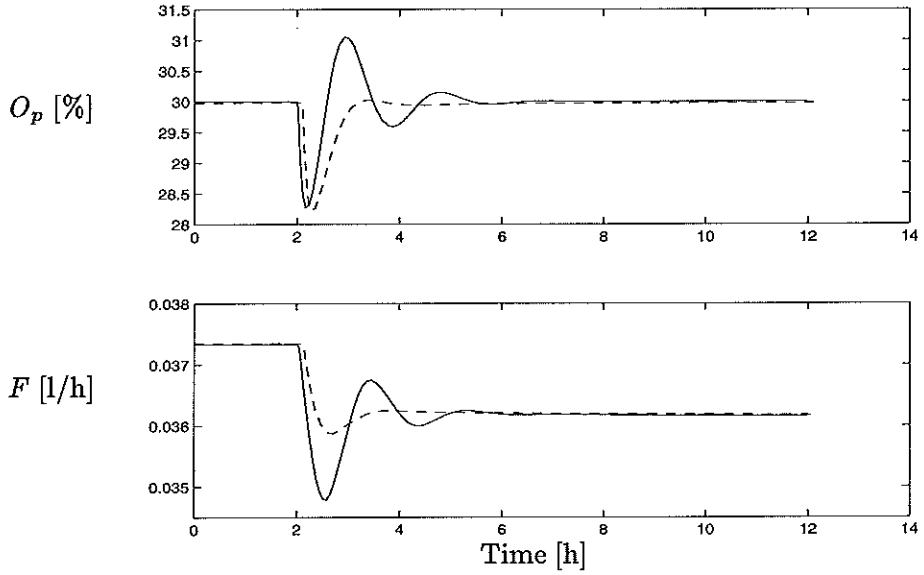

Figure 9 Disturbance in $K_L a$ at 8 hours. PID tuned for low glucose concentrations (solid) and for high glucose concentrations (dashed).

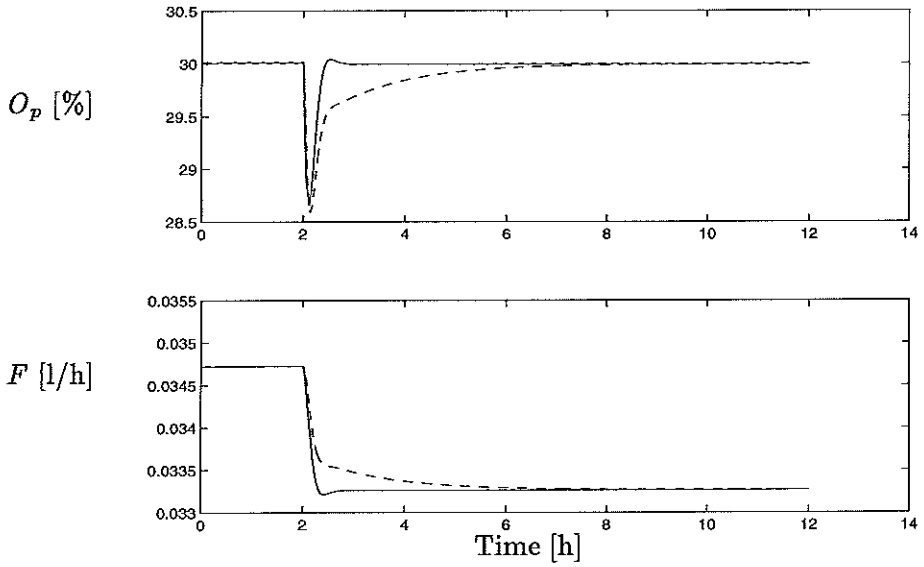

Figure 10 Disturbance in $K_L a$ at 24 hours. PID tuned for low glucose concentrations (solid) and for high glucose concentrations (dashed).

The controller designed for the beginning behaves well but gives a rather slow behavior at the end of the cultivation. The controller designed for the end works better around 24 hours but the system is oscillatory at the activation of the DO-stat. If one controller with fixed parameters is used, it should be tuned for the beginning. This would give a robustly stable closed-loop system but sluggish at the end. Gain scheduling should be used to achieve good performance at every time of the culture. The problem is that the adaptation is strongly correlated to the glucose concentration, which is not known.

Simple tuning procedures Good performances can be achieved if a good process model is available. However, in practice it is important to have simple tuning rules for experimental situations.

Tuning methods, such as Ziegler-Nichols, can be applied to get initial controller parameters. Manual adjustments can then be made to improve the performance. If the gain and the dominant time constant of the process is known from prior experiments, a preliminary tuning can be made as follows.

Approximating the process as

$$G(s) = \frac{K}{1+Ts}$$

the controller parameters can then be computed by using a pole placement method. A PI controller is described by:

$$G_c(s) = K_c(1 + \frac{1}{T_i s})$$

The characteristic equation for the closed loop system is thus

$$Ts^2 + (1 + KK_c)s + \frac{KK_c}{T_i} = 0$$

The controller parameters can be chosen such that the closed-loop characteristic equation is

$$s^2 + 2zws + w^2 = 0$$

K_c and T_i should be chosen as

$$K_c = \frac{2zwT - 1}{K}$$
$$T_i = \frac{2z}{w} - \frac{1}{Tw^2}$$

The process is in fact not well described by a first order model, one should adjust the coefficients to get good performance. Still the method can be reasonable if one chooses w close to $\frac{1}{T}$. One can also add a derivative part which yields better results.

3.3 Critical situations

It has been established that the DO-stat method can handle perturbations in the oxygen transfer coefficient. Aerobic conditions are maintained even when the oxygen transfer rate changes for any reason; the feed rate is manipulated to keep a constant DO level. However, problems may arise if the saturation of the respiratory system is reached, where one loses controllability. One can express oxygen and glucose dynamics in the case where q_g is beyond q_g^{crit}. Eq. 3 and Eq. 16 can be rewritten as

$$\begin{cases} \dfrac{dO}{dt} = K_L a(O^* - O) - q_o^{max} \cdot HX \\ \dfrac{dG}{dt} = \dfrac{F}{V} G_{in} - q_g(G) \cdot X \end{cases} \tag{20}$$

The oxygen uptake does not depend on the glucose concentration any more. It means that an increase in the flow rate will not directly give a decrease in DO. The controller will go on increasing the flow rate to no avail, because there is a decoupling between oxygen consumption and glucose supply. As a consequence, the glucose concentration in the broth will increase more and more and acetate will accumulate. The problem can get worse if the maximum glucose uptake rate is reached.

Several perturbations can give rise to the saturation of the respiratory system. Problems may appear if the DOT-feed strategy is activated with an inappropriate stirrer speed. An excessive agitation speed would result in an oxygen transfer rate larger than the oxygen uptake rate. The controller would then increase the feed flow rate to achieve the balance between the two previous rates. This can however be avoided by controlling the stirrer speed after the batch phase. Another kind of disturbances, namely the one originating from metabolism changes, may also occur in a cultivation. Indeed, perturbations such as decreases in q_g^{crit} or in the biomass, are frequently encountered, especially after induction of recombinant protein production. In the DO-stat strategy the controller reacts to these disturbances by increasing the feed rate.

A simulation shown in Figure 11 confirms the danger of a decrease in q_g^{crit}. As soon as the specific glucose uptake rate becomes larger than the critical value, DO goes up sharply. The fast increase in DO results in a prompt increase in the feed rate, and q_g increases to q_g^{max}. Glucose accumulates and acetate is produced. Provided that there is a saturation in the flow rate corresponding to the maximum pump speed and that an anti-windup system has been used, the situation comes back to a normal state. Indeed, the increasing biomass consume the accumulated substrate and when DO becomes lower than the setpoint, the feed rate decreases. An anti-windup system is required to reduce the time when the feed rate saturates. Without such a system, the control signal will remain saturated even when DO falls below the setpoint and glucose and acetate will accumulate further. See Appendix A for the structure used in the simulations. One can notice that the controller does not feed the cells between 11.7h and 13h. This comes from the fact that acetate is consumed and that the cells require oxygen to oxidate it.

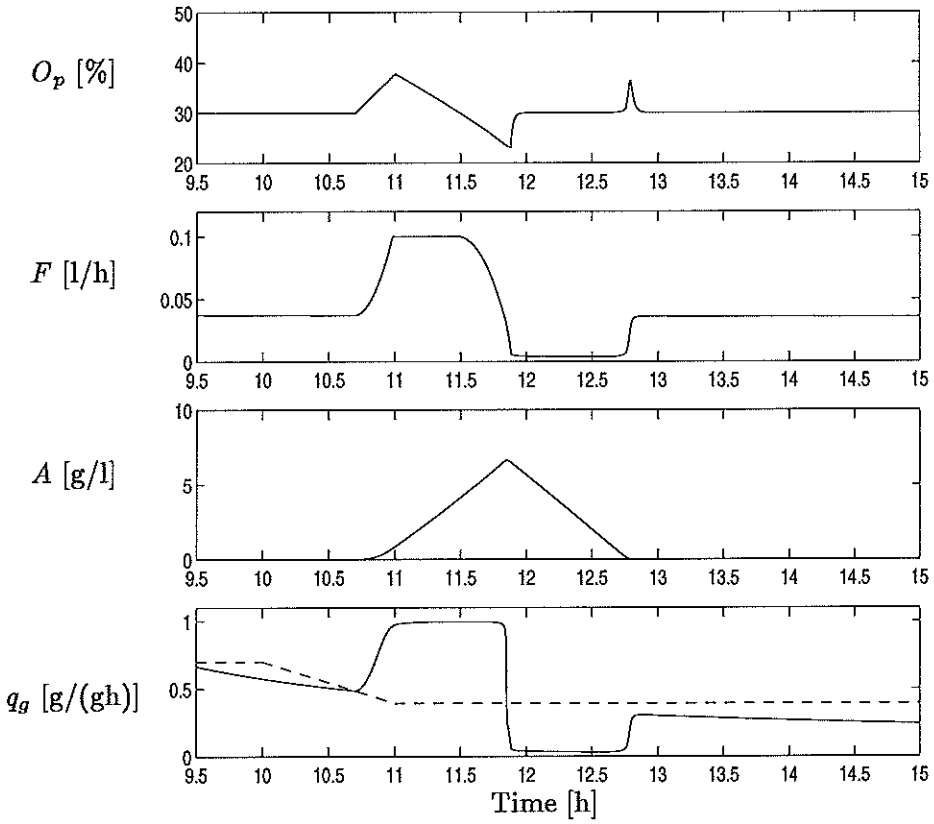

Figure 11 Simulation of the DO-stat strategy with a decrease in q_g^{crit} at time 10h. The feed flow rate is limited to 0.1 l/h.

Cell death is another perturbation that may be difficult to handle with the DO-stat strategy. If the amount of viable cells goes down, q_g increases continuously and the substrate accumulates until the saturation of the respiratory system occurs. As soon as q_g gets above q_g^{crit}, the dissolved oxygen concentration goes up sharply and the feed rate is significantly raised by the controller. Glucose accumulates and acetate is produced as it is shown in Figure 12. As long as the biomass goes down, DO increases and the feed rate saturates to its maximum flow.

In summary, the DO-feed strategy is a strategy, which is efficient for handling the limitation in the oxygen transfer capacity, but it may yield over-feeding when metabolic changes occur. The weak point is that one does not know the origin of the disturbance and disturbances in the metabolism may be falsely interpreted as a change in the oxygen transfer.

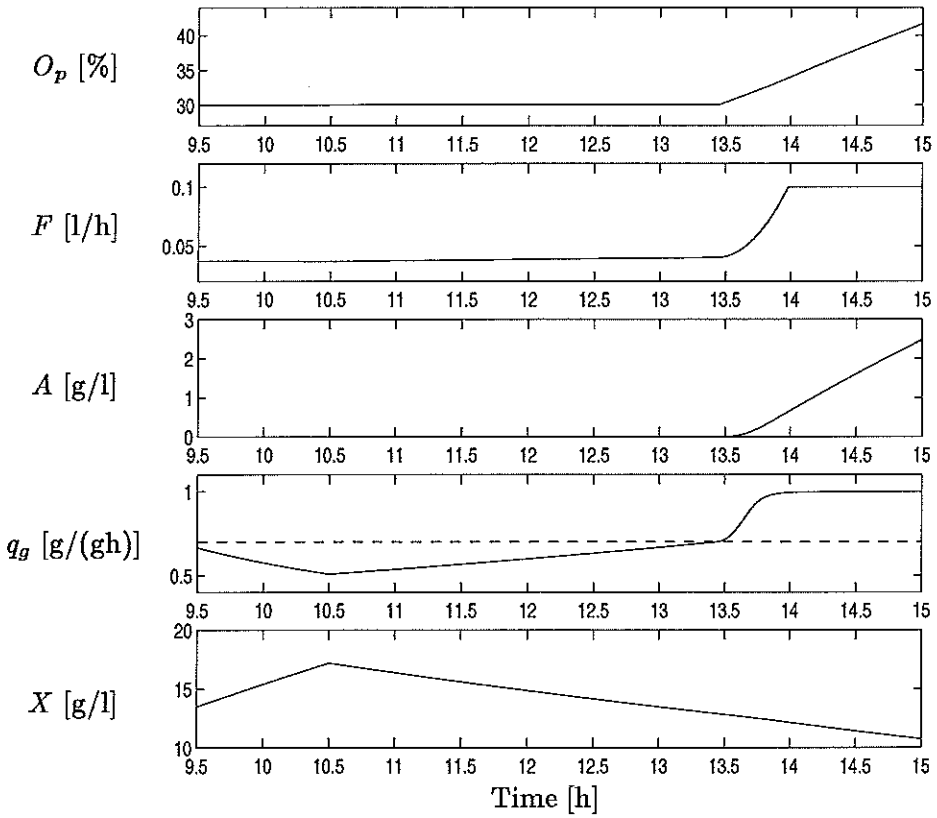

Figure 12 Simulation of the DO-stat strategy. After 10.5h cell mass decreases with a rate of $0.08h^{-1}$.

4. The early fed-batch phase

In the latter part of the cultivation (phase III), the feed rate is limited by the oxygen transfer capacity of the reactor. Indeed, the stirrer speed and the aeration rate will reach their maximum value. During the early fed-batch stage (phase II), the feed rate is instead limited by the cell metabolism. One cannot feed the cells more than the respiratory system allows it, overflow metabolism resulting in acetate by-product formation would then occur. If the critical growth rate μ_{crit} (or q_g^{crit}) where acetate formation starts, is known, it is possible to choose the feed rate so that $\mu < \mu_{crit}$. One can apply a fixed profile in the feed rate, or a profile in the stirrer speed combined with the DO-stat method. It is also possible to apply closed-loop control of μ. The problem is that the value of μ_{crit} is usually not known and that it may change during the cultivation. Another method is to use a probing feeding strategy, which finds the critical value of the growth rate, without knowing the value a priori.

4.1 Fixed profiles

We will investigate feeding strategies, which aim at achieving a cell growth at a predetermined rate μ_{set}.

Feed rate profile If all glucose is converted to cell mass and no disturbances occur, the exponential profile

$$F(t) = F(t_0) \cdot e^{\mu_{set}(t-t_0)}$$

will give an exponential growth with the growth rate μ_{set}

$$\begin{cases} X(t) = X(t_0) \cdot e^{\mu(t-t_0)} \\ \mu \approx \mu_{set} \end{cases}$$

Assuming that maintenance can be neglected and that glucose concentration is constant, Equation 5 gives an expression for the parameter $F(t_0)$:

$$F(t_0) = \frac{\mu_{set} X(t_0) V}{Y_{xg}^{ox} G_{in}}$$

A rather good estimation of the biomass after the batch phase is

$$X(t_0) = Y_{xg}^{ox} \cdot G_{init}$$

To avoid over-feeding, μ_{set} has to be chosen lower than μ_{crit}. Simulations have been carried out and results are shown in Figure 13 for the following μ_{set}

$$0.75\mu_{crit} \qquad \frac{\mu_{crit} + \mu_{max}}{2} \qquad \mu_{max}$$

The dissolved oxygen concentration has been maintained constant by controlling the stirrer speed with a PID controller.

One can see on the plots, that the exponential profile for F is efficient to control μ (or q_g). The specific glucose uptake rate is maintained at the desired value. The problem is that μ_{crit} is not always known and an underestimated μ_{crit} leads to over-feeding. For $\mu_{set} > \mu_{crit}$ acetate produced during the batch phase is not consumed, but more by-product is formed.

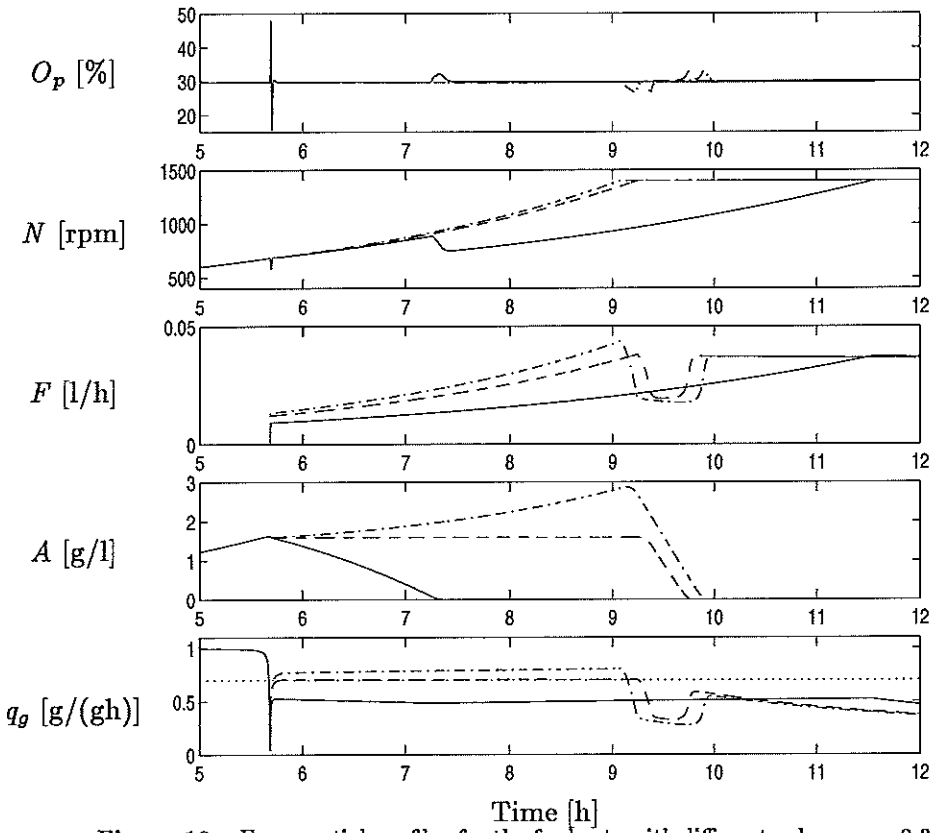

Figure 13 Exponential profiles for the feed rate with different values μ_{set}: 0.24 h^{-1} (solid), 0.35 h^{-1} (dashed), 0.38 h^{-1} (dotted). $\mu_{crit} = 0.32$ h^{-1}.

Profile for the stirrer speed We will now apply a predetermined profile in the stirrer speed and use the DO-stat strategy. As the oxygen transfer rate is increased by the profile in N, the feed rate is raised by the controller in order to keep DO constant. Equation 3 can give an idea about the profile that has to be applied to achieve an exponential growth. Assuming that DO is kept constant, we get

$$0 = K_L a(N)(O^* - O) - q_o H X$$

Under the assumption that $K_L a$ depends linearly of the stirrer speed, one can express N as:

$$N = N_0 + \frac{q_o H}{\alpha(O^* - O)} \cdot X$$

As many coefficients such as α or N_0 are not known, it is hard to control the specific growth rate at a predetermined value μ_{set}. Nevertheless, the following exponential profile is easy to perform:

$$N = N(t_0) e^{\mu_{set}(t - t_0)}$$

where $N(t_0)$ is the value of the stirrer speed at the end of the batch phase. Such profiles have been applied for 3 values μ_{set}:

$$0.25\mu_{crit} \qquad 0.5\mu_{crit} \qquad 0.6\mu_{crit}$$

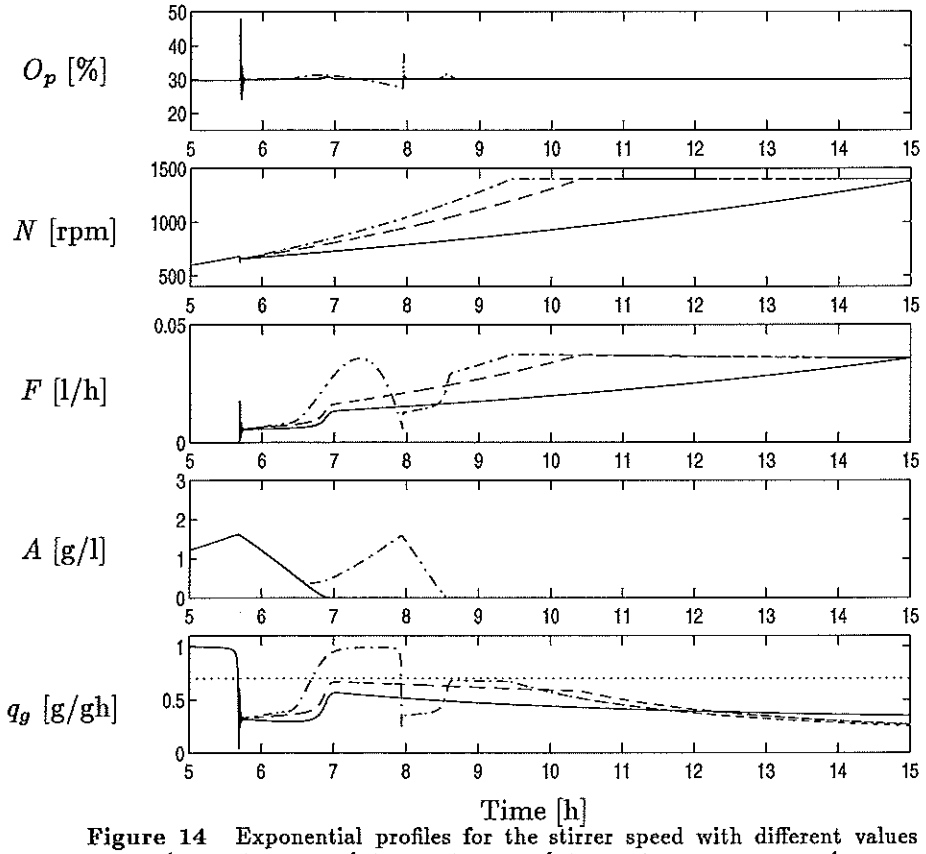

Figure 14 Exponential profiles for the stirrer speed with different values μ_{set}: 0.08 h^{-1} (solid), 0.16 h^{-1} (dashed), 0.2 h^{-1} (dotted). $\mu_{crit} = 0.32$ h^{-1}.

Results of simulations are shown in Figure 14.

Note that DO is not constant during the increase in N. This comes from the fact that a PID controller is not fast enough to cancel the effect of an exponential perturbation.

Poor results are obtained when μ_{set} approaches the critical value μ_{crit}. Indeed, acetate excreted during the batch phase is not consumed when $\mu_{set} > 0.5\mu_{crit}$, but more by-product is produced. The exponential profile is not appropriate to control the cell growth at a predetermined rate. A linear increase with an arbitrary slope may give better results. One should note that, if the profile does not lead to over-feeding, acetate is rapidly consumed in comparison with the profile for F. The DO-stat strategy does not provide cells with substrate if acetate is being consumed.

Conclusion Two characteristics have to be taken into account for the choice of the method: the ease of use and the safety. In term of complexity, both methods are on the same level. The profiles in F or N lead to two switches manual/automatic mode for the N-controller and 1 switch manual/automatic mode for the F-controller.

As far as the safety is concerned, the profile in F turns out to be better. Indeed, it has been observed that the profile in N is very sensitive to the choice of μ_{set}. Better results may be achieved with more information on the process. It seems difficult to increase the oxygen transfer rate with the help of the stirrer speed whereas the relationship $K_L a(N)$ is not known. Another problem is that one should not use a controller for the feed rate when there is risk of over-feeding. As a consequence, it seems reasonable to use a profile for

F while controlling dissolved oxygen tension with the stirrer speed.

One should however consider the results with care, because it has been assumed that μ_{crit} and other process parameters were known. One can exploit the data during the batch phase to estimate the maximum rate of growth μ_{max} and choose μ_{set} with a reasonable margin to avoid over-feeding. As far as the yield coefficients are concerned, they can usually be considered as known. Some changes may however occur in a cultivation, caused for instance by the induction. Some methods to estimate process parameters are given in Appendix B.

4.2 Pulse strategy

The pulse strategy is a strategy which achieves a feeding at a high growth rate without acetate formation, see [2]. The key idea is to detect acetate formation by exploiting that the respiratory system gets saturated. The principle consists in superimposing pulses to the feed rate and observing the DO response. If q_g is below q_g^{crit} an up pulse results in an increase in DO, whereas if q_g is beyond q_g^{crit} there is no reaction to pulses. The pulse responses can thus reveal if q_g is above or below q_g^{crit} (see Figure 15).

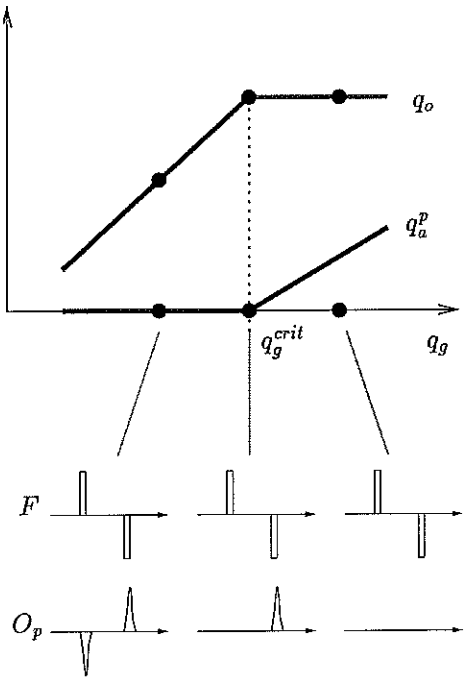

Figure 15 Pulse responses in DO for q_g below, at and above q_g^{crit}.

The feed rate is then adjusted so that cells grow at q_g^{crit}. The set of rules describing the adjustment of F is given in Figure 16.

Between two pulses a PID controller regulates the stirrer speed to bring DO back to the setpoint. As long as the stirrer speed has not reached the maximum value, the strategy achieves a feeding at q_g^{crit}. A simulation is shown in Figure 17. The acetate produced during the batch phase is initially consumed, which affects the pulse responses, and as a result the feed is decreased. This is not a problem because the acetate consumption is then accelerated. After the depletion of acetate, the probing controller achieves a feeding below q_g^{crit} until the stirrer speed saturates and then the DO-stat strategy is activated.

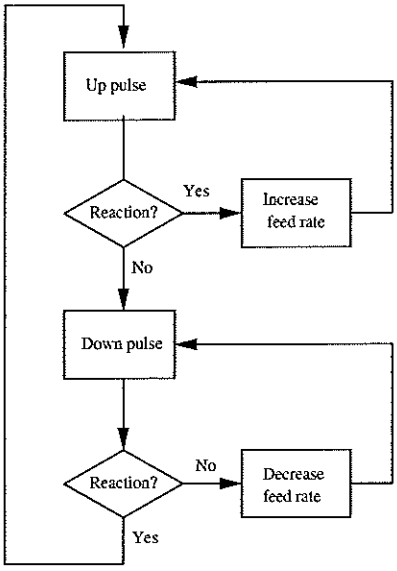

Figure 16 Control algorithm for feed rate adjustments.

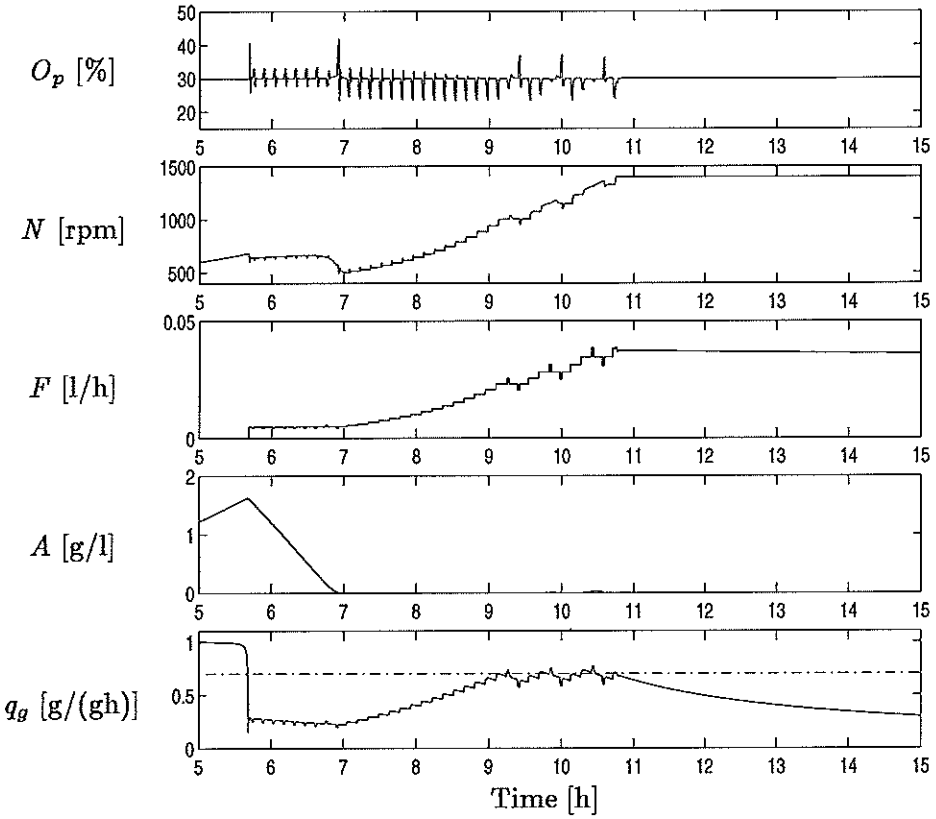

Figure 17 Simulation of the pulse strategy with activation of the DO-stat method when N becomes saturated.

In contrast to the previous methods, the pulse technique requires a minimum of information on the process and it also avoids over-feeding.

5. A combined feeding strategy

Our aim is now to develop a strategy, which can handle, at every time of the cultivation, both metabolic changes and the limitation in the oxygen transfer capacity. Acetate formation due to overflow metabolism can be detected by the pulse technique whereas the DO-stat strategy can handle the maximum oxygen transfer rate. The principle will consist in combining the advantages from these two methods.

5.1 Handling of metabolic changes

The probing strategy permits to avoid over-feeding by using the information from pulse responses in the DO signal. Some precautions have to be taken when the stirrer speed saturates. One cannot increase the feed rate any more if aerobic conditions are to be maintained. Furthermore, as DO cannot be controlled, false interpretations of DO responses could be made. To tackle this problem, a safety net has been introduced, see [5]. The feed rate is not allowed to increase when N is larger than 90% of the maximum. One will consequently perform only down pulses in this region. Figure 18 shows the behavior of the strategy when metabolic changes occur. Compared to the simulation in Figure 11, the amount of by-product that is produced is now really low.

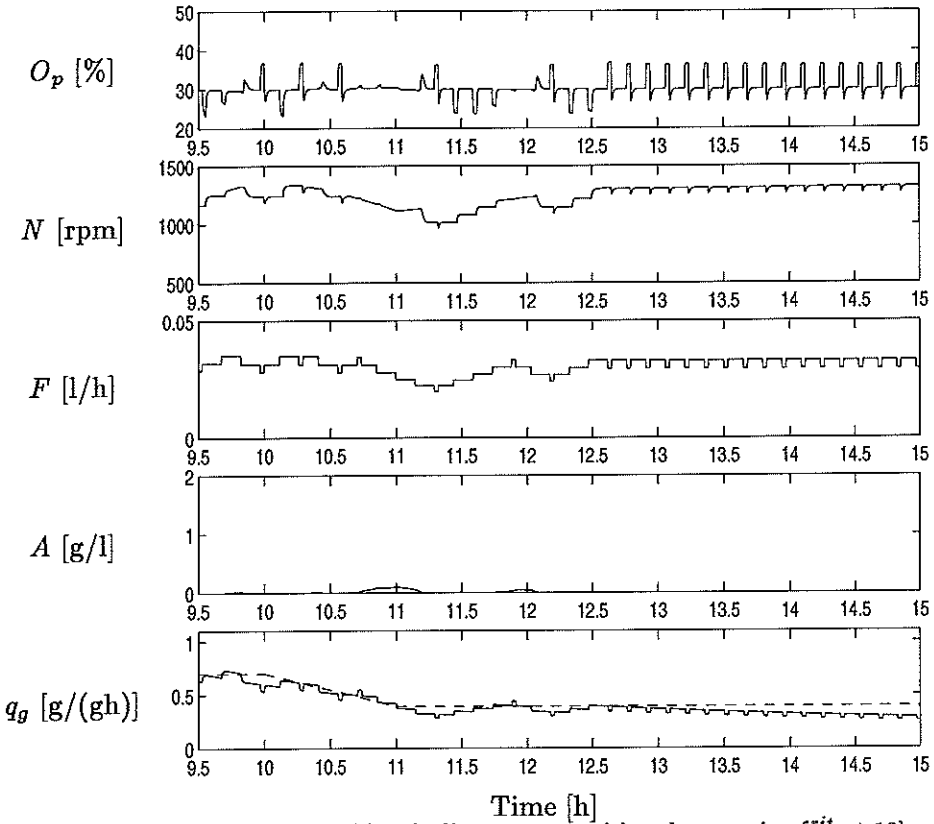

Figure 18 Probing feeding strategy with a decrease in q_g^{crit} at 10h.

The strategy with the safety net works well in case of metabolic disturbances, but it does not necessarily handle the limitation in the oxygen transfer. The simulation in Figure 19 shows the danger of changes in K_La.

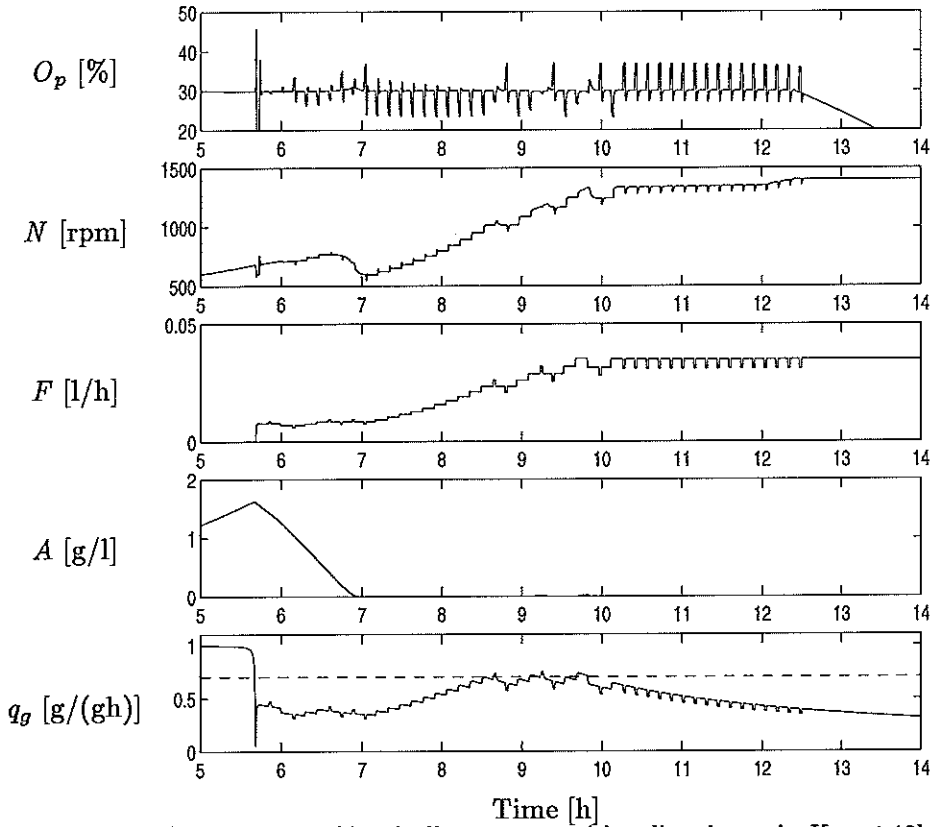

Figure 19 Probing feeding strategy with a disturbance in K_La at 12h.

After 12 hours, K_La is decreased and this gives a fall in DO such that aerobic conditions are not guaranteed any more. If acetate formation due to anaerobic conditions would have been modeled, one would have seen an accumulation of by-product when DO approaches zero.

To prevent false interpretations of pulse responses, no pulses are done when DO is below the setpoint.

5.2 Handling of limitation in oxygen transfer capacity

The DO-stat method can tackle problems related to the limited oxygen transfer capacity. The idea is now to use the feed rate as an additional control signal to control DO when the stirrer speed reaches its maximum. When the stirrer speed saturates between the pulses, a second PID controller manipulating the feed rate is activated for the control of DO. As soon as the stirrer speed is set lower than its maximum the controller for the feed rate is deactivated and gets in tracking mode. The controller using the stirrer speed is always active. Consequently, no excessive increase in F can occur. Any metabolic perturbations leading to an increase in DO will be handled with the controller for the stirrer speed. A simulation with a disturbance in the oxygen transfer coefficient is shown in Figure 20.

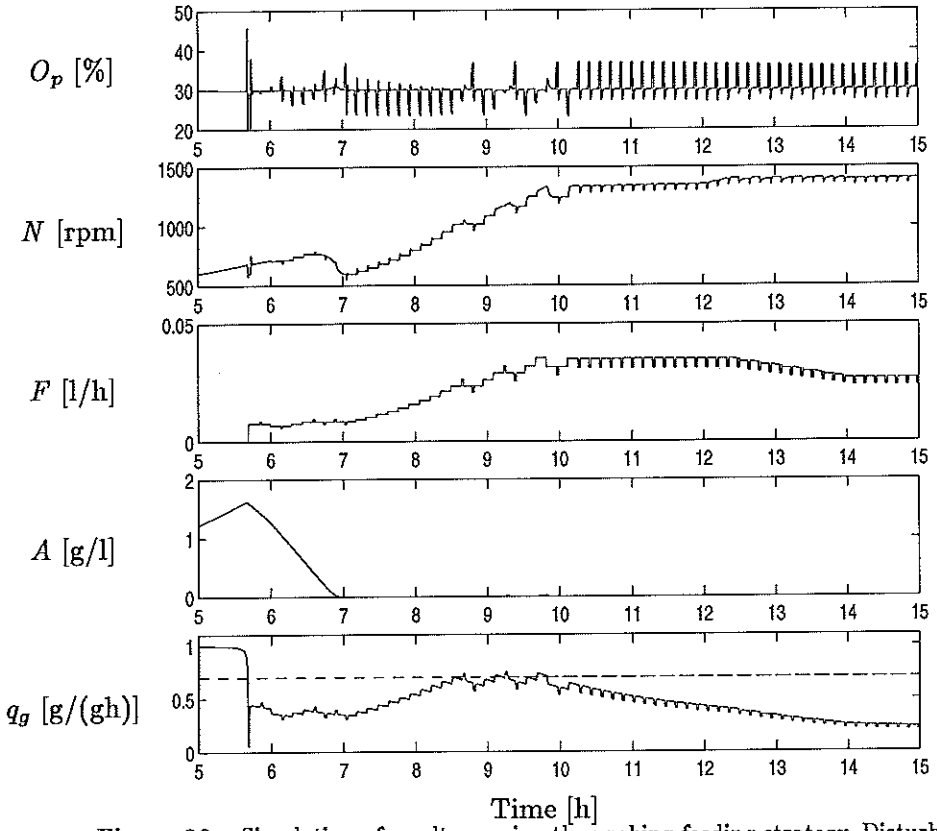

Figure 20 Simulation of a culture using the probing feeding strategy. Disturbance in K_La between 12h and 14h.

Aerobic conditions are now maintained when there is a change in the oxygen transfer rate. The feed rate is decreased by the controller and DO is kept at a constant level.

The combined feeding strategy is now able to handle perturbations in the metabolism as well as in the oxygen transfer rate. Figure 21 shows a simulation where both perturbations occur.

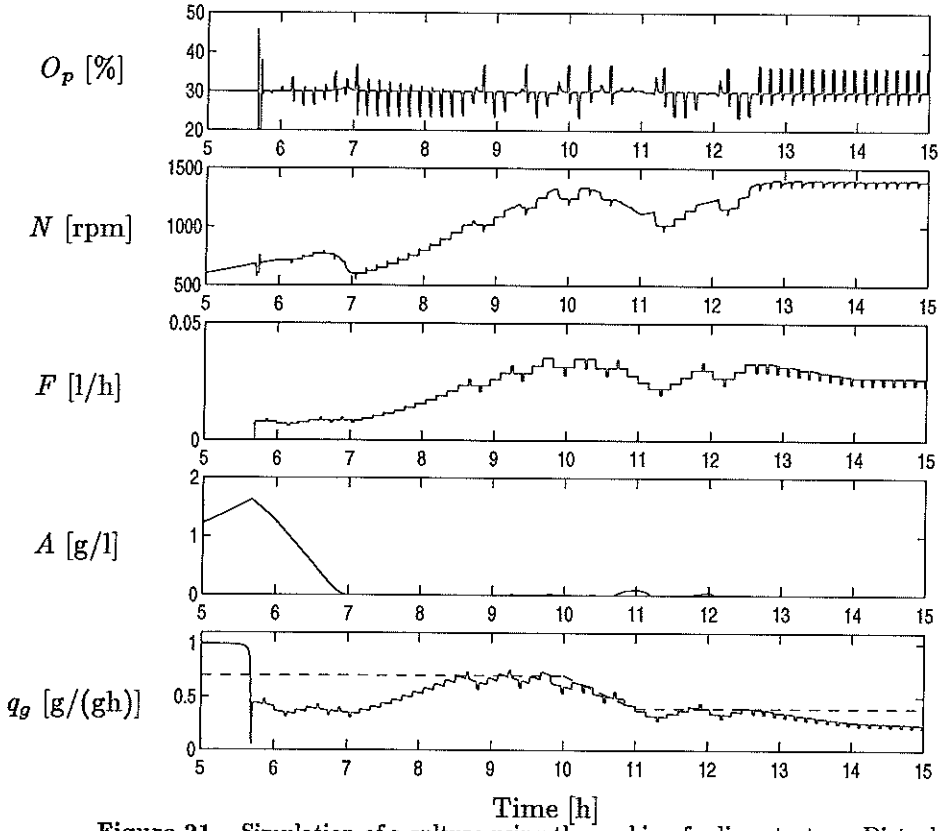

Figure 21 Simulation of a culture using the probing feeding strategy. Disturbance in q_o^{max} at 10h and in K_La between 12h and 14h.

6. Conclusion

Different feeding strategies for fed-batch cultures of *E. Coli* have been presented. The cultivation can be divided into different phases with their specific constraints requiring an appropriate strategy.

First, the DO-stat strategy, which can handle the limitation in the oxygen transfer capacity of the reactor has been studied. This method is typically activated when the stirrer speed reaches its maximum, that is in the later fed-batch phase. The feed rate is manipulated to keep a constant dissolved oxygen level and aerobic conditions are thus maintained. However, the strategy may cause over-feeding when changes in the metabolism occur. Simple tuning rules have also been derived for the design of the controller. It turned out that, in spite of process variations, a fixed controller may be used if it is tuned at the beginning of the fed-batch phase.

Then, several strategies that are used in the early fed-batch stage have been described. All of them tend to restrict the feed rate in order to avoid acetate formation from overflow metabolism. The problem is that the critical glucose concentration corresponding to the onset of acetate formation is not known. A probing strategy using a pulse technique can however overcome this difficulty. It achieves a feeding at a high growth rate and prevents from over-feeding when metabolism changes occur. The major advantage with the pulse strategy is that a minimum of a priori information on the process is required. However, it does not guarantee aerobic conditions.

Finally, a new strategy combining the advantages of the DO-stat strategy and the pulse strategy has been suggested. Simulations show that this strategy is able to avoid acetate formation due to overflow metabolism and anaerobic conditions in spite of different disturbances. With this approach it is possible to use the same feeding strategy throughout the cultivation.

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A. APPENDIX

The integrator in a PID controller is essential to achieve good performance, but it gives rise to problems in some cases. If the control system operates in different modes, it is necessary to avoid switching transients. One has to ensure that the integrator in the PID is reset to a proper value before switching. The smooth transition can be achieved by the circuit shown in Figure 22. With this system the controller tracks the control signal such that the controller output will be close to the manual value at every time.

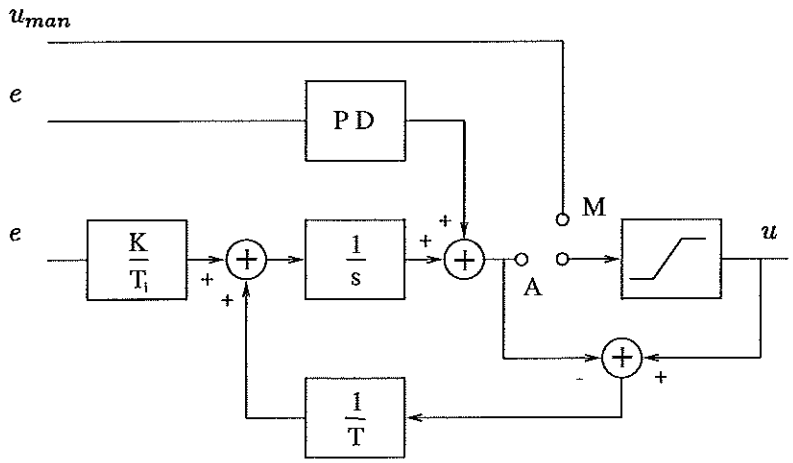

Figure 22 PID controller with tracking system.

This system is also able to avoid integrator windup. When the error is very large, the integral part of the controller goes up to a very large value and the actuator saturates. When the error changes sign it can take considerable time until the integral assumes a normal value again. The control signal remains saturated even if no control action should be given. The tracking system resets the integrator when the output saturates. The integral part is recomputed so that the control signal is at the saturation limit. See [6] for more details.

B. APPENDIX

- Estimation of the maximum rate of growth

  During the batch phase, the glucose concentration is high enough to consider that $q_g = q_g^{max}$, and the resulting rate of growth is

  $$\mu = (q_g^{crit} - q_m)Y_{xg}^{ox} + (q_g^{max} - q_g^{crit})Y_{xg}^{fe} = \mu_{max}$$

  As μ is constant, integration of Equation 7 between t_1 and t_2 yields the following expression

  $$\mu_{max} = \frac{ln\frac{X_2}{X_1}}{t_2 - t_1}$$

  Some measurements of the biomass using for instance optical density can give a rather good estimate of μ_{max}.

- On-line estimation of the rate of growth

  Several methods have been developed to estimate the specific growth rate. If we assume that the variation in the biomass is proportional to that of product formation or nutrient uptake, we can write

  $$dX = YdP$$

  Since,

  $$\mu dt = \frac{dX}{X}$$

  we can express μ as a function of the ammonia consumption (we also could use carbon dioxide formation). Assuming that the yield coefficient Y is constant, we get

  $$\frac{dX}{X} = \frac{YdR}{YR} = \frac{dR}{R}$$

  Integration of the previous equation leads to

  $$\mu = \frac{d(lnR)}{dt}$$

  Then, we need to measure continuously the amount of ammonia dosed to the vessel. An easy way to achieve it, avoiding an expensive balance equipment, has been proposed in [7]. The principle consists in storing the accumulated dosing time, which is proportional to the total amount of solution added.

- On-line estimation of the yield coefficients

  In most cases, yield coefficients are not constant over the cultivation, and they need to be estimated. On-line estimation of yield coefficients can be done by utilizing macroscopic and elemental balances, see [8]. The

process of growth with ammonia as the nitrogen source can be described by the following chemical reaction :

$aC_xH_yO_z$	+	bO_2	+	$cNH_3 - - - >$
carbon source		*oxygen*		*ammonia*
$C_\alpha H_\beta O_\gamma N_\delta$	+	dH_2O	+	eCO_2
biomass		*water*		*carbon dioxide*

Formulae of the carbon source and cell biomass are assumed to be known. The yield coefficients for oxygen and carbon dioxide are related to the stoichiometry coefficients as follows

$$Y_{xo} = \frac{MW}{32b}$$
$$Y_{xco2} = \frac{MW}{44e}$$

with MW denoting the cell mass. Elemental balances for C, H, O, and N yields

$$4xb + (2z - y - 4x)e = 2\gamma x + y\alpha + 3x\delta - x\beta - 2z\alpha$$

We can also derive an expression for the overall rate of growth using the two previous yields

$$R = \frac{32Y_{xo}}{V}(Q_{in}c_{o,in} - Q_{out}c_{o,out})$$
$$R = \frac{44Y_{xco2}}{V}(Q_{out}c_{co2,out} - Q_{in}c_{co2,in})$$

Then, yield coefficients can be estimated on-line, and calculation of the specific growth rate can be done.