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A MODIFIED PROBING FEEDING STRATEGY: CONTROL ASPECTS

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Abstract The paper presents a fermentation technique, which combines the advantages of the probing feeding strategy and the temperature limited fed-batch technique. The early phase of the cultivation is run under glucose limited conditions by using the original probing technique. When the maximum oxygen transfer capacity of the reactor is reached, the temperature is decreased to lower the oxygen demand. A slight glucose excess, achieved by downwards probing pulses, guarantees that the temperature is the only limiting factor. To achieve a good control of the dissolved oxygen a mid-ranging controller manipulating the stirrer speed and the temperature is used. A model describing the temperature influence on the cell metabolism is derived to facilitate the controller design. The feeding strategy is illustrated by an experiment.

1. INTRODUCTION

E. coli is a common host organism for production of recombinant proteins. It is a well-known bacterium that can be quickly grown to high cell density. The main problem encountered when cultivating *E. coli* is the accumulation of the by-product acetate. Formation of acetate occurs in two situations: under anaerobic conditions or under fully aerobic conditions by overflow metabolism, that is when the carbon source in the medium is in excess. To limit the carbon source in the reactor it is usually fed continuously. When the carbon source is the limiting factor, the technique is called substrate limited fed-batch. One feeding strategy that can be used to dose the substrate feed and that avoids acetate accumulation is the probing feeding strategy described in (Åkesson *et al.* (2001a)).

However a cultivation technique based only on the manipulation of the feed is not enough, it usually results in the release of endotoxins into the media, as noticed in (Rozkov (2001)) and (Han (2002)). Endotoxins are unfavorable since it complicates the downstream processing. To minimize the release one can use the temperature limited fed-batch technique described in (Silfversparre *et al.* (2002)). The method consists of decreasing the cultivation temperature to control the oxygen consumption rate and thereby avoid oxygen limitation. Also, the substrate has to be fed in excess in order to prevent substrate limitation.

The main obstacle with the temperature limited fed-batch technique is to achieve a non-growth limiting glucose concentration in the reactor without accumulating acetic acid, since an on-line glucose sensor is usually not available.

A new fermentation technique combining the advantages of the substrate limited fed-batch and the temperature limited fed-batch methods is described in the paper. In section 2, a brief description of the probing feeding strategy is given. The influence of the temperature is investigated in section 3 where a model of the bioreactor is derived. In section 4 a modified probing feeding technique is presented where the temperature is used to lower the oxygen demand of the bacteria. One experiment with the new technique is shown in section 5.

2. THE PROBING FEEDING STRATEGY

Usually glucose is used as the substrate and the probing feeding strategy leads to a cultivation that is somewhat glucose limited. The feeding strategy makes use of the following principle: under glucose limited conditions pulses added to the feed give rise to changes in the glucose uptake rate. These variations affect the oxygen uptake rate that can be seen in the dissolved oxygen signal, which is usually measured. The information from the dissolved oxygen signal is used in a feedback algorithm that adjusts the feed rate after each pulse.

- When the response in the dissolved oxygen is large enough, the feed rate is increased proportionally to the size of the pulse response.
- When there is no visible response in the dissolved oxygen, the feed rate is decreased with a fixed proportion.

The strategy is illustrated in figure 1, which shows a part of a cultivation. The feed rate is first increased since there are large responses in the dissolved oxygen signal and thus no acetate accumulation. The absence of response to the third pulse indicates that acetate is produced and the feed rate is therefore decreased. The dissolved oxygen is controlled between the pulses using the stirrer speed. A gain-scheduled PID with respect to the stirrer speed is used and a setpoint value of 30 % is chosen. During a feed pulse the stirrer speed is frozen. Otherwise it would have been difficult to quantify the response in the dissolved oxygen signal.

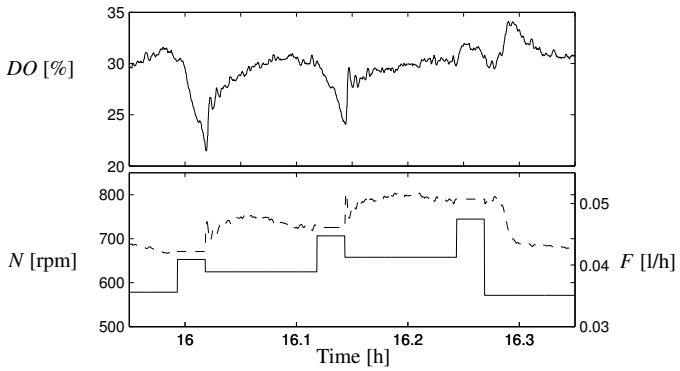

Fig. 1 A part of an experiment where the probing feeding strategy is used. From top: dissolved oxygen *DO*, feed *F* and stirrer speed *N* (dashed).

When the maximum oxygen transfer capacity of the reactor is reached i.e. the maximum stirrer speed, the probing feeding strategy decreases the feed rate to keep the reactor working under aerobic conditions. The decrease is done at a constant rate:

$$\frac{dF}{dt} = -\gamma F$$

The strategy has been successfully used for *E. coli* cultivations on 3 l up to 12 m^3 reactors, see (Ramchuran *et al.* (2002)), (Velut *et al.* (2002)). It has also been tested on other microorganisms presenting similar overflow metabolism phenomenon, see (de Maré *et al.* (2003)).

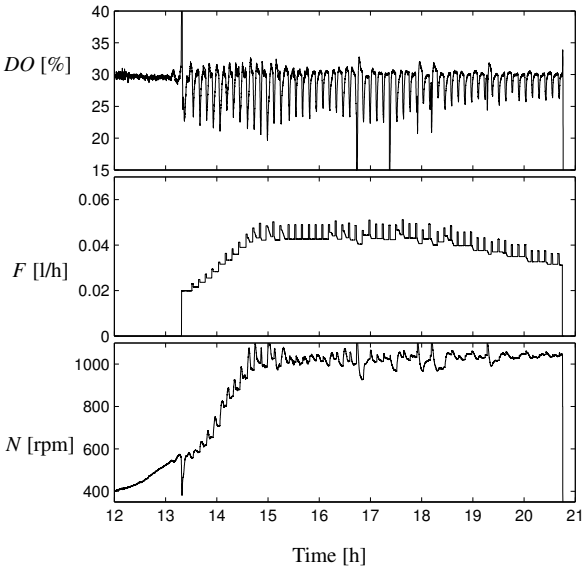

Fig. 2 A cultivation where the original probing feeding strategy is used. The fed-batch part of the cultivation is shown. From top: dissolved oxygen *DO*, Feed *F* and the stirrer speed *N*. At t = 16.7 h and t = 17.5 h antifoam is added which has a large impact on the dissolved oxygen.

Figure 2 shows an experiment performed in a 3 l reactor where the probing feeding strategy is used. Here the maximum stirrer speed is reached 2 hours after the feed started. Then the feed is decreased for about 6 hours. The low feed rate may lead to starvation and endotoxins formation, (Rozkov (2001)) and (Han (2002)).

An alternative way to lower the oxygen demand of the bacteria would be to decrease the temperature, see (Bauer and White (1976)).

3. PROCESS MODEL

To investigate the influence of the temperature on the process a model was derived. A model of a fed-batch cultivation described in (Xu *et al.* (1999)) and (Åkesson *et al.* (2001b)) is modified to incorporate temperature dependence.

The mass balance equations for the media volume V, the glucose concentration G, the acetic acid concentration A, the cell mass X and the oxygen concentration

C_o are:

$$\frac{dV}{dt} = F$$
$$\frac{d(VG)}{dt} = FG_{in} - q_g(G)VX$$
$$\frac{d(VA)}{dt} = q_a(G,A)VX$$
$$\frac{d(VX)}{dt} = \mu(G,A)VX$$
$$\frac{d(VC_o)}{dt} = K_La(N)V(C_o^* - C_o) - q_o(G,A)VX$$

Into the model an equation describing the outlet gas concentration O_2 is added (Enfors and Häggström (1994)):

$$\frac{d(V_gO_2)}{dt} = Q(O_{2i} - O_2) - K_LaV(C_o^* - C_o)\frac{RT}{PM}$$

Q is the gas flow into the reactor, R the gas-constant, M the molar mass, P the pressure and T the temperature. V_g is the gas volume in the reactor and it is calculated as $V_{tot} - V$ where V_{tot} is the total volume of the reactor. The saturated dissolved oxygen in the medium C_o^* is calculated as follows

$$C_o^* = \frac{O_2P_{tot}}{O_{2i}P_{cal}H}$$

Henry's law gives the dissolved oxygen concentration DO in %:

$$DO = HC_o$$

The relation between K_La and the stirrer speed N is approximated as

$$K_La(N) = \alpha(N - N_0)$$

The equations for the growth rate μ and the uptake-rates q_a, q_g, q_o are given in the appendix.

The temperature dependence of the growth rate is incorporated into the model using Arrhenius law. A decrease in the medium temperature from 37^{o}C to 25^{o}C has been reported to lower the growth rate by half, see (Pirt (1985)) and (Esener *et al.* (1983)). Since the growth rate is proportional to the glucose uptake rate q_g, one can write $q_g^{max}(T)$ as

$$q_g^{max}(T) = q_{g,37}^{max} e^{-50(\frac{1}{T} - \frac{1}{37})}$$
$$q_g^{max}(25) \approx 0.5 q_{g,37}^{max}$$

The uptake rates q_a and q_o are changed in a similar fashion and also the maintenance coefficient q_{mc} (Esener *et al.* (1983)).

We strive for a model that is as simple as possible, so the influence of the temperature on K_La and the solubility of oxygen is neglected. The resulting effect on the oxygen transfer is small in the range 20 ^{o}C to 40^{o}C (Enfors and Häggström (1994)).

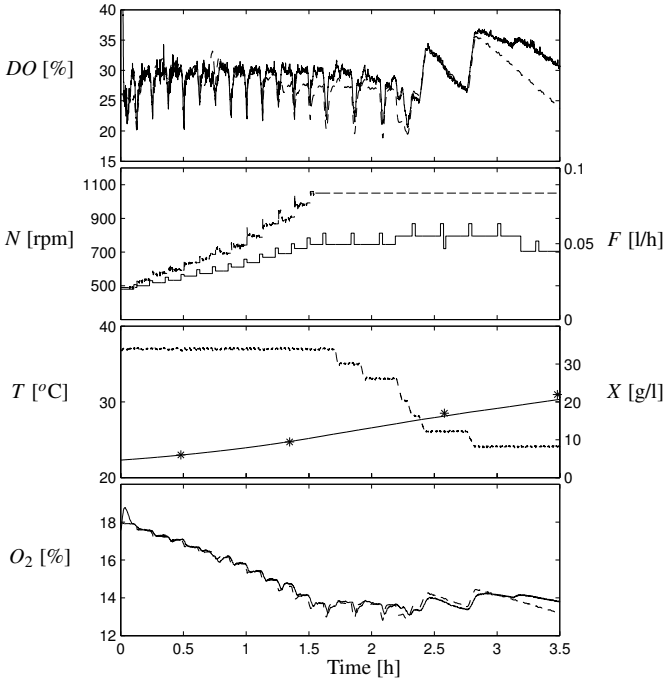

Fig. 3 Simulation of the model together with experimental data. From top: dissolved oxygen DO (simulation dashed), stirrer speed (dashed) N and feed F, temperature T (dashed) and cell mass X (experimental data '*'), oxygen concentration in the outlet gas O_2 (simulation dashed). Time [h] after feed-start.

Simulation of a cultivation is shown in figure 3 together with experimental data.

The values of the parameters in the model are listed in table 1 in appendix. In the experiment the temperature is lowered from 37 to 24 ^{o}C stepwise. As seen the process model fits remarkably well to the experimental data. In the literature (Pirt (1985)) it is mentioned that the behavior of the growth-rate drastically changes below 25 ^{o}C. This explains the poorer fit the last 30 min. The model is still good enough for the purpose of designing a dissolved oxygen - temperature controller

4. COMBINATION OF THE PROBING FEEDING STRATEGY AND THE TEMPERATURE LIMITED FED-BATCH TECHNIQUE

Our idea here is to use the original probing strategy, described in section 2, until the maximum oxygen transfer capacity of the reactor is reached. Then the temperature instead of the feed is decreased to lower the oxygen demand. To achieve a slight glucose excess in the reactor the up-pulses superimposed to the feed are shifted to down-pulses when the maximum stirrer speed is reached.

4.1 *Feed control*

The same feed controller as described in the original probing control strategy is used until the maximum

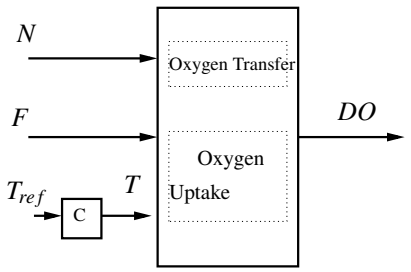

Fig. 4 A description of the process. Stirrer speed, N, Feed, F, Temperature T, Temperature reference T_{ref}, dissolved oxygen DO and a temperature controller C. C is typically a pulse width modulation of the cold and hot water flows.

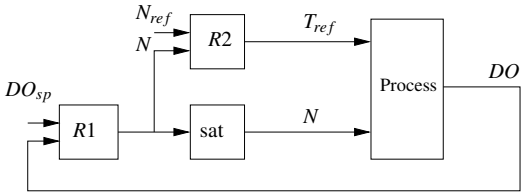

Fig. 5 A block-diagram over the mid-ranging controller. Sat stands for saturation. R1 is used even when 'only' N is controlling the dissolved oxygen, a gain scheduled PID. R2 is a PID controller.

stirrer speed is reached. Then the up-pulses superimposed to the feed are shifted to down pulses. When a down-pulse is made the dissolved oxygen signal will increase instead of decrease if the cultivation is glucose limited. The feed rate is adjusted as before depending on the size of the response in the dissolved oxygen. With down pulses a slight glucose excess in the reactor can be achieved. At a given feed rate a down-pulse may indeed lead to a response in the dissolved oxygen when an up-pulse would not. The glucose excess is important since the goal in this part of the cultivation is to let the temperature be the limiting factor and not the glucose concentration.

4.2 *Dissolved oxygen control*

Aerobic conditions should be maintained during the entire cultivation. For that purpose three control variables can be manipulated between the pulses: the stirrer speed N, the feed rate F and the temperature T, see Figure 4. The stirrer speed affects the oxygen transfer in the reactor whereas the feed rate and the temperature act on the oxygen uptake rate.

Control allocation From a control point of view, dissolved oxygen control is a regulation problem where the oxygen supply should be in balance with the oxygen consumption by the cells. The probing strategy determines the feed rate that maximizes the glucose uptake with respect to the respiratory capacity of the cells. The oxygen transfer and consequently the agitation speed should follow the cell growth to keep a constant oxygen concentration.

Since the oxygen transfer capacity of the reactor is limited, it is necessary to manipulate the oxygen consumption rate to keep the oxygen balance. The temperature or the feed rate should consequently be decreased. It is preferable to use the temperature since a larger decrease in the feed rate may lead to starvation. At high cell densities the heat production is high and both the temperature and the flow of the cooling water introduce limitations. When the maximal cooling capacity of the reactor is reached, the feed rate can be used to control the dissolved oxygen. Thus the feed rate should be used only when both the the stirrer speed and the temperature are saturated.

Mid-ranging The model derived in the previous section reveals that the dissolved oxygen-temperature dynamics is comparable to the dissolved oxygen-stirrer one. However, cooling of the reactor introduces additional dynamics. The resulting dynamics from T_{ref} to DO is much slower than the DO-stirrer one. Fast disturbances in the dissolved oxygen such as feed adjustments and antifoam addition cannot be quickly handled by the temperature. Dissolved oxygen control based on the sole manipulation of the temperature would result in a poor performance. One way to handle this problem is shown in figure 5. Here a so-called mid-ranging controller structure, see (Allison and Isaksson (1998)), is chosen to control the dissolved oxygen. The first controller R_1 manipulates the stirrer speed N and it is tuned to handle the fast disturbances. The second controller R_2 manipulates the temperature to keep the stirrer speed at N_{ref}, below N_{max}. The second controller R_2 is much slower than R_1 and takes typically care of slow disturbances like cell growth. The second loop involving the temperature is activated once the stirrer speed has reached N_{ref}. The model derived in Section 3 can be used to determine a good value for N_{ref}. A typical short term perturbation in DO should not saturate the stirrer speed.

5. EXPERIMENT

The feeding strategy was evaluated by simulations using the model from section 3. Then it was implemented and tested on a 3 l bioreactor. Figure 6 shows an experiment run with the new technique. At $t \approx 13.5\ h$ the initial glucose amount from the batch phase is totally consumed and the glucose starts to be fed into the reactor. After 1.5 h of feeding the stirrer speed reaches N_{ref} and the temperature starts to decrease. The pulses in the feed are performed downwards to achieve a slight glucose excess. The initial decrease in the temperature does not affect the dissolved oxygen concentration. This can be explained by the model which predicts a lack of authority when the oxygen uptake rate is not saturating. Induction occurs at $t = 15\ h$. When the dissolved oxygen is stabilized at 30 %, no pulse responses are visible. The feed rate is therefore decreased by the algorithm. At $t = 16\ h$ pulse responses

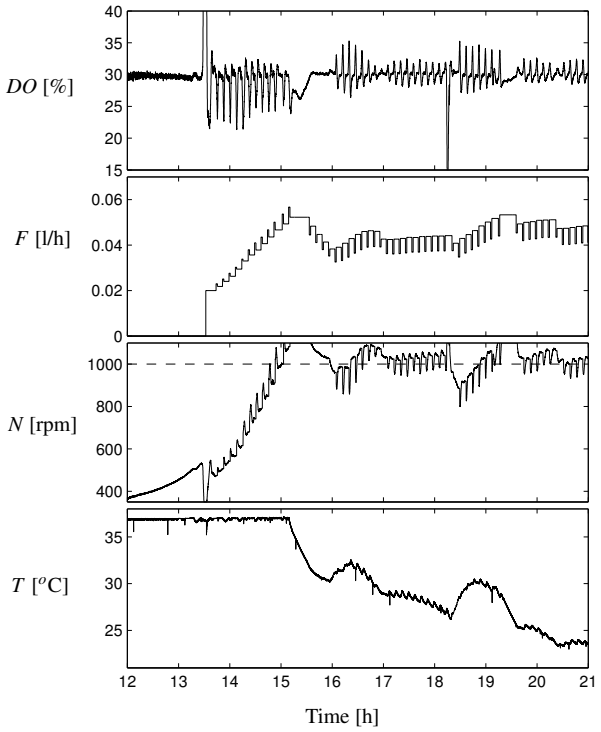

Fig. 6 Experiment using the modified probing strategy together with the temperature limited fed-batch technique. The fed-batch part of the cultivation is shown. From top: *DO* dissolved oxygen, *F* feed, *N* stirrer speed, *T* temperature. N_{ref} is dashed. At t = 18.2 h antifoam is added which has a large impact on the dissolved oxygen signal.

are seen in the oxygen signal, which indicates that glucose becomes limiting again. Unlike the substrate-limiting technique, over-feeding can easily occur after the maximum stirrer speed is reached. Indications of glucose excess from the probing pulses led to a decrease in the feed at four occasions. This shows the importance of the glucose feeding in temperature limited fed-batch cultivations. It is interesting to compare the feed profiles from figures 2 and 6. In the first experiment, a 50% decrease in the feed was necessary to keep the reactor working in aerobic conditions. In the second experiment, the temperature was instead lowered and it was never necessary to decrease the feed to control the dissolved oxygen. This should be a less stressful way to limit the oxygen demand of the bacteria, as indicated by less foaming.

6. CONCLUSION

A cultivation strategy combining the probing feeding strategy and the temperature limited fed-batch technique was presented. The strategy was implemented and compared to a cultivation using the original probing feeding strategy. The temperature instead of glucose was the limiting factor in the late phase of the cultivation. This should be less stressful for the bacteria.

A model of the process was derived and compared to experimental data in order to design the controllers. To achieve a good control of the dissolved oxygen a mid-ranging controller manipulating the stirrer speed and the temperature was used.

7. ACKNOWLEDGMENT

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9. APPENDIX

The temperature dependency is introduced in the maximal specific uptake rates. The maximal uptake rates $q_a^{c,max}(T)$, $q_g^{max}(T)$, $q_o^{max}(T)$ and the maintenance coefficient $q_{mc}(T)$ are given by:

$$q_a^{c,max}(T) = q_{a,37}^{c,max} e^{-50(\frac{1}{T}-\frac{1}{37})} = q_{a,37}^{c,max} f(T)$$

$$q_g^{max}(T) = q_{g,37}^{max} f(T), \quad q_{mc}(T) = q_{mc,37} f(T),$$

$$q_o^{max}(T) = q_{o,37}^{max} f(T)$$

The uptake rates for acetic acid and glucose are modeled by Monod kinetics:

$$q_a^{c,pot}(A,T) = q_a^{c,max}(T)\frac{A}{k_a + A}$$

$$q_g(G,T) = q_g^{max}(T)\frac{G}{k_s + G}$$

Part of the glucose is used for maintenance:

$$q_m(T) = \min(q_g(G,T), q_{mc}(T))$$

Symbol	Value	Description
G_{in}	500 g/l	glucose conc. in feed
H	14000	Henrys const.
k_s	0.01 g/l	sat. const. for gluc. uptake
k_a	0.05 g/l	sat. const. for acet. uptake
α	3 $(\text{hRPM})^{-1}$	oxygen transf. const.
N_0	289 RPM	oxygen transf. const.
$q_{a,37}^{c,max}$	0.2 g/gh	max. spec. acet. uptake
$q_{g,37}^{max}$	1.5 g/gh	max. spec. glucose uptake
$q_{g,37}^{crit}$	1.25 g/gh	crit. glucose uptake
$q_{mc,37}$	0.06 g/gh	maintenance coefficient
$q_{o,37}^{max}$	0.66 g/gh	max. spec. oxygen uptake
Y_{ag}	0.55 g/g	acetate/glucose yield
Y_{oa}	0.55 g/g	oxyg./acet. yield
Y_{og}	0.50 g/g	oxyg./gluc. yield for growth
Y_{om}	1.07 g/g	oxyg./gluc. yield for maint.
Y_{xa}	0.4 g/g	biomass/acet. yield
Y_{xg}^{ox}	0.51 g/g	oxidative biomass/gluc.
Y_{xg}^{fe}	0.15 g/g	fermentative biomass/gluc.
O_2i	21 %	oxyg. conc. in the inlet gas
P_{cal}	1 atm	pressure when calib.
P_{tot}	1 atm	pressure during cult.
V_{tot}	3 l	total reactor volume
Q	2.4 l/min	gasflow
M	32 g/mol	Molar mass

Table 1 Parameters in the model

For clarity purposes the argument T is omitted in the following equations. The acetic acid formation q_a and the growth uptake q_{gg} are described by:

$$q_a = q_a^p - q_a^c, \qquad q_{gg} = q_g - q_m$$

where q_a^p is the production of acetic acid and q_a^c stands for the acetic acid consumption. Splitting into an oxidative flow and a fermentative flow gives:

$$q_{gg}^{ox} = \min((q_o^{max} - q_m Y_{om})/Y_{og}, q_{gg})$$

$$q_{gg}^{fe} = q_{gg} - q_{gg}^{ox}$$

The specific acetate production, acetate consumption, growth rate and oxygen uptake rate are given by the following equations:

$$q_a^p = q_{gg}^{fe} Y_{ag}$$

$$q_a^c = \min(q_a^{c,pot}, (q_o^{max} - q_{gg}^{ox} Y_{og} - q_m Y_{om})/Y_{oa})$$

$$\mu = q_{gg}^{ox} Y_{xg}^{ox} + q_{gg}^{fe} Y_{xg}^{fe} + q_{ac} Y_{xa}$$

$$q_o = q_{gg}^{ox} Y_{og} + q_m Y_{om} + q_{ac} Y_{oa}$$

A consequence of the model assumptions is that μ and all q's can be written on the form $q(T) = q_{37} f(T)$. The saturation in the oxygen uptake rate therefore occurs for the same values of G and A independently of T.