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A Probing Strategy for Substrate Feeding in *Escherichia coli* Cultivations

Abstract

Large-scale production of many proteins can today be made using genetically modified microorganisms. One of the most frequently used host organisms is the bacterium *Escherichia coli*. A problem encountered during cultivations of *E. coli* is the accumulation of the by-product acetate, which tends to reduce growth and protein production. Formation of acetate can be avoided by a proper substrate feeding strategy, but most strategies require considerable process knowledge to work well. The main problem is that many important process variables cannot be measured on-line, which complicates the design and realization of a feedback strategy.

This thesis presents a novel feedback strategy for substrate feeding in cultivations of *E. coli*. The key idea is to exploit a characteristic change in the cell metabolism at the onset of acetate formation. By superimposing short pulses in the substrate feed rate, on-line detection of acetate formation can be made using a standard dissolved oxygen sensor. Several experiments confirm the validity of this detection method.

A simple feedback algorithm is used to adjust the feed rate to avoid acetate formation while maintaining a high cell growth rate. The feasibility of the feeding strategy is demonstrated by simulations and tuning rules that require a minimum of process specific information are derived.

The feeding strategy requires good control of the concentration of dissolved oxygen. Variations in oxygen dynamics during a cultivation may cause problems if a controller with fixed parameters is used. A control approach based on PID control and gain scheduling from the stirrer speed is suggested.

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

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Mats Åkesson

1

Introduction

Many pharmaceuticals are today produced using genetically modified microorganisms. Recombinant DNA technology makes it possible to insert foreign DNA, encoding a desired substance, into a host organism. The modified, or recombinant, cells are then cultivated to large numbers and made to produce the foreign substance, often a protein. To achieve reproducible results, well-defined cultivation conditions are a prerequisite and automatic control is routinely used for control of temperature, pH, and dissolved oxygen. A difficulty in monitoring and control of biotechnical processes, however, is that many important process variables cannot be measured on-line. This fact has triggered much research concerning new sensor and sampling technologies, but the problem can also be addressed by improving and extending the use of existing sensors.

The bacterium *Escherichia coli* is a frequently used host organism for production of recombinant proteins. It has many advantages, such as being well characterized and supporting growth to high cell densities, but also drawbacks. One of the problems encountered in *E. coli* cultivations is the formation of the metabolic by-product acetate. Accumulation of acetate has been reported to reduce both cell growth and recombinant protein production. Fed-batch processes, where additional substrate is fed during the cultivation, is often a preferred way of production. The substrate feeding can then be scheduled to avoid acetate formation. The design of a feeding strategy that is robust against process variations require a feedback solution. However, the realization of this is complicated by the lack of cheap and reliable on-line sensors

for the relevant process variables.

This thesis presents a feedback substrate feeding strategy for fed-batch cultivations of *E. coli*. The key idea is to exploit a characteristic change in the metabolism at the onset of acetate formation. This change can be detected from the responses in dissolved oxygen to pulses superimposed in the substrate feed rate. A simple feedback algorithm is then used to adjust the feed rate to avoid acetate formation while maintaining a high cell growth rate. Attractive features are that dissolved oxygen is a standard on-line measurement and that tuning of the control algorithm can be made with a minimum of process specific information. The original idea for the pulse technique has earlier been used at Pharmacia & Upjohn, see [Axelsson *et al.*, 1996].

1.1 Publications

The thesis consist of this overview and summary together with the following papers:

Paper I – A Pulse Feeding Strategy for Fed-batch Cultivations of Escherichia coli

A substrate feeding strategy for fed-batch cultivations of *E. coli* is presented in this paper. Experiments under steady-state conditions have indicated that an apparent maximum in the specific oxygen uptake is reached concomitantly with the onset of acetate formation. Assuming that these observations are valid under transient conditions, the key idea is to detect this saturation in the dissolved oxygen responses to pulses superimposed in the substrate feed rate. A simple feedback algorithm is then used to adjust the feed rate to avoid acetate formation while maintaining a high cell growth rate. The feasibility of the presented strategy is demonstrated by simulations and tuning rules that require a minimum of process specific information are presented.

The paper is submitted for journal publication as

ÅKESSON, M., P. HAGANDER, and J. P. AXELSSON (1998): "A pulse feeding strategy for fed-batch cultivations of *Escherichia coli*." Submitted.

A shorter conference version was published in

ÅKESSON, M., P. HAGANDER, and J. P. AXELSSON (1997): "A pulse technique for control of fed-batch fermentations." In *Proceedings of 1997 IEEE Conference on Control Applications*. Hartford, Connecticut, USA.

Paper II – Acetate Formation and Dissolved Oxygen Responses to Feed Transients in Escherichia coli Cultivations

This paper confirms the validity of the approach in Paper I. It is demonstrated, theoretically and experimentally, how on-line detection of acetate formation in *E. coli* cultivations can be made with a standard dissolved oxygen sensor by superimposing short pulses to the substrate feed rate. Starting from two hypotheses for acetate formation, relations between glucose uptake, oxygen uptake and acetate production on the cell level are derived using metabolic flux networks. Both hypotheses predict a "saturation" in the oxygen uptake when the acetate formation starts. Simulations show that this gives a clear change in the dissolved oxygen response to transients in the substrate feed rate. The predicted effects were verified in fed-batch cultivations of two recombinant *E. coli* strains, both before and after induction of recombinant protein production.

The paper is submitted for journal publication as

ÅKESSON, M., E. NORDBERG KARLSSON, P. HAGANDER, J. P. AXELSSON, and A. TOCAJ (1998): "Acetate formation and dissolved oxygen responses to feed transients in *Escherichia coli* cultivations." Submitted.

A shorter conference version will appear in

ÅKESSON, M., A. TOCAJ, P. HAGANDER, and J. P. AXELSSON (1998): "Acetate formation and dissolved oxygen responses to feed transients in *Escherichia coli* fermentations: Modeling and experiments." Accepted to *the 7th International Conference on Computer Applications in Biotechnology*. Osaka, Japan.

Paper III – Control of Dissolved Oxygen in Stirred Bioreactors

The performance of the substrate feeding strategy in Paper I is dependent on tight control of the dissolved oxygen concentration. This paper discusses control of dissolved oxygen when the oxygen supply

is manipulated by variation of the mechanical agitation. The process dynamics may change significantly during a cultivation and a control approach based on PID control and gain scheduling is suggested.

The paper is published in

ÅKESSON, M. and P. HAGANDER (1998): "Control of dissolved oxygen in stirred bioreactors." Technical Report ISRN LUTFD2/TFRT--7571--SE. Department of Automatic Control, Lund Institute of Technology, Sweden.

1.2 Thesis Outline

First, some background material on recombinant protein production and cultivations of microorganisms is given in Chapter 2. In the following three chapters, the main topics of the papers are reviewed and some extensions given. Finally, in Chapter 6, the thesis is concluded and ideas for future work are outlined.

2

Background

The human utilization of microorganisms has a long tradition. For centuries, microorganisms have been exploited in the production of feedstocks like cheese, wine, soy sauce, and bread. Originally, this was of course made without knowledge of the concept microorganism. With the progress in microbiology, genetics, molecular biology and chemical engineering, the field of applications is today far more diverse. Examples range from waste-water treatment to production of pharmaceuticals, and more recently, functional foods.

This thesis deals with processes where the bacterium *Escherichia coli* is used for production of foreign, or recombinant, proteins. The purpose of this chapter is to provide some background material for the reading of the following chapters and papers. More thorough treatments can be found in for instance [Pirt, 1975; Bailey and Ollis, 1986; Enfors and Häggström, 1994].

2.1 Production of Recombinant Proteins

The cellular DNA contains genetic instructions for the behavior and the composition of a living cell. Thus, changes in the DNA imply that the properties of the organism are modified. In the end of the 1970s, methodologies emerged that permitted precise alterations of DNA molecules and these scientific breakthroughs largely increased the potential of biotechnical applications.

By introducing a DNA sequence coding for a foreign protein into a

microorganism, a "cell factory" for synthesis of the this protein can be created. Cultivation of the recombinant cells to large numbers, then make it possible to produce the protein in amounts that would otherwise be difficult or even impossible to obtain from a natural source. Human growth hormone and human insulin are two examples of products that are nowadays produced in an industrial scale using recombinant microorganisms.

Cloning

The first step in the development of a process for recombinant protein production is the cloning of the protein in interest into an appropriate production strain. In bacterial cells, a foreign gene is usually inserted into a *plasmid*, which is a small circular DNA molecule that can exist independently of the bacterial chromosome. The recombinant plasmid is then introduced into the host organism.

The presence of the plasmid impose an additional metabolic burden on the host cell, especially when the recombinant protein is produced [Bentley *et al.*, 1990], and plasmid-free cells will tend to outgrow the plasmid carrying ones. It is therefore common that the plasmid encodes a selective marker that favors the plasmid-containing cells. This can for instance be done by including a gene giving resistance for an antibiotic and then including the antibiotic in the growth medium.

Normally the plasmid also contains a promoter region controlling the expression of the recombinant gene. Inducible promoters give the possibility to start the recombinant protein production from an external signal such as temperature shift or addition of an inducer substance.

Cultivation

The actual production begins with a cultivation phase, where the number of cells is increased, sometimes by several orders of magnitude. As the product is not useful for the host cell, product synthesis reduces the capacity for growth and survival. A common strategy is therefore to first grow the cells to a high cell density and then induce expression of the recombinant protein. Intracellular concentrations of the recombinant protein may then reach 20 to 40 percent of the cell dry weight. The cultivation process will be further discussed in Section 2.2.

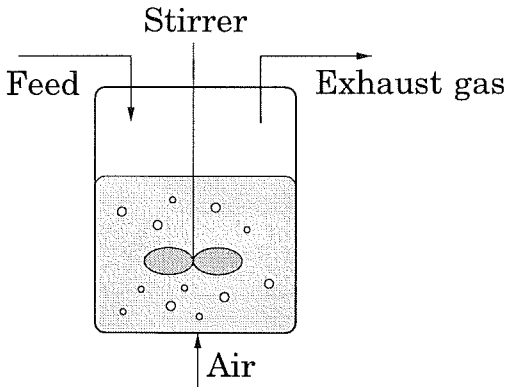

Figure 2.1 A stirred bioreactor with incoming feed flow.

Product recovery

In the recovery phase, the product concentration is increased from fractions of a percent in the harvest broth up to 90–100% purity after the final steps. The separation techniques involved depend on the properties of the product, the location of the product (intra- or extracellular), process size, and also of the value of the product. Typical operations are cell disruption, filtration, centrifugation, solvent extraction, and precipitation. Various types of chromatography are commonly used for purification of high-value pharmaceuticals.

2.2 Cultivation of Microorganisms

Bioreactor and instrumentation

The cultivation process takes place in a vessel called bioreactor, or fermenter. In the reactor, there is a liquid containing cells and nutrients. There are many types of bioreactors, but the most frequently used is the stirred tank reactor, see Figure 2.1. Well-mixed conditions are obtained through agitation with a mechanical stirrer. In aerobic processes, air or oxygen is sparged into the liquid. Reactor sizes range from laboratory-scale reactors of 1 to 10 liters to large-scale production reactors of several cubic meters. The reactor should operate under aseptic conditions and must therefore be sterilizable. Small reactors can be put into an autoclave, while larger reactors have steam jackets for in-place sterilization.

In many cultivations, the cell growth eventually becomes restricted by limitations in oxygen transfer and cooling capacity of the reactor. As an example, the microbial heat evolution, that has to be cooled away, may in a 10 m^3 reactor amount to over 100 kW [Kieran, 1996].

On-line measurements that are routinely available are often confined to physical and chemical properties of the liquid medium (pH, temperature, dissolved oxygen tension, etc.) and the exhaust gas (CO_2 and O_2 content). A limiting factor is that an *in situ* sensor must be steam sterilizable. The fact that many important process variables are difficult to measure on-line has led to much research concerning new sensors and sampling techniques, see [Schügerl *et al.*, 1996]. Indirect measurements can also be obtained by combining information from existing sensors [Wang *et al.*, 1977; Stephanopoulos and San, 1984].

To maintain a suitable environment in the bioreactor, control loops for temperature, pH, and dissolved oxygen are standard. The use of automatic control is otherwise somewhat restricted due to the lack of reliable on-line sensors.

Modes of operation

A bioreactor may be operated in batch, continuous or fed-batch mode. In batch cultures, all substrate components are added initially, except for oxygen and pH-controlling agents. Typically, the cells grow exponentially until some substrate is exhausted or the concentration of a by-product becomes inhibiting.

In contrast to the batch culture, continuous cultures are mostly operated at steady state with an incoming substrate feed flow in balance with an outgoing flow. The substrate feed rate is normally growth-limiting and this gives a possibility to control the cell growth.

Most industrial processes are run in fed-batch mode. After an initial batch phase, one of the substrates is fed at a growth-limiting rate. By manipulation of the feed rate, it is possible to avoid limitations in oxygen transfer and cooling as well as limitations in the metabolism causing by-product formation. In this way, high cell densities can be achieved. To reduce the volume increase, the incoming feed is often highly concentrated which may cause problems with substrate gradients in the reactor.

Cultivation media

The cultivation medium should provide raw materials for cell growth and product synthesis. From the desired final cell density, the cellular composition and the energy required to build cell mass, it is possible to compute the required amounts of carbon, nitrogen, phosphorus, trace elements and so on. A *defined* medium is a mixture of chemicals with a well-known composition whereas *complex* media also contain less well-defined substrates like various protein hydrolysates. The carbon/energy source is usually a sugar such as glucose.

It is often impossible to include all desired substrate components in the initial medium. High salt concentrations may be inhibitory and high carbon/energy source concentrations tend to give excessive by-product formation. The fed-batch technique is well suited to avoid such problems. Nitrogen is often provided continuously by using ammonia for regulation of pH.

2.3 Fed-batch Cultivation of Recombinant E. coli

The bacterium *Escherichia coli*, occurring naturally in the intestines of humans and mammals, is one of the common host organisms for recombinant protein production. As many other bacteria, *E. coli* can grow fast and is fairly robust with respect to cultivation conditions. It is also one of the most studied organisms and it is genetically well characterized. A drawback with bacteria is that they cannot be used for synthesis of some of the more complicated proteins found in higher organisms. Other problems encountered with *E. coli* are the production of endotoxins and the degradation of the foreign protein by destructive enzymes.

The product is often accumulated intracellularly which makes the productivity proportional to the final cell density. Fed-batch cultivation with glucose as main substrate is the standard approach to achieve high cell densities. The cultivation is mostly divided into a growth phase and a production phase using an inducible expression system.

Acetate formation

One of the problems encountered in *E. coli* cultivations is the formation of the metabolic by-product acetate. The accumulation of acetate has been reported to reduce cell growth and also to reduce recombinant protein production [Bauer *et al.*, 1990; Luli and Strohl, 1990; Bech Jensen and Carlsen, 1990].

Acetate is produced when *E. coli* is grown under oxygen-limited conditions but may also occur under fully aerobic conditions at high growth rates and/or high glucose uptake rates. It is often described as an overflow phenomenon in the central metabolic pathways. Saturations in the respiratory system [Andersen and von Meyenburg, 1980] or the TCA-cycle [Majewski and Domach, 1990] have been suggested as possible explanations.

A number of strategies have been developed to avoid acetate accumulation [Lee, 1996]. The concentration of acetate can be reduced using dialysis, but this kind of process is difficult to scale up. A common approach is instead to restrict the growth rate such that acetate formation is avoided. In fed-batch cultures, the substrate feed rate can be chosen to control the growth rate and this will be further discussed below. The growth rate can also be reduced by decreasing the cultivation temperature.

The choice of cultivation medium is an important factor. Complex media tend to give acetate formation at lower growth rates than defined media [Meyer *et al.*, 1984]. By substituting glucose with glycerol, acetate formation can be reduced. Acetate production also depends on the particular *E. coli* strain used. Metabolic engineering, manipulation of the metabolic pathways using recombinant DNA technology, have been used as a tool to develop *E. coli* strains that produce less acetate, see for instance [Chou *et al.*, 1994].

Substrate feeding strategies

The substrate feeding strategy is a major determinant for productivity and quality in fed-batch production of recombinant proteins. Underfeeding will give low cell growth and hence low productivity. On the other hand, overfeeding often results in accumulation of acetate. To reduce or avoid acetate formation, a number of feeding strategies have been developed, for an overview see [Lee, 1996; Yee and Blanch, 1992].

A common approach is to control the specific growth rate, for instance through fixed feeding profiles [Paalme *et al.*, 1990] or feedback from indirect measurements [Riesenberg *et al.*, 1991]. However, the critical growth rate, where acetate formations starts, must then be known. This rate may also change during a cultivation, especially when expression of the recombinant protein is induced, see Paper II.

Another approach is to use feedback from the acetate concentration to determine the feed rate as in [Shimizu *et al.*, 1988; Turner *et al.*, 1994]. This approach can in principle handle process variations but cheap and reliable on-line sensors for acetate are still not commercially available.

Control of the substrate concentration could be another way to avoid acetate formation. Coarse control of the glucose concentration can be achieved by intermittent feeding at the depletion of glucose, detected as a peak in dissolved oxygen [Mori *et al.*, 1979] or in pH [Robbins and Taylor, 1989]. Most of the time, however, glucose will be present in excess and thus acetate formation will occur. In [Kleman *et al.*, 1991], feedback from the glucose concentration was used to adjust the glucose feed rate. Production of acetate was reduced but not avoided. The problem is that the critical glucose concentrations for *E. coli* are very low and accurate on-line measurements in this range are not available today. Even if a measurement was available, the critical concentration has to be known *a priori*.

Sufficient oxygen supply can be guaranteed by manipulating the substrate feed rate in order to maintain a constant dissolved oxygen concentration [Konstantinov *et al.*, 1990; Riesenberg *et al.*, 1990]. However, these strategies do not avoid acetate formation unless assumptions on oxygen uptake and the critical growth rate are fulfilled.

The lack of appropriate on-line measurements complicates the design of a robust feeding strategy and many strategies are therefore based on in-direct measurements. In order to work well, however, considerable process knowledge is required.

2.4 Process Model

To evaluate and simulate the behavior of a substrate feeding strategy, a model of the cultivation process is required. We will now formulate

a dynamic model of a fed-batch cultivation of *E. coli*, with glucose as main substrate, in a small-scale bioreactor. It is assumed that pH and temperature are constant and that well-mixed conditions apply. Dilution effects from base/acid addition and effects of volume drawn for samples are neglected. Linearized equations for the glucose and oxygen dynamics, that give valuable insights of the process behavior, are also presented.

Nonlinear model

Balance equations Component-wise mass balances for the bioreactor give the following equations

$$\begin{aligned}
\frac{dV}{dt} &= F \\
\frac{d(VX)}{dt} &= \mu(G) \cdot VX \\
\frac{d(VG)}{dt} &= FG_{in} - q_g(G) \cdot VX \\
\frac{d(VO)}{dt} &= K_L a(N) \cdot V(O^* - O) - q_o(G) \cdot HVX
\end{aligned}$$

where G, X, O, V are the glucose concentration, the cell concentration, the dissolved oxygen tension, and the liquid volume, respectively. Further, F, G_{in}, H, O^* denote the feed rate, the glucose concentration in the feed, the proportionality constant relating dissolved oxygen concentration with dissolved oxygen tension, and the dissolved oxygen tension in equilibrium with the oxygen in gas bubbles.

Kinetic expressions The specific glucose uptake, q_g, is taken to be of Monod type

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

which describes a smoothly saturating glucose uptake. Part of the glucose uptake is assumed to be used for non-growth associated maintenance purposes. This is here modeled by a constant flow q_m.

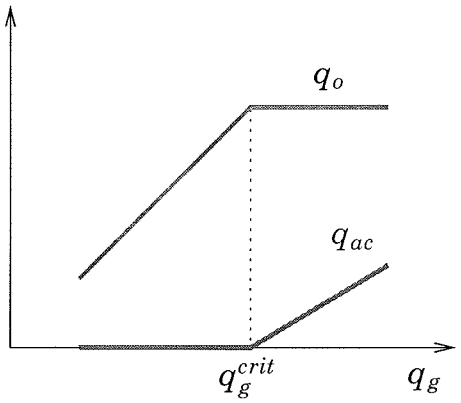

Figure 2.2 Relations between glucose uptake, q_g, oxygen uptake, q_o, and acetate production, q_{ac}.

Oxygen is used to metabolize the glucose, and the specific oxygen uptake q_o depends on the specific glucose uptake q_g. Under steady-state conditions, it has been observed that the specific oxygen uptake rate reaches an apparent maximum q_o^{max} at the onset of acetate formation [Andersen and von Meyenburg, 1980; Reiling *et al.*, 1985]. Above the corresponding glucose uptake rate q_g^{crit}, excess glucose is fermented with acetate as main by-product, see Figure 2.2.

Mathematically, this limitation in the specific oxygen uptake rate can be formulated as

$$q_o(G) = \begin{cases} (q_g - q_m)Y_{og} + q_m Y_{om}, & q_g < q_g^{crit} \\ q_o^{max}, & q_g \geq q_g^{crit} \end{cases}$$

with the yield coefficients for growth Y_{og} and maintenance Y_{om}.

Similarly, the specific growth rate, μ, is described as

$$\mu(G) = \begin{cases} (q_g - q_m)Y_x^{ox}, & q_g < q_g^{crit} \\ (q_g^{crit} - q_m)Y_x^{ox} + (q_g - q_g^{crit})Y_x^{fe}, & q_g \geq q_g^{crit} \end{cases}$$

Due to the production of acetate, the yield from the glucose that is fermented, Y_x^{fe}, is somewhat lower than Y_x^{ox}.

Oxygen transfer To cover a broad range of stirrer speeds, the volumetric transfer coefficient, $K_L a$, is often taken to be proportional to the square or the cube of the stirrer speed, N. However, in the working

range of the reactor, $K_L a$ is approximated as an affine function of the stirrer speed

$$K_L a(N) = \alpha \cdot (N - N_0)$$

where $N > N_0$.

Dissolved oxygen sensor Dissolved oxygen tension is measured with a dissolved oxygen probe that adds significant dynamics to the system. The probe is modeled as a first-order system with time constant T_p.

$$T_p \frac{dO_p}{dt} + O_p = O$$

If the assumption of well-mixed conditions is not valid, it may be appropriate to add another time constant as well as a time delay [Dang *et al.*, 1977].

Linearized model

The process dynamics may change significantly during a cultivation. Valuable insight into this can be obtained from a linearized description.

In glucose-limited fed-batch cultures, the pool of glucose in the bioreactor is small compared to the rates of supply and consumption. If the glucose supply is shut off, most of the glucose in the reactor will be consumed within seconds. This is also true for dissolved oxygen, due to the low solubility of oxygen in the cultivation medium. The dynamics for deviations in glucose and oxygen is thus rather fast, and have time constants that are in the order of seconds.

The cell growth, on the other hand, takes place in a time scale of hours. Over shorter periods of time, V and X can be considered to be constant. Around a trajectory, the differential equations for glucose and oxygen may then be approximated by the linear expressions

$$T_g \frac{d\Delta G}{dt} + \Delta G = K_g \Delta F$$

$$T_o \frac{d\Delta O}{dt} + \Delta O = K_o \Delta G + K_n \Delta N$$

where the gains and the time constants are given by

$$
\begin{aligned}
T_g &= \left(\frac{\partial q_g}{\partial G}X\right)^{-1} \\
T_o &= (K_L a)^{-1} \\
K_g &= \frac{G_{in}}{V}\left(\frac{\partial q_g}{\partial G}X\right)^{-1} = \frac{G_{in}T_g}{V} \\
K_o &= -Y_{og}H\frac{\partial q_g}{\partial G}X(K_L a)^{-1} = -\frac{Y_{og}HT_o}{T_g} \\
K_n &= [O^* - O]\frac{\partial K_L a}{\partial N}(K_L a)^{-1}
\end{aligned}
$$

These parameters vary during a cultivation and, typically, the time constants T_g and T_o decrease with increasing biomass. The expression for K_o is valid as long as the limitation in the oxygen uptake is not reached.

2.5 Summary

Large-scale production of many proteins can today be made using genetically modified microorganisms. One of the frequently used host organisms is the bacterium *Escherichia coli* which can be grown to high cell densities in fed-batch cultivations. A problem encountered during cultivations of *E. coli* is the accumulation of the by-product acetate, which tends to reduce growth and protein production. Formation of acetate can be avoided by a proper substrate feeding strategy, however, most strategies require considerable process knowledge to work well. The main problem is that many of the interesting process variables, such as glucose and acetate, cannot be measured on-line in the relevant concentration ranges. This complicates the design and realization of a feedback strategy that is robust to process variations.

3

A New Substrate Feeding Strategy

As was pointed out in the previous chapter, one of the difficulties encountered when *E. coli* is used for recombinant protein production is the formation of acetate. In Paper I, a feeding strategy that aims to avoid acetate formation while maintaining a high growth rate is presented. The key idea is to exploit a characteristic change in the relation between oxygen uptake and glucose uptake at the onset of acetate formation. This makes it possible to obtain on-line detection of acetate formation using a standard dissolved oxygen probe. A simple feedback algorithm is then used to adjust the glucose feed rate. No knowledge of the critical growth rate for acetate production is required and process variations, due to for instance recombinant protein production, can also be handled.

3.1 Detection of Acetate Formation

An apparent saturation in the specific oxygen uptake rate at the onset of acetate formation has been reported in [Andersen and von Meyenburg, 1980; Reiling *et al.*, 1985]. Assuming that this saturation is valid also under transient conditions, we will now discuss how it can be exploited to detect acetate formation.

In glucose-limited cultivations, an increased glucose feed rate F will give rise to an increase in the glucose uptake rate q_g. As the cells

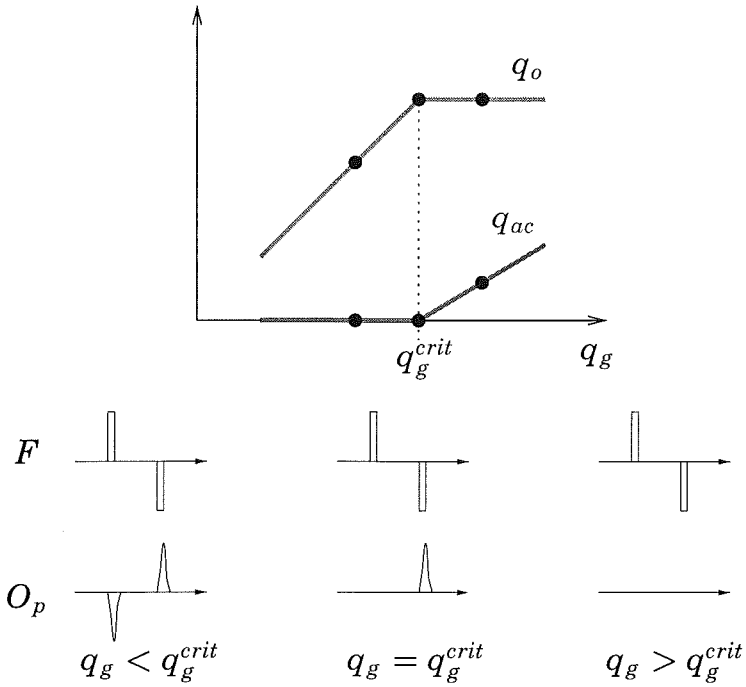

Figure 3.1 Idealized pulse responses for q_g below, at, and above q_g^{crit}.

use oxygen to metabolize the glucose, this implies an increase in the oxygen uptake. This, in turn, will cause a decreased dissolved oxygen level that can be detected in the dissolved oxygen measurement O_p. Similarly, a decrease in the feed rate would lead to an increase in the dissolved oxygen. The solubility of oxygen in the cultivation medium is low, which makes dissolved oxygen a sensitive indicator to changes in the supply and uptake of oxygen.

Superimposed pulses in the feed rate will thus give rise to transients in the dissolved oxygen signal. However, when the glucose uptake q_g exceeds q_g^{crit}, the oxygen uptake saturates. As a consequence, the oxygen response to feed pulses will change character. Figure 3.1 shows idealized pulse responses for q_g below, at, and above q_g^{crit}.

It is clear that the pulse responses can be used to determine if q_g is above or below q_g^{crit}, and hence to detect if acetate is being produced or not. Note that it is a qualitative change that is detected, and consequently no values on stoichiometric coefficients or other process parameters have to be assumed or known.

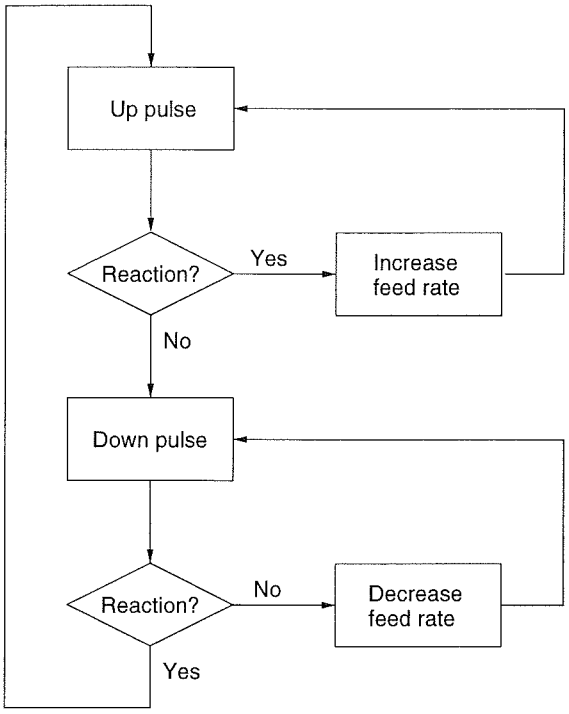

Figure 3.2 Flow diagram of the control algorithm.

3.2 Feedback Algorithm

The information in the dissolved oxygen response to feed pulses will now be used to adjust the glucose feed rate to achieve feeding at q_g^{crit}. This is optimal in the sense that it gives the highest possible growth rate without acetate formation. When more knowledge of the process is available, it can be beneficial to combine the feed algorithm with a pre-determined feed profile.

Control algorithm

At each cycle of the algorithm, a pulse is given to obtain information about acetate formation. The feed rate is then adjusted in order to achieve feeding at q_g^{crit}. Pulse generation and feed rate adjustment is governed by a set of rules that can be described by the flow diagram in Figure 3.2. A reaction to an up pulse is said to occur if O_p is below a level O_{low}. Similarly, a reaction to a down pulse is defined from O_p exceeding O_{high}. The rule-based control algorithm can be interpreted as a three-level incremental controller. An example of a cycle in the

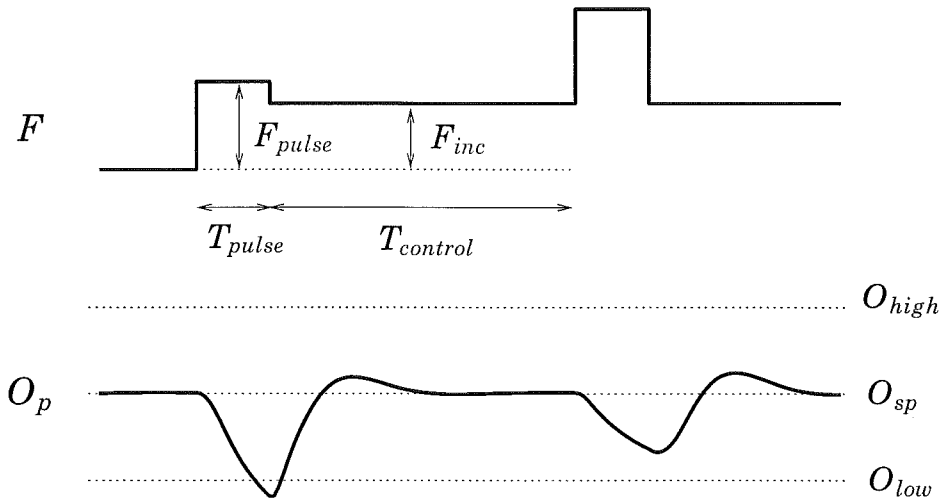

Figure 3.3 Example of a cycle in the algorithm. During the time $T_{control}$ between two pulses, O_p is regulated by a PID controller manipulating the stirrer speed.

algorithm is shown in Figure 3.3 where also the algorithm parameters are defined.

Closed-loop system

During the feed pulses, the stirrer speed in the reactor is fixed. Between two pulses, the stirrer speed is manipulated by a PID controller regulating O_p. This ensures that O_p is at the same level, O_{sp}, at the start of each pulse. Moreover, this will avoid that the cell growth becomes limited by oxygen. To achieve good performance in the feed algorithm it is important to have a short cycle time. The cycle time depends on how fast the the oxygen set-point can be regained after a pulse and thus high performance is required in the PID loop.

The closed-loop system is a multivariable setup where two inputs, N and F, are computed from the same output variable O_p, see Figure 3.4. However, the loops take turns in using the measurement and are thus separated in time. The PID loop is switched between on- and off-mode and tracking is used to avoid transients when the controller mode is changed. To improve the performance in the PID loop, feedforward from F can be used.

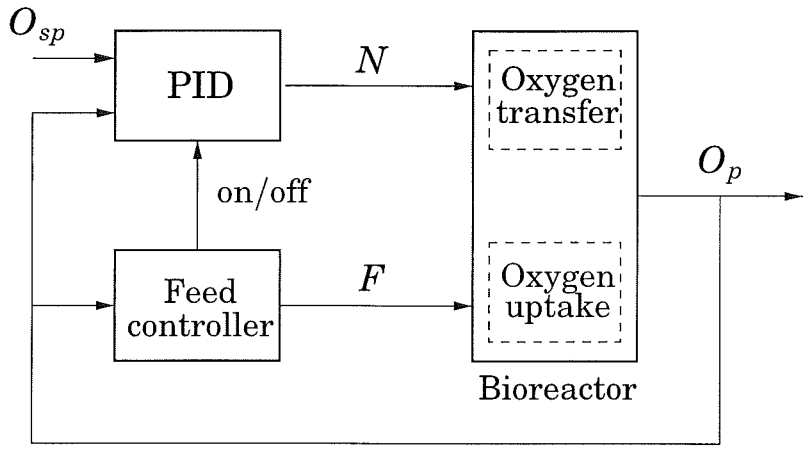

Figure 3.4 Block diagram of the closed-loop system.

Tuning rules

The algorithm has several parameters that affect the performance of the feeding strategy and in real applications it is important to have simple guidelines for tuning. In Paper I, tuning rules have been derived based on the linearized process model. Only a minimum of process knowledge is required which is important especially when the feeding strategy is used for new and poorly known processes. The process specific information that is needed is an upper bound of the overall time constant in the dynamics from glucose feed rate to dissolved oxygen measurement. It is also assumed that a well-tuned PID controller is available and that an upper bound for settling time in this loop is known.

Stability analysis

The choice of the parameter F_{inc}, which can be interpreted as the gain in the three-level controller, is a tradeoff between speed and stability. A simplified stability analysis is obtained from an automata description, [Booth, 1967].

The control algorithm is conveniently described as a state automaton with two states, *Up* and *Down*, corresponding to what the next probing pulse will be. Viewed from the controller, the process can also be described as an automaton. The process then has three states, *Above*, *At*, and *Below*, referring to that q_g is above, at, or below q_g^{crit}, see Figure 3.5. Computation of the synchronous composition for the two interconnected automata gives an equivalent automaton for the

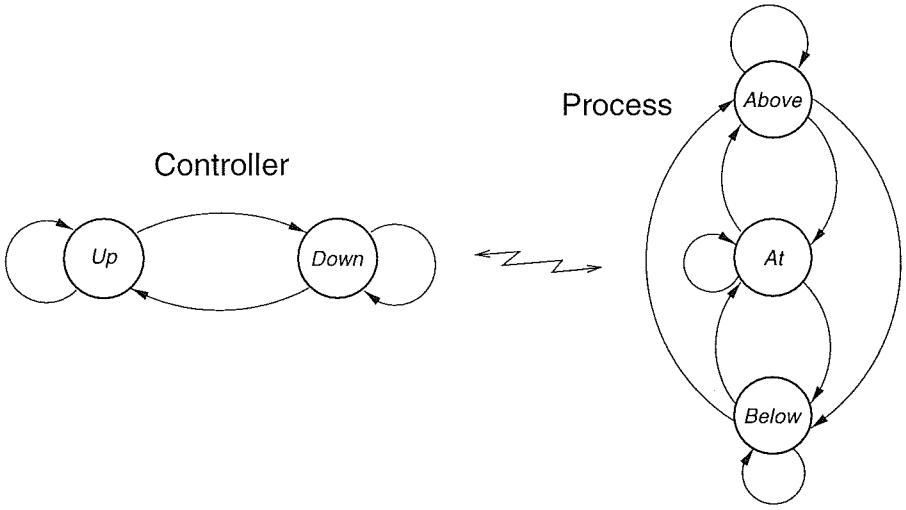

Figure 3.5 Automata description of control algorithm and process.

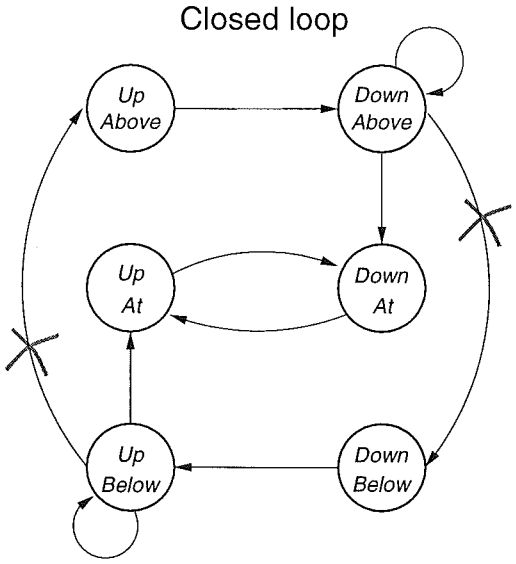

Figure 3.6 Automata description of the closed-loop system. Stability can be guaranteed if the two transitions marked with a cross are prohibited.

closed-loop system, see Figure 3.6.

The desired process state, *At*, corresponds to the two states in the middle. Instability in the sense that the process state oscillates between *Above* and *Below* is avoided by prohibiting the two transitions marked with a cross. These correspond to feed rate changes that take the process from a state below q_g^{crit} to above q_g^{crit} and vice versa. The

stability condition can be shown to be equivalent to the condition

$$F_{inc} < F_{pulse}$$

In other words, the feed increments should be smaller than the pulse size. However, this is a very restrictive condition and in practice, the probability for oscillations is low, even if F_{inc} is larger than F_{pulse}.

3.3 Simulations

The performance of the presented strategy will now be exemplified. The algorithm is simulated together with the nonlinear model from Chapter 2. Parameters in the algorithm are chosen according to the guidelines and are given in Paper I.

In the first example, see Figure 3.7, the initial feed rate after a batch phase is chosen too low. When the feed is started, the algorithm increases the feed rate until q_g^{crit} is reached. After the initial transient, the algorithm keeps q_g approximately constant at the critical level q_g^{crit}. After two hours, the value on q_g^{crit} is lowered but the algorithm detects the change and adjust the feed accordingly. This illustrates the ability to track changes in q_g^{crit} that may occur during a cultivation, see Paper II.

The second example illustrates the behavior when the control algorithm is used together with a pre-determined feed profile, see Figure 3.7. The feed-forward action from the profile clearly improves the overall behavior. We note that the compensation for the change in q_g^{crit} is somewhat slower than in the previous example. In practice, however, the changes in process parameters such as q_g^{crit} are probably less drastic than in the simulation.

An example given in Paper I, demonstrated the robustness of the strategy. The model parameters were changed such that the time constant for the overall dynamics was largely underestimated and the amplitude of the oxygen responses became lower than expected. As a result, the algorithm misinterpreted this for a saturated oxygen uptake and the glucose uptake deviated more from q_g^{crit} than in the previous examples. However, acetate formation was avoided and the performance was certainly acceptable.

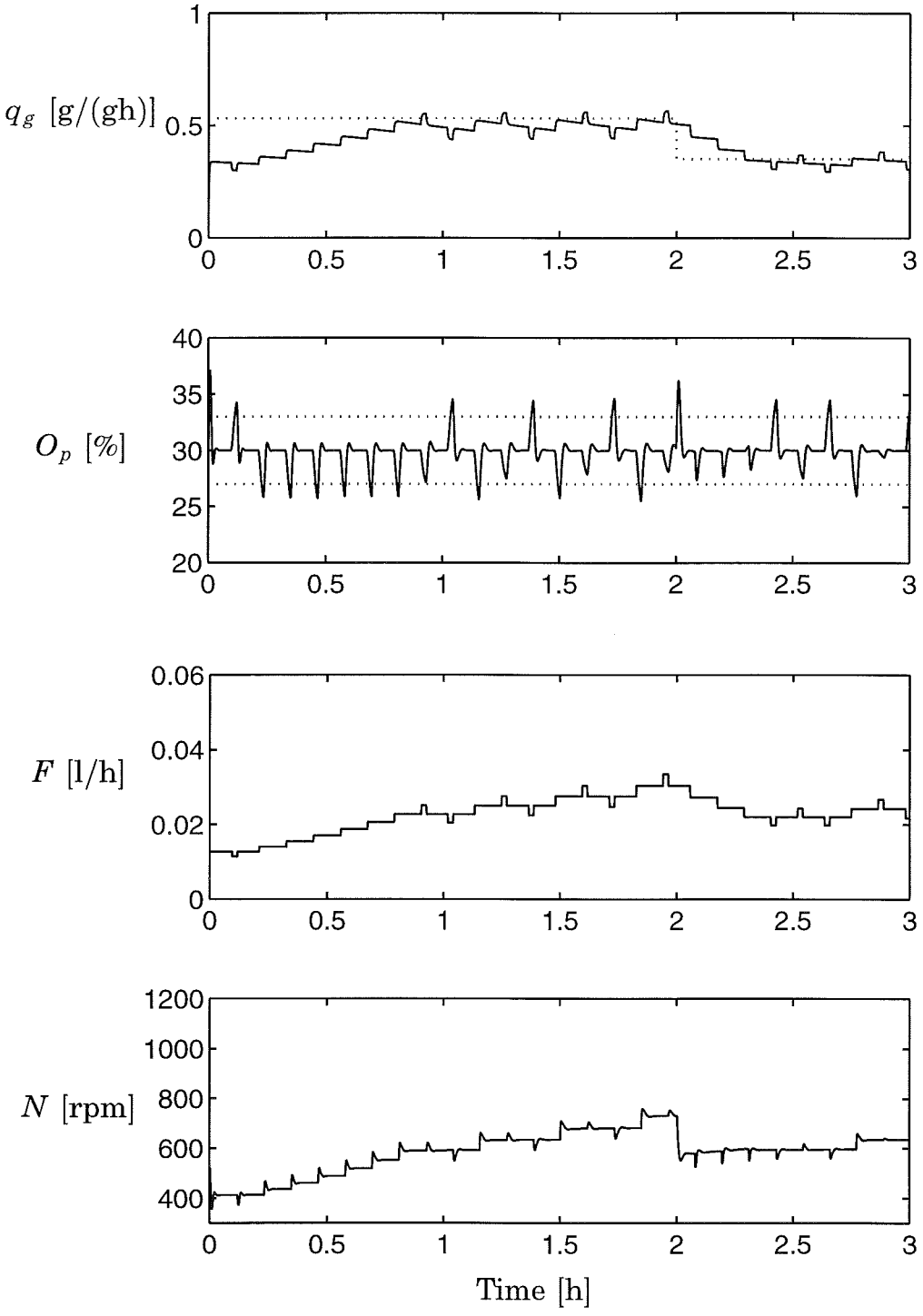

Figure 3.7 Simulation where the initial feed rate is chosen too low. After two hours q_g^{crit} is changed. The dotted lines indicate the critical glucose uptake, q_g^{crit}, and the reaction levels, O_{high} and O_{low}.

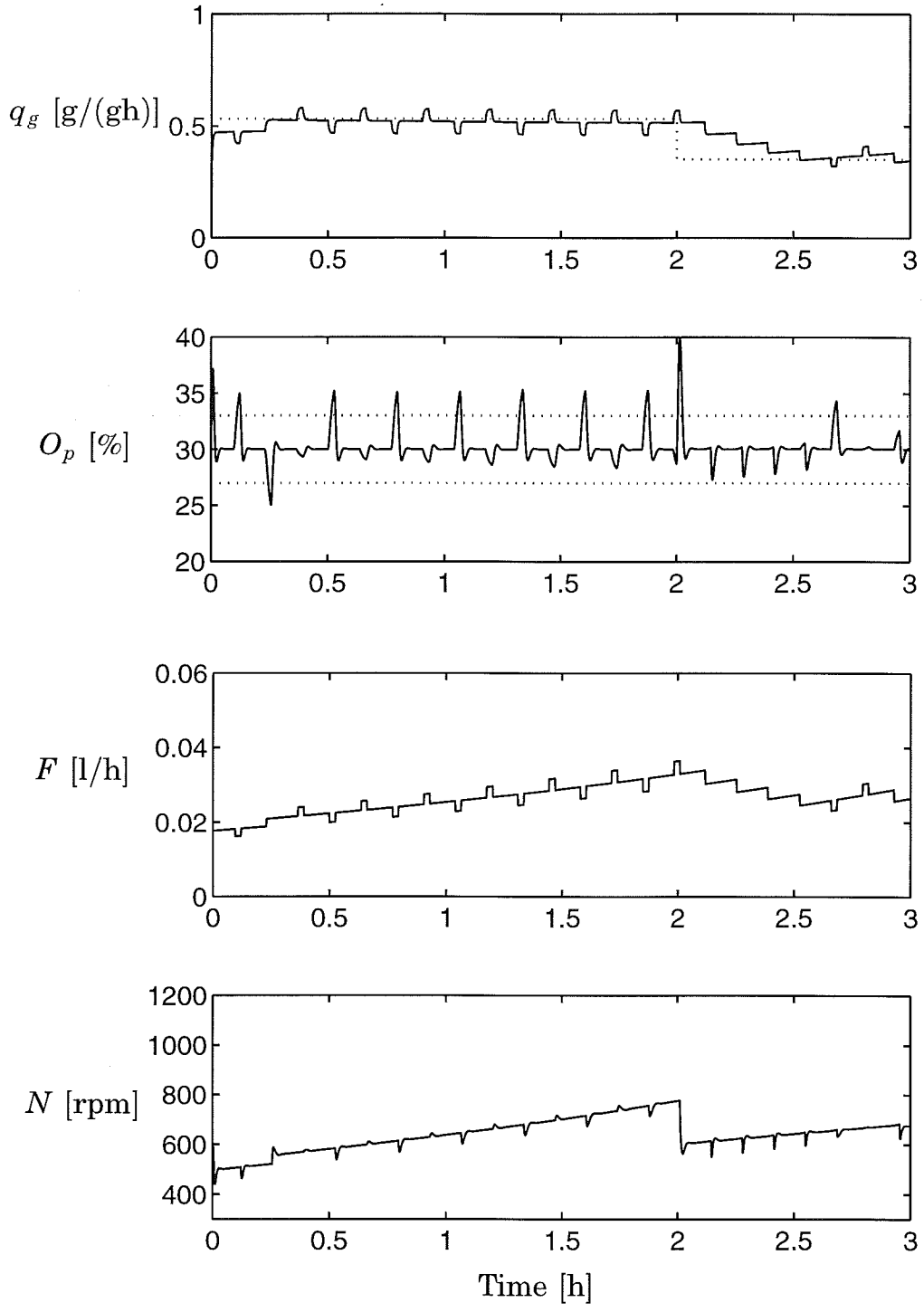

Figure 3.8 Simulation where control algorithm is combined with an exponential feed profile. After two hours q_g^{crit} is changed. The dotted lines indicate the critical glucose uptake, q_g^{crit}, and the reaction levels O_{high} and O_{low}.

3.4 Problems and Safety Nets

Problems may arise if the stirrer speed reaches its minimum or maximum. Then, the PID controller can no longer keep O_p at the desired set-point which may cause erroneous interpretations of the pulse responses. An example of this is given in Figure 3.9 where a runaway situation occurs when the maximum stirrer speed is reached.

This calls for a change of strategy when a saturation of the stirrer is detected. If the maximum stirrer speed is reached, a strategy where the feed rate is manipulated to give a constant dissolved oxygen level could be used, for example, the one in [Riesenberg *et al.*, 1990]. This guarantees a sufficient oxygen supply, but it does not necessarily avoid acetate formation. By combining this strategy with down pulses in the feed rate, however, it would be possible to detect acetate formation and then switch back to the probing strategy.

In the case where the minimum stirrer speed is reached, a similar technique could be used to regain the setpoint O_{sp} but, again, this does not guarantee that acetate formation is avoided. In practice this event is most likely to happen at startup due to a low initial feed rate.

Sporadic process disturbances or anti-foam additions, see Paper III, may also cause problems. Deviations in O_p from O_{sp} at the start of a feed pulse can in principle be solved by delaying the pulse until O_p has settled at O_{sp}. Perturbations can also occur during a pulse, and a misinterpretation of the pulse response may then result. By using area calculations instead of level-checking, the evaluation of the pulse response could be robustified.

3.5 Discussion

A feedback strategy for substrate feeding in cultivations of recombinant *E. coli* has been presented. The aim was to avoid acetate formation while maintaining a high growth rate. Major advantages with the strategy are that changes in the critical glucose uptake can be handled and that no prior knowledge of its value is required. It is also attractive that the strategy is based on information in the dissolved oxygen signal, which is a standard measurement. Simple tuning rules that require only a minimum of process specific information are pro-

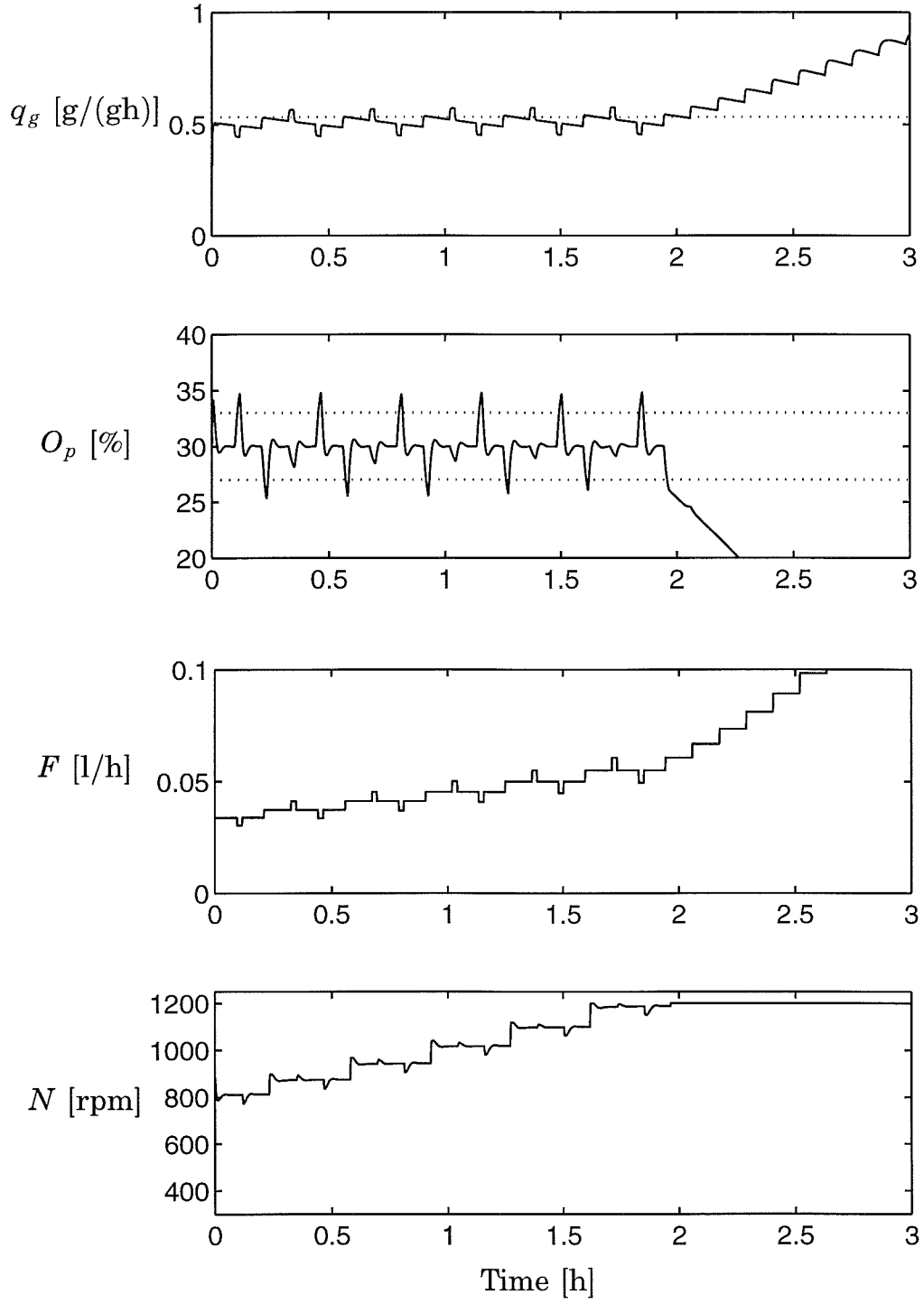

Figure 3.9 When the maximum stirrer speed is reached, O_p falls below O_{sp}. As a consequence, all responses to up pulses are above O_{high} and the feed rate is increased too much.

vided. This makes the strategy well suited for new and poorly known processes. A short discussion of appropriate safety nets was also given.

Superimposed pulses in the glucose feed rate were used to detect a qualitative change in the dissolved oxygen response at the onset of acetate formation. Similar ideas have appeared in other contexts. Detection of a change in the quotient between carbon dioxide production and oxygen uptake has been used to avoid ethanol formation in yeast cultivations [Wang *et al.*, 1977]. The idea of introducing deliberate process perturbations in a control loop to gain information can also be found in the areas of extremum control and dual control, see [Åström and Wittenmark, 1995], and in [Yongaçoglu *et al.*, 1982], a pulse technique was used for feed-rate control in an activated sludge process with a toxic substrate.

The presented strategy relies on an assumption that there is a limitation in the oxygen uptake at the onset of acetate formation. The validity of this assumption is verified experimentally in Paper II. If acetate has accumulated, for instance after an initial batch phase, effects from its consumption may affect the oxygen responses. This have been neglected, so far, but should be further investigated. To obtain good performance it is also important to have tight control of the dissolved oxygen level. This problem is further investigated in Paper III.

4

Acetate Formation and Dissolved Oxygen Responses

In both batch and continuous cultivations of *E. coli* it was observed that the onset of acetate formation coincided with a maximum in the specific oxygen uptake rate [Andersen and von Meyenburg, 1980; Reiling *et al.*, 1985]. In Paper I, a substrate feeding strategy was proposed that detects this saturation by analyzing the response in dissolved oxygen to superimposed pulses in the substrate feed rate. It was assumed that the saturation in the oxygen uptake is present also under transient conditions. The validity of this assumption is investigated in Paper II.

Starting from two hypotheses for acetate formation, relations between glucose uptake, oxygen uptake and acetate production on the cell level are derived using metabolic flux networks. Both hypotheses predict a "saturation" in the oxygen uptake coinciding with the start of acetate formation. Simulations show that this gives a clear change in the dissolved oxygen response to transients in the substrate feed rate. Experiments with two recombinant *E. coli* strains confirm the theoretical results.

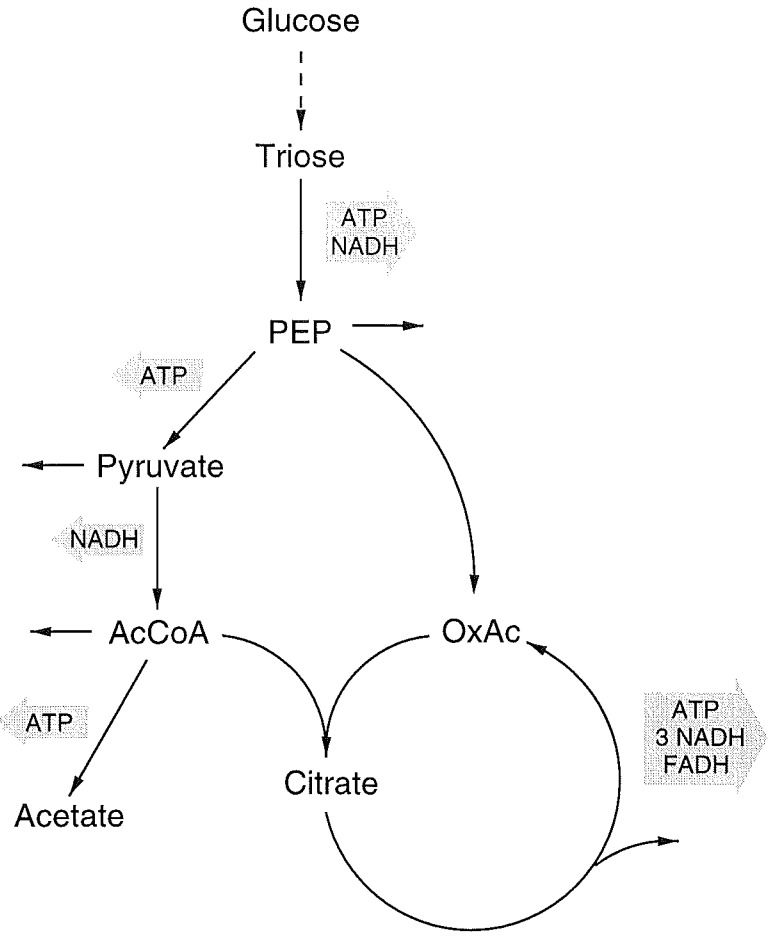

Figure 4.1 Simplified description of the central metabolic pathways in *E. coli* under aerobic conditions. The empty arrows indicate fluxes to precursor metabolites. Fluxes of carbon dioxide have been omitted.

4.1 Metabolic Modeling

The central metabolic pathways in a cell is the "engine" providing energy, reducing power, and precursor metabolites for cell growth. A simplified description of the glycolysis and the tricarboxylic acid (TCA) cycle in *E. coli* under aerobic conditions is shown in Figure 4.1. The incoming glucose flux is converted to carbon dioxide, precursors, and by-products such as acetate. The biochemical reactions also result in production of reducing equivalents (NADH,FADH) and energy carriers (ATP). Most of the energy production, however, comes from the respiratory chain where the reducing equivalents are re-oxidized using oxygen.

Acetate formation

Formation of acetate, when *E. coli* is grown under fully aerobic conditions, typically occurs at high growth rates and/or high glucose uptake rates. The acetate production is thought of as an overflow phenomenon where flux of AcetylCoA is directed to acetate, via acetylphosphate, instead of entering the TCA-cycle.

In [Andersen and von Meyenburg, 1980] it was suggested that the respiratory system, where NADH is re-oxidized, has a limited capacity. As flux to the TCA-cycle results in more NADH production than does flux to acetate, redirection of AcetylCoA flux to acetate would be necessary to avoid accumulation of NADH when the respiration saturates. Another explanation that has been suggested is that the TCA-cycle has a limited capacity, and that this limitation is reached before that of the respiration [Majewski and Domach, 1990]. When the TCA-cycle saturates, increasing glucose uptake will again result in flux from AcetylCoA to acetate. However, in this case, NADH production and respiration can increase further until the maximum respiration capacity or the maximum glucose uptake is reached.

Metabolic relations

In [Majewski and Domach, 1990], a flux network over parts of the central metabolic pathways was used to derive relations between triose flux and acetate production for the two explanations mentioned above. Assuming that the cells tend to maximize ATP production, a constrained optimization problem was formulated. Acetate production was predicted when constraints in the respiration or the TCA-cycle were reached.

From the analysis in [Majewski and Domach, 1990], it is straightforward to compute also the corresponding NADH fluxes for the two hypotheses. Assuming that the oxygen consumption is proportional to the NADH production and that the glucose flux is proportional to the triose flux, relations between glucose uptake, acetate production and oxygen consumption can be obtained. Qualitative results are shown in Figure 4.2.

When the glucose uptake, q_g, exceeds a critical level, q_g^{crit}, acetate formation starts. For both the respiratory and the TCA-cycle limitation, a characteristic change in the relation between q_g and q_o is pre-

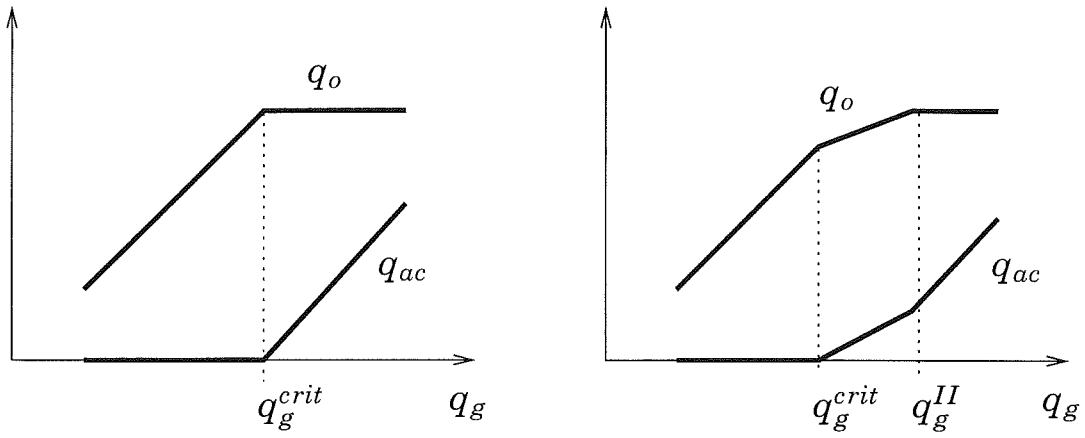

Figure 4.2 Relations between specific glucose uptake, q_g, specific oxygen uptake, q_o, and specific acetate production, q_{ac}, in the case of respiratory limitation (left) and TCA-cycle + respiratory limitation (right).

dicted. The main difference between the hypotheses is that the TCA-cycle limitation predicts an increase in q_o even after onset of acetate production. The oxygen uptake q_o continues to increase until the glucose uptake exceeds a second level q_g^{II} where the respiratory limitation is reached.

Simulations

A comparison of the dissolved oxygen response to pulses in the feed rate predicted by the two hypotheses, respiratory and TCA-cycle limitation, will now be made. Mathematical expressions for the derived relations, assumed to be valid under transient conditions, are incorporated in the dynamic model for a bioreactor in Chapter 2. Simulations of the dissolved oxygen response to pulses in the feed rate are made for the two cases. In Figures 4.3, 4.4 and 4.5, simulations for glucose uptakes, below, at, and above acetate formation are shown.

Both hypotheses predict a change in the oxygen responses at the onset of acetate formation and with the model parameters used, see Paper II, the quantitative differences are small. However, a qualitative difference is that the TCA-limitation model predicts a small oxygen response to an up pulse in feed even when acetate is produced, see Figures 4.4 and 4.5.

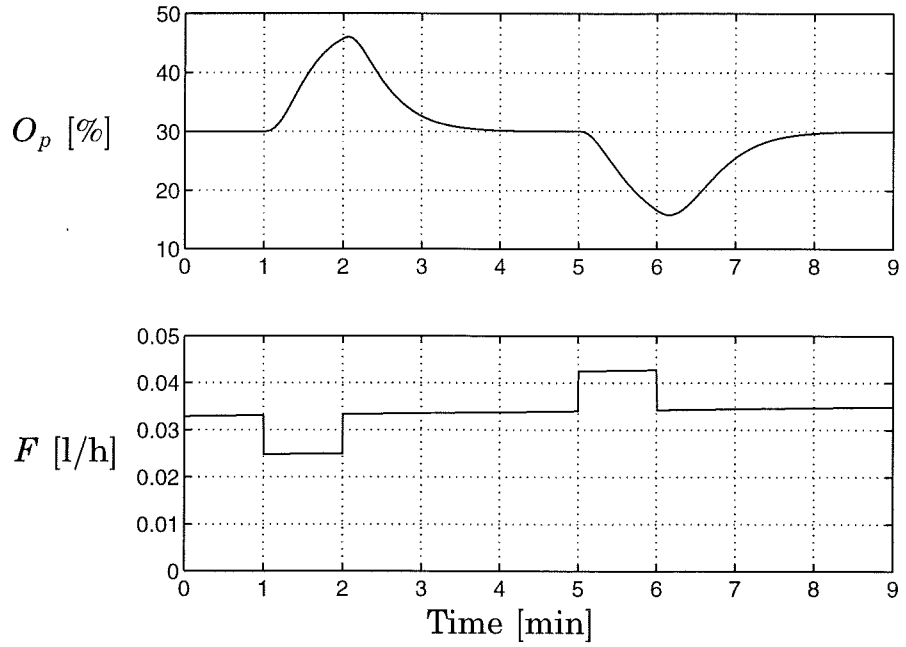

Figure 4.3 Simulation of response in dissolved oxygen response O_p to pulses in feed rate F *below* onset of acetate formation, $q_{ac} = 0$, $q_g = 0.8$ g/(gh) $< q_g^{crit}$. Respiratory limitation (solid) and TCA-cycle + respiratory limitation (dashed).

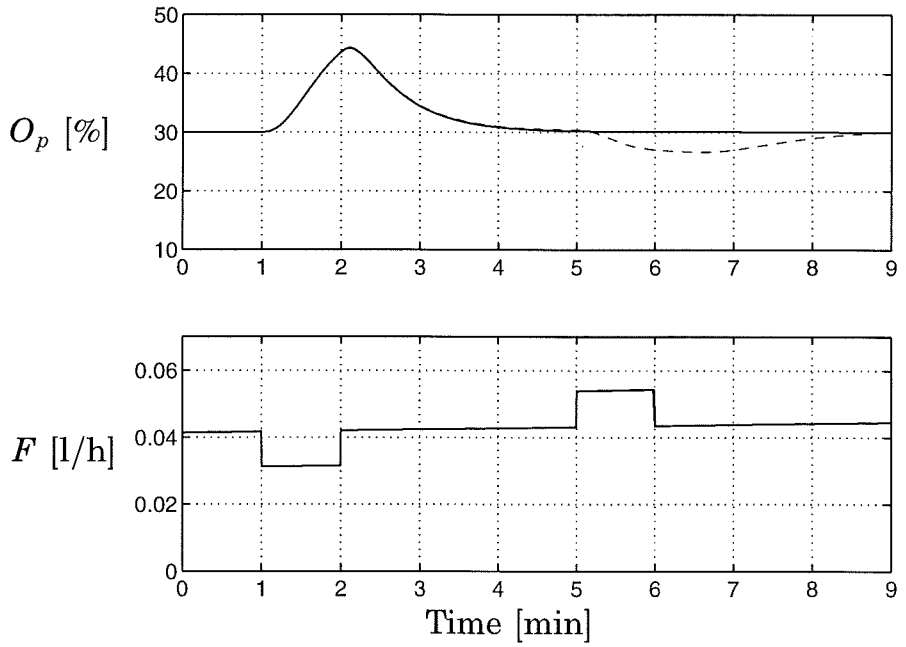

Figure 4.4 Simulation of response in dissolved oxygen response O_p to pulses in feed rate F *at* the onset of acetate formation, $q_{ac} = 0$, $q_g = 1.0$ g/(gh) $= q_g^{crit}$. Respiratory limitation (solid) and TCA-cycle + respiratory limitation (dashed).

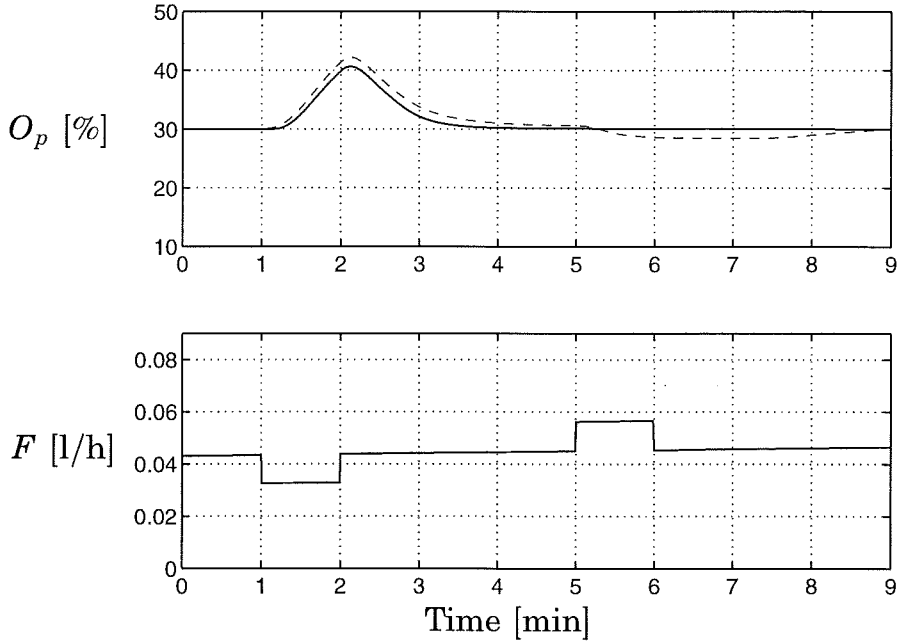

Figure 4.5 Simulation of response in dissolved oxygen response O_p to pulses in feed rate F just *above* onset of acetate formation, $q_{ac} > 0$, $q_g^{crit} < q_g = 1.05$ g/(gh) $< q_g^{II}$. Respiratory limitation (solid) and TCA-cycle + respiratory limitation (dashed).

4.2 Experiments

To verify the results from the simulations, cultivations were made with two recombinant strains, *E. coli* TOPP1 and *E. coli* BL21(DE3). Pulse experiments for different glucose uptake rates were performed at well-controlled oxygen levels. During the pulse experiments, the PID controller regulating dissolved oxygen was set in manual mode which resulted in drifts in the dissolved oxygen. To facilitate the comparison with the simulations, linearly detrended data is shown together with the original data.

Experiments were performed both before and after induction of recombinant protein production. As was predicted in the simulations, the oxygen response to up pulses in the feed rate disappeared when acetate was produced.

Three consecutive pulse experiments after induction are shown here. In the first experiment, displayed in Figure 4.6, q_g was about 0.54 g/(gh) and no acetate production could be observed. Clear responses to both up and down pulses were obtained. When q_g increased

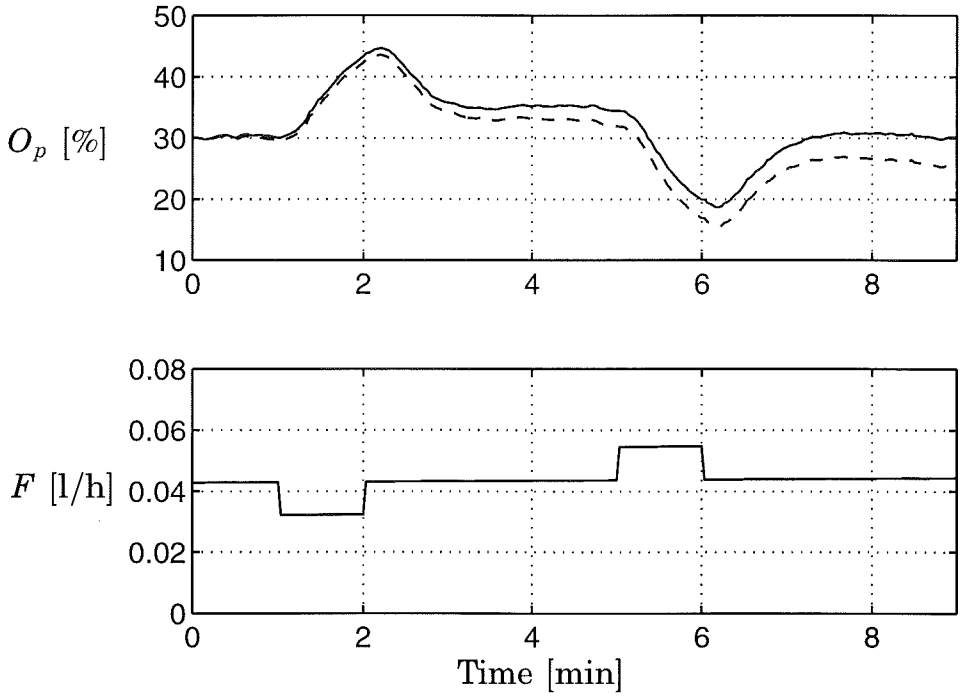

Figure 4.6 Experiment with *E. coli* BL21(DE3) after induction. Response in dissolved oxygen O_p to pulses in feed rate F. No acetate formation. $q_g \approx 0.54$ g/(gh). Detrended (solid) and original data (dashed).

to 0.59 g/(gh), the response to an up pulse was broadened and the amplitude was somewhat reduced, see Figure 4.7. Still no accumulation of acetate was detected. Figure 4.8 shows the third experiment where q_g was approximately 0.63 g/(gh). Almost no response to up an up pulse resulted and shortly after this experiment, an increased acetate concentration was detected.

4.3 Summary

From the experimental results, it is clear that the dissolved oxygen response to transients in the glucose feed rate changes character at the onset of acetate formation. This supports the key assumption in the substrate feeding strategy in Paper I.

The two metabolic models, assuming limitations in the respiration and/or the TCA-cycle, give a good description of the experimental results. However, from the experiments it is not possible to distinguish

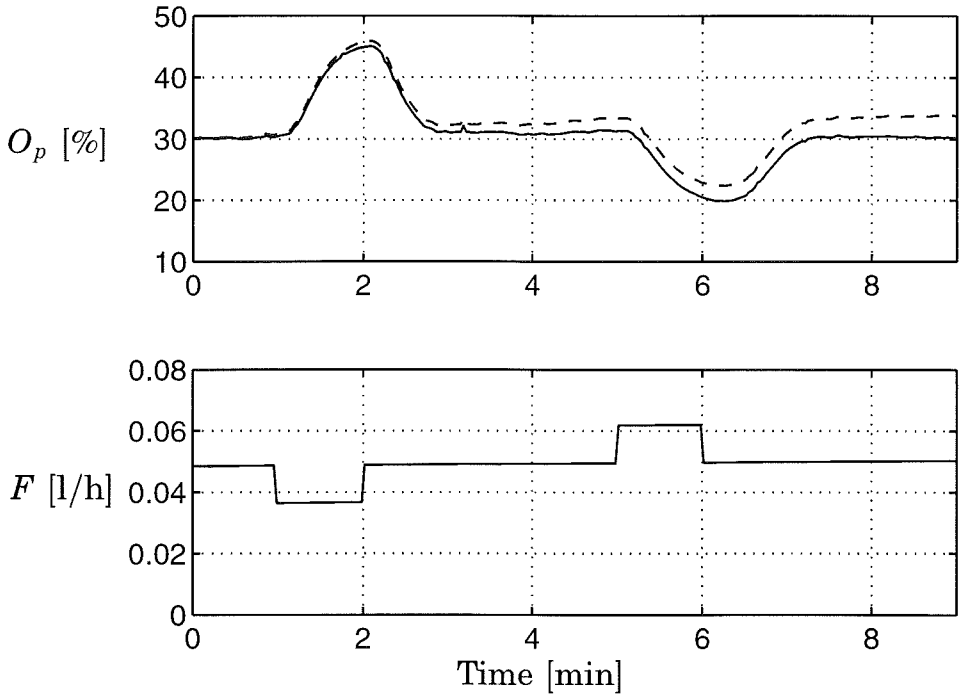

Figure 4.7 Experiment with *E. coli* BL21(DE3) after induction. Response in dissolved oxygen O_p to pulses in feed rate F. No acetate formation. $q_g \approx 0.59$ g/(gh). Detrended (solid) and original data (dashed).

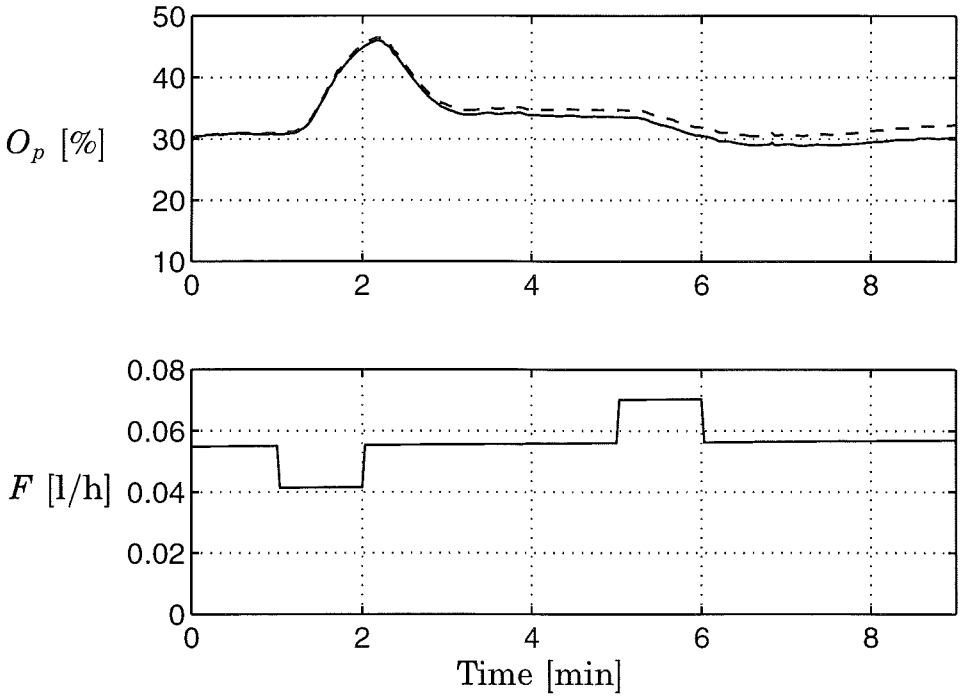

Figure 4.8 Experiment with *E. coli* BL21(DE3) after induction. Response in dissolved oxygen O_p to pulses in feed rate F. Just before onset of acetate formation. $q_g \approx 0.63$ g/(gh). Detrended (solid) and original data (dashed).

between the two hypotheses. In practice, it is also likely that the difference between the two models is evened out. For example, concentration gradients in the reactor will tend to average out the sharp bottleneck to the left in Figure 4.2.

5

Dissolved Oxygen Control

Many biotechnical processes, for instance cultivations of recombinant *E. coli*, require aerobic conditions. A simple way to provide enough oxygen supply is to keep a constant dissolved oxygen concentration. It is often sufficient to maintain the dissolved oxygen concentration above a certain level, and the performance demands on the dissolved oxygen control are then moderate. In the substrate feeding strategy presented in Paper I, however, tight control of dissolved oxygen is important for the overall performance.

In this chapter, based on Paper III, we discuss control of dissolved oxygen in a bioreactor when the stirrer speed is used as the control variable. In batch and fed-batch cultivations, the oxygen dynamics varies significantly with the operating condition. This may cause tuning problems when controllers with fixed parameters are used and high performance is desired. A control strategy based on PID control and gain scheduling from the stirrer speed is suggested.

5.1 Control Problem

From a control point of view, dissolved oxygen control is a regulation problem where the main disturbance is the oxygen consumption due to cell metabolism. In continuous and fed-batch cultivations, this disturbance is strongly correlated to the substrate feed rate. In a stirred bioreactor, the oxygen supply can be varied in many ways; by manipulating the air flow rate, the oxygen content in the incoming air,

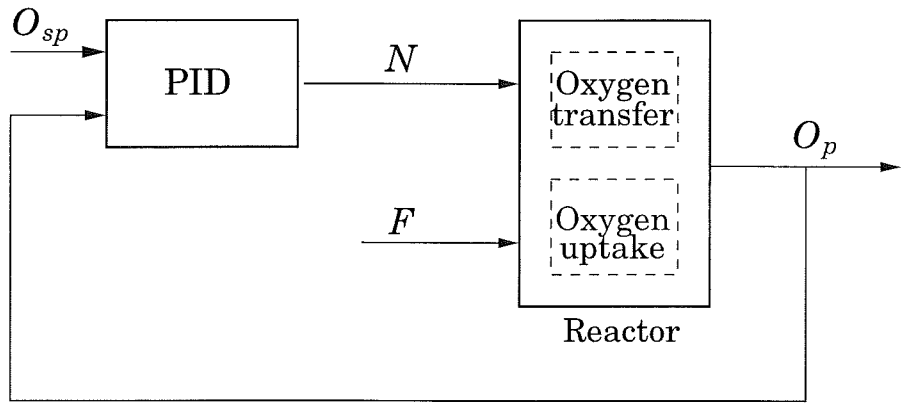

Figure 5.1 Dissolved oxygen control loop where the dissolved oxygen signal O_p should be kept at the set-point O_{sp}. By manipulating the stirrer speed N the oxygen transfer to the reactor can be varied. In fed-batch and continuous cultivations, the oxygen consumption depends on the substrate feed rate F.

the reactor pressure, or the stirrer speed. We will here consider control of dissolved oxygen using the stirrer speed as control signal, see Figure 5.1. Apart from the microbial oxygen consumption, other disturbances affecting the process include temperature changes, foaming, and addition of surface active components. At low turbulence levels, gas bubbles at the surface of the dissolved oxygen sensor may give significant sensor noise.

Process variations

In continuous cultivations, the process is operated at steady-state conditions and good performance can be expected using controllers with fixed parameters. When the reactor is run in batch or fed-batch mode, the process characteristics vary significantly with important process variables, like cell mass, substrate concentration, and oxygen uptake. Controllers with fixed parameters tend to show stability problems for low oxygen uptake rates, that is, low stirrer speeds, and sluggish control at high oxygen uptake rates. Similar difficulties occur in dissolved control of activated sludge processes in waste-water treatment, where the air flow rate is used as control signal, see for instance [Lindberg, 1997].

5.2 Analysis

Insight into the reported tuning problems can be gained from a linearized process model. First, we briefly recall the relevant model equations from Chapter 2. Values of the model parameters corresponds to a laboratory-scale bioreactor and are given in Paper III.

Process model

Neglecting effects from dilution, the dynamics in the dissolved oxygen tension O can be described as

$$\frac{dO}{dt} = K_L a(N) \cdot (O^* - O) - \underbrace{q_o \cdot HX}_{=d}$$

The oxygen consumption term is considered as a load disturbance d.

In the practical working range of the stirrer and for a fixed air flow rate, the volumetric oxygen transfer coefficient, $K_L a$, can be modeled as a linear function of the stirrer speed, N.

$$K_L a(N) = \alpha \cdot (N - N_0)$$

The probe that is used to measure the dissolved oxygen tension contributes significantly to the process dynamics. It is modeled as

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

with O_p denoting the measured dissolved oxygen tension. The time delay τ is short, and it was neglected in the design and analysis of the feed control algorithm in Chapter 3.

Linearized model

Around an operating point, the equation for the dissolved oxygen dynamics may be linearized as

$$T_o \frac{d\Delta O}{dt} + \Delta O = K_n \Delta N - K_d \Delta d$$

where the parameters are given by

$$T_o = (K_L a)^{-1}$$
$$K_n = [O^* - O]\frac{\partial K_L a}{\partial N}(K_L a)^{-1}$$
$$K_d = (K_L a)^{-1}$$

The transfer function from the stirrer speed ΔN to the oxygen measurement ΔO_p becomes

$$G_{on}(s) = \frac{K_n e^{-s\tau}}{(1 + sT_o)(1 + sT_p)}$$

Here τ and T_p depend on the probe and can be considered to be constant during a cultivation. On the other hand, K_n and T_o are both proportional to the inverse of $K_L a$, which may vary significantly. In Figure 5.2, a Bode plot of G_{on} at three different stirrer speeds illustrate how the process dynamics is affected. When $K_L a$ increases with the stirrer speed, the low-frequency gain decreases and the phase lag in the low- and mid-frequency region increases. This explains the tuning difficulties with stability problems at low stirrer speeds and sluggish behavior at high stirrer speeds.

The high-frequency behavior is unaffected by the process variations and in principle a robust design can be obtained with a high-bandwidth controller. In practice, however, this approach will be prohibited by measurement noise.

5.3 Control Design

Fixed-parameter controllers

If a fixed controller is used there will be a tradeoff between stability robustness at low stirrer speeds and achievable performance at high stirrer speeds. This can be illustrated by the following example.

Using the Kappa-Tau tuning method, see [Åström and Hägglund, 1995], two PID controllers are designed to work well at 400 and 1100

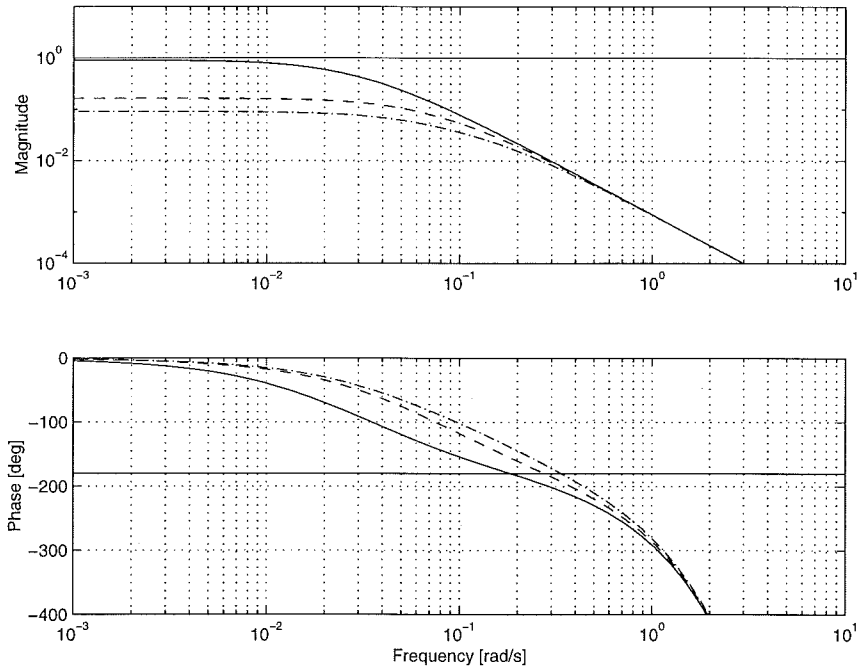

Figure 5.2 Bode plot of the transfer function from stirrer speed to measured dissolved oxygen tension at three different stirrer speeds; 400 rpm (solid), 750 rpm (dashed), 1100 rpm (dash-dotted).

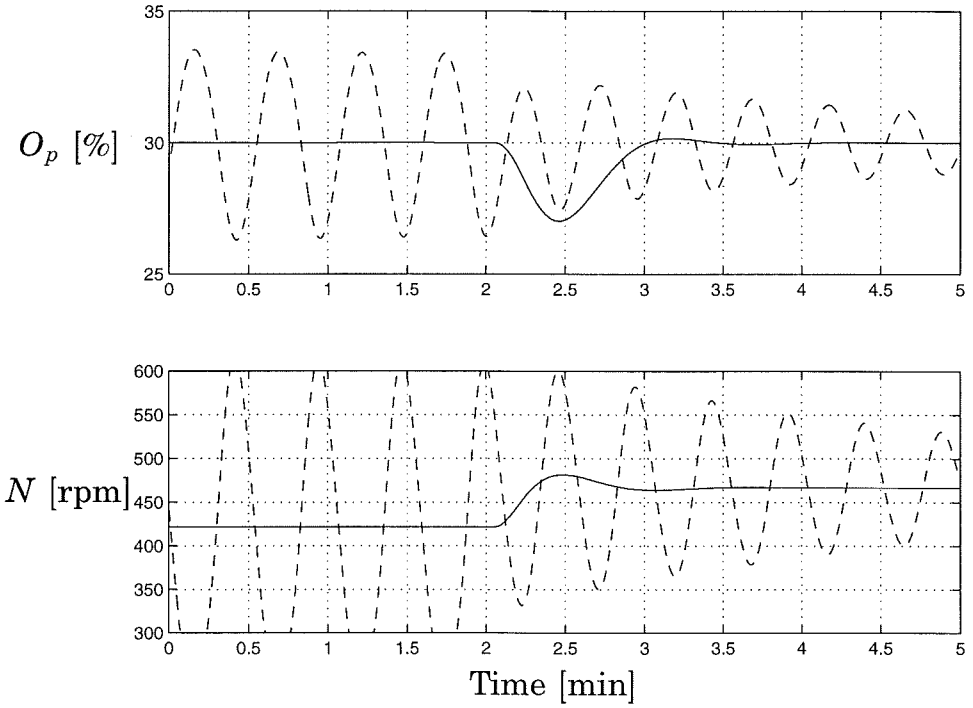

Figure 5.3 Closed-loop simulation around 400 rpm. After two minutes, a step change in the load is made. Results with PID controller tuned for 400 rpm (solid) and 1100 rpm (dashed).

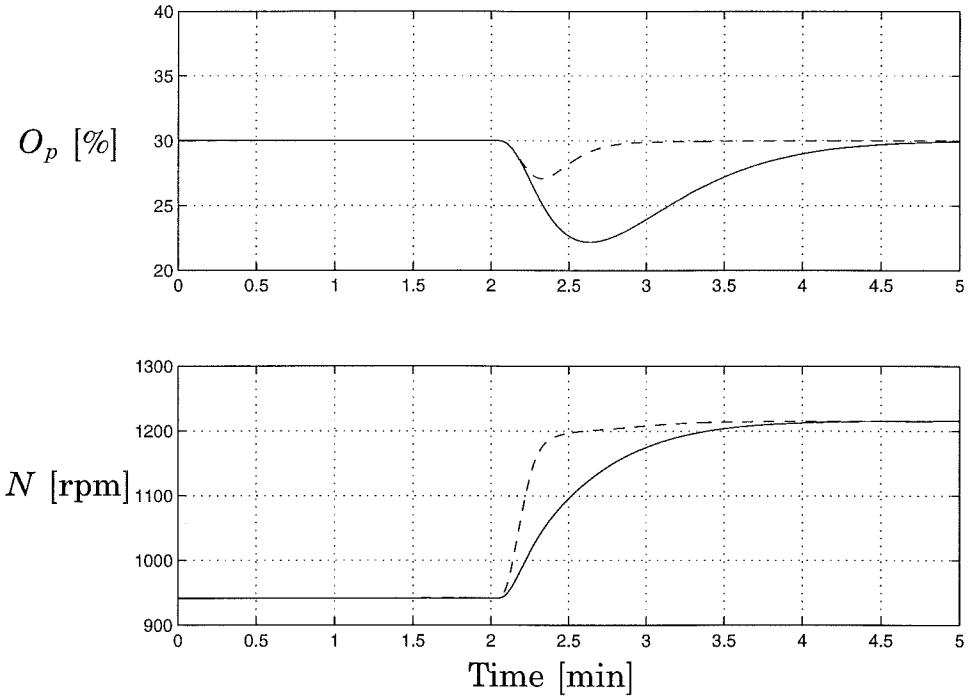

Figure 5.4 Closed-loop simulation around 1100 rpm. After two minutes, a step change in the load is made. Results with PID controller tuned for 400 rpm (solid) and 1100 rpm (dashed).

rpm respectively. Simulations of the closed-loop behavior at 400 rpm and 1100 rpm respectively, are shown in Figures 5.3 and 5.4. The controller designed for 400 rpm behaves, as expected, well around 400 rpm but is unnecessarily slow at 1100 rpm. On the other hand, the controller designed for 1100 rpm gives an almost unstable closed-loop system when used at 400 rpm.

If stability in the presence of process variations is the most important objective, the tuning should be made for the lowest K_La that is expected, that is, at the lowest stirrer speeds. This gives a robustly stable closed-loop system at the expense of a sluggish response at higher K_La values.

Adaptive controllers

To obtain the same performance at all operating points, without trading off robustness, the controller should depend on the operating conditions. Approaches based on PID control together with gain scheduling from the oxygen uptake rate and PID control combined with feed-

forward from the oxygen uptake rate were presented in [Cardello and San, 1988]. On-line tuning of PID controllers based on output variance [Lee *et al.*, 1992] and parameter estimation from off-gas analysis [Levisauskas, 1995] have also been suggested. Indirect adaptive control, that is, parameter estimation and subsequent controller design, have been tested in [Lee *et al.*, 1991] and [Hsiao *et al.*, 1992].

A simple gain-scheduling approach

As the process dynamics changes with K_La, this is a natural scheduling variable for adjustment of the controller parameters, but on-line estimation of K_La is then required. As K_La is dependent on the stirrer speed, a simpler approach is to use gain scheduling from the stirrer speed itself. This method is straightforward to implement in an industrial control system. A drawback is that this technique does not capture changes in K_La due to other sources than the stirrer speed, for instance, foaming, surface active compounds and viscosity variations. In processes where such effects are important, a method based on estimation of K_La is preferable.

5.4 Experiments

The approach with gain scheduling from the stirrer speed was tested in a 3 liter laboratory bioreactor. The controller was implemented in the industrial control system SattLine from Alfa Laval Automation AB, Malmö, Sweden. A standard module for PID control with facilities for gain scheduling and auto-tuning was used. The working range for the stirrer was divided into three regions and in each region, controller parameters were obtained using the auto-tuner function. Load response experiments were made at the end of a cultivation of recombinant *E. coli*, see Figures 5.5 and 5.6. At this stage, there was substantial foaming in the reactor and anti-foam chemicals which affect the oxygen transfer had to be added several times.

Good disturbance rejection was achieved throughout the operating range. However, some tuning problems could be hinted at in the lower part of the mid-region. This indicates that it could be beneficial to divide the working range into more operating regions or to change the

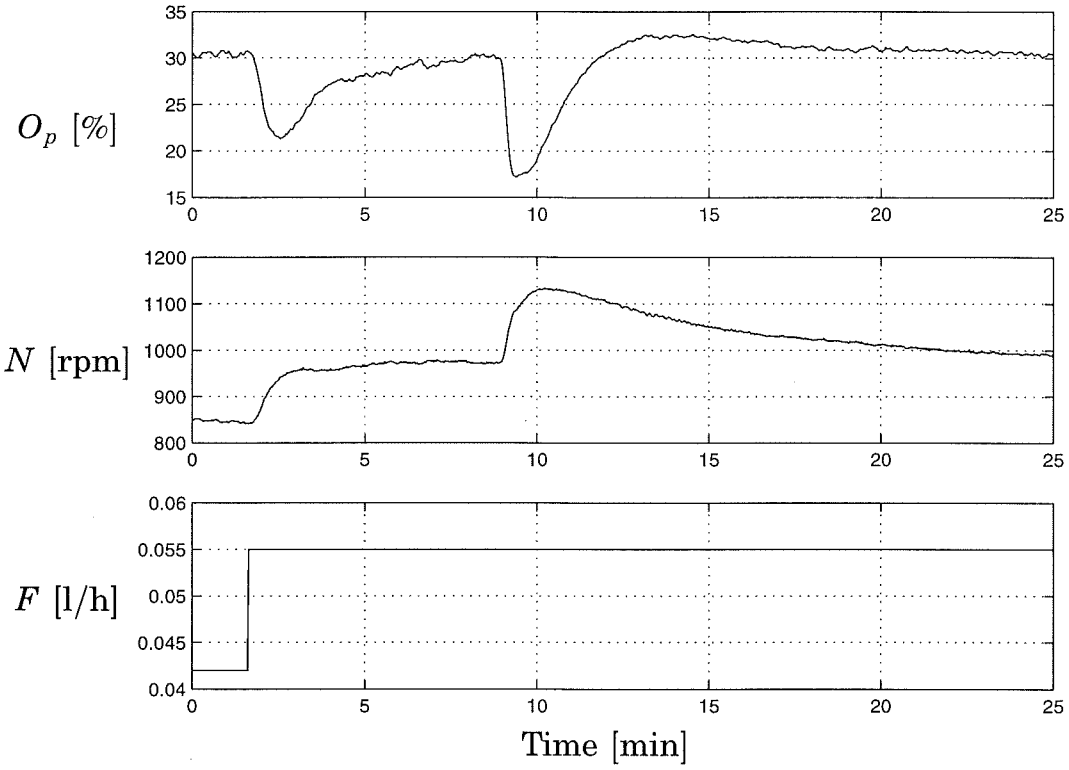

Figure 5.5 Step load change followed by anti-foam addition. At 920 rpm, the controller changed from the mid to the high operating region. The set-point O_{sp} was fixed at 30 %.

partitioning of the operating regions. From the expressions for K_n and T_o, it can be seen that most of the process variation take place at lower $K_L a$ values. This can also be seen in the controller parameters obtained from auto-tuning experiments. This suggests that the partitioning of the operating regions should be denser in the lower part of the working range.

Disturbances caused by anti-foam addition were well handled but did take considerable time to eliminate completely. At lower stirrer speeds, the excitation from sporadic disturbances, possibly due to air bubbles adhering to the sensor, was clearly important.

5.5 Summary

Control of dissolved oxygen using the stirrer speed as control variable was discussed. When high performance is required, variations in the

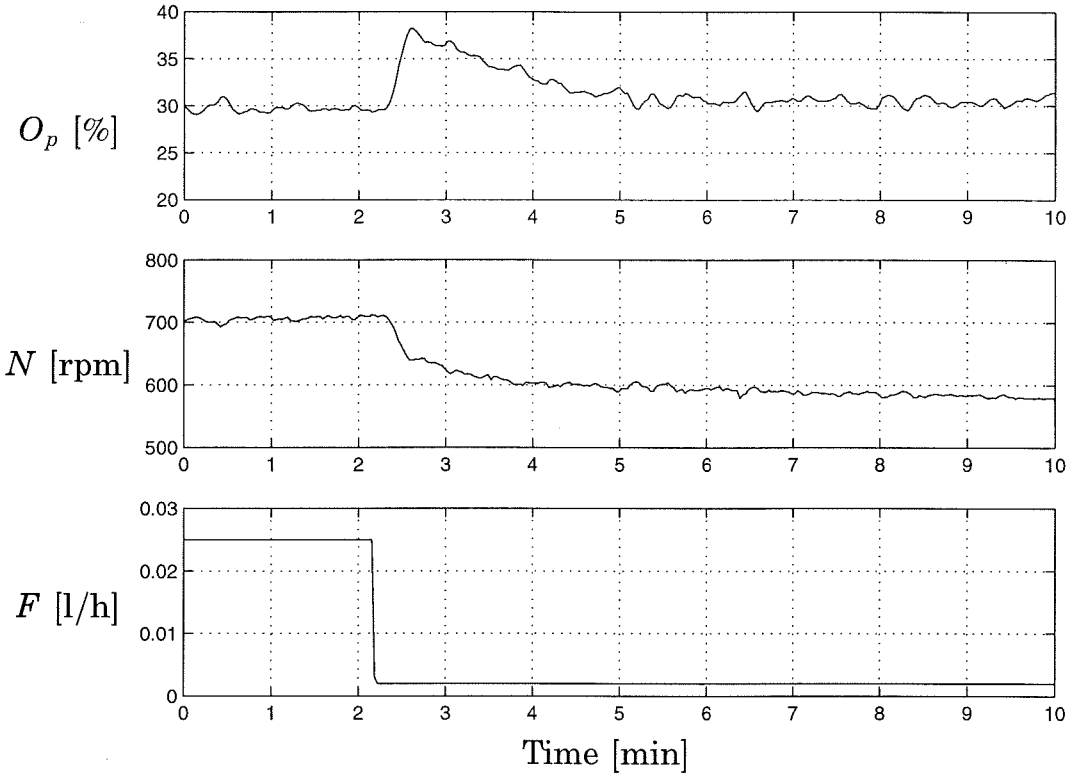

Figure 5.6 Step load change in the lower part of the mid operating region. The low region was never entered. The set-point O_{sp} was 30 %.

process dynamics may cause tuning problems. Gain scheduling from the stirrer speed was suggested as a solution.

The working range for the stirrer in a laboratory scale reactor was partitioned into three regions and in each region relay auto-tuning was used to obtain parameters for a PID controller. Settling times to step load disturbances below 7 minutes could be achieved under all operating conditions. With better partitioning of the operating regions and improved tuning methods, the settling times can probably be reduced significantly.

Disturbances caused by anti-foam additions took longer time to eliminate. Effects cold be seen up to 15 minutes after the addition. It was observed that the excitation from sporadic disturbances was pronounced at low stirrer speeds.

6

Conclusions

The production of recombinant proteins in *Escherichia coli* cultivations can be significantly reduced by accumulation of the metabolic by-product acetate. This thesis has discussed how feedback control of the substrate feed rate can be used to avoid acetate accumulation. The main contributions of the thesis are

- a method for on-line detection of acetate formation in fed-batch cultivations of *E. coli*.
- a feedback strategy for substrate feeding in *E. coli* cultivations.
- a simple approach to high-performance control of dissolved oxygen in stirred bioreactors.

The key idea in the detection method is to exploit a saturation in the specific oxygen uptake at the onset of acetate formation. By superimposing short pulses in the substrate feed rate, a standard dissolved oxygen sensor can be used to detect production of acetate.

In the substrate feeding strategy, the pulse detection method is combined with a simple feedback algorithm. The feedback compensation can handle variations in the process, for example, changes in the critical glucose uptake rate. Tuning rules that require a minimum of information and assumptions about the process make the presented strategy well suited also for new and poorly known processes.

The feeding strategy puts high demands on the performance of the dissolved oxygen control. Variations in oxygen dynamics during a cultivation may cause problems if a controller with fixed parameters is used. To solve the problem with the varying process dynamics, a control

approach based on PID control and gain scheduling is suggested. The scheduling variable is the stirrer speed, which makes the approach straightforward to implement as no parameter estimation or off-gas analysis is required.

Ideas for future work

So far, the performance of the feeding strategy, together with the tuning rules, has not been investigated experimentally. Implementation of the control algorithm is therefore a natural direction to follow. This will surely imply that further attention should be paid to the issue of safety nets. For instance, the change of strategy that is required when the limitations in the stirrer speed is reached is an interesting problem.

The present study has only considered acetate production. If acetate is present in the medium, for example after an initial batch phase, it can be also be consumed by the cells. Effects from the concomitant oxygen consumption may affect the behavior of the algorithm which should be further investigated. This may be important especially in large scale reactors where well-mixed conditions no longer can be assumed. Production and consumption of acetate may then occur simultaneously in different locations in the reactor.

The evaluation of the dissolved oxygen responses is based on level-checking. This may cause problems in processes where the influence from measurement noise or other disturbances is important. More robust evaluation measures may then be required. An alternative evaluation of the pulse responses could, for instance, be based on area calculations.

The feed control algorithm and the mechanism for detection of acetate formation are simple and rather robust. However, it would be interesting to look into the possibilities to use more information from the dissolved oxygen response.

7

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Paper I

A Pulse Feeding Strategy for Fed-batch Cultivations of *Escherichia coli*

Mats Åkesson, Per Hagander, and Jan Peter Axelsson

Abstract

A strategy for substrate feeding in fed-batch cultivations of *Escherichia coli* is presented. The aim is to avoid acetate formation while maintaining a high growth rate. The key idea is to superimpose pulses in the substrate feed rate and to evaluate the response in the dissolved oxygen signal. This gives information of acetate formation that is used for feedback control of the feed rate. Tuning rules that require only a minimum of process specific information are given and simulation experiments show the feasibility of the presented strategy.

1. Introduction

A major determinant for productivity and quality in fed-batch production of recombinant proteins is the substrate feeding strategy. Underfeeding will give low cell growth and hence low productivity. On the other hand, overfeeding often results in production of undesirable by-products. For instance, *Escherichia coli*, one of the most frequently used host organisms, tends to produce acetate which has been reported to inhibit growth and to reduce production of the recombinant protein [Bech Jensen and Carlsen, 1990].

To reduce or avoid acetate formation, a number of feeding strategies have been developed, see [Lee, 1996; Yee and Blanch, 1992]. A common approach is to control the specific growth rate, μ, for instance through fixed feeding profiles [Paalme *et al.*, 1990; Korz *et al.*, 1995] or feedback from off-gas analysis [Riesenberg *et al.*, 1991]. Another approach is to use feedback from the acetate concentration to determine the feed rate as in [Shimizu *et al.*, 1988; Turner *et al.*, 1994]. A third category of strategies are based on control of the substrate concentration, either using direct measurements [Kleman *et al.*, 1991] or indirect measurements from pH [Robbins and Taylor, 1989] or dissolved oxygen [Mori *et al.*, 1979]. Sufficient oxygen supply can be guaranteed by manipulating the substrate feed rate in order to maintain a constant dissolved oxygen concentration. The stirrer speed may be fixed or varied [Konstantinov *et al.*, 1990; Riesenberg *et al.*, 1990].

In this article we present a feeding strategy that aims to avoid acetate formation while maintaining a high growth rate. An advantage is that only a minimum of process specific information is required, and this also makes it well suited for new expression systems. The basic idea has earlier been presented in [Åkesson *et al.*, 1997] and similar ideas are used for control of a process with a growth inhibitory substrate in [Yongaçoglu *et al.*, 1982].

2. Detection of Acetate Formation

Acetate formation under aerobic conditions is believed to occur due to a limitation in the capacity to oxidize glucose. Saturation of the TCA-cycle and/or the electron transport chain have been suggested as possi-

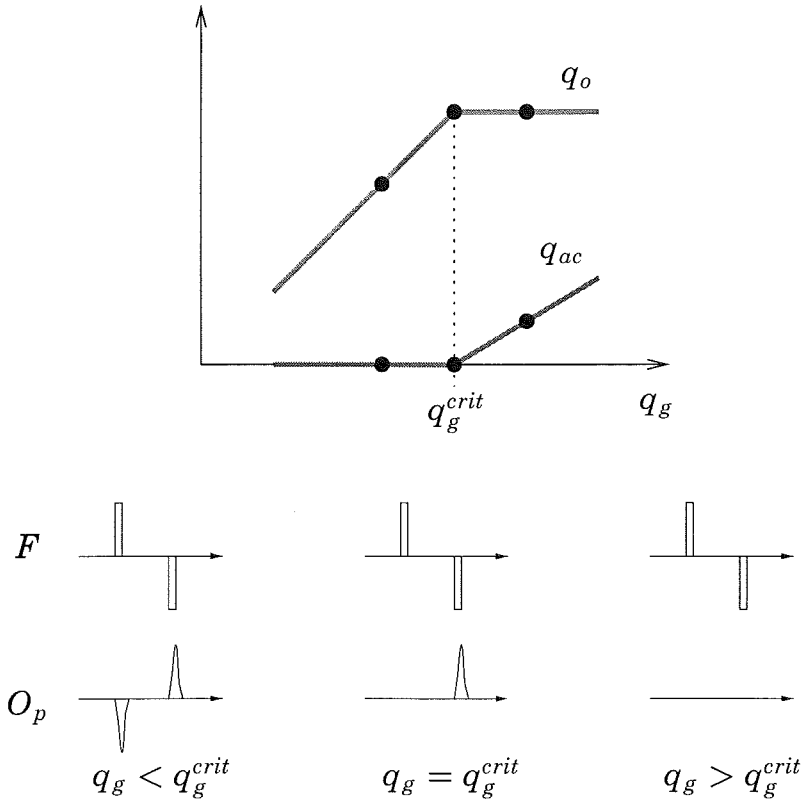

Figure 1 Relations between glucose uptake, q_g, oxygen uptake, q_o, and acetate production, q_{ac}. Pulses in feed rate F and idealized responses in measured dissolved oxygen O_p for q_g below, at, and above q_g^{crit}.

ble explanations, see for instance [Majewski and Domach, 1990]. Above a critical specific glucose uptake rate, q_g^{crit}, excess glucose is fermented with acetate as main by-product. Under steady-state conditions, it has been observed that the specific oxygen uptake rate reaches an apparent maximum q_o^{max} when the acetate formation starts [Andersen and von Meyenburg, 1980; Reiling *et al.*, 1985]. Corresponding relations between glucose uptake, oxygen uptake and acetate formation are illustrated in Figure 1. Experiments supporting that these relations are valid also under transient conditions are presented in [Åkesson *et al.*, 1998]. The key idea is now to exploit the characteristic change in the relation between oxygen uptake and glucose uptake at the onset of acetate formation. This change, and hence acetate formation, can be detected by superimposing short pulses in the glucose feed rate.

Under glucose-limited conditions, a short pulse increase in the feed rate, F, gives rise to a pulse increase in the glucose uptake rate, q_g.

Below q_g^{crit}, oxygen is used to metabolize the glucose, and there will ultimately be a pulse decrease in the dissolved oxygen concentration that can be detected in dissolved oxygen measurement O_p. Similarly, a pulse decrease in the feed rate would lead to a pulse increase in the dissolved oxygen. Above q_g^{crit}, the oxygen uptake is saturated and the oxygen response to feed pulses will change character. Figure 1 shows idealized pulse responses for q_g below, at, and above q_g^{crit}. Indirect information about acetate formation can thus be obtained from the dissolved oxygen response to superimposed feed pulses. This information is used for feedback control of the feed rate in the proposed strategy.

3. Process Model

A mathematical model is formulated with emphasis on the relation between substrate feed rate, stirrer speed and dissolved oxygen concentration under transient conditions. It is assumed that pH and temperature are constant and that well-mixed conditions apply. Dilution effects from base/acid addition and sampling are neglected. The model is used for simulation of the proposed feeding strategy under various cultivation conditions. A linearized model, that gives valuable insights for tuning of parameters in strategy, is also presented.

Nonlinear Model

Balance equations Component-wise mass balances for the bioreactor give the equations

$$\begin{aligned}
\frac{dV}{dt} &= F \\
\frac{d(VX)}{dt} &= \mu(G) \cdot VX \\
\frac{d(VG)}{dt} &= FG_{in} - q_g(G) \cdot VX \\
\frac{d(VO)}{dt} &= K_L a(N) \cdot V(O^* - O) - q_o(G) \cdot HVX
\end{aligned}$$

where G, X, O, V are, respectively, the glucose concentration, the cell concentration, the dissolved oxygen tension, and the liquid volume.

Further, F, G_{in}, H, O^* denote, the feed rate, the glucose concentration in the feed, the proportionality constant relating dissolved oxygen concentration with dissolved oxygen tension, and the dissolved oxygen tension in equilibrium with the oxygen in gas bubbles.

Kinetic expressions The glucose uptake, q_g, is taken to be of Monod type

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

which describes a smoothly saturating glucose uptake. Part of the glucose uptake is assumed to be used for maintenance purposes. This is here modeled by a constant flow q_m.

The relation between specific oxygen uptake rate q_o and q_g can be formulated as

$$q_o(G) = \begin{cases} (q_g - q_m)Y_{og} + q_m Y_{om}, & q_g < q_g^{crit} \\ q_o^{max}, & q_g \geq q_g^{crit} \end{cases}$$

with the yield coefficients for growth Y_{og} and maintenance Y_{om}.

Similarly, the specific growth rate, μ, is described as

$$\mu(G) = \begin{cases} (q_g - q_m)Y_x^{ox}, & q_g < q_g^{crit} \\ (q_g^{crit} - q_m)Y_x^{ox} + (q_g - q_g^{crit})Y_x^{fe}, & q_g \geq q_g^{crit} \end{cases}$$

Due to the production of acetate, the biomass yield from the glucose that is fermented, Y_x^{fe}, is believed to be somewhat lower than the oxidative yield Y_x^{ox}.

Oxygen transfer To cover a broad range of stirrer speeds, the volumetric transfer coefficient, $K_L a$, is often taken to be proportional to the square or the cube of the stirrer speed, N. However, in the working range of the reactor, $K_L a$ is well approximated by a linear function of the stirrer speed

$$K_L a(N) = \alpha \cdot (N - N_0)$$

q_g^{max}	1.0 g/(gh)	Y_x^{fe}	0.2 g/g
k_s	10 mg/l	G_{in}	600 g/l
q_m	0.06 g/(gh)	H	14000 (l%)/g
q_g^{crit}	0.53 g/(gh)	O^*	100 %
Y_{og}	0.5 g/g	α	1.4 1/(h · rpm)
Y_{om}	1.07 g/g	N_0	150 rpm
Y_x^{ox}	0.5 g/g	T_p	50 s

Table 1 Default parameter values in the model.

Dissolved oxygen sensor Dissolved oxygen tension is measured with a dissolved oxygen probe which adds significant dynamics to the system. The probe is modeled as a first-order system with time constant T_p

$$T_p \frac{dO_p}{dt} + O_p = O \tag{1}$$

which is reasonable under well-mixed conditions [Dang *et al.*, 1977].

Parameter values The parameter values were adjusted using experimental data, provided by Pharmacia & Upjohn, from cultivations of inducible *E. coli*. Default values are shown in Table 1. Note that the values for q_g^{crit} and Y_x^{fe} could not be estimated from the process data. A comparison between simulated model output and experimental data when pulses are given in the feed rate are shown in Figure 2. The offset is due to the approximation in $K_L a$.

Linearized Model

Over shorter periods of time, V and X are approximately constant. The dynamics for deviations in glucose and oxygen may then be approximated with the linear equations

$$T_g \frac{d\Delta G}{dt} + \Delta G = K_g \Delta F \tag{2}$$

$$T_o \frac{d\Delta O}{dt} + \Delta O = K_o \Delta G + K_n \Delta N \tag{3}$$

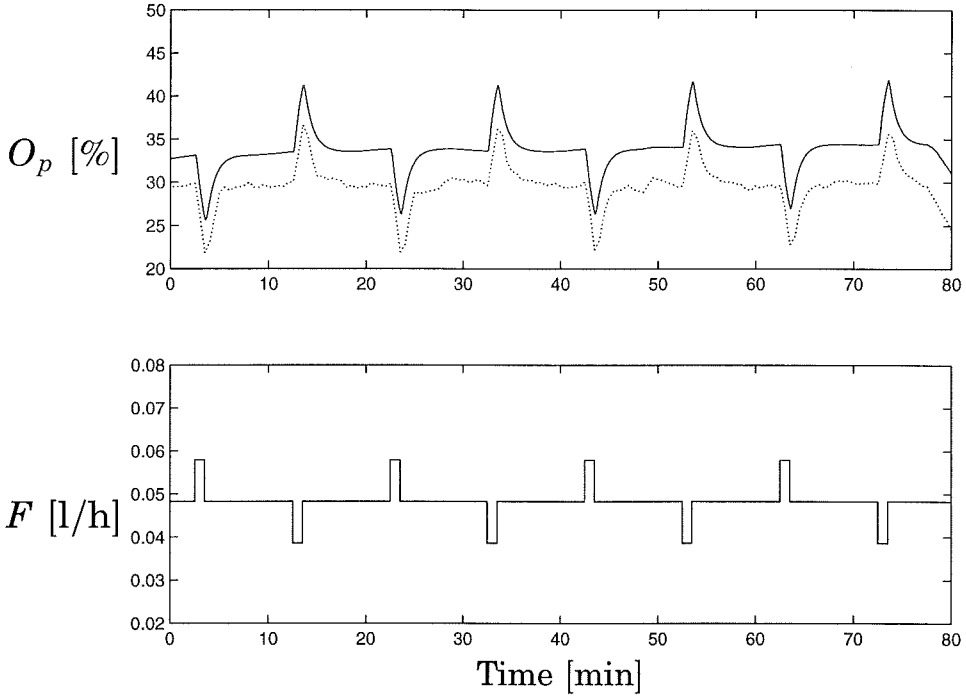

Figure 2 Comparison between model (solid) and experimental data (dotted). The offset is due to the approximation in $K_L a$.

where the gains and the time constants are given by

$$T_g = \left(\frac{\partial q_g}{\partial G} X\right)^{-1}$$

$$T_o = (K_L a)^{-1}$$

$$K_g = \frac{G_{in}}{V}\left(\frac{\partial q_g}{\partial G} X\right)^{-1} = \frac{G_{in} T_g}{V}$$

$$K_o = -Y_{og} H \frac{\partial q_g}{\partial G} X (K_L a)^{-1} = -\frac{Y_{og} H T_o}{T_g}$$

$$K_n = [O^* - O]\frac{\partial K_L a}{\partial N}(K_L a)^{-1}$$

These parameters vary significantly during a cultivation. Typically, the time constants T_g and T_o decrease with increasing biomass. The expression for K_o is valid as long as the limitation in the oxygen uptake is not reached.

The three first-order linear differential equations (1)–(3) give an approximate description of the relation between F, N and O_p that will be used to derive tuning rules for the feeding strategy.

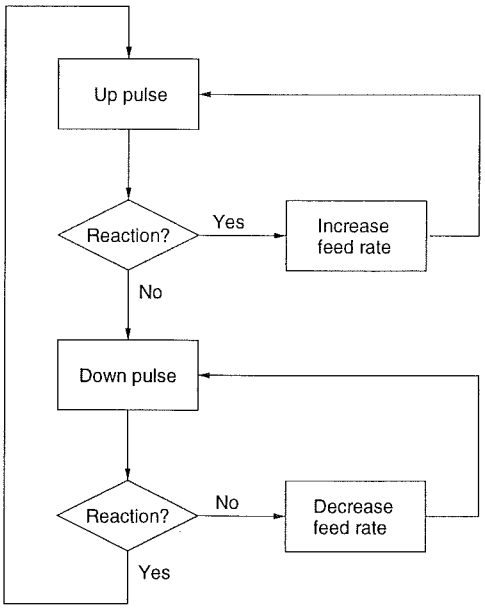

Figure 3 Flow diagram of the control algorithm.

4. Feeding Strategy

The key idea is to superimpose pulses in the feed rate and to evaluate the response in the dissolved oxygen signal. It was discussed how this gives information of acetate production, or rather, indicates if the specific glucose uptake rate q_g is above or below q_g^{crit}. The feed rate is then adjusted to achieve feeding at q_g^{crit}, which is optimal in the sense that it gives the highest possible growth rate without acetate formation. A major advantage with the presented approach is that changes in q_g^{crit} can be handled and that no prior knowledge of its value is required.

Control Algorithm

At each cycle of the algorithm, a pulse is given to obtain information about the current q_g. The feed rate is then adjusted in order to achieve feeding at q_g^{crit}. Pulse generation and feed rate adjustment is governed by a set of rules that can be described by the flow diagram in Figure 3. A reaction to an up pulse is said to occur if O_p is below a level O_{low}. Similarly, a reaction to a down pulse is defined from O_p exceeding O_{high}. Between the pulses, O_p is regulated by a PID controller manipulating the stirrer speed. This ensures that O_p is at the same level, O_{sp}, at the start of each pulse. Moreover, this will avoid that the cell

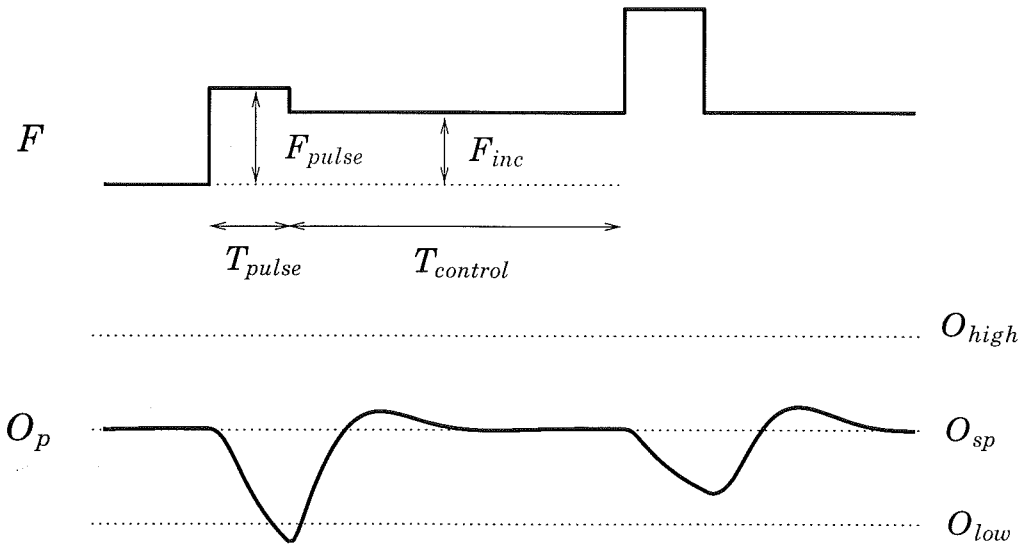

Figure 4 Example of a cycle in the algorithm.

growth becomes limited by oxygen.

An example of a cycle in the algorithm is shown in Figure 4 where also the algorithm parameters are defined. Guidelines for how to select these will be given below.

Tuning

For a feeding strategy to be successful in practice, it is important to have good and simple rules for tuning of its parameters. The linearized model will now be used to derive such guidelines.

It is assumed that a well-tuned PID controller for the stirrer is used. The only process parameter required is an upper bound for the overall time constant in the relation between the feed rate and the dissolved oxygen measurement. An estimate can be obtained from the sum of the following quantities:

- The time constant of the dissolved oxygen probe T_p, which is straightforward to determine.
- An upper bound for the oxygen time constant $T_o^{max} = 1/K_L a^{min}$. This parameter depends mainly on the reactor and the medium and can thus be estimated beforehand. However, variations due

to anti-foam addition and changes in medium composition may be significant.

- An upper bound for the glucose time constant around q_g^{crit}, T_g^{max}. This parameter depends on the cell properties. The worst case occurs at low cell concentrations and a default value of $T_g^{max} = 30$ seconds can be used for most *Escherichia coli* strains.

Pulse length The pulse length, T_{pulse}, is chosen such that the oxygen response to a feed pulse is seen clearly below the limitation in the oxygen uptake. It should thus be longer than the process time constants T_o, T_g and T_p. This also reduces the effects of the time-varying process characteristics. However, a longer pulse length also gives longer time between pulses which could affect the control performance negatively. A straightforward choice is

$$T_{pulse} = T_o^{max} + T_g^{max} + T_p \tag{4}$$

Typically, T_{pulse} will be in the order of 1 minute.

Settling time The time interval $T_{control}$ must be long enough for O_p to return to the setpoint O_{sp} before a new pulse is given. Thus, $T_{control}$ must be chosen longer than the settling time of the dissolved oxygen/stirrer loop. Times in the order of 5 minutes are reasonable using PID control. The motivation below for the choice of F_{inc} also assumes $T_{control} > T_{pulse}$.

Reaction levels The reaction amplitude $O_{reac} = O_{high} - O_{sp} = O_{sp} - O_{low}$, should be chosen large enough for the algorithm to be unaffected by the background variability in O_p. On the other hand, larger reaction amplitudes requires larger pulse heights, which may be undesirable. With a good dissolved oxygen measurement and a well-tuned PID-controller a default choice of

$$O_{reac} = 3\% \tag{5}$$

is a reasonable compromise.

Pulse height The pulse height, F_{pulse}, must give an oxygen response that exceeds the reaction levels. In steady state, the gain from variations in feed rate to variations in measured dissolved oxygen is given by

$$K = K_g K_o = -\frac{Y_{og} H G_{in}}{K_L a V} = \\ = -\frac{O^* - O}{F\left(1 + \frac{q_m}{q_g}\frac{Y_{om} - Y_{og}}{Y_{og}}\right)} \approx -\frac{O^* - O}{F}$$

In stationarity, the amplitude of the oxygen response away from O_{sp} would be

$$O_{pulse} = |K| \cdot F_{pulse} \approx \frac{O^* - O_{sp}}{F} F_{pulse}$$

Accounting for the finite pulse time, a better estimate can be obtained as

$$O_{pulse} \approx 0.63 \frac{O^* - O_{sp}}{F} F_{pulse}$$

The process dynamics has here been approximated by a lumped time constant equal to the sum of the three process time constants. It is then used that T_{pulse} is chosen as the worst-case estimate of this time constant.

To make O_{pulse} clearly larger than the reaction level, we take

$$F_{pulse} = \kappa_p \frac{O_{reac}}{O^* - O_{sp}} F \\ \kappa_p > \frac{1}{0.63} = 1.59 \tag{6}$$

A default value around $\kappa_p = 2.5$ is suggested.

Feed increments The size of the feed increments, F_{inc}, can be thought of as the "controller gain". Stability, in the sense that the

controller does not oscillate between increments and decrements, can be shown to hold if

$$F_{inc} < F_{pulse}$$

This is equivalent to

$$F_{inc} = \kappa_i \frac{O_{reac}}{O^* - O_{sp}} F \qquad (7)$$
$$\kappa_i < \kappa_p$$

In practice, the probability for oscillations is low even if κ_i is slightly larger than κ_p. Increasing κ_i admits the controller to react faster on changes and disturbances. It may thus be used as a tuning knob, trading off speed versus stability. The recommended default value is $\kappa_i = \kappa_p$.

Note also that the maximum specific growth rate that the feed algorithm can give is bounded by

$$\mu_{lim} = \frac{\kappa_i O_{reac}}{(O^* - O_{sp})(T_{pulse} + T_{control})}$$

For the process used in the simulations, the default choice of the control parameters give $\mu_{lim} \approx 1.0$ 1/h, well above the estimated μ_{max}.

5. Results

The performance of the presented strategy will now be exemplified. The algorithm is simulated together with the full model. Parameters in the algorithm are chosen according to the guidelines in the previous section which yields: $T_{pulse} = 1.4$ min, $T_{control} = 5.5$ min, $O_{reac} = 3\%$, $\kappa_p = \kappa_i = 2.5$.

In the first example, see Figure 5, the chosen initial feed rate after a batch phase is too low. When the feed is started, the algorithm increases the feed rate until q_g^{crit} is reached. After the initial transient, the algorithm keeps q_g approximately constant at q_g^{crit}. After two hours, the value on q_g^{crit} is lowered but the algorithm detects the

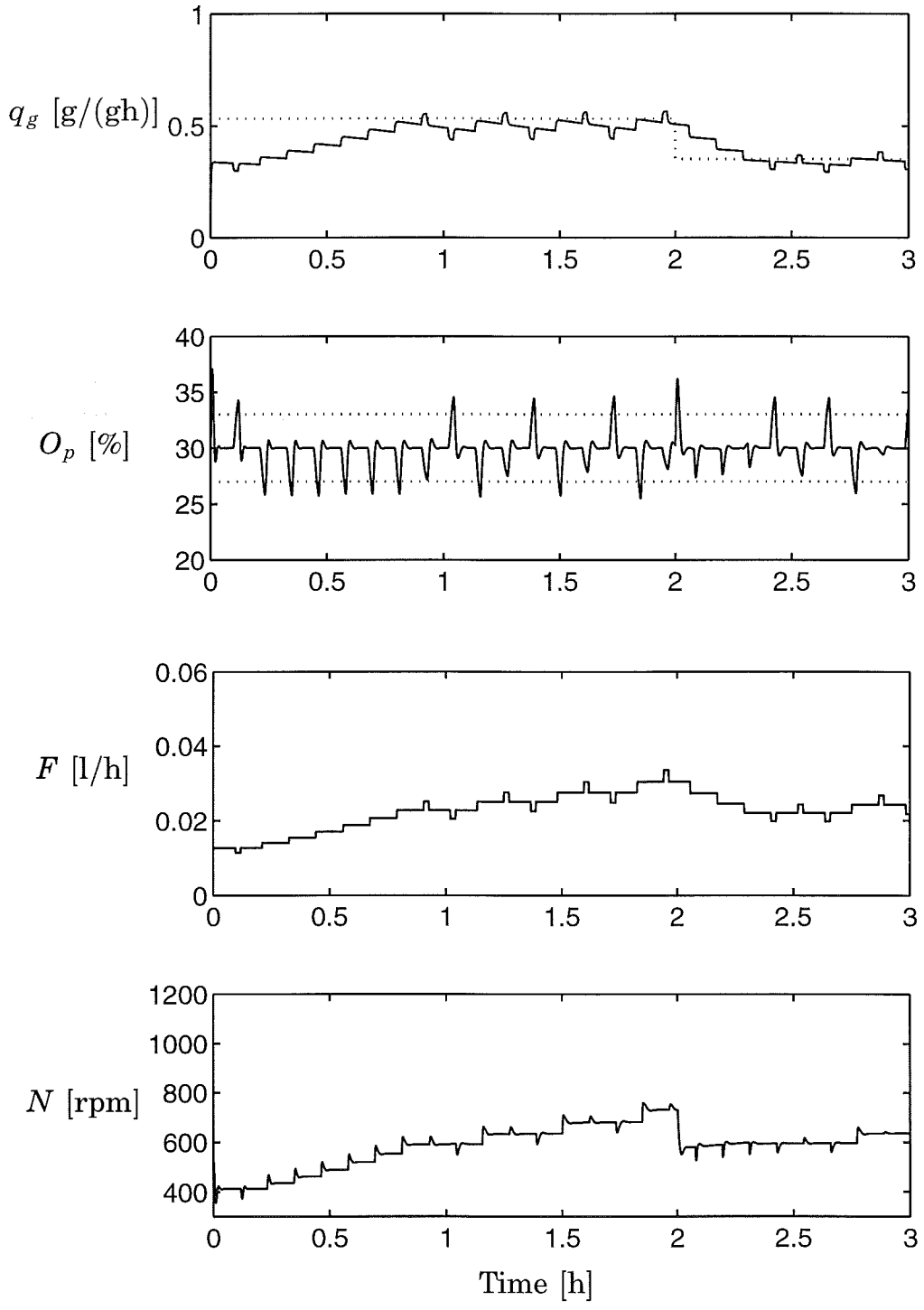

Figure 5 Simulation where the initial feed rate is chosen too low. After two hours q_g^{crit} is changed. The dotted lines indicate the critical glucose uptake, q_g^{crit}, and the reaction levels, O_{high}, O_{low}.

change and adjust the feed accordingly. This illustrates the ability to track changes in the culture.

The second example shown in Figure 6 demonstrates the robustness of the strategy. The same tuning is used but the model parameters are now changed such that the assumptions behind the tuning rules are violated. Choosing $q_g^{crit} = 0.73$ g/(gh), $k_s = 0.015$ g/l and $Y_x^{ox} = 0.4$ g/g, makes T_g^{max} underestimated and the amplitude of the oxygen responses are lower than expected. As a result, the algorithm misinterprets this as a saturated oxygen uptake, and q_g tends to deviate more from q_g^{crit} than in the previous example. The increased time for the initial transient is due to the increased value of q_g^{crit}. The performance is clearly acceptable even though the underlying assumptions are violated.

6. Discussion

It has been shown how the use of a standard measurement can be extended. By superimposing pulses in the feed rate, indirect information of acetate formation can be obtained from the dissolved oxygen signal. This was used for feedback adjustments of the feed rate.

Feeding at the critical glucose uptake without prior information of its value could be attained by the proposed strategy. This could be achieved even when the critical glucose uptake was changed. Tuning of the algorithm is based on an upper bound for the overall time constant in the relation between the feed rate and the dissolved oxygen measurement. This requires only a minimum of process specific information, which makes the strategy well suited for new and unknown processes. When more knowledge about the process is available, the performance may be improved by combining the strategy with a predetermined profile.

To achieve good performance, it is important to have short times between consecutive pulses. The performance of the dissolved oxygen control can then be a limiting factor. Disturbances in the dissolved oxygen concentration, for instance from anti-foam addition, may cause problems if a fixed time between pulses is used. A safety net checking that the dissolved oxygen is at the desired setpoint before a new pulse is given should be included. When the maximum stirrer speed is

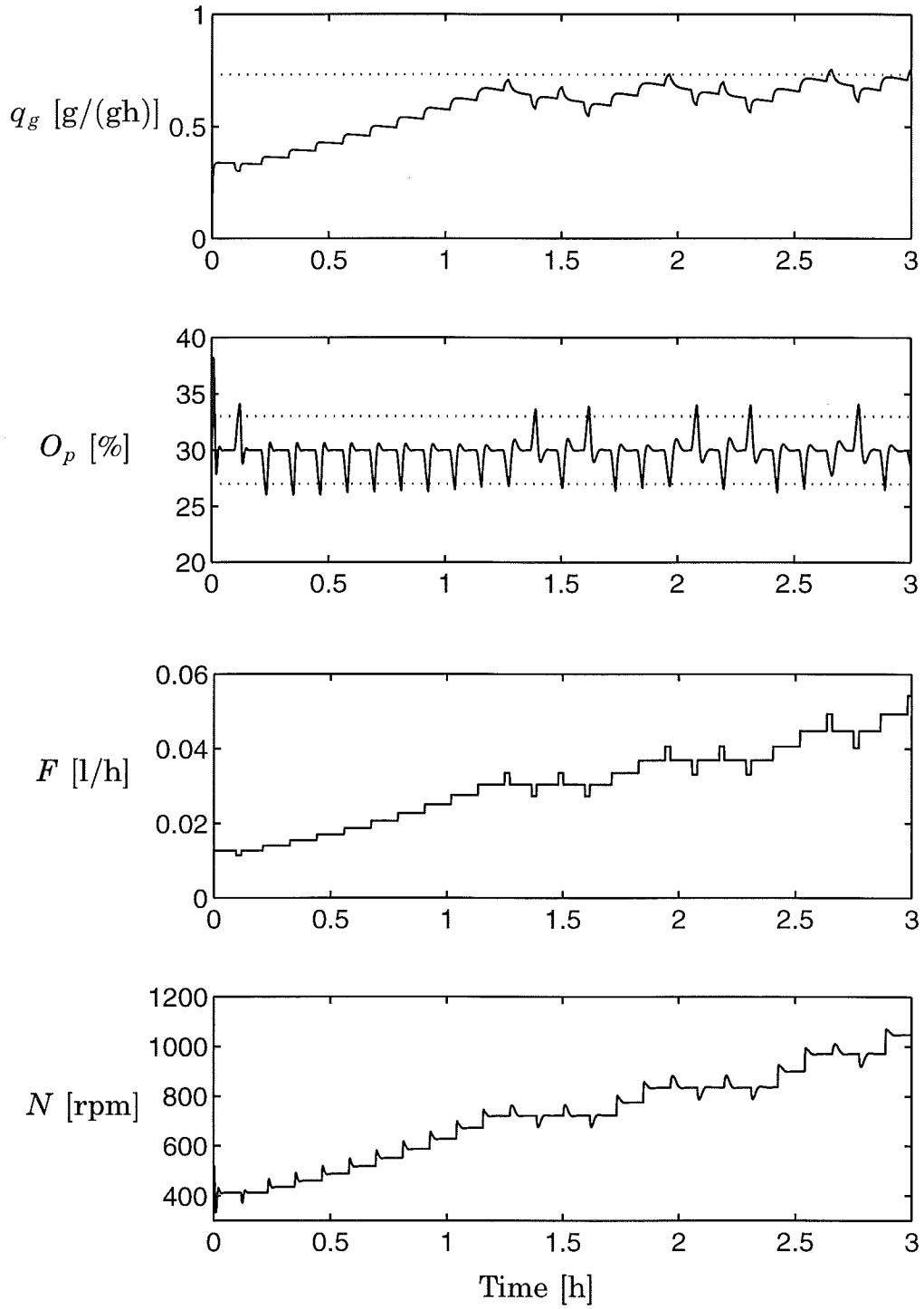

Figure 6 Simulation with changed model parameters such that the assumptions behind the tuning rules are violated. The initial feed rate is chosen too low. The dotted lines indicate the critical glucose uptake, q_g^{crit}, and the reaction levels, O_{high}, O_{low}.

reached, it is no longer possible to keep the desired setpoint in the dissolved oxygen. It would then be necessary to switch to another strategy, for instance the one in [Riesenberg *et al.*, 1990].

So far, only the production of acetate has been considered. If acetate has accumulated, for instance after an initial batch phase, effects from its consumption may affect the oxygen responses. This should be further investigated.

Acknowledgments

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Nomenclature

F	glucose feed rate [l/h]
F_{pulse}, F_{inc}	pulse height and increments in control algorithm [l/h]
G, G_{in}	glucose concentration in reactor and feed medium [g/l]
H	constant [(l%)/g]
K_g, K_n, K_o	gains in linearized process model [(gh)/l^2], [%/rpm], [(l%)/g]
K_La	volumetric oxygen transfer coefficient [1/h]
k_s	Monod saturation constant [g/l]
N	stirrer speed [rpm]
N_0	constant [rpm]
O, O_p	real and measured dissolved oxygen tension [%]
O_{high}, O_{low}, O_{reac}	reaction levels and reaction amplitude in control algorithm [%]
O_{sp}	setpoint for dissolved oxygen [%]
O^*	dissolved oxygen tension in equilibrium with air bubbles [%]
q_{ac}	specific acetate production rate [g/(gh)]
q_g, q_g^{max}	specific and maximum specific glucose uptake rate [g/(gh)]
q_g^{crit}	critical specific glucose uptake rate [g/(gh)]
q_m	specific glucose consumption rate for maintenance [g/(gh)]
q_o, q_o^{max}	specific and maximum specific oxygen uptake rate [g/(gh)]

T_g, T_o	time constants in linearized process model [h]
T_p	time constant for dissolved oxygen probe [h]
T_{pulse}, $T_{control}$	time intervals in control algorithm [h]
V	liquid volume [l]
X	biomass concentration [g/l]
Y_{og}, Y_{om}	growth and maintenance oxygen yield [g/g]
Y_x^{ox}, Y_x^{fe}	oxidative and fermentative biomass yield [g/g]
α	constant [1/(h · rpm)]
κ_p, κ_i	parameters in control algorithm [1]
μ, μ_{max}	specific and maximum specific growth rate [1/h]
μ_{lim}	maximum specific growth rate in control algorithm [1/h]

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Paper II

Acetate Formation and Dissolved Oxygen Responses to Feed Transients in *Escherichia coli* Cultivations

Mats Åkesson, Eva Nordberg Karlsson, Per Hagander, Jan Peter Axelsson, and Anita Tocaj

Abstract

Recombinant protein production in *Escherichia coli* can be significantly reduced by acetate accumulation. It is demonstrated how on-line detection of acetate production can be made with a standard dissolved oxygen sensor by superimposing short pulses to the substrate feed rate. Two hypotheses for acetate formation in *E. coli* are used to obtain simplified relations between glucose uptake, oxygen uptake and acetate production. At the onset of acetate formation, both hypotheses predict a "saturation" in the specific oxygen consumption rate. This is in agreement with experimental observations previously made in continuous and batch cultivations. Assuming that the relations are valid also under transient conditions, models for dissolved oxygen responses to transients in substrate feed rate are derived. The models predict a clear change in the character of the transient response when acetate formation starts. The predicted effects were verified in fed-batch cultivations of *E. coli* TOPP1 and *E. coli* BL21(DE3), both before and after induction of recombinant protein production.

1. Introduction

Escherichia coli is one of the most frequently used host organisms for recombinant protein production. Fed-batch cultivations is a common method to obtain high cell densities and thereby high productivity. One of the problems encountered, is the formation of by-products such as acetate. Accumulation of acetate has been reported to inhibit growth, [Luli and Strohl, 1990], and to reduce recombinant protein production, [Bech Jensen and Carlsen, 1990]. To reduce or avoid acetate formation, a number of substrate feeding strategies have been developed, see [Lee, 1996; Yee and Blanch, 1992].

A typical problem in monitoring and control of microbial cultivations is that many important process variables cannot be measured on-line. This has triggered much research and development concerning new sensors, see for instance [Schügerl *et al.*, 1996]. Another way of addressing the problem is to improve and to extend the use of existing sensors [Wang *et al.*, 1977; Stephanopoulos and San, 1984]. In this paper we will demonstrate how a standard dissolved oxygen sensor can be used for on-line detection of undesirable acetate formation. The key idea is to exploit a characteristic change in the relation between oxygen uptake and glucose uptake at the onset of acetate formation. This change can be detected by superimposing short pulses in the glucose feed rate. An attractive feature is that no assumptions on stoichiometric coefficients or model parameters are required.

2. Acetate Production

Formation of acetate, when *E. coli* is grown under fully aerobic conditions, typically occurs at high growth rates and/or glucose uptake rates. The acetate production is thought of as an overflow phenomenon where flux of AcetylCoA is directed to acetate, via acetylphosphate, instead of entering the TCA-cycle. In batch and continuous cultivations, it was observed that the specific oxygen uptake rate reached an apparent maximum at the onset of acetate formation [Andersen and von Meyenburg, 1980; Reiling *et al.*, 1985]. It was suggested that the respiratory system, where NADH is reoxidized, has a limited capacity. As flux to the TCA-cycle results in more NADH production than does flux

to acetate, redirection of AcetylCoA flux to acetate would be necessary to avoid accumulation of NADH when the respiration saturates. Another explanation that has been suggested is that the TCA-cycle has a limited capacity, and that this limitation is reached before that of the respiration, [Majewski and Domach, 1990]. When the TCA-cycle saturates, increasing glucose uptake will again result in flux from AcetylCoA to acetate. However, in this case, NADH production and respiration can increase further until the maximum respiration capacity or the maximum glucose uptake is reached.

In [Majewski and Domach, 1990], a flux network over parts of the central metabolic pathways was used to derive relations between triose flux and acetate production for the two explanations mentioned above. Assuming that the cells tend to maximize ATP production, a constrained optimization problem was formulated. Acetate production was predicted when constraints in the respiration or the TCA-cycle were reached. These ideas were extended in [Ko *et al.*, 1993; Ko *et al.*, 1994] and [Varma *et al.*, 1993] where the models also include acetate formation due to oxygen limitation.

3. Simulation Model

We will now derive two models for how dissolved oxygen in a bioreactor responds to transients in the feed rate. First, considerations on the cell level are used to derive relations between glucose uptake, acetate production and oxygen consumption. These relations are then incorporated in a macroscopic model of a bioreactor.

Metabolic Relations

From the analysis in [Majewski and Domach, 1990], it is straightforward to compute also the corresponding NADH fluxes for the two hypotheses. Assuming that the oxygen consumption is proportional to the NADH production and that the glucose flux is proportional to the triose flux, relations between glucose uptake, acetate production and oxygen consumption can be obtained. Qualitative results are shown in Figure 1. When the glucose uptake, q_g, exceeds a critical level, q_g^{crit}, acetate formation starts. For both the respiratory and the TCA-cycle

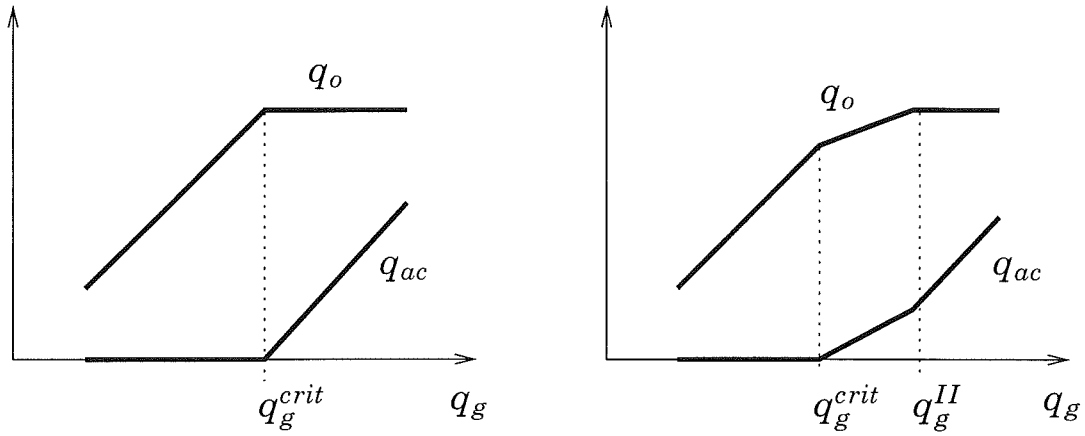

Figure 1 Relations between specific glucose uptake, q_g, specific oxygen uptake, q_o, and specific acetate production, q_{ac}, in the case of respiratory limitation (left) and TCA-cycle + respiratory limitation (right).

limitation, a characteristic change in the relation between q_g and q_o is predicted. The main difference between the hypotheses is that the TCA-cycle limitation predicts an increase in q_o even after onset of acetate production. The oxygen uptake q_o continues to increase until the glucose uptake exceeds a second level q_g^{II} where the respiratory limitation is reached. This gives a smoother transition to the apparent maximum in the specific oxygen uptake rate.

For the case of a respiratory limitation, the specific oxygen uptake, q_o, can be described mathematically as

$$q_o(G) = \begin{cases} q_g Y_{og}, & q_g < q_g^{crit} \\ q_g^{crit} Y_{og}, & q_g \geq q_g^{crit} \end{cases}$$

with the yield constant Y_{og}. When the TCA-cycle saturates before the respiration, three regions have to be treated; no saturation, TCA-cycle saturation, and respiratory limitation. This can be written as

$$q_o(G) = \begin{cases} q_g Y_{og}, & q_g < q_g^{crit} \\ q_g^{crit} Y_{og} + (q_g - q_g^{crit}) Y_{og}^{II}, & q_g^{crit} < q_g \leq q_g^{II} \\ q_g^{crit} Y_{og} + (q_g^{II} - q_g^{crit}) Y_{og}^{II}, & q_g \geq q_g^{II} \end{cases}$$

where Y_{og}^{II} is the incremental oxygen/glucose yield during the TCA-cycle saturation.

Glucose Uptake

The specific glucose uptake rate, q_g, is taken to be of Monod type

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

which describes a smoothly saturating glucose uptake. However, the findings in [Neubauer *et al.*, 1995] may indicate that the relation between glucose concentration and uptake is not static.

Bioreactor Model

Assuming that the expressions for oxygen uptake and glucose uptake are valid under transient conditions, these are inserted in a dynamic model of a bioreactor. During the time scale of interest, about 10 minutes, cell concentration and volume are considered to be constant.

Mass balances for glucose and oxygen then give the following equations

$$\frac{d(VG)}{dt} = FG_{in} - q_g(G) \cdot VX$$
$$\frac{d(VO)}{dt} = K_L a(N) \cdot V(O^* - O) - q_o(G) \cdot HVX$$

where G, X, O, and V are, respectively, the glucose concentration, the cell concentration, the dissolved oxygen tension, and the liquid volume. Further, F, G_{in}, H, and O^* denote, the feed rate, the glucose concentration in the feed, the proportionality constant relating dissolved oxygen concentration with dissolved oxygen tension, and the dissolved oxygen tension in equilibrium with the oxygen in gas bubbles. The volumetric oxygen transfer coefficient, $K_L a$, is approximated as an increasing linear function of the stirrer speed, N.

The dissolved oxygen tension is measured with a dissolved oxygen probe. The probe is modeled as a first-order system with time constant T_p.

$$T_p \frac{dO_p}{dt} + O_p = O$$

which is reasonable under well-mixed conditions [Dang *et al.*, 1977].

Parameter	Value	Parameter	Value
q_g^{max}	1.34 g/(gh)	Y_{og}	0.5 g/g
k_s	10 mg/l	Y_{og}^{II}	0.25 g/g
q_g^{crit}	1.0 g/(gh)	G_{in}	500 g/l
q_g^{II}	1.1 g/(gh)	H	14000 (l%)/g
O^*	100 %	T_p	20 s
$VX(0)$	20 g	$V(0)$	2.0 l

Table 1 Parameter values used in simulations.

4. Simulations

The oxygen response to pulses in the feed rate that is predicted by the two models is now investigated. Model parameter values that are used are found in Table 1 together with initial values for cell mass and volume.

Figures 2, 3 and 4 show simulations for increasing glucose uptakes; below, at, and above the onset of acetate formation. Below acetate formation, a clear response in dissolved oxygen is seen to both up and down pulses in the glucose feed rate F, see Figure 2. When the onset of acetate formation is reached, see Figure 3, the response to an up pulse in F is clearly reduced due to the characteristic knee in the specific oxygen uptake. For a glucose uptake slightly above acetate formation, there is also a reduction in the response to a down pulse, Figure 4. When the glucose uptake is increased even further, both models have a severe limitation in the respiration and no oxygen response will be seen.

Both models predict a change in the oxygen responses at the onset of acetate formation and with the model parameters used, the quantitative differences between the models are small. However, a qualitative difference is that the TCA-limitation model predicts an oxygen response to an up pulse in feed even when acetate is produced, see Figures 3 and 4. If the simulation results are valid, it is clear that it is possible to detect the change in the relation between glucose uptake and oxygen uptake, and hence acetate formation, by making pulses in the glucose feed rate. This will now be examined experimentally.

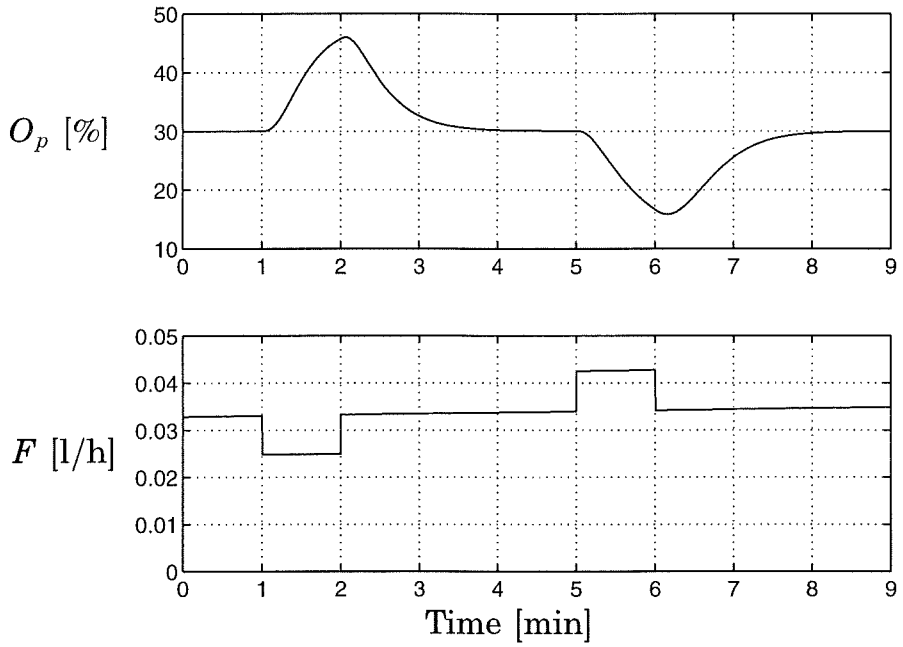

Figure 2 Simulation of response in dissolved oxygen response O_p to pulses in feed rate F *below* onset of acetate formation, $q_{ac} = 0$, $q_g = 0.8$ g/(gh) $< q_g^{crit}$. Respiratory limitation (solid) and TCA-cycle + respiratory limitation (dashed).

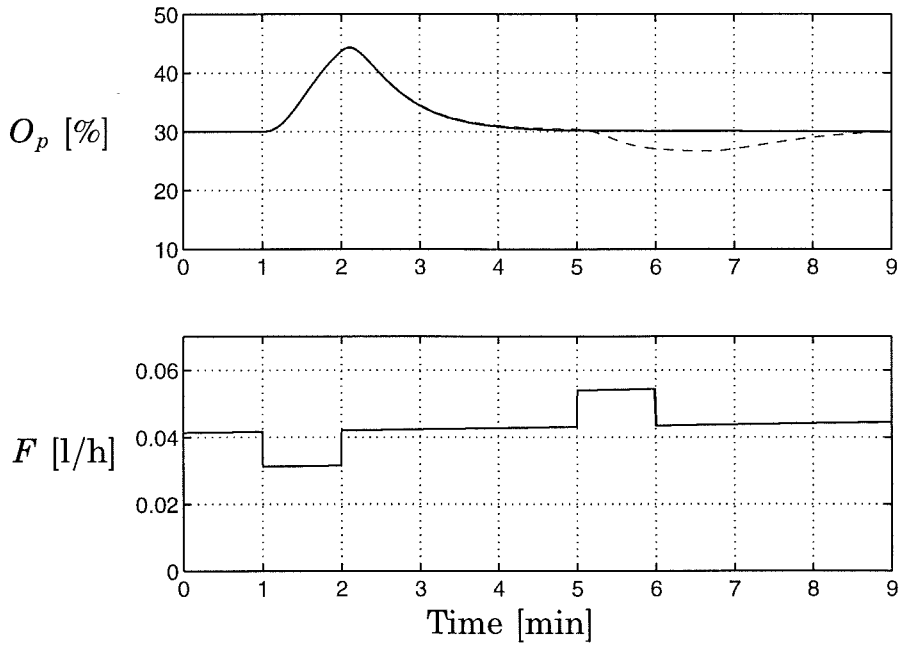

Figure 3 Simulation of response in dissolved oxygen response O_p to pulses in feed rate F *at* the onset of acetate formation, $q_{ac} = 0$, $q_g = 1.0$ g/(gh) $= q_g^{crit}$. Respiratory limitation (solid) and TCA-cycle + respiratory limitation (dashed).

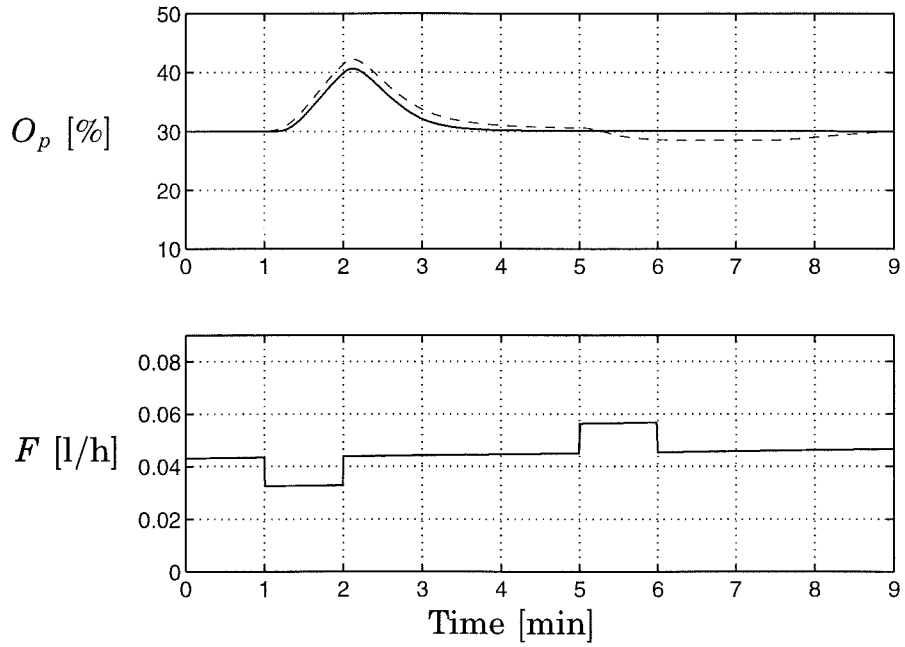

Figure 4 Simulation of response in dissolved oxygen response O_p to pulses in feed rate F just *above* onset of acetate formation, $q_{ac} > 0$, $q_g^{crit} < q_g = 1.05$ g/(gh) $< q_g^{II}$. Respiratory limitation (solid) and TCA-cycle + respiratory limitation (dashed).

5. Materials and Methods

Microorganisms

Two recombinant *E. coli* strains with different plasmids were used. *E. coli* TOPP1 (Stratagene, La Jolla, CA, USA) carrying a plasmid pHD389 with a protein L gene fragment inserted [Tocaj *et al.*, 1995]. During these experiments the recombinant protein was not expressed. *E. coli* BL21(DE3) [Studier and Moffatt, 1986] with plasmid pBRMX14 derived from the vector pET25b (Novagen, Madison, Wis) and encoding a xylanase (Xyn1ΔN) [Nordberg Karlsson *et al.*, 1998].

Bioreactor equipment and cultivation conditions

Media composition The media for the batch and fed-batch cultivations were composed as described in Table 2.

Media component	TOPP1	BL21(DE3)	Feed
Glucose (g/l)	10	10	500
$(NH_4)_2SO_4$ (g/l)	2.0	2.0	-
KH_2PO_4 (g/l)	1.6	-	-
K_2HPO_4 (g/l)	-	14.6	-
$Na_2HPO_4 \cdot (2H_2O)$ (g/l)	6.6	-	-
$NaH_2PO_4 \cdot (H_2O)$ (g/l)	-	3.6	-
$(NH_4)_2$-H-citrate (g/l)	0.5	0.5	-
1M $MgSO_4$ (ml/l)	2.0	2.0	50
Trace element (ml/l)	2.0	2.0	10
Ampicillin (g/l)	0.1	0.1	-
Adekanol (ml/l)	0.05	0.05	-

Table 2 Media composition. The trace element solution used was that of [Holme *et al.*, 1970]. Adekanol (antifoam), was also added when needed (0.1 ml).

Inoculum The inoculum was prepared in 100 ml of the media in Table 2, but without antifoam. It was grown in a baffled shake-flask for 10 h, at 30°C (TOPP1) or 37°C (BL21(DE3)), on a rotary shaker (GFL 1092, Burgwedel, Germany).

Fed-batch cultivations The cultivations were carried out in a 3 l bioreactor (Chemoferm FLC-B-3, Hägersten, Sweden) with an initial volume of 2.0 l. Data logging, dissolved oxygen (DO) control and feed-pump control were implemented using the SattLine control system (Alfa Laval Automation AB, Malmö, Sweden). Temperature was 37°C (BL21(DE3)) or 30°C (TOPP1) and pH was kept at 7.0 by titration with 6.7 M ammonia. DO was measured with a galvanic electrode calibrated to 100% at air saturation at 30°C and 37°C, respectively, in the cultivation media. The DO was kept at 30% by automatic control of the stirrer speed, using a PID controller. Aeration was set to approximately 1 vvm. Oxygen and carbon dioxide content in the exhaust gas stream were recorded by a paramagnetic oxygen analyzer (Series 1100, Servomex) and a CO_2 analyzer (Binos 1.1, Leybold-Heraeus), respectively. The feed vessel was placed on a balance for registration

of feed addition. The feed period was started upon depletion of the initial substrate, indicated by a peak in the DO, and the feed was then added according to exponential feed-rate profiles as described below. Expression of the recombinant protein in BL21(DE3) was initiated by adding IPTG (1 mM).

Feed-rate profile The feed rate was varied to obtain different specific growth rates μ_{set}. In each interval the feed rate was increased exponentially as

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

where t_0 is the start of the interval. The value of $VX(t_0)$ was precalculated from the total amount added glucose assuming that the biomass/glucose yield Y_{xg} was 0.5 g/g. Pulses with a duration of 1 min were superimposed to the nominal feed rate. The pulse amplitude was ± 25 % of the nominal feed rate.

Analyses

Sampling and sample treatment The samples were withdrawn through a septum at the bottom of the fermenter, using evacuated blood collecting tubes, 3 ml Venoject (Terumo, Madrid, Spain). Samples for glucose or metabolite determinations were collected in tubes containing perchloric acid. These samples, used for enzymatic determination of glucose and acetic acid, were centrifuged ($15000 \times g$) to remove the cell fraction. Supernatants were kept frozen (-20°C), and prior to analysis the samples were thawed, heated (80°C, 15 min), and centrifuged ($15000 \times g$).

Optical density (OD) The OD was measured at 620 nm. Samples were diluted with 0.9% NaCl at OD values exceeding 0.5.

Acetic acid Acetic acid concentrations were determined enzymatically using a test kit (no. 148261, Boehringer Mannheim).

Glucose Glucose concentration was determined enzymatically using a test kit (no. 716251, Boehringer Mannheim), according to the analytical procedure described by [Larsson and Törnkvist, 1996].

Xylanase activity Samples were taken at 30 minute intervals during the induction phase. The xylanase activity was determined according to the procedure in [Nordberg Karlsson *et al.*, 1998].

Cell dry weight calculations (cdw) For *E. coli* TOPP1, the cdw was calculated using a standard curve (cdw vs. OD) obtained from several batch cultivations without induction. One OD unit was equal to 0.44 g cdw/l.

For *E. coli* BL21(DE3), the cdw was determined by centrifuging (15000×g) quadruple samples (1 ml) in preweighed Eppendorf tubes. The pellets were washed with 1 ml 0.9% NaCl and dried overnight (105°C). One OD unit was equal to 0.52 g cdw/l.

6. Results

To verify the results from the simulations, cultivations were made with two recombinant strains, *E. coli* TOPP1 and *E. coli* BL21(DE3). Different glucose uptake rates were obtained by changing the feed rate between different exponential profiles. In this way, periods with and without acetate production could be achieved. For each exponential feeding period, pulse experiments were performed at well-controlled oxygen levels. During the pulse experiments the PID controller was set in manual mode and the stirrer speed was kept constant. This gave a decreasing trend in the dissolved oxygen as F was increasing exponentially. In the experiments with BL21(DE3) this effect could be reduced by increasing the stirrer speed in accordance with the feed rate. To facilitate the comparison with the simulations, data where linear trends have been removed are shown together with the original data.

E. coli TOPP1

The fed-batch part from a cultivation of *E. coli* TOPP1 is shown in Figure 5. Acetate produced in the initial batch phase was consumed during the first part of the fed-batch phase. The glucose concentration was kept below 50 mg/l except during the period with the highest μ_{set} when it temporarily increased to about 350 mg/l.

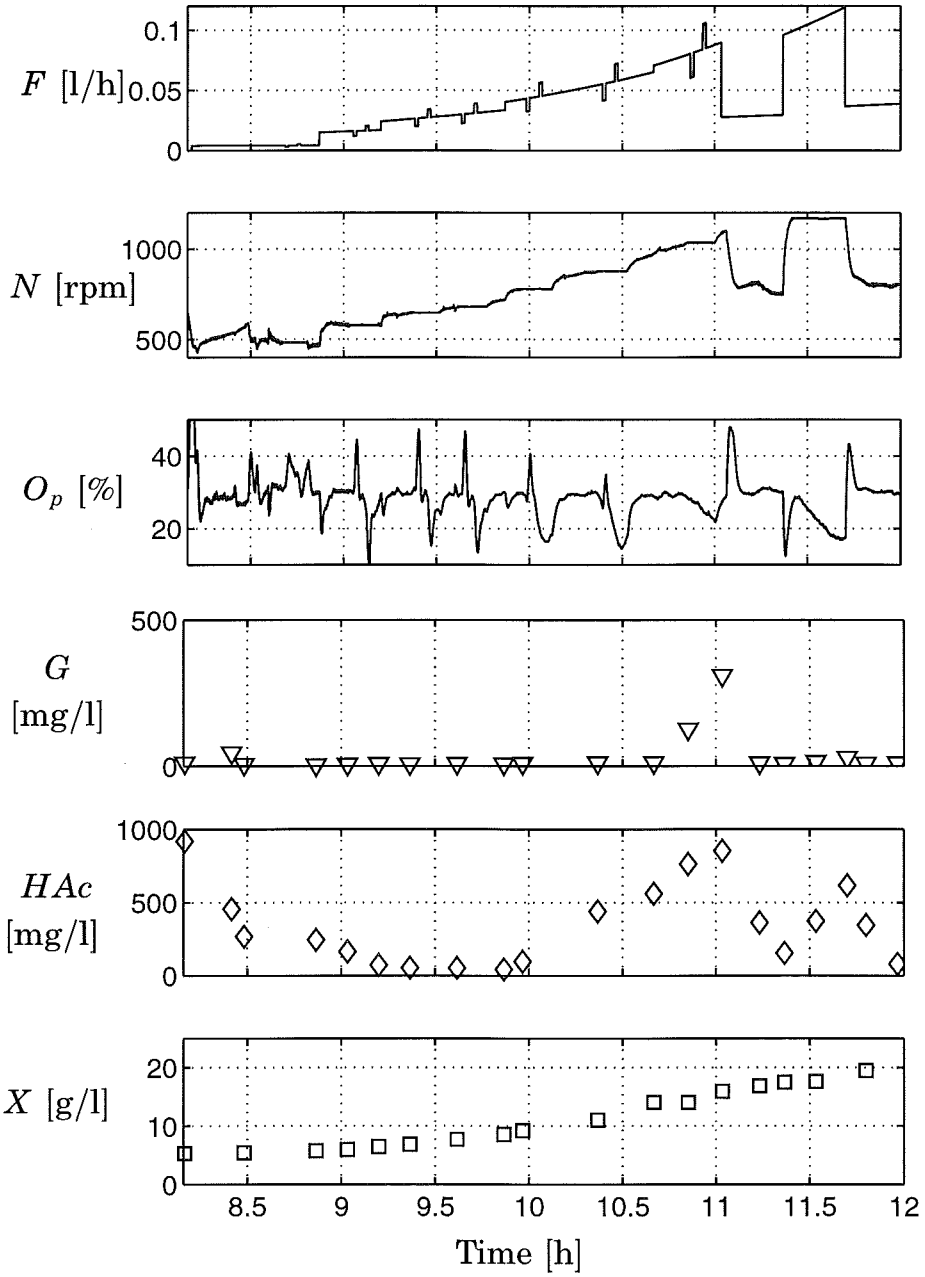

Figure 5 Fed-batch part from an experiment with *E. coli* TOPP1. From top to bottom: glucose feed rate F, stirrer speed N, dissolved oxygen tension O_p, glucose concentration G, acetate concentration HAc, and cell concentration X.

Pulse experiments A pulse experiment for a moderate glucose uptake rate, $q_g \approx 0.66$ g/(gh), is shown in Figure 6. No acetate production could be detected and the glucose concentration was kept low. A clear response in dissolved oxygen is seen to both the up and the down pulse in the feed rate.

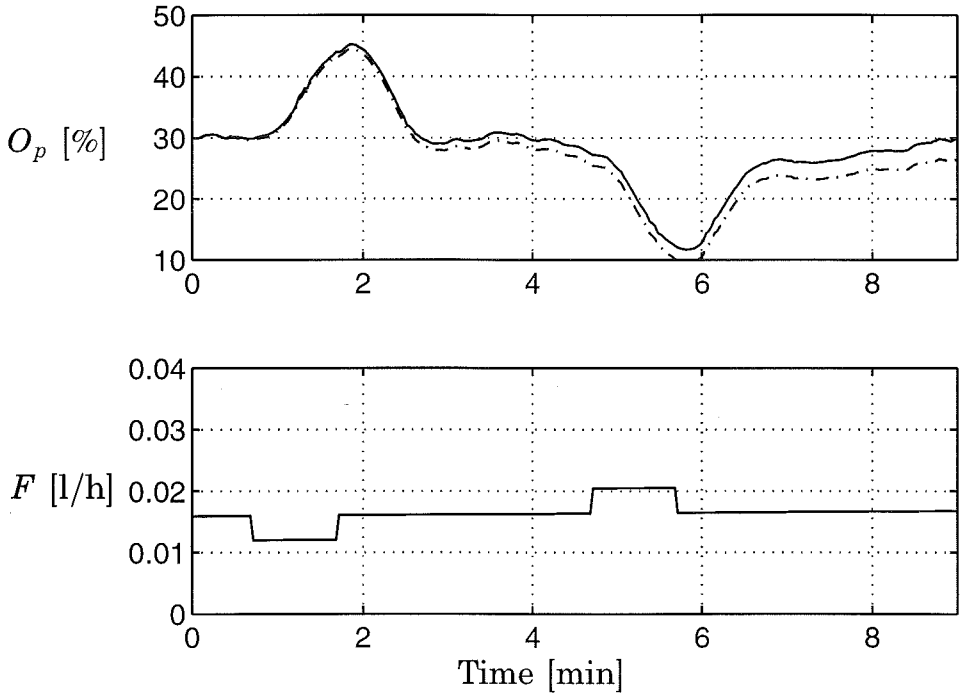

Figure 6 Experiment with *E. coli* TOPP1. Response in dissolved oxygen O_p to pulses in feed rate F. No acetate formation, $q_g \approx 0.66$ g/(gh). $X \approx 6$ g/l. Detrended (solid) and original data (dash-dotted).

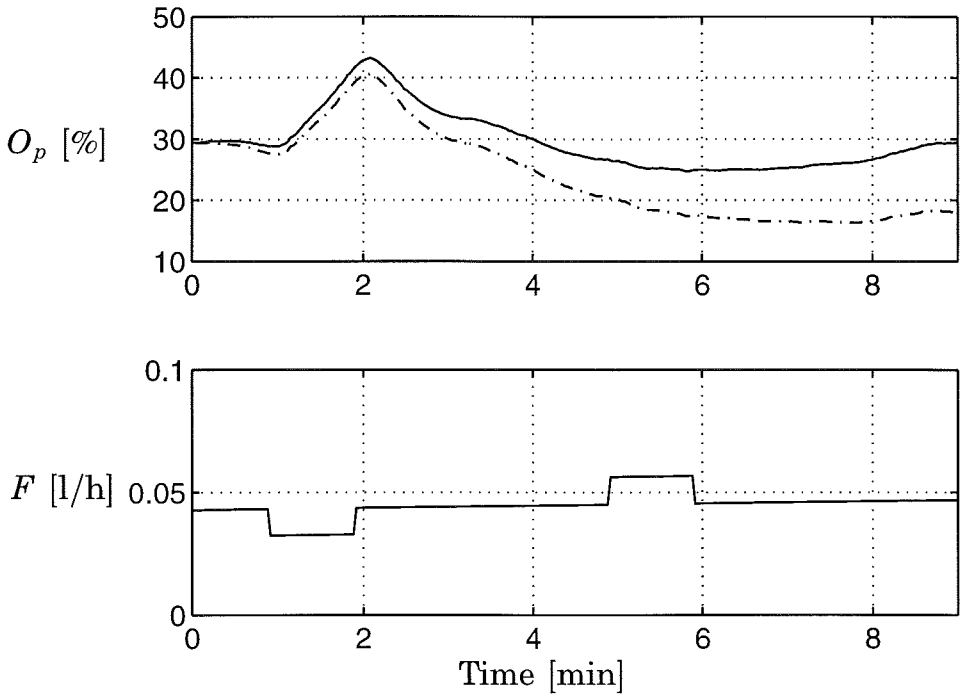

Figure 7 Experiment with *E. coli* TOPP1. Response in dissolved oxygen O_p to pulses in feed rate F during acetate formation. $q_g \approx 1.26$ g/(gh). $X \approx 9$ g/l. Detrended (solid) and original data (dash-dotted).

Figure 7 shows an experiment made during acetate production. The specific glucose uptake rate is rather high, $q_g \approx 1.26$ g/(gh), but the glucose concentration was still low. As was predicted by the simulations, the response to the down pulse is now slightly reduced and no reaction is seen to the up pulse started at 5 minutes.

E. coli BL21(DE3)

The fed-batch part of an experiment with *E. coli* BL21(DE3) is shown in Figure 8. The acetate concentration after the batch phase was negligible and the concentrations of acetate as well as glucose were low throughout the fed-batch phase. Before the induction phase, acetate production could be detected for a specific glucose uptake rate around 1.22 g/(gh).

Prior to induction μ_{set} was decreased to 0.25 h^{-1}. Expression of the foreign protein was induced after 8.6 h and a reduction of the specific growth rate down to 0.11 h^{-1} was observed, see Figure 9. While μ_{set} was still kept at 0.25 h^{-1}, the specific glucose uptake rate q_g increased from 0.50 g/(gh) to 0.63 g/(gh). During the same period, it was also observed that the ratios between oxygen uptake and carbon dioxide production to the glucose uptake q_o/q_g and q_{co}/q_g increased. At approximately 11 h, a fourfold increase in the acetate concentration was detected indicating that acetate had been produced. In the final part of the cultivation, μ_{set} was set to 0.15 h^{-1}. The xylanase activity obtained, see Figure 8, shows that a high production level was achieved.

Pulse experiments Pre-induction pulse experiments gave responses similar to those obtained with TOPP1. Three consecutive pulse experiments after induction, starting at 9.5 h, are shown in Figures 10, 11 and 12. In the first experiment, see Figure 10, q_g was about 0.54 g/(gh) and no acetate production could be observed. Clear responses to both up and down pulses were obtained. When q_g increased to 0.59 g/(gh), the response to an up pulse was broadened and the amplitude was somewhat reduced, Figure 11. Still no accumulation of acetate was detected. Figure 12 shows the third experiment where q_g was approximately 0.63 g/(gh). Almost no response to an up pulse resulted and shortly after this experiment, an increased acetate concentration was detected.

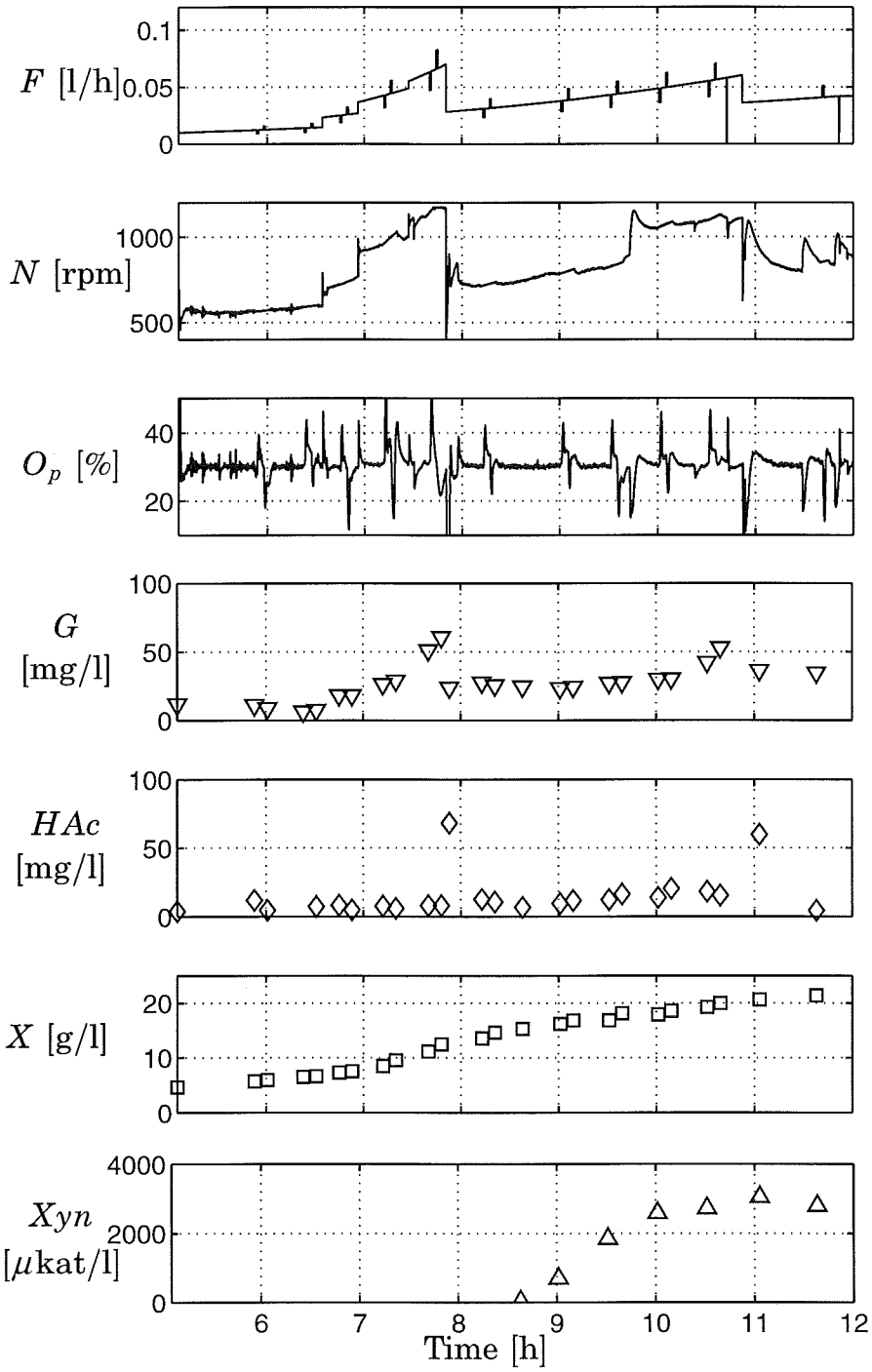

Figure 8 Example of fed-batch part of an experiment with *E. coli* BL21(DE3). From top to bottom: glucose feed rate F, stirrer speed N, dissolved oxygen tension O_p, glucose concentration G, acetate concentration HAc, cell concentration X, and recombinant protein detected as xylanase activity Xyn.

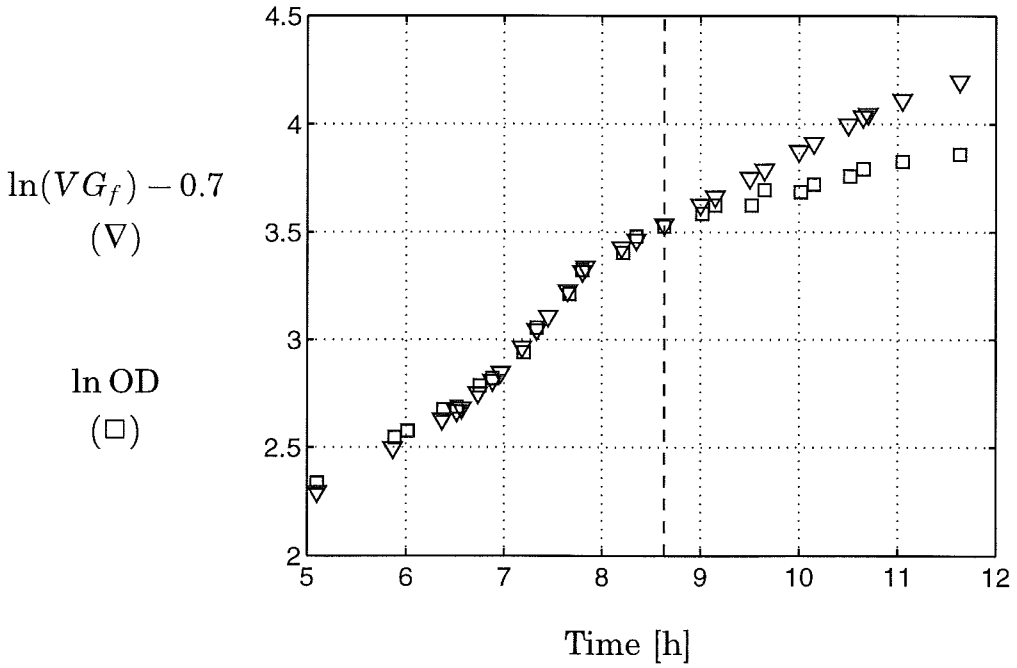

Figure 9 Logarithms of total amount fed glucose VG_f (∇) and optical density (□). Time for induction is indicated with the dashed line.

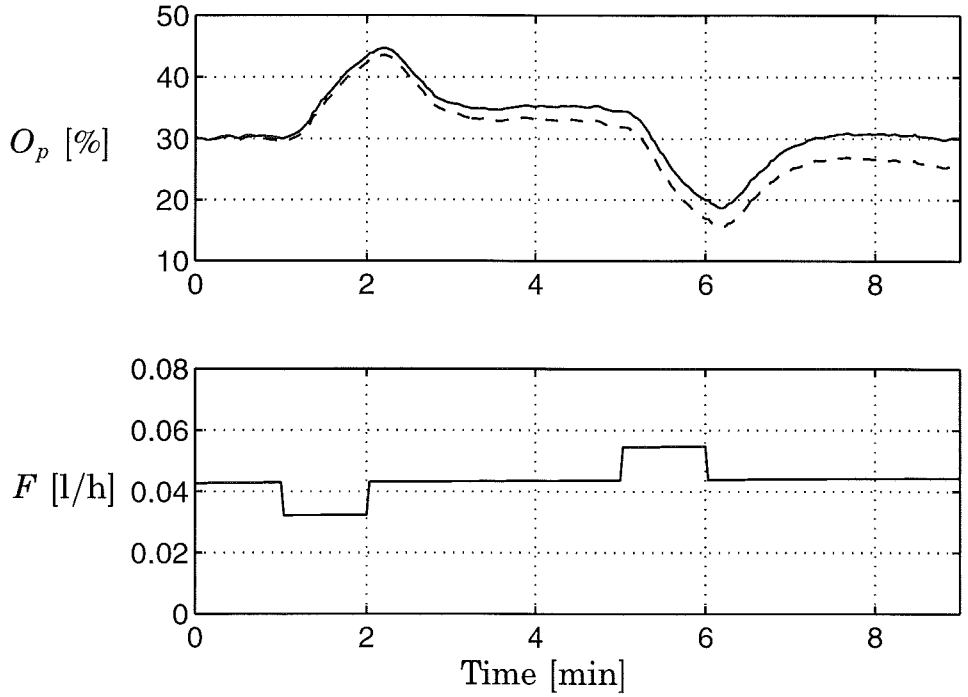

Figure 10 Experiment with *E. coli* BL21(DE3) after induction. Response in dissolved oxygen O_p to pulses in feed rate F. No acetate formation. $q_g \approx 0.54$ g/(gh). $X \approx 20$ g/l. Detrended (solid) and original data (dashed).

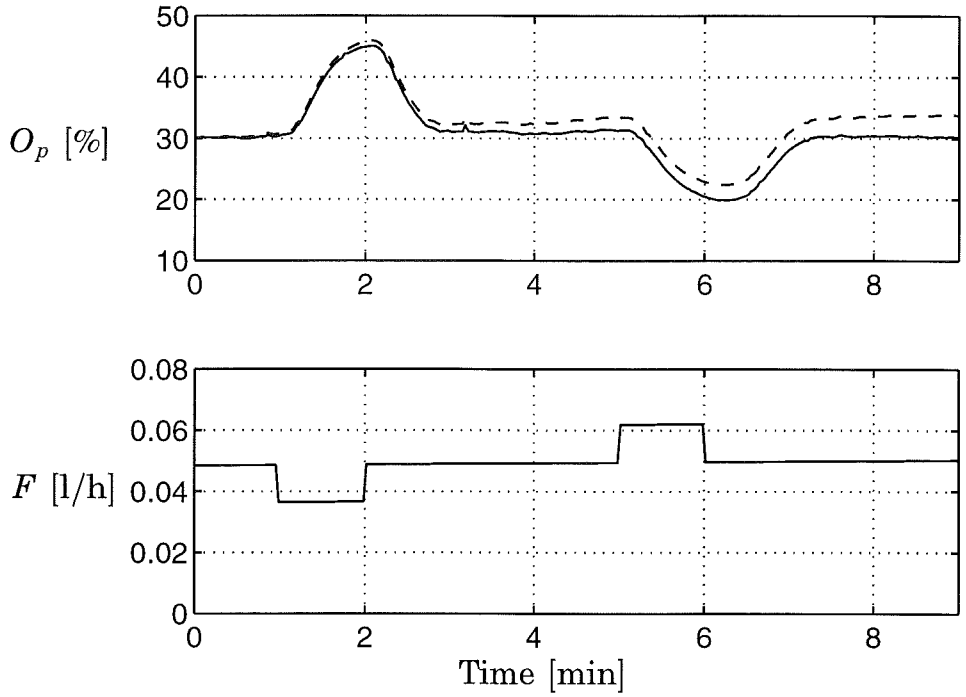

Figure 11 Experiment with *E. coli* BL21(DE3) after induction. Response in dissolved oxygen O_p to pulses in feed rate F. No acetate formation. $q_g \approx 0.59$ g/(gh). $X \approx 21$ g/l. Detrended (solid) and original data (dashed).

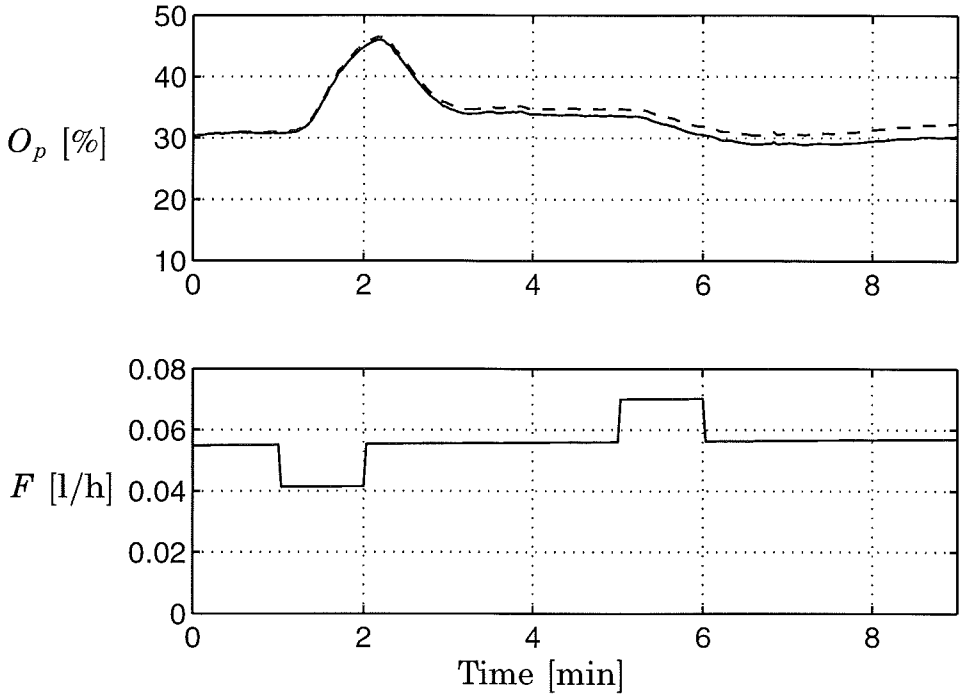

Figure 12 Experiment with *E. coli* BL21(DE3) after induction. Response in dissolved oxygen O_p to pulses in feed rate F. At onset of acetate formation. $q_g \approx 0.63$ g/(gh). $X \approx 22$ g/l. Detrended (solid) and original data (dashed).

7. Discussion

From the experimental results, it is clear that the dissolved oxygen response to transients in the glucose feed rate change character with the onset of acetate formation. This supports the idea of a change in the relations between glucose and oxygen uptake at the onset of acetate formation. The results also indicate that this change take place in a time scale of seconds or faster.

An important consequence is that it is possible to detect acetate formation from the dissolved oxygen response to superimposed transients in the glucose feed rate. It is important to note that it is a qualitative change that is detected, and consequently no values on stoichiometric coefficients or other process parameters have to be assumed or known. Furthermore, as it is the formation of acetate that is detected, it is possible to discover, and hence avoid, accumulation of acetate at an early stage.

Two hypotheses for acetate formation in *E. coli*, limitations in the respiration and/or the TCA-cycle, were used to derive models for dissolved oxygen responses to feed rate transients. Even though a number of approximations and assumptions were made, both models give a good description of the experimental results. However, from the experiments it is neither possible to validate nor to reject any of the hypotheses. It is clear that the experimental results can be simulated well using the simple respiratory limitation model. In practice, it is also likely that the difference between the two models is evened out. For example, concentration gradients in the reactor will tend to even out the sharp bottleneck predicted by the respiratory limitation.

The models, based on the flux models from [Majewski and Domach, 1990], give valuable insight about phenomena observed in these and other experiments. In the cultivations with *E. coli* BL21(DE3), the critical specific glucose uptake rate rate was significantly lower after induction ($q_g^{crit} \approx 0.63$ g/(gh)) than before ($q_g^{crit} \approx 1.22$ g/(gh)). At the same time the respiration and carbon dioxide production gradually increased which indicates an increased energy consumption due to the recombinant protein production. This is in accordance with the model which predicts a lowered value of q_g^{crit} if the cell yield to ATP is reduced. Another common observation is that q_g^{crit} is lower in complex than in defined media, see for instance [Meyer *et al.*, 1984]. When complex

media are used, the need for precursors from the central metabolic pathways is reduced. If the precursor fluxes in the model are reduced, a decreased q_g^{crit} results as well.

So far, only the production of acetate has been considered in the model. If consumption of acetate can occur under transient conditions, the concomitant oxygen consumption may affect the oxygen responses. This could for instance explain the broadened response to the up pulse seen in Figure 11. It would thus be interesting to extend the model to include also consumption of acetate.

E. coli BL21 has earlier been reported to accumulate low acetate concentrations [Shiloach *et al.*, 1996] and was therefore suggested as a suitable host for recombinant genes. This is confirmed by our experiments, where we obtained very low acetate concentrations and good production levels. Complex media are often used to improve the productivity of the recombinant protein. We achieved good protein production using a defined medium. In general, defined media imply easier downstream purification and more reproducible cultivation conditions. Complex media have also been reported to increase the acetate formation [Meyer *et al.*, 1984]. An interesting way to increase productivity, while maintaining well-defined cultivation conditions, is instead to add only the amino acids that are over-represented in the recombinant protein [Ramírez and Bentley, 1993].

8. Conclusion

Experiments with two recombinant *E. coli* strains, before and after induction, show that the dissolved oxygen response to pulses in the substrate feed rate undergoes a characteristic change at the onset of acetate formation. This gives an attractive way to obtain on-line detection of acetate formation using a standard dissolved oxygen probe.

Acknowledgments

This work was financially supported by Pharmacia & Upjohn and EU-Biotech program (BIO4-CT96-0488).

Nomenclature

F	glucose feed rate [l/h]
G, G_{in}	glucose concentration in reactor and feed medium [g/l]
H	constant [(l%)/g]
$K_L a$	volumetric oxygen transfer coefficient [1/h]
k_s	Monod saturation constant [g/l]
N	stirrer speed [rpm]
N_0	constant [rpm]
O, O_p	real and measured dissolved oxygen tension [%]
O^*	dissolved oxygen tension in equilibrium with air bubbles [%]
q_{ac}	specific acetate production rate [g/(gh)]
q_g, q_g^{max}	specific and maximum specific glucose uptake rate [g/(gh)]
q_g^{crit}	critical specific glucose uptake rate [g/(gh)]
q_g^{II}	second critical specific glucose uptake rate [g/(gh)]
q_o	specific oxygen uptake rate [g/(gh)]
T_p	time constant for dissolved oxygen sensor
V	liquid volume [l]
VG_f	total amount fed glucose [g]
X	biomass concentration [g/l]
Y_{og}, Y_{og}^{II}	oxygen to glucose yields [g/g]
Y_{xg}	biomass to glucose yield [g/g]
α	constant [1/(h · rpm)]
μ	specific growth rate [1/h]
μ_{set}	specific growth rate in feed profile [1/h]

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Paper III

Control of Dissolved Oxygen in Stirred Bioreactors

Mats Åkesson and Per Hagander

Abstract

This report discusses control of dissolved oxygen in a bioreactor where the oxygen supply is manipulated using the stirrer speed. In batch and fed-batch cultivations the operating conditions change significantly which may cause tuning problems. Analysis using a linearized process model shows that the process dynamics is mainly affected by changes in the volumetric oxygen transfer coefficient $K_L a$. To account for the process variations, a control strategy based on PID control and gain scheduling from the stirrer speed is suggested. This approach is straightforward to implement in an industrial control system. Experimental results from a laboratory reactor are presented.

1. Introduction

Today, many commercial products are produced using microorganisms. Living cells are grown to large numbers and made to produce a desired substance, often a protein. The cells are kept in a bioreactor where several control loops ensure that important process parameters, such as pH and temperature, stay within specified operating conditions. In aerobic processes, it is also important to provide the culture with oxygen. This is often solved by keeping a constant dissolved oxygen concentration.

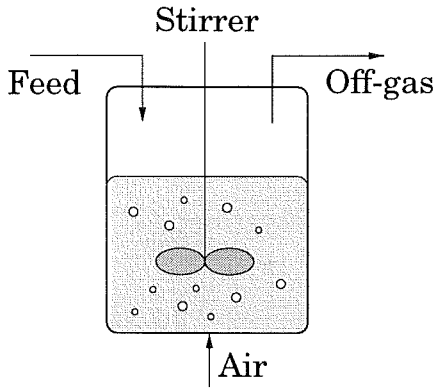

Figure 1 Stirred bioreactor.

Figure 1 shows a drawing of a stirred bioreactor. In the reactor there is a liquid medium that contains cells and substrates. Air is sparged into the liquid and well-mixed conditions are obtained through agitation with a mechanical stirrer. The oxygen supply can be varied by manipulating air flow rate, oxygen content in the incoming air, reactor pressure, and stirrer speed. In this report, we will discuss how to control dissolved oxygen using the stirrer speed as control signal.

Control Problem

When controlling dissolved oxygen in a bioreactor, it is often sufficient to keep the dissolved oxygen above a certain level. In some applications the performance requirements are higher, for instance when elevated oxygen levels are toxic for the microorganisms. Tight control is a key element in the substrate-dosage control scheme presented in [Åkesson *et al.*, 1997] and it may also be beneficial for process supervision. If the

dissolved oxygen level is kept constant, the control input can be used as an indicator of the biological activity, see for instance [Lee *et al.*, 1996].

From a control point of view, dissolved oxygen control is a regulation problem where the main disturbance is the oxygen consumption due to cell metabolism. Other disturbances affecting the process include temperature changes, foaming, and addition of surface active components. At low turbulence levels, measurement errors may occur due to gas bubbles that adhere to the sensor surface, see [Heinzle *et al.*, 1986]. In [Yano *et al.*, 1981], a classification of these disturbances is used to derive a rule-based control strategy. Controllers of PI-, and PID-type, where the parameters are obtained from open loop experiments, have for instance been presented in [Court, 1988] and [Clark *et al.*, 1985].

In continuous cultivations, the process is operated at steady-state conditions and good performance can be expected using controllers with fixed parameters. However, when the reactor is run in batch or fed-batch mode, important process variables, e.g., cell mass, substrate concentration, oxygen uptake etc., are no longer constant and the process characteristics may vary significantly. Many authors have reported tuning difficulties when controllers with fixed parameters are used, see [Court, 1988], [Lee *et al.*, 1991] and [Cardello and San, 1988]. Typical observations are stability problems for low uptake rates and sluggish control at high uptake rates. Similar difficulties occur in dissolved control of activated sludge processes in waste-water treatment, where the air flow rate is used as control signal, see for instance [Lindberg, 1997] and [Holmberg *et al.*, 1989].

To overcome the problems with the varying process dynamics, various adaptive control schemes have been suggested. Approaches based on PID control together with gain scheduling from the oxygen uptake rate and PID control combined with feed-forward from the oxygen uptake rate were presented in [Cardello and San, 1988]. On-line tuning of PID controllers based on output variance [Lee *et al.*, 1992] and parameter estimation from off-gas analysis [Levisauskas, 1995] have also been suggested. Indirect adaptive control, that is, parameter estimation and subsequent controller design, have been tested in [Lee *et al.*, 1991] and [Hsiao *et al.*, 1992].

Outline

The outline of the report is as follows. In Section 2, a model of the oxygen dynamics is derived. Analysis of a linearized model shows that the oxygen dynamics depends critically on the volumetric oxygen transfer coefficient $K_L a$. The implications for control are discussed in Section 3. A control strategy that is straightforward to implement in an industrial process control system is suggested. Section 4 presents experimental results from dissolved oxygen control in a laboratory scale bioreactor during a cultivation of recombinant *Escherichia coli* bacteria. Finally, in Section 5 some conclusions are given.

2. Process Model

A model of the dissolved oxygen dynamics in a laboratory scale bioreactor will be derived. It is assumed that well-mixed conditions apply and that any mechanical or electrical dynamics from control signal to stirrer speed are negligible. The parameters in the model are adjusted to describe a 3 liter laboratory bioreactor according to experimental data. A linearized model is derived for analysis purposes.

Nonlinear Model

Oxygen balance Mass-balance for the dissolved oxygen in the reactor yields the following differential equation

$$\frac{d(VC_o)}{dt} = K_L a(N) \cdot V(C_o^* - C_o) - q_o \cdot VX$$

where V, C_o, X, denote liquid volume, dissolved oxygen concentration, and cell concentration, respectively. The first term models the oxygen transfer from air bubbles to liquid. Here, $K_L a$ is the volumetric oxygen transfer coefficient which is dependent on the stirrer speed, N, and C_o^* denotes the oxygen concentration in the liquid layer around the air bubbles. The second term describes the oxygen consumption due to the biological activity. The specific oxygen consumption rate q_o is a function of substrate uptake rate and hence it will depend on the substrate feed rate.

Contributions from incoming and outgoing flows have been neglected because the oxygen solubility in water is very low. A similar argument can be used to neglect concentration due to dilution effects and the oxygen equation can be rewritten as

$$\frac{dC_o}{dt} = K_L a(N) \cdot (C_o^* - C_o) - q_o \cdot X$$

In practice, most sensors do not measure the oxygen concentration but the dissolved oxygen tension, a quantity proportional to the oxygen partial pressure. A dissolved oxygen tension of 100 % corresponds to a solution where the oxygen partial pressure is in equilibrium with air, that is, an oxygen saturated solution. The dissolved oxygen tension O is related to the dissolved oxygen concentration through Henry's law

$$O = H \cdot C_o$$

where the constant H depends on the oxygen solubility, see [Popović *et al.*, 1979] and [Pirt, 1975]. In the sequel, the common literature value for water $H = 14000\ \%\,\mathrm{l\,g^{-1}}$ will be used. In a laboratory reactor it is also reasonable to assume that O^* is close to 100 %. The oxygen dynamics can now be described as

$$\frac{dO}{dt} = K_L a(N) \cdot (O^* - O) - \underbrace{q_o \cdot HX}_{=d}$$

From now on, the oxygen consumption term will be considered as a load disturbance d.

Volumetric oxygen transfer coefficient For a fixed air flow rate, the volumetric oxygen transfer coefficient, $K_L a$, can be modeled as a function of the stirrer speed, N. Commonly used expressions are of the form $K_L a \sim N^\gamma$, for instance $K_L a \sim N^3$ as suggested in [Pirt, 1975]. To obtain good mixing, the stirrer speed is in practice never below a minimum value. In the working range, it is then reasonable to approximate the stirrer dependence with a linear expression

$$K_L a(N) = \alpha \cdot (N - N_0)$$

For the 3 l laboratory bioreactor, $\alpha = 0.92$ h^{-1}rpm^{-1} and $N_0 = 323$ rpm give a good approximation for stirrer speeds between 350 rpm to 1200 rpm. These values were obtained using the K_La-estimation technique suggested in [Van't Riet, 1979] for different stirrer speeds. Calculating the oxygen transfer rate, OTR, from off-gas analysis, K_La can be estimated as

$$K_La = \frac{H \cdot OTR}{O^* - O}$$

Except for air flow rate and stirrer speed, the oxygen transfer is also affected by viscosity, temperature, foaming etc., [Pirt, 1975]. Addition of antifoam chemicals also tend to give a temporary decrease in K_La.

Dissolved oxygen sensor Dissolved oxygen tension is measured with a probe in the reactor liquid. As will be seen, the probe contributes significantly to the oxygen dynamics. For large deviations, the probe tends to respond faster for up responses than for down responses, [Lee and Tsao, 1979]. The sensitivity of the probe does also increase with temperature. In large reactors, where mixing problems cannot be neglected, the probe placement may be important for the oxygen control performance [Belfares *et al.*, 1989].

For non-viscous and well-mixed systems the probe may be modeled as a linear first order system and possibly a time delay

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

with O_p denoting the measured dissolved oxygen tension. However, for viscous systems and for low stirrer speeds, it may be necessary to add a second time constant, see [Dang *et al.*, 1977].

For the probe used in the laboratory reactor a time constant of $T_p \approx 20$ s was estimated. The time delay was less than the data logging period of 2 s. In the sequel $\tau = 2$ s will be used.

Linearized Model

Around a trajectory, small variations in the dissolved oxygen dynamics may be described by the linearized equation

$$T_o \frac{d\Delta O}{dt} + \Delta O = K_n \Delta N - K_d \Delta d$$

where the parameters are given by

$$T_o = (K_L a)^{-1}$$
$$K_n = [O^* - O]\frac{\partial K_L a}{\partial N}(K_L a)^{-1}$$
$$K_d = (K_L a)^{-1}$$

The transfer function from the stirrer speed ΔN to the oxygen measurement ΔO_p becomes

$$G_{on}(s) = \frac{K_n e^{-s\tau}}{(1 + sT_o)(1 + sT_p)}$$

Here τ and T_p depend on the probe and are considered to be constant during a process run. On the other hand, K_n and T_o are both proportional to the inverse of $K_L a$ which may vary significantly. In Figure 2, a Bode plot of G_{on} at three different stirrer speeds illustrate the changing process dynamics. When $K_L a$ increases with the stirrer speed, K_n and

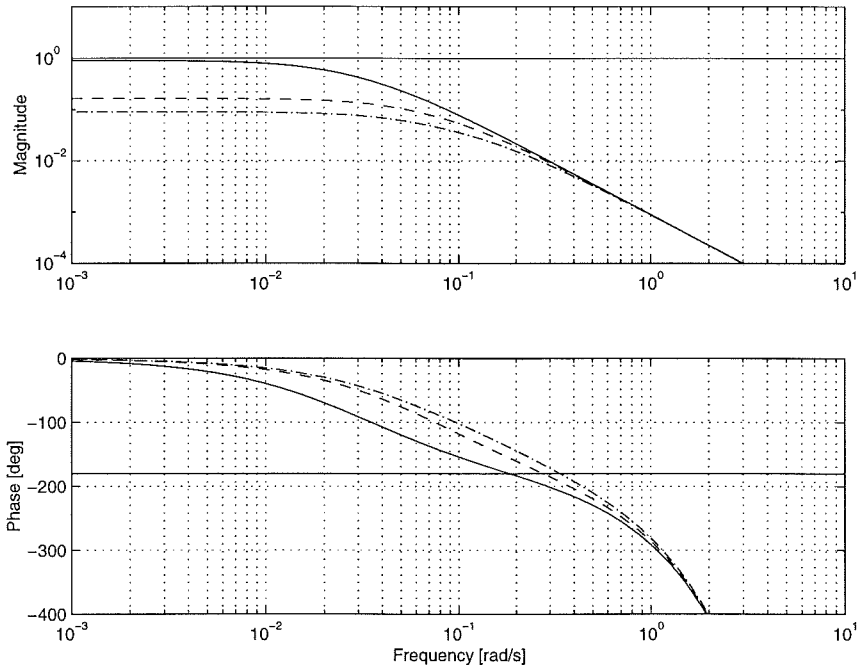

Figure 2 Bode plot of the transfer function from stirrer speed to measured dissolved oxygen tension at three different stirrer speeds; 400 rpm (solid), 750 rpm (dashed), 1100 rpm (dash-dotted).

T_o decrease which will decrease the low-frequency gain and increase the phase in the low- and mid-frequency region. The high-frequency region is unaffected.

In summary, the lower the K_La, the higher is the process gain and the larger is the phase lag. Note also that the gain K_n depends on the linearization point in dissolved oxygen, i.e., the chosen set-point.

3. Control Design

The process model derived in the previous section will now be used to discuss the dissolved oxygen control problem. Design and analysis of controllers of PID-type are made based on the linearized model. The non-linear model is used for simulations of the closed-loop systems, see Figure 3. A control strategy based on gain scheduling from the stirrer speed is suggested.

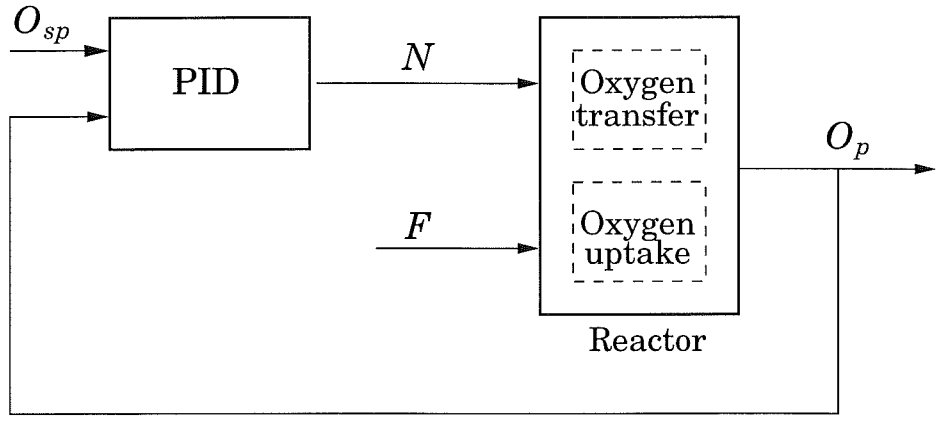

Figure 3 Dissolved oxygen control loop where the dissolved oxygen signal O_p should be kept at the set-point O_{sp}. By manipulating the stirrer speed N the oxygen transfer to the reactor can be varied. In fed-batch and continuous cultivations, the oxygen consumption depends on the substrate feed rate F.

Fixed-parameter Controller

We will now examine the behavior of controllers with fixed parameters when the process dynamics change. Using the Kappa-Tau tuning method, see [Åström and Hägglund, 1995], two PID controllers are designed to work well at 400 and 1100 rpm respectively. The resulting

	K	T_i	T_d
400 rpm	12.9 rpm/%	24.6 s	5.7 s
1100 rpm	48.1 rpm/%	12.5 s	3.0 s

Table 1 PID parameters for the laboratory reactor using the Kappa-Tau tuning method with $M_s = 1.4$.

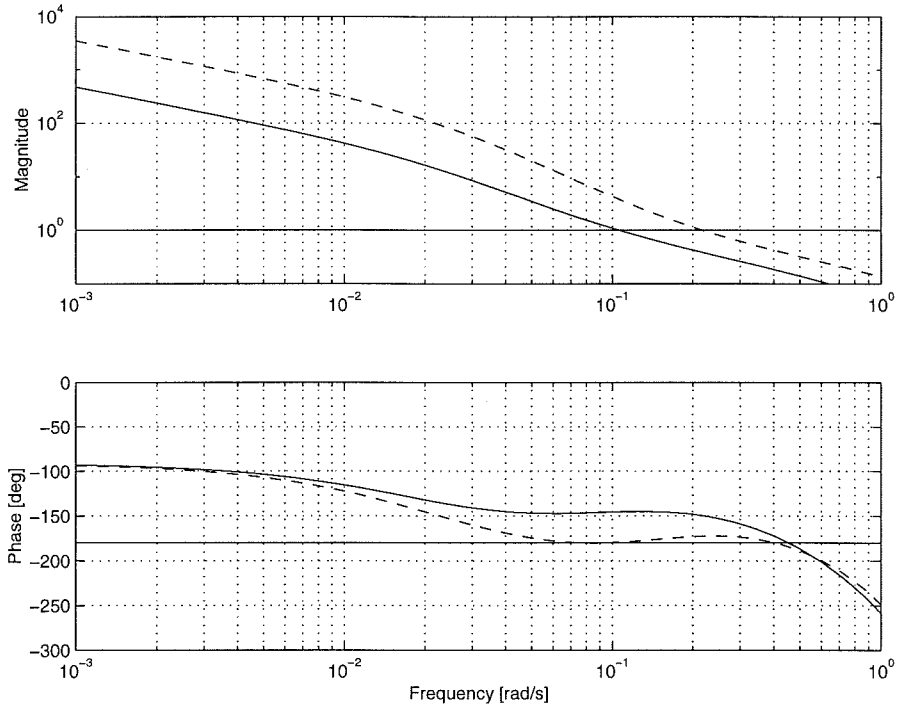

Figure 4 Bode plot of the open-loop transfer function at 400 rpm. PID controller tuned for 400 rpm (solid) and 1100 rpm (dashed).

controller parameters can be found in Table 1. In both cases, low-pass filtering of the derivative term with a time constant $T_d/5$ is used.

Figure 4 shows the open-loop Bode diagram when the two controllers are operated around 400 rpm. The controller designed to work at 1100 rpm, which has higher gain and more integral action, seems to give a closed-loop system that is close to instability. This is confirmed when simulating the two systems, see Figure 5. The controller designed for 1100 rpm gives an oscillating system while the controller designed for 400 rpm gives well damped system that responds quickly to a step load change.

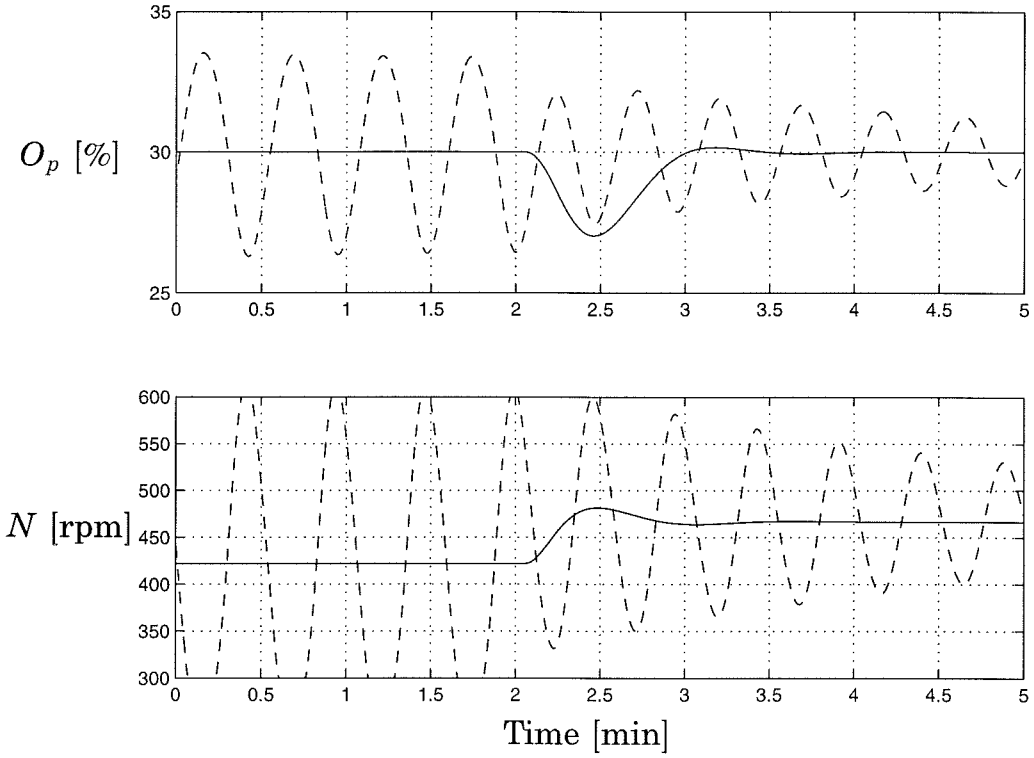

Figure 5 Closed-loop simulation around 400 rpm. After two minutes, a step change in the load is made. Results with PID controller tuned for 400 rpm (solid) and 1100 rpm (dashed).

Around 1100 rpm, the process gain has decreased and both controllers give a well damped closed-loop system, see Figure 6. From the Bode diagram, it can also be seen that the controller designed for 1100 rpm gives a faster system with better load disturbance rejection. Again, this is confirmed when the closed-loop systems are simulated, see Figure 7. The controller designed for 1100 rpm is considerably faster without being oscillatory.

Thus, when a controller with fixed parameters is used, there is a trade-off between stability and performance. If stability in the presence of process variations is the most important objective, the tuning should be made for the lowest K_La that is expected, i.e., at the lowest stirrer speeds. This gives a robustly stable closed-loop system at the expense of a sluggish response at higher K_La values.

It was pointed out that the K_La variations do not affect the high-frequency region. A high-gain feedback controller with fixed parameters could then, in principle, yield a closed-loop system insensitive to

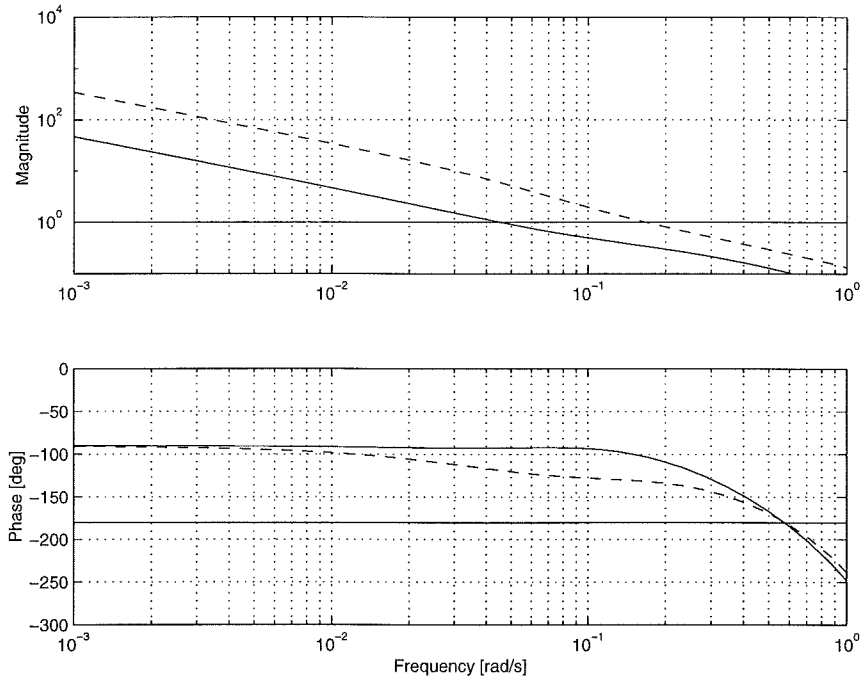

Figure 6 Bode plot of the open-loop transfer function at 1100 rpm. PID controller tuned for 400 rpm (solid) and 1100 rpm (dashed).

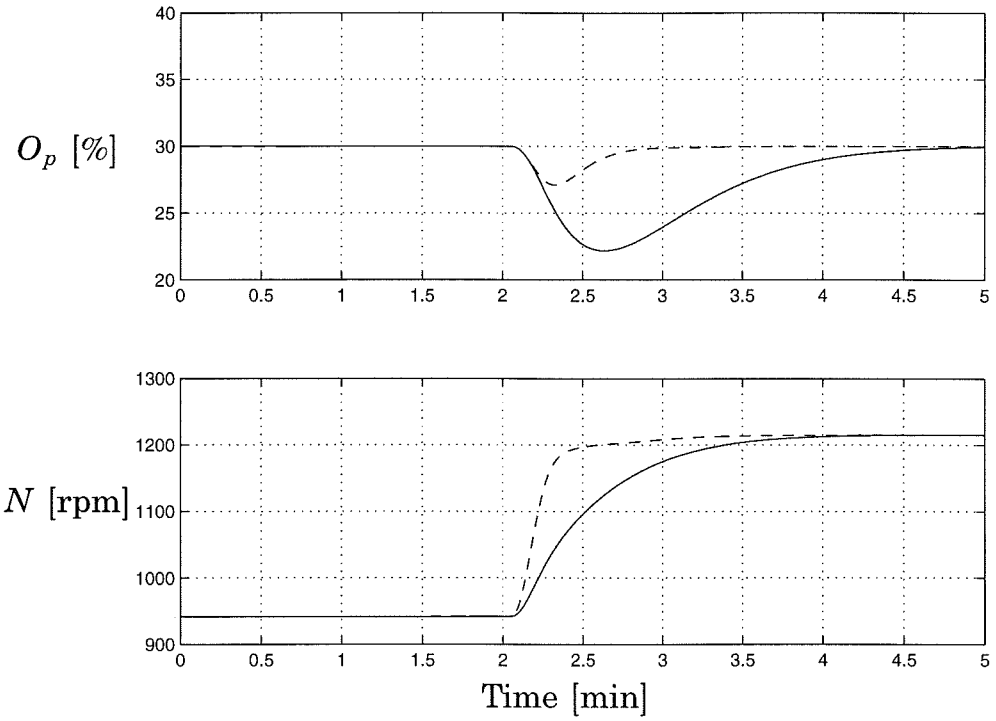

Figure 7 Closed-loop simulation around 1100 rpm. After two minutes, a step change in the load is made. Results with PID controller tuned for 400 rpm (solid) and 1100 rpm (dashed).

the variations. However, in practice this alternative is not feasible. For instance, the influence of measurement noise would be too large.

As pointed out in Section 2, the chosen set-point O_{sp} also affects the process behavior. If a large span of set-points are to be used this may have to be accounted for. Good robustness is obtained if the controller is tuned at the lowest set-point where the process gain is highest.

Gain Scheduling

To obtain the same performance at all operating points, without trading off robustness, the controller should depend on the operating conditions. One way to do this is to use gain scheduling [Åström and Hägglund, 1995]. As the process dynamics changes with K_La, this is a natural scheduling variable. An alternative and a slight variation of gain scheduling, is to use exact linearization, see [Lindberg, 1997]. Both methods require on-line estimation of K_La, which for instance can be done using off-gas measurements.

As K_La is dependent on the stirrer speed, a simpler approach is to use gain scheduling from the stirrer speed itself. This method is straightforward to implement in an industrial control system and will be used in the experiments in the next section. A drawback is that this technique does not capture K_La changes due to other sources than the stirrer speed, e.g., foaming, surface active compounds, viscosity changes etc. In processes where such effects are important, a method based on estimation of K_La is preferable.

Feed-forward

In fed-batch and continuous cultivations, the oxygen consumption is dependent on the substrate feed rate. Feed-forward action from the feed rate can then be used to improve the control performance. For instance, in fed-batch cultivations with exponential growth, the oxygen consumption increase exponentially. This kind of disturbance is hard to reject for an ordinary PID controller without feed-forward.

The dynamics from feed rate to oxygen consumption is often unknown but a static relation between feed rate and stirrer speed can be obtained from recorded process data.

4. Experiments

This section describes dissolved oxygen control in a 3 liter laboratory fermenter during fed-batch fermentations of recombinant *Escherichia coli*.

Control Strategy and Implementation

As was suggested in the previous section, a PID controller combined with gain scheduling from the stirrer speed was used. The working range for the stirrer was divided into three regions; 350–600 rpm, 600–900 rpm, and 900–1200 rpm. A hysteresis of ±20 rpm was introduced to avoid oscillations around the transition regions when changing from one region to another. No feed-forward action was used during the experiments.

The controller was implemented in the SattLine system from Alfa Laval Automation AB, Malmö, Sweden. A standard module for PID control with facilities for gain scheduling and auto-tuning was used. Anti-reset windup and bumpless parameter changes are also included in the module. When derivative action was used, the derivative term was low pass filtered with a time constant $T_d/6$. A sampling time of 0.5 s was chosen according to the guidelines in [Åström and Wittenmark, 1997]. Limitations in the choice of sampling interval may impose restrictions on achievable performance, especially when derivative action is needed.

For each of the operating regions, the auto-tuner function in the controller was used to obtain controller parameters. The load was varied by changing the feed rate F. A constant feed rate gives an approximately constant oxygen consumption. Relay experiments made at the end of a cultivation are shown in Figure 8. The resulting controller parameters are given in Table 2.

Results

The control algorithm was tested using the same reactor but with another strain of *E. coli*. In the mid operating region, the controller parameters obtained from the relay experiments gave a behavior that was somewhat oscillatory. The derivative time T_d was therefore increased from 0.0 s to 1.0 s. Load response experiments were made at the end of the cultivation. At this stage, there was substantial foaming in the

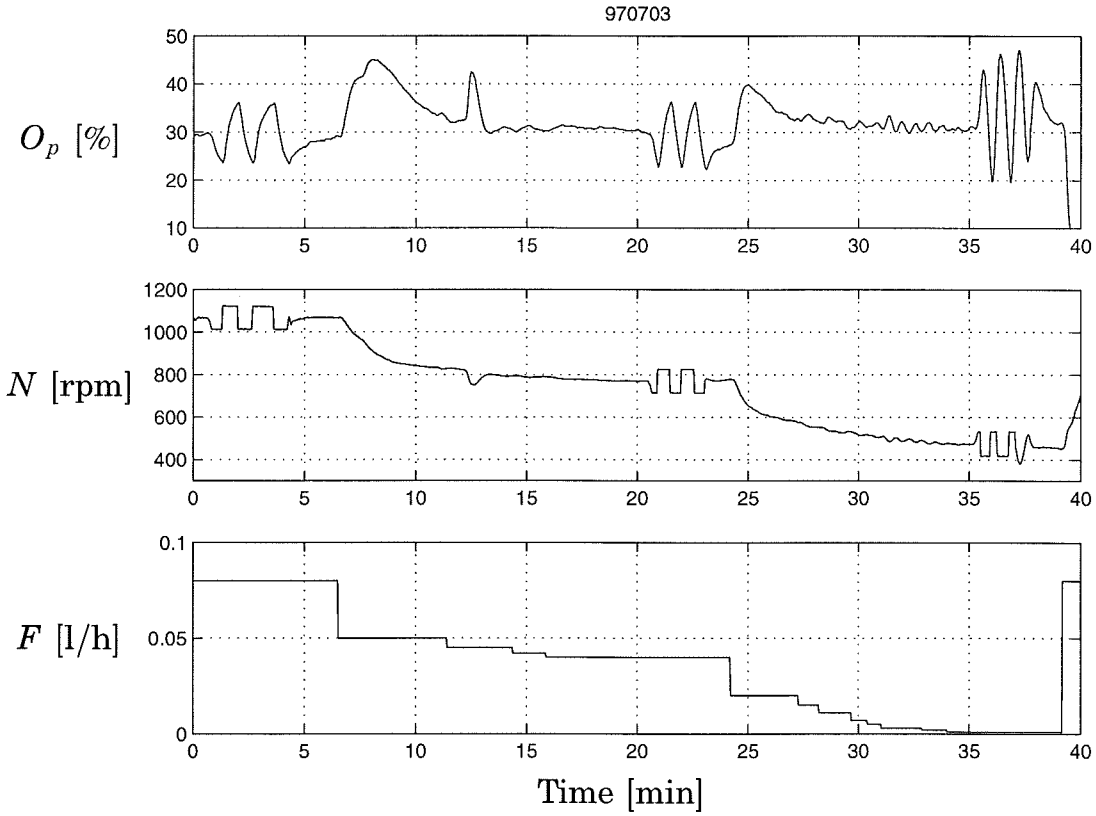

Figure 8 Relay experiments at three different operating points. The load has been varied by changing the feed rate F.

	K	T_i	T_d
450 rpm	3.2 rpm/%	29.4 s	4.7 s
750 rpm	6.3 rpm/%	40.8 s	0.0 s
1050 rpm	6.8 rpm/%	61.2 s	0.0 s

Table 2 PID parameters for the laboratory reactor obtained from relay auto-tuning experiments.

reactor and anti-foam chemicals which affect the oxygen transfer had to be added several times.

In Figure 9, the behavior at higher stirrer speeds is shown. First, a step increase was made in the substrate feed rate F. This gives an approximate step change in the oxygen consumption. During this experiment, the controller changed from the mid to the high operating region. After about 9 minutes, an anti-foam addition was made which

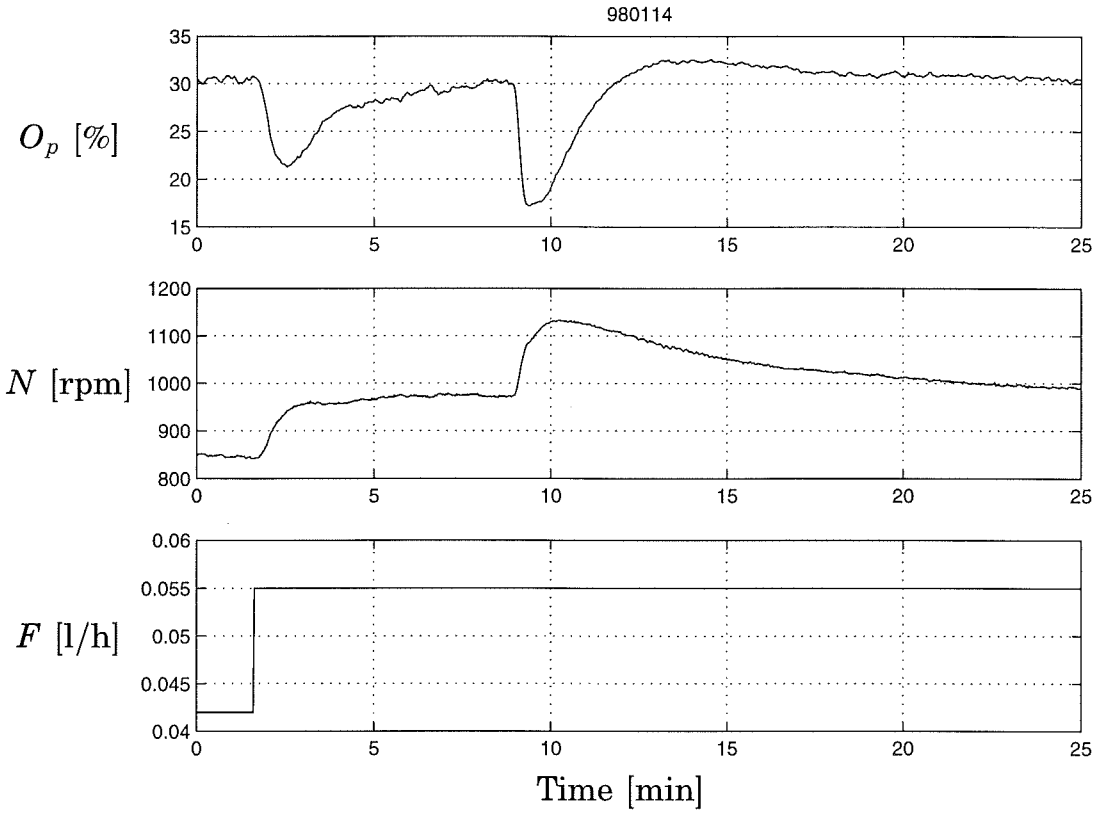

Figure 9 Step load change followed by anti-foam addition. At 920 rpm, the controller changed from the mid to the high operating region. The set-point O_{sp} was fixed at 30 %.

immediately reduced the oxygen transfer and the dissolved oxygen level O_p dropped. The controller compensated for this by increasing the stirrer speed N. The effect of the anti-foam on the oxygen transfer decayed slowly, which caused O_p to stay slightly above the set-point O_{sp} for a while. This can be thought of as the response to a ramp disturbance.

Figure 10 illustrates the response to a load change in the lower part of the mid operating region. The low region was never entered. The disturbance was eliminated faster than in the previous experiments but the variations in the output were substantially larger. Small damped oscillations can be hinted at. One explanation for this could be that the mid-controller was approaching the lower limit for its applicability. There also seemed to be more excitation from measurement "noise", possibly due to gas bubbles adhering to the sensor surface, see [Heinzle *et al.*, 1986].

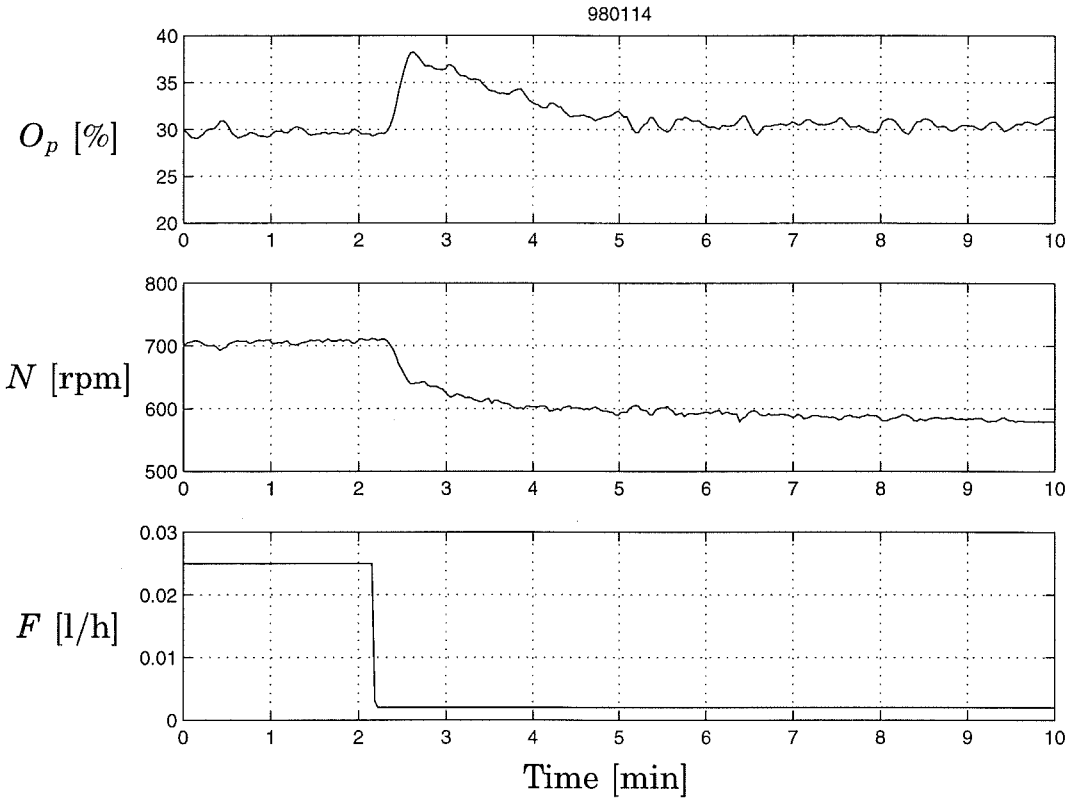

Figure 10 Step load change in the lower part of the mid operating region. The low region was never entered. The set-point O_{sp} was 30 %.

The experiments indicate that the approach with gain scheduling from the stirrer speed works well. Good disturbance rejection was achieved throughout the operating range. However, some tuning problems were encountered in the lower part of the mid-region. This indicates that it could be beneficial to divide the working range into more operating regions or to change the partitioning of the operating regions. From the expressions for K_n and T_o, and also the obtained controller parameters, it can be seen that most of the process variation take place at lower $K_L a$ values. This suggests that the partitioning of the operating regions should be denser in the lower part of the working range. One could also improve the robustness by tuning the controllers in the lower part of each region.

Disturbances caused by anti-foam addition were well handled but did take considerable time to eliminate completely. At lower stirrer speeds, the excitation from sporadic disturbances, possibly due to air bubbles adhering to the sensor, seemed important.

5. Conclusions

This report has treated dissolved oxygen control in bioreactors when the stirrer speed is used as control input. In batch and fed-batch cultivations, the process characteristics may vary significantly and many authors have reported tuning problems when using controllers with fixed parameters. From a model of the process, it can be seen that the variations in the oxygen dynamics are due to changes in the volumetric oxygen transfer coefficient $K_L a$.

If the performance needs are moderate, a PID controller with fixed parameters can be used. Maximum robustness against process variations is obtained if the controller is tuned for the lowest $K_L a$-values that are likely to occur. This gives a stable closed-loop system under all operating conditions but the response will be sluggish at operating points with high $K_L a$-values. When the performance demands are higher, gain-scheduling can be used to obtain similar performance at all operating points. The controller parameters should then depend on $K_L a$. As $K_L a$ is affected by the stirrer speed, a simpler approach is to use the stirrer speed as the scheduling variable. In fed-batch and continuous cultivations, the load or the oxygen consumption is determined by the substrate feed rate. Feed-forward from the feed rate could then improve the overall control behavior.

Experiments with dissolved oxygen control in a laboratory bioreactor were performed during cultivations of *E. coli* bacteria. A PID controller combined with gain scheduling from the stirrer speed was tested. The controller was implemented using standard modules in an industrial control system. The approach was found to work well, yet some tuning problems were detected. This suggested that the partitioning of the operating regions should be made differently or that more operating regions may be useful. To improve the robustness further, the tuning should be made in the lower part of each interval. During the experiments anti-foam additions were made. The resulting disturbances were well handled but effects from the anti-foam could be observed for quite some time after the additions. It was also noted that sporadic disturbances, and their influence on the control, was more significant at low stirrer speeds.

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