Extremum-Seeking Control of Industrial-Scale Fermentation Processes

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Abstract

The work presented here is based on two papers, both pertaining to perturbation-based control strategies in industrial fed-batch fermentation processes.

The first paper describes a new control strategy for avoiding overflow metabolism in the exponential growth phase of a fermentation, based on analysis of the frequency spectrum of the dissolved oxygen measurement following a periodic perturbation in the feed rate. A controller based on this strategy was tested in pilot scale, where it gave higher specific growth rates and lower concentrations of overflow metabolites during the exponential growth phase of the process compared to a reference strategy currently used to control the process, resulting in approximately 30 % higher biomass concentrations (w/w) 8 h after inoculation in two different processes utilizing different strains. Adding excess substrate at different points in time showed that the controller can detect and respond to excess substrate. In a set-up with inoculum volume decreased to 1/3 of its normal value, the controller compensated for the decreased feed demand whereas the reference strategy caused excessive accumulation of overflow metabolites leading to process failure.

The second paper is based on an experimental study in an industrial production-scale (>100 m^3) process, in which sinusoidal perturbations in the feed rate were applied to evaluate the applicability of perturbation-based control strategies and to model the process for the purpose of such strategies. The results indicated that perturbations in the feed rate of the process can give rise to measurable responses in dissolved oxygen measurements without decreasing process productivity and that a second-order model can be used to describe feed and oxygen dynamics in the process. The perturbation frequency range 3.33-5 mHz was identified as suitable for utilization of the model for perturbation-based control and a simple example of an observer is given to illustrate how the model can be used in on-line control.

Acknowledgments

I would like to thank my supervisors, Charlotta Johnsson, Gunnar Lidén, Jonas Andersson and Tore Hägglund, for all the advice and encouragement they have given me in my research. Without Jonas' support of research cooperation between Novozymes and Lund University, this project would not have been possible.

My colleagues at the Department of Automatic Control have contributed to a pleasant environment to work in, where interesting discussions regarding research and other topics are often brought up. For this I am particularly thankful to the people in the process control group and to the colleagues whom I share offices with. Per Hagander has been of help by discussing previous research within the field and by pointing me to publications of interest. Leif Andersson has aided me in many LaTeX-related matters, including the layout of this thesis and the papers presented in it.

This project has been performed in cooperation with Novozymes A/S, which has granted access to industrial pilot and production facilities in the studies presented here. In the pilot scale studies in Bagsværd, Henning Fugmann has helped by explaining and reprogramming the process control system while Stuart Stocks has contributed with biotechnical insights. In the production scale studies in Kalundborg, Frederik Riisgaard has helped by sharing his expertise regarding the production processes and Henrik Nørgaard has patiently reprogrammed the process control and data collection system.

This work was carried out within the framework of the Process Industrial Centre at Lund University (PIC-LU) and supported by the Swedish Foundation for Strategic Research (SSF), in cooperation with Novozymes A/S. The author is a member of the LCCC Linnaeus Center and the eLLIIT Excellence Center at Lund University.

Contents

1

Introduction

Industrial processes utilizing microorganisms for production, commonly referred to as fermentation processes, are used today to produce a wide variety of products from industrial bulk chemicals to food and feed ingredients to specialized pharmaceuticals. Although genetic engineering for development of new microbial strains represents a major factor behind improved processes, auxiliary systems such as process control play an important part in achieving processes with a higher productivity and yield. As a single fermentation represents significant values, avoiding process failures is of high importance in industry.

The success of a fermentation process is a result of the intrinsic properties of the strain used and the time dependent environmental conditions in the fermentor tank. The purpose of fermentation control is to ensure the proper environmental conditions at each point in time throughout the process. For best process performance, control must be robust, i.e. be able to handle unforeseen variations such as variation in substrate quality and disturbances in the process.

A major difficulty in control of fermentation processes is the lack of sensors for a number of key process parameters, particularly in an industrial environment where many measurements which are possible in laboratory-scale processes with defined media are not applicable due to the use of complex media in industrial processes. In addition, some of the measurements which can be made are sometimes only performed with long intervals, making them less useful for on-line control.

Another issue is the fact that although many aspects of microbial metabolism and growth are known today, many complex biochemical processes which are part of these are still not fully understood. This increases the uncertainty in process behaviour, sometimes leading to a view of fermentation processes as highly unpredictable with little to no possibility to explain the occurrence of certain phenomena in processes. Although modelling, prediction and control of microbial processes is clearly possible, this view illustrates the challenges in fermentation control.

A number of different approaches to fermentation control exist, more or less suitable for application in industrial processes. The aim of the current project is to investigate how perturbation-based control can be used in industrial fermentations. Since this project is carried out in close collaboration with Novozymes it has had unique opportunities for calibration and validation in pilot scale and also for experimental studies in production scale.

2

Background

2.1 Microbial metabolism in a process perspective

Many microorganisms have very short generation times, down to less than 10 minutes [Eagon, 1962]. Under optimal conditions their numbers increase exponentially and due to their short generation times the total biomass can increase hundred- and even thousandfold in less than an hour. This is a desirable feature in industrial processes, as it means that only a small number of organisms (inoculum) must be present at the start of the process in order to achieve high productivity within a short time.

The metabolism of an organism can be divided into two parts, catabolism and anabolism. Catabolism involves the uptake of compounds which can supply the organism with the raw materials and energy required for its metabolism and the transformation of these into the building blocks and energy carriers which the organism can use efficiently. Anabolism involves the creation of larger compounds which serve a purpose within the organism, such as proteins, lipids and carbohydrates, from the building blocks and energy carriers produced in catabolism.

Microorganisms can be aerobes, meaning that they require oxygen to grow, obligate anaerobes, meaning that oxygen is toxic to them, or facultative anaerobes, meaning that they can switch between aerobic and anaerobic metabolism. Usage of oxygen allows more efficient use of energy-containing substrates through oxidative metabolism, and is therefore desirable in industrial processes unless a certain product is sought for which is only produced anaerobically.

Overflow metabolism

The capacity of the oxidative metabolism of a microorganism is limited, meaning that non-oxidative metabolism can occur even under aerobic conditions if more substrate is consumed than can be metabolized oxidatively. This phenomenon is labelled overflow metabolism, illustrated in figure 2.1.

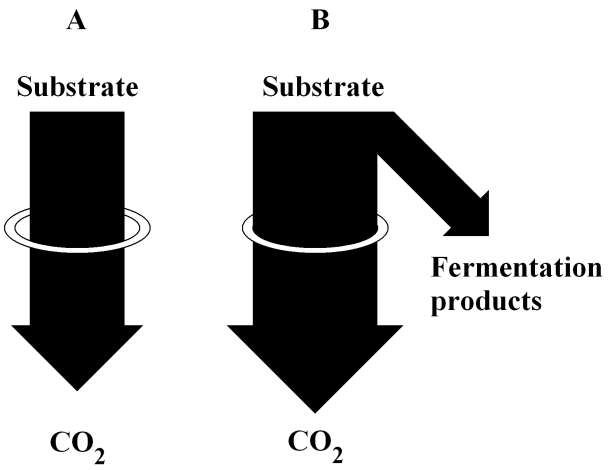

Figure 2.1 Illustration of overflow metabolism. In case A, the oxidative capacity is not limiting and no overflow metabolism is occurring, the only carbon-containing compound excreted by the organism is CO_2. In case B, the oxidative capacity is limiting and overflow metabolism takes place; the excess substrate is converted into fermentation products such as acetic acid, lactic acid, ethanol or other organic compounds.

Overflow metabolism fills a purpose in that it allows a higher growth rate than if metabolism would be constrained by the limitation in oxidative metabolism. However, whereas the end products of oxidative metabolism are energy, water and carbon dioxide, non-oxidative metabolism also leads to production of one or more organic compounds, for instance lactic acid, acetic acid or ethanol. These contain energy which has not been utilized by the organism, meaning that metabolism becomes less efficient. More importantly, although these are excreted by the organism, they inhibit its growth and at high concentrations they can cause the death of the entire cell culture. For these reasons, overflow metabolism is typically undesirable.

Overflow metabolism is mainly a problem when rapid growth of microorganisms is desired. Ideally, for fastest possible growth without production of overflow metabolites, the oxidative metabolism should be operating at full capacity while no overflow metabolism should occur. This means maintaining a substrate uptake rate exactly corresponding to the critical substrate uptake rate where the oxidative metabolism becomes saturated; a higher substrate uptake rate causes overflow metabolism while a lower rate leads to lower utilization of the oxidative metabolism and hence to lower growth rate.

Product formation

The metabolites produced by a microorganism can be formed through a great number of different metabolic pathways, which can be more or less active in different stages of growth. A distinction is made between primary and secondary metabolites; the former are compounds formed as a direct result of microbial growth and reproduction, while production of the latter is not directly coupled to growth.

In many cases, the desired product of a fermentation process is a secondary metabolite. If so, in the case of genetically engineered organisms, a key gene needed for production is commonly designed so that its activation requires the presence of a certain compound in the medium. This compound is not present at the start of the process, meaning that the microorganisms will not devote energy to production of this secondary metabolite and instead use it for faster growth. When the biomass concentration has grown sufficiently high, the compound is added and metabolism is switched from focusing on growth to focusing on production. This approach is labelled induction, as the key gene is induced by addition of a compound.

2.2 *Bacillus licheniformis*

The microorganisms utilized in the processes investigated in this study are bacteria of different strains in the *Bacillus licheniformis* species. This species is widely spread in the environment and common in most soils, as well as in agricultural products such as cereals [Boer et al., 1994].

B. licheniformis is closely related to the *B. subtilis* species, which is one of the most well-studied microorganisms in existence [Rey et al., 2004]. The genetic code of *B. licheniformis* is well characterized and there exist highly developed transformation and gene replacement technologies for use in the species, meaning that it is considered highly suitable for production of recombinant proteins, i.e. proteins which are not produced by wild-type strains and require genetic engineering of the organism [Demain and Vaishnav, 2009].

The first permission for industrial use of genetically engineered strains of this species was given in 1989 [Boer et al., 1994] and today it is used extensively in industrial fermentation processes along with others in the *Bacillus* genus [Schallmey et al., 2004]. Among the reasons for this are a fast growth rate and the ability to excrete proteins into the fermentation medium [Schallmey et al., 2004], the latter simplifies product recovery significantly in cases where the product is among these proteins. It is considered as generally non-pathogenic in humans [Boer et al., 1994], although the existence of toxin-producing strains has been shown [Salkinoja-Salonen et al., 1999; From et al., 2005].

Products of industrial *B. licheniformis* processes include proteases, α-amylase, penicillinase, β-mannanase and various pectinolytic enzymes [Rey et al., 2004]. The temperature properties of the α-amylase produced by *B. licheniformis* has been of great interest; the *B. licheniformis* version of this enzyme, which breaks α-1-4-linkages in carbohydrate chains to make them more accessible to the microorganism, can operate at temperatures up to 95 °C and withstand temperatures over 100 °C degrees for short periods. This makes it highly suitable for industrial liquification of starches. [Schallmey et al., 2004]

2.3 The industrial fermentor

An industrial fermentor is designed to provide a means of efficient microbial growth and production, while being cost-efficient to run. To achieve this, it must provide the best possible environment for the microbial processes for which it is used.

A fermentor used in industrial production is typically near-cylindrical, with a volume up to several hundred cubic metres [Hermann, 2003]. Transport issues become more important as fermentor volume increases. One common type of fermentor is the stirred tank reactor (STR), in which one or more agitators are used to stir the fermentation broth in order to facilitate transportation of various compounds within it. This type of fermentor is illustrated in figure 2.2, giving the general design as well as detailing the important components.

Mixing

Many different types of agitators for use in industrial fermentors exist, with different shapes providing different advantages and disadvantages. It is typically required that a short mixing time is achieved, meaning that spatial inhomogeneities in the broth are smoothed out as fast as possible. One problem which can arise is that although mixing times are short in most of the fermentor, there can exist "dead zones" where mixing effects are very weak. This can be caused by the geometry of the fermentor in combination with other factors.

Aeration

For fermentation processes requiring the presence of oxygen, air is added through a sparger at the bottom of the fermentor and let out through a tube near the top. Diffusion of oxygen into the broth liquid and mixing of the broth transports oxygen to the microorganisms. As these consume oxygen and produce carbon dioxide, the levels of the former are lower and the levels of the latter higher in the outlet gas than in the inlet air. Dissolved

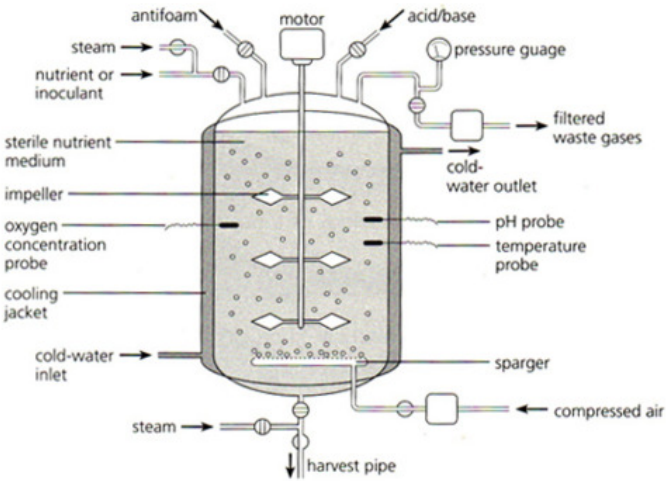

Figure 2.2 Illustration of a stirred tank fermentor showing the core parts of the tank as well as inlets, outlets and probes.

oxygen in the broth can be measured on-line using electrodes or optodes and sometimes concentrations of oxygen and carbon dioxide in the outlet gas can be measured using a variety of gas analysis equipment [Gnoth et al., 2008].

Temperature and pH

Microbial metabolism requires uptake of certain compounds and release of others, which changes the pH of the broth. Typically, broth pH is decreased due to release of weak acids from the microorganisms. Similarly, the reactions of metabolism generate heat, leading to an increased broth temperature. A certain microorganism has a rather small pH and temperature span within which it can sustain efficient growth and production and it is therefore important to monitor these parameters, which can be done on-line using electrodes. Acid or base is added to compensate for changes in pH while excess heat is cooled off through a cooling jacket on the outside of the fermentor and/or cooling coils inside the fermentor, through which cold water is run.

Fermentation medium

Microbial growth requires a number of different compounds to be available for uptake by the microorganisms. The substrate must supply carbon, nitrogen and many other compounds, as well as compounds from which the organisms can extract energy.

Fermentation media are commonly separated into two types, defined and complex. Defined media consist of certain chemicals in known amounts,

meaning that its exact composition is known. Complex media consist of raw materials whose exact composition is not known, such as molasses or soy meal, and where the composition can vary from one batch of substrate to another.

Carbohydrates are excellent sources of carbon, which also act as energy sources and are typically the main substrate, while nitrogen can be supplied through base addition if ammonia (NH_3) is used.

On-line measurement of the main substrate in a fermentor has been shown possible in academic applications [Brooks et al., 1988; Kleman et al., 1991], but this is considered difficult in industrial applications due to robustness problems [Johnston et al., 2002]. In addition, due to the multitude of carbohydrates present in complex media used in industry, measurement of the main substrate may not be sufficient to measure the total availability of carbohydrates in the medium.

2.4 Substrate addition in fed-batch fermentations

A fermentation can be performed in one of three different modes:

- **Batch mode**, where all substrate used in the fermentation is present in the starting medium and no addition of substrate occurs during the fermentation.
- **Fed-batch mode**, where feed containing one or several substrates is added throughout the fermentation.
- **Continuous mode**, where feed is added and fermentation medium withdrawn throughout the fermentation.

Industrial fermentations are commonly performed in fed-batch mode. This has the advantage over batch fermentations in that it allows control of microbial growth through the rate of feed addition to the process, while being easier to operate than continuous fermentations. By supplying the right amounts of substrate, fast growth and production can be maintained while avoiding production of undesired by-products due to overflow metabolism.

A fed-batch fermentation can be divided into two phases. In the first phase, the amount of biomass and its corresponding feed demand determines the desired feed rate. In this phase as fast microbial growth as possible is desired; after a short period at the start of the phase where the microorganisms adjust to the medium in the fermentor, they can grow exponentially. Therefore, this phase can be referred to as the phase of exponential growth.

In the second phase, microbial growth must be constrained due to transport limitations in the fermentor. Typically, either the oxygen transport capacity is not sufficient to supply a larger biomass with enough oxygen, or

the heat transport capacity is not sufficient to cool off a larger biomass in order to avoid an increase in temperature. This phase commonly coincides with induction in cases where it is used.

2.5 Sensing and feed rate control in fed-batch fermentations

The main control challenge in many fed-batch fermentations is how to control the feed rate to achieve a desirable substrate uptake rate during the exponential growth phase (see section 2.1). As described in section 2.3, many other parameters such as pH and temperature must be controlled but these can easily be measured on-line. In many industrial fermentations the possibilities for on-line measurement of substrate and biomass concentrations are severely limited [Gnoth et al., 2008], meaning that the control problem is mainly an issue of sensing. The control issue is further complicated as the total oxidative capacity of the microbial culture is not constant but rather grows with the biomass, i.e. exponentially. Some approaches to feed rate control, often described in literature and/or employed in practice, are outlined here.

Open-loop control

Based on prior knowledge of the process, feed addition during the exponential growth phase can be pre-programmed as a function of time. It is the simplest possible approach from a control perspective, but can still require extensive studies of the process. This approach does not allow for taking process variations into account, which is a significant disadvantage as fermentation processes can have batch-to-batch variations due to for instance the quality of inoculum (initial biomass) and complex medium [O'Connor et al., 1992]. Although its disadvantages are well known it is used due to its simplicity coupled with the difficulties in sensing for on-line feedback methods.

Mechanistic model-based methods

More or less advanced mechanistic models of the fermentor and the metabolism of the microorganism can be used to facilitate control. Mechanistic indicates that the model seeks to emulate the system by use of the laws and relations which have been derived to describe its individual components.

When the whole system model is known and values of the controlled parameters are assumed to be measurable, control can be formulated as an optimization problem as has been done for various fed-batch processes [Banga et al., 2003]. Methods of this type can give good results in simulations [Smets and Van Impe, 2002], but their usefulness in industrial applications is typically limited [Gnoth et al., 2008]. The model predictive control

(MPC) framework is one means which has been suggested for controlling fermentations using models of this type [Ashoori et al., 2009].

As measurement of all important states is generally not possible in bioprocesses, mechanistic models can be used in observers for estimation of unknown states from measurements which in turn allows control of these states. This is often referred to as "soft sensing" as it combines sensors with a software model for estimation. This approach can be used for estimation of a large number of states, depending on which measurements are available [Luttmann et al., 2012], and such methods based on mechanistic models have been successfully implemented for estimation of key variables in fed-batch fermentation processes [Chéruy, 1997; Sundström and Enfors, 2008], as well as in industrial scale batch processes [Golobič et al., 2000]. When good estimates of variables such as concentrations of biomass, substrates and products are available, the control problem becomes much simpler.

Data-driven model-based methods

This approach utilizes historical process data to derive data-driven models and control laws. This means that in difference to mechanistic modelling, models are of black-box type and not based on known principles. Artificial neural networks (ANNs), which can be used to provide nonlinear models describing the relationship between inputs and outputs [Lennox et al., 2001], is a data-driven method which has received much focus in regard to fermentation control [Lee et al., 1999]. Many different variants of this method have been suggested [Azlan Hussain, 1999; Tian et al., 2002; Xiong and Zhang, 2004], some of which have been successfully implemented for estimation of unmeasurable states in fermentations with defined carbon sources [Valdez-Castro et al., 2003; Gnoth et al., 2008], but are naturally limited to processes for which a high amount of historical data exists [Gnoth et al., 2008].

Data-driven models can be used for soft sensing in fermentations in the same way as mechanistic models [Luttmann et al., 2012]. Similarly, they too can be used in model predictive control and it has been suggested how this can be done in fermentation processes [Kiran and Jana, 2009].

A development of artificial neural networks is hybrid modelling, wherein artificial neural networks are combined with known fundamental mechanistic relationships. The aim of this is to achieve the advantage of artificial neural networks in not requiring a mechanistic model, while decreasing its disadvantage in requiring high amounts of historical data for model training. This method has also been successfully implemented for estimation of key variables in fed-batch fermentations. [Gnoth et al., 2008]

Extremum-seeking methods

Methods of this type are based on the use of variations in one or more input signals to the process (typically the feed rate in the case of fermentation control). The response to these perturbations in the measured signals is used to determine the current state of the system and the appropriate control action to take. [Dewasme et al., 2011]

Purely perturbation-based extremum seeking methods exist, which assume no system model, however these require direct measurement of all parameters which are to be controlled. Although this is the case in certain biotechnical applications [Dochain et al., 2011; Trollberg and Jacobsen, 2012], it is not so in the case of growth control in fed-batch fermentations.

Other methods use perturbations to achieve excitation of the system and assume some process model which allows estimation of states which are not measured directly. A number of different methods of this type exist, utilizing different perturbation types and control laws [Åkesson, 1999; Åkesson and Hagander, 2000; de Maré, 2006; Henes and Sonnleitner, 2007; Dewasme et al., 2009; Dewasme et al., 2011; Vargas et al., 2012; Johnsson et al., 2013].

These methods also differ in regard to which measurements they utilize for estimation. Some utilize only measurement of dissolved oxygen in the broth [Åkesson, 1999; Åkesson and Hagander, 2000; de Maré, 2006; Henes and Sonnleitner, 2007; Johnsson et al., 2013] while others utilize gas flow measurements [Vargas et al., 2012] and yet others utilize measurements of biomass and substrate concentrations [Dewasme et al., 2009; Dewasme et al., 2011]. Naturally, utilization of a higher number of measurements enables better control but requires fast and robust on-line probes for such measurements if they are to be implemented successfully. Several different extremum-seeking methods have been successfully implemented in fed-batch fermentation processes [Åkesson, 1999; Åkesson and Hagander, 2000; de Maré, 2006; Henes and Sonnleitner, 2007; Johnsson et al., 2013].

require only standard measurements for which fast and reliable probes are commonly available in industrial fermentations [Åkesson, 1999; Henes and Sonnleitner, 2007] while others require more advanced measurements for which such probes are often not available [Dewasme et al., 2011; Vargas et al., 2012].

3

Publications

This licentiate thesis is based on two papers. They are listed below with a description of their contributions to the field and the roles of their authors in each paper.

Paper I

Johnsson, O., J. Andersson, G. Lidén, C. Johnsson, and T. Hägglund (2013). "Feed rate control in fed-batch fermentations based on frequency content analysis". *Biotechnology progress* **29**:3, pp. 817–824.

This study presents a new extremum-seeking strategy for controlling aerated fed-batch fermentations and shows its implementation in pilot scale. The strategy is based on the same basic principles as previously known control strategies (probing control) but expands the concept with continuous on-line tracking of feed demand and by not requiring rectangular-shaped pulses.

The author came up with the idea for the strategy, wrote the software for its implementation, performed the experimental work for evaluating it and was the main author of the manuscript. J. Andersson, G. Lidén, C. Johnsson and T. Hägglund discussed the strategy and experimental results with the author and assisted in writing the manuscript. J. Andersson also provided technical input regarding the processes in which the strategy was implemented.

Paper II

Johnsson, O., J. Andersson, G. Lidén, C. Johnsson, and T. Hägglund. "Modelling of the oxygen level response to feed rate perturbations in an industrial scale fermentation process". Submitted to *Journal of Biotechnology*.

In this study, modelling of feed and oxygen dynamics in an industrial production-scale fermentation process was performed, based on experiments performed in the process. Results showed the feasibility of extremum-seeking

control strategies in the process and that for certain perturbation frequencies, a simple model can be used to describe the dynamics of the process.

The author designed the experiments performed in the study, performed the analysis of resulting data and modelling of the process and was the main author of the manuscript. J. Andersson, G. Lidén, C. Johnsson and T. Hägglund discussed the experiment design, results and modelling with the author and assisted in writing the manuscript.

Additional publications

Other related publications by the author are listed below.

Johnsson, O., J. Andersson, and C. Johnsson (2011). "Probing control in B. licheniformis fermentations". In: *Proc. 18th World Congress of the Int. Federation of Automatic Control (IFAC). Milano, Italy*, pp. 7132–7137.

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This section contains the bibliography for chapters 1 and 2. Bibliographies of the included papers are found at the end of their respective re-print.

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Paper I

Feed rate control in fed-batch fermentations based on frequency content analysis

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Abstract

A new strategy for controlling substrate feed in the exponential growth phase of aerated fed-batch fermentations is presented. The challenge in this phase is typically to maximize specific growth rate while avoiding the accumulation of overflow metabolites which can occur at high substrate feed rates. In the new strategy, regular perturbations to the feed rate are applied and the proximity to overflow metabolism is continuously assessed from the frequency spectrum of the dissolved oxygen signal. The power spectral density for the frequency of the external perturbations is used as a control variable in a controller to regulate the substrate feed. The strategy was implemented in an industrial pilot scale fermentation set up and calibrated and verified using an amylase producing *Bacillus licheniformis* strain. It was shown that a higher biomass yield could be obtained without excessive accumulation of harmful overflow metabolites. The general applicability of the strategy was further demonstrated by implementing the controller in another process utilizing a *Bacillus licheniformis* strain currently used in industrial production processes. Also in this case a higher growth rate and decreased accumulation of overflow metabolites in the exponential growth phase was achieved in comparison to the reference controller.

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1. Introduction

Many production microorganisms in the biotechnology industry show overflow metabolism, i.e. the excretion of metabolic by-products, such as ethanol, acetate and lactate, at high substrate uptake rates. [Tempest and Neijssel, 1979] This is well-known for the commonly used bacterium Escherichia coli [Neijssel et al., 1980] and the yeast Saccharomyces cerevisiae. [Sonnleitner and Käppeli, 1986] but also for the bacterium Bacillus licheniformis which is a host organism for e.g. amylase production. [Voigt et al., 2004] Overflow metabolism allows for increased short-term growth, but is undesirable in many industrial processes. [El-Mansi, 2004] The by-products not only decrease the yield directly by diverting substrate but furthermore often influence the process negatively by inhibition effects caused by the by-products; a well-known example is the inhibitory effects on growth caused by too high concentrations of acetic acid. [Luli and Strohl, 1990] These compounds can not only inhibit growth but also reduce the production of heterologous proteins. [Jensen and Carlsen, 1990] Each of these effects may severely impact the productivity of fed-batch fermentations.

In fed-batch production processes it is therefore important to maintain a feed rate low enough to avoid overflow metabolism. At the same time, as high feed rate as possible is desired to obtain a high volumetric productivity. The challenge in feed rate control is thus to find a feed rate which gives as high growth rate as possible, while avoiding the saturation in oxidative capacity or other rate limiting steps in the respiratory metabolism.

The maximum desirable feed rate is determined by overflow metabolism only in the first phase of the fed-batch cultivation, i.e. before oxygen or heat transfer limitations set in. This first phase will here be referred to as the exponential growth phase. At sufficiently high biomass concentration, the oxygen demand of the microorganisms for maintaining exponential growth at the desired specific growth rate will exceed the oxygen transfer capacity of the fermentor system. From this point and onwards, the maximum allowable substrate feed rate will simply be determined by the maximum volumetric oxygen transfer rate. It deserves to be pointed out that although the overflow metabolism problem is mostly relevant in the exponential growth phase, this phase is highly critical for the entire process. Overflow metabolism - or carbon starvation - in the exponential growth phase can have a severe inhibiting effect on cell growth and product formation and can even cause a complete process failure in the latter phase of the process.

In practice, it is difficult to obtain an optimal feed rate for three reasons. The first reason is that the optimal feed rate depends on the total amount of biomass, which increases exponentially, meaning that the rate spans a large range. Secondly, the concentrations of biomass, substrate and overflow metabolite can be difficult to measure in-situ. Finally, even if the biomass

concentration is precisely monitored, its maximum oxidative capacity can vary due to metabolic shifts. [Swartz, 1996]

Observers (software incorporating a model of the system to make estimates of its state from various measurements) can be developed to estimate these concentrations, but require a well-tuned model and typically require some measurements for which good in situ probes are not widely used today such as substrate concentration and/or assumptions of constant yield parameters. [Chéruy, 1997; Selişteanu et al., 2008; Dewasme and Vande Wouwer, 2008; Riesenberg and Guthke, 1999; Gnoth et al., 2008] A further complication is that industrial processes typically utilize complex media containing several carbon sources, meaning that even if the concentration of the principal carbon source could be measured reliably, it would not necessarily be sufficient to predict when overflow metabolism occurs due to the presence of additional carbon sources. Another option for obtaining good process control is to use artificial neural networks (ANN) and similar methods. These are popular in some implementations, but have the disadvantage that they require large amounts of historical data from the process in order to be useful [Riesenberg and Guthke, 1999; Gnoth et al., 2008].

An alternative strategy, which circumvents these problems, is to instead measure the system response to variations in the feed rate, a so-called "probing control" approach. [Åkesson, 1999] A suitable response variable is the dissolved oxygen saturation (DO) in the fermentation broth, as consumption of oxygen is directly coupled to the occurrence of overflow metabolism. Robust, fast and precise probes for measuring dissolved oxygen are routinely used in the fermentation industry, making this a highly suitable measurement upon which to base feed rate control.

The DO response to perturbations in the feed rate has been used for control purposes by [Åkesson, 1999], and - with some modifications - by e.g. [Whiffin et al., 2004], [Velut, 2005], [de Maré, 2006] and [Henes and Sonnleitner, 2007]. Common for all of these strategies is that tracking of the optimal feed rate is not continuous. Instead, singular perturbations are performed and after such a perturbation, the DO response is evaluated and the feed rate changed at a discrete point in time. After a certain delay, during which DO values are not used to track the optimal feed rate, a new perturbation is performed and the process is repeated. This typically gives a piecewise linear feed rate trend.

In the current work, a different method of analysis of the response to external feed rate perturbations is used. The frequency spectrum of the DO signal is calculated and the power spectral density for frequencies close to that of the externally applied disturbances is used to derive a control variable upon which to base a regulator. This allows continuous tracking of the optimal feed rate and avoids the risk inherent in probing control that a disturbance in DO during a pulse will have a significant effect on the feed rate. The method has

	Symbol	Unit
Controller variables		
Dissolved oxygen saturation	DO	%
Content of relevant frequency	C	Power units
Relevant frequency of perturbations	ω_{rel}	Hz
Feed rate	F	L/h
Controller parameters		
Sampling frequency	h	Hz
Filter length	m	Sampling points
Frequency band width	d	Hz
Controller gain	K	L/h/power units
Controller integral time	T_i	s
Off-line measurements		
Substrate concentration	s	g/L
Acetate concentration	A	g/L
Biomass concentration	X	g/L

Table 1. Nomenclature.

been implemented, tuned and demonstrated in pilot scale (0.550 m^3) fed-batch production of amylase using a model strain of Bacillus licheniformis as well as in a second proprietary fermentation process. The fermentations were characterized in terms of overflow metabolites, biomass yield and volumetric productivities.

2. Theoretical aspects

2.1 Nomenclature

Throughout this work, a number of different parameters are used to describe the system and the controller. Their descriptions and units can be found in table 1.

2.2 Basic principles of the controller

The dynamics from the input signal (feed rate) to output signal (DO measurement) mainly depend on five processes: substrate delivery, substrate mixing, microbial uptake of substrate and oxygen, oxygen mixing and oxygen measurement. Mixing can be described using first order dynamics, and the dynamics of substrate delivery and oxygen measurement can be included in this process. The kinetics of the oxidative metabolism in an aerobic environment is well described by a saturation kinetics model, as the oxygen uptake rate q_o is determined by and stoichiometrically coupled to the substrate uptake rate q_s - as long as the maximum oxidative capacity of the cell is not exceeded. The substrate uptake rate at which the maximum q_o is exactly reached at fully respiratory conditions is referred to as $q_{s,crit}$. This is illus-

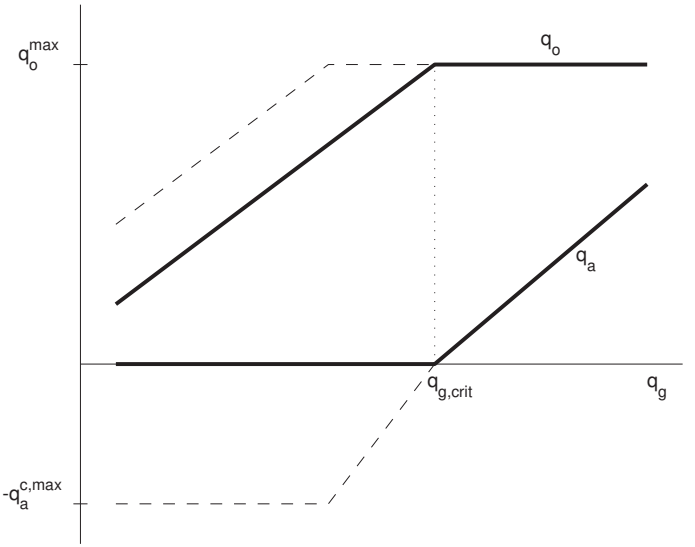

Figure 1. The relation between glucose uptake rate and the rates of oxygen uptake and acetate production, showing the limitation of oxidative metabolism at $q_{g,crit}$ which is marked with a dotted line. Dashed lines indicate uptake of acetate, which can only occur if acetate is present and the glucose uptake rate sufficiently low. [Åkesson, 1999]

trated in figure 1 for a case in which glucose is the limiting substrate and acetate the overflow metabolite, as in many *B. licheniformis* processes.

As perturbations to the feed rate occur the substrate concentration in the broth will change, which in turn will lead to a change in substrate uptake rate. This will lead to one of three different outcomes:

1. q_s is at all points above $q_{s,crit}$. As the oxidative capacity is limiting, no change in q_o will be observed.

2. q_s crosses $q_{s,crit}$ at least once during the perturbation. A change in q_o will occur, but will be limited by the saturation.

3. q_s is at all points below $q_{s,crit}$. The saturation will have no influence on the outcome and the change in q_o will be the highest possible (limited only by the amplitude of the perturbation).

For outcome 2, the size of the change in q_o is related to the distance from q_s to $q_{s,crit}$. For outcomes 1 and 3, it is possible to determine which of these that has occurred, giving the sign and minimum value of the distance from q_s to $q_{s,crit}$. The principle behind the control strategy is that perturbations to the feed rate can be repeated with a known time interval (i.e. the signal has a known, constant frequency). The output signal will have a different shape than the input signal, but its frequency will be the same.

Rather than using the *DO* signal directly to determine the effect of the perturbations, as done in the probing control strategy [Åkesson, 1999], the frequency spectrum of the *DO* signal is here obtained through Fourier transformation and studied. As noise in the *DO* signal typically occurs in a different frequency band than that of the perturbations, these are easily separated and it becomes possible to get a direct measure of the influence of the perturbations. A larger content of the relevant frequency indicates that the average value of q_s over time is below $q_{s,crit}$, while a smaller content indicates that q_s is on average above $q_{s,crit}$. This means that a controller provided with a suitable setpoint for the content of the relevant frequency can be used to keep q_s at $q_{s,crit}$ using the feed rate as the manipulated variable.

3. Controller details

The content of a certain frequency in a signal can be described by its power spectral density (PSD) To dampen the effect of minor process disturbances, the summed PSD of a small band of frequencies around the relevant frequency was used rather than that of one specific frequency (cf. figure 2). Furthermore, to reduce noise this value was filtered using a very simple filter giving the average value over a predefined interval of sampling points as the output. To achieve a good measurement of the system's response to perturbations, the sampling points used by the filter should cover at least one period of the perturbation and preferably more, this is however a tradeoff against the speed of the controller as including more data points will make the filter respond more slowly to changes in the process. Equation (1), where PSD_ω denotes the power spectral density of a frequency ω and d is the predefined size of the frequency band around the relevant frequency ω_{rel}, shows the calculation of the frequency content C at the current sampling point k, filtered over m points. Using the variable C to determine the status of the process will be referred to as frequency content analysis (FCA).

$$C_k = \frac{1}{m} \sum_{i=k-m}^{k} \left(\sum_{\omega=\omega_{rel_i}-d/2}^{\omega_{rel_i}+d/2} PSD_{\omega_i} \right) \tag{1}$$

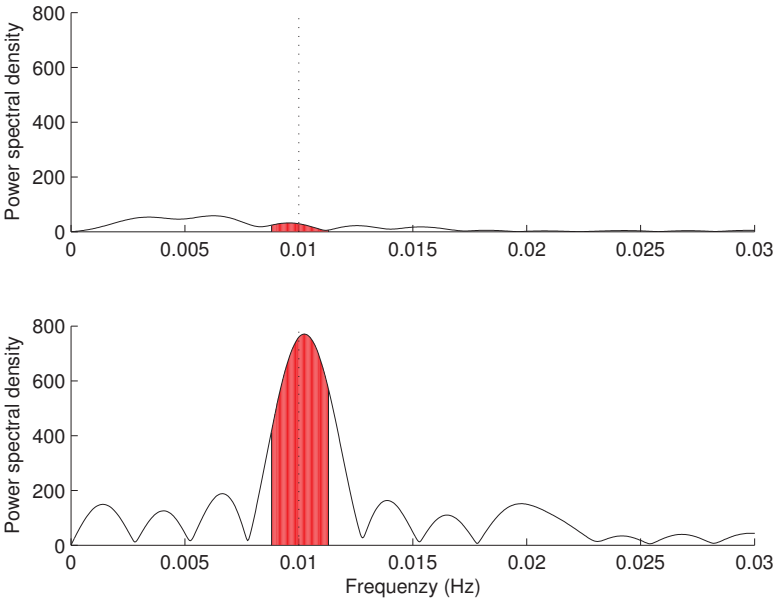

Figure 2. Frequency spectra of the *DO* signal. The upper diagram is from a point in time when perturbations have little influence on *DO*, while the lower diagram is from a point where perturbations have large influence. *C*, the summed content of frequencies in a band around the frequency of the perturbations in the input (in this case 0.01 Hz, marked with a dotted line), is shown in red.

A small value of C indicates that the system is close to the saturation caused by oxidative limitation whereas a large value indicates that the system is far below this level (cf. figure 1) and therefore, in order to control the feed rate of the process using FCA it is natural to employ proportional feedback control. Such control is typically coupled with integral control in order to avoid static errors. In this implementation the microbial consumption of feed can be considered as a disturbance on the system and as this disturbance follows microbial growth, i.e. increases exponentially, an integrating controller will not be sufficient to avoid static errors. This was not considered a major problem as the true aim of the controller was not to follow a setpoint for C but to increase the feed rate to keep up with the demands of the exponentially growing consumption without excessive overflow metabolism. Nonetheless, to partially counteract the exponentially growing consumption, an integral term was added. The main solution used to solve this problem

was however to add gain scheduling to the controller.

Equation (2) shows how the feed rate, F, is changed based on C and C_{sp}. Its form is similar to that of a discrete-time PI controller with sampling rate h, gain K and integral time T_i. A gain scheduling factor, here chosen as in equation (3), is necessary as the dynamics of the process change with increasing volume and biomass concentration.

$$F_k = \frac{K}{h}\left((C_{sp} - C_k) + \frac{h}{T_i}\sum_{1\leq j\leq k}(C_{sp} - C_j)\right) \tag{2}$$

$$K = K_0\sqrt{\frac{F_{k-1}}{F_{min}}} \tag{3}$$

To avoid dissolved oxygen limitation while also avoiding an unnecessarily high agitator speed (AG) or aeration rate (AR) a parallel control system is run controlling DO (using a setpoint of 20 %) using AG and AR as manipulated variables. A PI controller using gain scheduling from the AG value is used to control AG, when it reaches its maximum value the PI controller switches to controlling AR instead. In difference to the original probing control scheme, the DO controller and the feed rate controller are active at the same time. Instead, the parameters of the DO controller are set so that it is comparatively slow and will not significantly influence perturbations in DO at the relevant frequency. This is possible as the feed rate controller based on FCA does not require strict adherence to the baseline defined by the DO setpoint. The FCA controller is only used until the maximum capacity of the fermentor (oxygen transfer, pH regulation and/or cooling capacity) is reached. At this point the exponential growth phase is over and overflow metabolism no longer a major issue.

4. Material and methods

4.1 Fermentation equipment

A number of 0.550 m^3 pilot scale fermentors (0.350 m^3 fill volume) described in [Albæk et al., 2008] were used. The feed system of each fermentor is discontinuous, with pulses of feed liquid being injected into the fermentor at a rate and with a volume intended to achieve the same total feed volume as the integral of the feed rate setpoint curve. While this makes it practically impossible to implement a sine wave perturbation on the feed rate, which would be the most natural thing to do for frequency response analysis, the feed pulses themselves can be seen as regular perturbations if they are performed with a constant (or near-constant) time interval.

The control system for feed addition in allows variation in the time between each feed pulse. As outlined in Theoretical aspects, the controller can

handle some variation in frequency but in order to avoid too large variations a minimum time of 100 s between pulses is enforced.

4.2 Strain and growth conditions

In all experiments except where noted otherwise, the recombinant amylase-producing B. licheniformis strain SJ4628, derived from DN286 [Fleming et al., 1995], was used. This is an older, low-yielding industrial strain developed by Novozymes A/S. In one series of experiments a newer, high-yielding strain currently used by Novozymes A/S was used to study whether the strategy could easily be transferred to processes using other strains.

For propagation, frozen bacteria were grown on agar plates and then used to inoculate a seed fermentor of the same type as the main fermentors, using 300 kg of a medium containing: soy meal, 110 g/kg; $Na_2HPO_4{\cdot}2H_2O$, 5 g/kg; antifoam agent (Pluronic/Dowfax 63 N10), 1.67 ml/kg. The seed fermentor was run for 16 h at 37° C, pH 7 (± 0.2), using linear ramps for agitator speed and aeration rate starting at 125 rpm and 180 l/min and reaching 375 rpm and 300 l/min at 10 h after which these were constant.

Except where noted otherwise, 33 kg broth from the seed fermentor was used to inoculate the main fermentors. In fermentations utilizing the SJ4628 strain, 300 kg of a defined medium was used and the fermentors were fed with 64 % w/w glucose. The medium contained: K_2HPO_4, 7 g/kg; $Na_2HPO_4{\cdot}2H_2O$, 7 g/kg; K_2SO_4, 5 g/kg; $MgSO_4{\cdot}7H_2O$, 4 g/kg; $(NH_4)_2SO_4$, 4 g/kg; citric acid, 0.78 g/kg; $CaCO_3$, 1 g/kg; trace metal mix, 0.5 g/kg. The trace metal mix contained 16 % w/w $MnSO_4{\cdot}H_2O$, 63 % w/w $FeSO_4{\cdot}7H_2O$, 7 % w/w $CuSO_4{\cdot}5H_2O$, 14 % w/w $ZnSO_4{\cdot}7H_2O$. In fermentations utilizing the proprietary strain, a complex, proprietary medium was used and the fermentors were fed with 64 % sucrose.

4.3 Software implementation

The fermentors were controlled using the commercial DeltaV process control system, which allowed on-line measurement of dissolved oxygen saturation (DO), pH, temperature (T) and the concentration of oxygen and carbon dioxide in the outlet gas, allowing for calculation of oxygen uptake rate (OUR) and carbon dioxide emission rate (CER). The script used to control the fermentors was developed in MATLAB and run on a separate computer, using an MX OPC server to allow it to write setpoints to and read measured data from the DeltaV system. The controller was used to control the feed rate (F), the aeration rate (AR) and the agitator speed (AG) of the processes. PSD of the frequencies in the DO signal was calculated using the Fast Fourier Transform (FFT) function in MATLAB. 2000-point FFT was used over an interval of 100 datapoints, to increase smoothness of the calculated frequency spectrum.

For all experiments, the following parameters were used: $h = 0.5$ Hz, $m = 100$ sampling points, $d = 0.0025$ Hz. The value of m was chosen so that the filter would include two periods of the perturbation; this was considered a suitable tradeoff between noise rejection and speed. ω_{rel} varied but was in general between 0.009 and 0.01 Hz, the reason for this being that the feeding mechanism used in the fermentors only allowed poor following of its desired value which was 0.01 Hz.

4.4 Experiment design

Experiments were performed with the purpose of regulator tuning, evaluation of the process control strategy and testing its generality as described below. Where applicable, comparisons to a reference controller currently used in industrial fermentations were made; the reference controller utilized a PI design regulating DO by manipulating F, with predefined ramps limiting the maximum and minimum values of F during the exponential growth phase.

Regulator tuning Analysis of data from previous fermentations indicated that a C_{sp} of 400 would be suitable to achieve fast growth without excessive production of overflow metabolites. Three fermentations were performed using a similar setup with C_{sp} set to 400, 500 and 600 respectively. During the exponential growth phase, the parameters K and T_i were tuned manually to achieve smooth, yet fast, control of the feed rate.

Evaluation of the strategy To evaluate the strategy, three fermentations using a standard setup were performed; two utilizing the FCA feeding strategy and one reference, using a control system currently implemented in industrial processes of this type. A similar evaluation, with one fermentation utilizing FCA control and one reference fermentation, was also performed in a setup where the inoculation volume was 1/8 of its normal value to simulate a situation where the viable cells in the inoculation volume are fewer or the lag phase longer than expected. This can normally cause severe inhibition of cells due to excessive accumulation of acetate. In a final fermentation, large pulses of feed were added at certain points in time via a separate feeding system to study the controller's response to accumulated glucose.

Test of generality The FCA strategy was tested on a strain currently used in industrial production processes rather than the strain used for developing the strategy. Two fermentors employed the FCA strategy and a third was used as a reference. The aim was to test whether the control strategy could easily be transferred to such processes and give the same performance as in processes using SJ4628, and to assess its performance in a process using complex medium.

4.5 Off-line measurements

Off-line measurements of substrate and acetic acid were made using enzymatic kits: sucrose by "Sucrose Assay Kit", Sigma, Product code SCA-20; glucose by "D-Glucose", R-biopharm, Cat. No. 10 716 251 035; acetate by "Acetic acid", R-biopharm, Cat. No. 10 148 261 035. Cell concentration was calculated through measurement of cell dry mass, performed as follows. 7-10 g cell broth was poured in a pre-dried, weighed test tube and total weight was noted. The remaining volume of the tube was filled with distilled water, it was centrifuged at 2800 rpm for 20 min, the pellet was rinsed with distilled water and centrifuged again at 2800 rpm for 20 min after which the pellet was dried in an oven overnight. Cell dry weight concentration was defined as the weight of the pellet divided by the weight of the broth. Three samples were made for each measurement point in each fermentation. Dry weight measurements were not carried out for two hours after inoculation as non-bacterial solid matter from the inoculation volume would distort measurements greatly, an effect which may also be present to a smaller extent in measurements from two hours and forward.

5. Results and discussion

5.1 Regulator tuning

Over the first 3.5 hours of the fermentations, manual tuning of the controller took place in order to give a smooth trajectory of F (cf. first part of figure 3). Based on the shape of this feed rate curve, tuning was considered successful. Glucose and acetate levels were low in all fermentations as seen in figure 3, indicating that values of C_{sp} in the chosen interval (400 - 600) would not give excessive overflow metabolism. For future experiments a C_{sp} value of 400 was therefore chosen, as higher values would give more conservative feed profiles. In the fermentation labelled FCA 1:2 volumetric growth of biomass was significantly slower than in the others, with a very long lag phase after inoculation. This does however not influence the conclusions drawn from this experiment.

5.2 Evaluation of the strategy

The two FCA-controlled fermentations gave very similar results, with almost identical feed rate curves as can be seen in figure 4, showing that the FCA strategy provides good repeatability of fermentations. A comparison between these two and the reference showed that while neither gives any major acetate accumulation, the increase in biomass concentration from 2 to 10 h was 33 % respectively 24 % higher (cell dry weight) in the FCA-controlled fermentations compared to the reference, whereas none of the fermentations exhibit

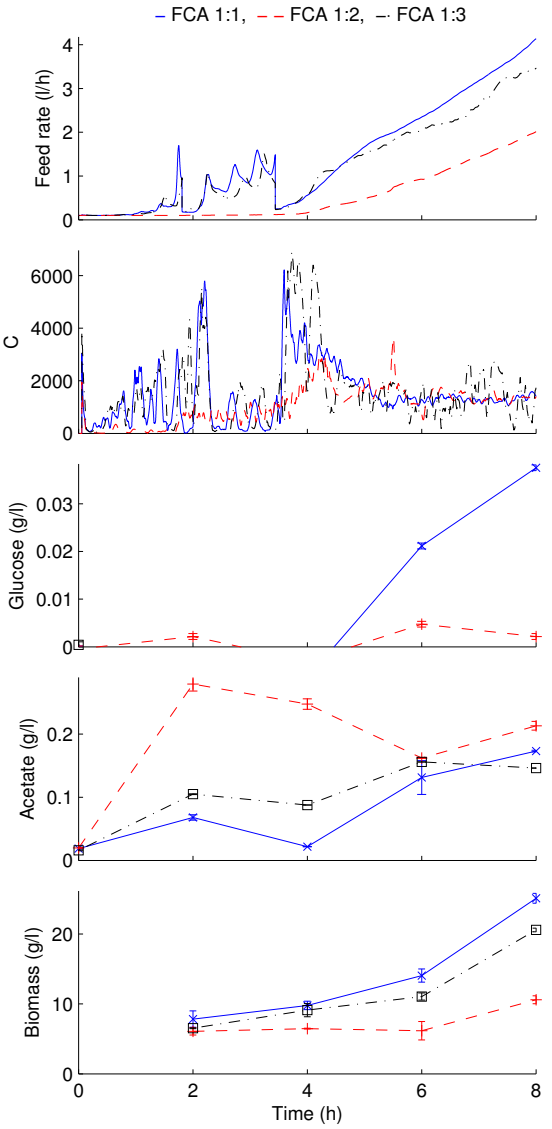

Figure 3. Exponential growth phase of fermentations FCA 1:1-1:3, all of which were controlled using the FCA controller using different setpoints. From 0 to 3.5 h tuning of the feed rate controller was performed; after 3.5 h tuning was considered successful based on the behaviour of the feed rate curve after this point.

excessive production of acetate (cf. figure 4). It is important to note that the controller is not designed with the purpose of C to reach its reference value, rather it is the difference between these which drives the increase in F.

In the setup using 1/8 of the normal inoculation volume, the FCA-controlled fermentation showed a considerably lower slope of the feed rate than that of the reference initially (cf. figure 5). Acetate accumulation in the FCA-controlled fermentation was higher than in the standard setup but was kept below levels considered harmful, this was due to the uptake rate of acetate in this organism being low and C_{sp} not being perfectly adjusted to the process. In the reference fermentation high concentrations of acetate (>1 g/l) were accumulated (cf. figure 5). Although initial biomass was higher in the reference, the increase in biomass concentration was significantly slower than in the FCA-controlled fermentation towards the end of the exponential growth phase when acetate levels were high, as can be seen in figure 5. This indicates that growth was inhibited by acetate; at 18 h after inoculation, this caused complete process failure in the reference fermentation. This shows that while the standard controller cannot respond to overflow metabolism, the FCA controller can do so and avoid excessive accumulation of overflow metabolites caused by it, thereby avoiding process failure.

In the fermentation where large feed pulses were added externally, the controller responded by decreasing the feed rate within 5 minutes after an external feed pulse, showing that it can rapidly detect the onset of overflow metabolism (cf. figure 6). After the feed added by the pulse was consumed, the feed rate increased again without oscillations. It returned to a value close to where, tracing an exponential growth profile, it would be had the pulse not been added and continued following an exponential profile. This shows that the controller can also rapidly detect that overflow metabolism has ceased and switch back to increasing the feed rate at a pace suitable for keeping up with the increasing biomass concentration.

Two of the feed additions in this fermentation occurred 30 minutes or less before an acetate measurement point (at 4 and 8 hours); these points show increased acetate levels but not harmfully so, despite the volume of extra feed added being very high compared to the feed rate. For instance, at 3.5 h 0.75 l was added while the feed rate was 1 l/h, i.e. the additional amount was equivalent to the total feed added during 45 minutes at that feed rate. The response to the pulse at 3.5 h, whose size corresponds to 45 minutes of feeding at the current feed rate, was over in less than 1 h indicating an efficient controller response.

Theoretically, the most efficient controller response would be to completely switch off the controlled feed as soon as extra feed was added and then immediately return it to levels corresponding to exponential growth when the extra feed had been consumed. This indicates that there is some room for improvement of the controller, so that the gain for negative changes

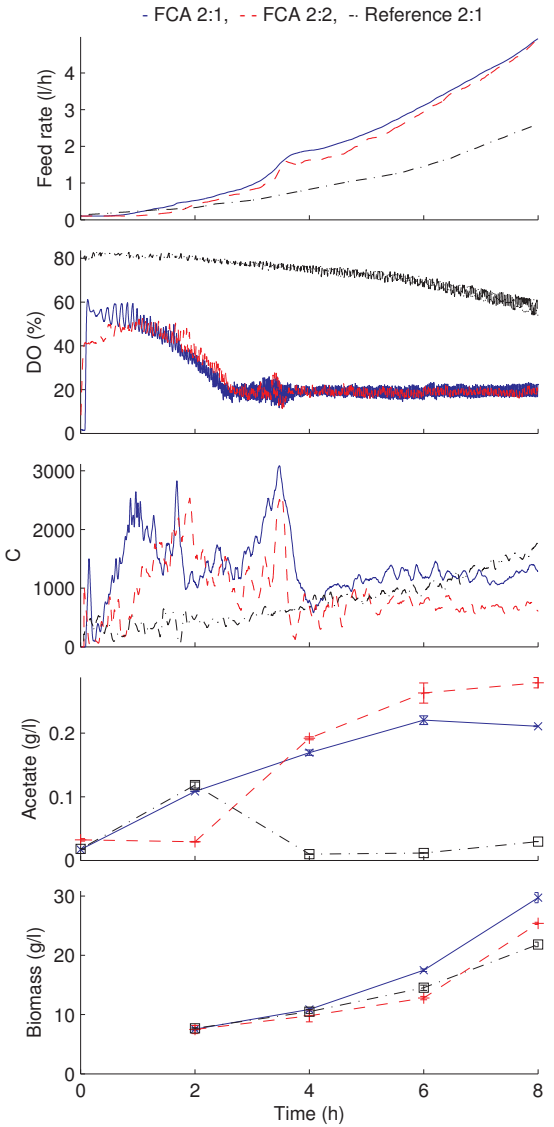

Figure 4. Fermentations FCA 2:1 and 2:2 were controlled using the FCA controller ($C_{sp} = 400$), while the fermentation titled Reference 2:1 used the reference control strategy to enable a comparison. Glucose was measured at the same points in time as acetate and was below the detection limit (0.8 mg/l) at all points.

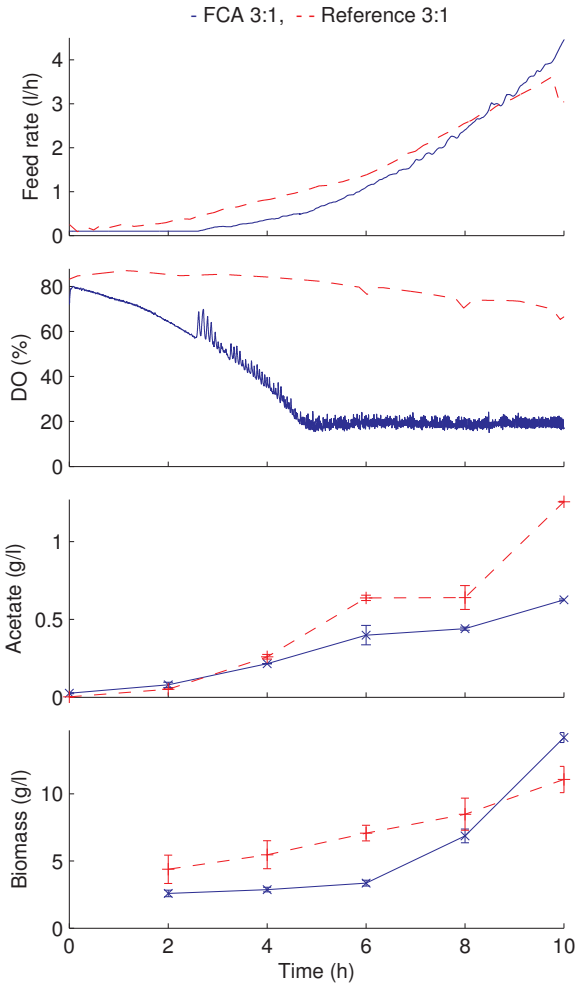

Figure 5. Controller behaviour in fermentations with decreased inoculation volume, simulating a situation where the viable cell concentration in the inoculum is lower than expected or the lag phase of the organisms is longer than expected. Fermentation FCA 3:1 was controlled using the FCA controller and Reference 3:1 by the reference strategy. In both fermentations, the inoculation volume was 1/8 of its normal value in order to challenge the FCA controller and enable a comparison to the reference during these conditions.

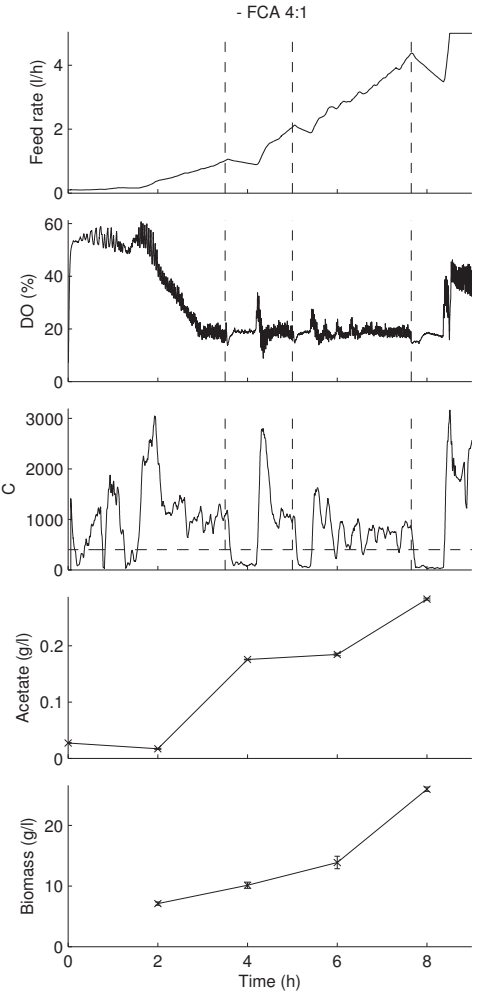

Figure 6. Study of the controller response to excess substrate. In fermentation FCA 4:1, external additions of feed were used to disturb the process in order to study the FCA controller's response to such disturbances. For C, a horizontal dashed line indicates the setpoint. Vertical dashed lines in the plots of F, DO and C indicate the times for external feed additions. After each external addition of feed, the fast dynamics in DO disappeared (seen as less "noise" in the DO signal), indicating that the regular feed pulses do not cause a response. Hence, C was lowered to a value well below its setpoint for a while and during this period F was decreased.

of F is higher. This can be accomplished using a nonlinear transformation of C, although finding the most suitable transformation would require extensive system identification and tuning.

5.3 Test of generality

In the first four hours of these fermentations, tuning of the feed rate controller proved necessary as the increase in biomass concentration was significantly faster than in previous fermentations. The FCA strategy could detect the presence of complex medium at the start of the fermentation, shown by very low values of C early in the fermentation, which ensures that F is kept at its minimum to minimize overflow metabolism (cf. figure 7).

Due to the complex medium containing carbon sources for the bacteria, some overflow metabolism is unavoidable and all fermentations show accumulation of low levels of acetate during the first 2 h after inoculation as seen in figure 7. However, the FCA-controlled fermentations moved away from overflow metabolism and showed decreased acetate levels sooner than the reference.

Volumetric productivity of biomass is higher in the fermentations using the FCA strategy (biomass concentration at the onset is about the same as in the reference but 30 % higher at 8 h). This, along with low acetate levels, shows that the oxidative capacity has been utilized efficiently while excessive overflow metabolism has been avoided.

6. Conclusions

In this study a new feeding strategy for aerated fed-batch fermentations has been presented, aimed at giving as high levels of oxidative metabolism as possible in the exponential growth phase while avoiding excessive overflow metabolism leading to undesirably high levels of by-products. Its basic principle is the same as for the pre-existing probing control strategy and has the advantage that only dissolved oxygen measurements are needed, but it improves this concept by use of frequency analysis. This allows continuous tracking of a feed rate corresponding to the substrate uptake rate and decreases the sensitivity to disturbances compared to probing control as disturbances occurring at most frequencies do not affect the measurements.

A simple feed rate controller based on this strategy has been tested in pilot scale fermentations (0.550 m^3), which has shown that it can achieve a significantly higher volumetric productivity of biomass than in a reference process while production of harmful by-products has been minor. For fermentations with a lower initial viable cell mass, it can detect the decreased feed demand and adjust the feed rate accordingly, where the reference controller (used today in industrial fermentations of this type) causes complete

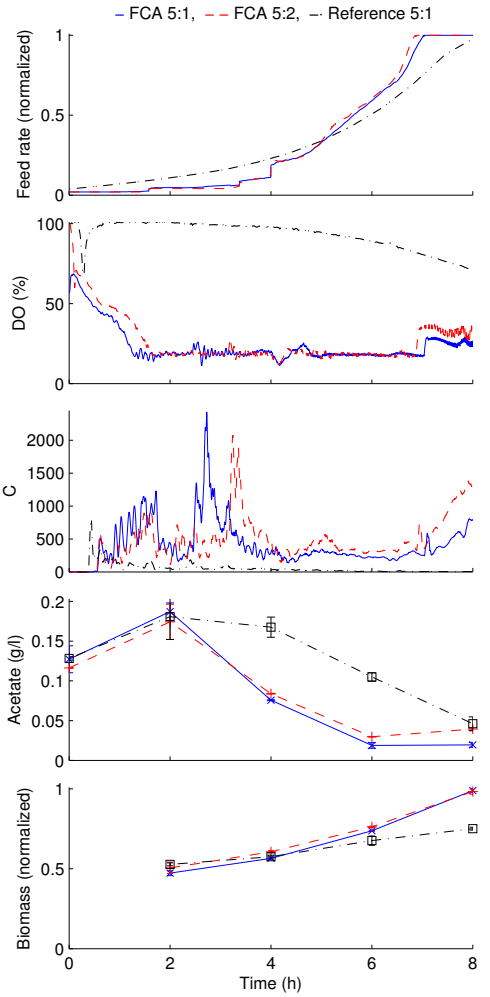

Figure 7. FCA control applied to a current production process scaled down to pilot scale. Fermentations FCA 5:1 and FCA 5:2 used the FCA strategy, while Reference 5:2 used the reference strategy. From 0 to 4 h re-tuning of the FCA controller was necessary in order to adjust it as the *B. licheniformis* strain used here was faster-growing than that which the controller was previously tuned for, which caused discrete changes to the feed rate. Re-tuning was not necessary in the reference fermentation as its parameters could be altered on beforehand to suit this process, using a priori knowledge. In the fermentations using the FCA controller, F reached its highest allowed level at 7 h; this limit was set so that saturation of the fermentor's oxygen and heat transport capacity was avoided.

process failure through excessive formation of overflow metabolites. The controller response to excess substrate in the medium has been tested and it has been shown that it can detect and compensate for this by temporarily decreasing the feed rate. While there is room for improvement of the controller by improved tuning and finding a suitable nonlinear transformation of the measured variable, the new concept as such has shown itself successful.

Although most practical testing has been carried out in a process using an old production strain from Novozymes A/S growing on defined medium, one series of experiments was carried out in a process utilizing an industrial strain currently used in large-scale enzyme production growing on complex medium to study the feasibility of the strategy in such processes. The strategy gave significantly higher growth of biomass in this process as well, although retuning of controller parameters was necessary as this strain is faster-growing than the old strain.

7. Acknowledgements

This work was carried out within the framework of the Process Industrial Centre at Lund University (PIC-LU) and supported by the Swedish Foundation for Strategic Research (SSF) and the Lund Centre for Control of Complex Engineering Systems (LCCC), in cooperation with the Novozymes A/S fermentation pilot plant in Bagsværd, Denmark where all fermentations were performed.

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Paper II

Modelling of the oxygen level response to feed rate perturbations in an industrial scale fermentation process

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Abstract

A study of the feasibility of perturbation-based control methods in industrial fed-batch fermentations based on experiments in industrial production scale fermentors (>100 m^3) is presented, as well as modelling of the relation between substrate feed rate and dissolved oxygen level in such a process. Several different types of perturbation-based control methods have been suggested for control of this type of process but it has been reported that perturbations in the feed rate may cause decreased productivity in fermentations. The results of this study show that perturbations in the feed rate of production scale fermentations can achieve significant dissolved oxygen level responses without decreased productivity. A model based on data for dissolved oxygen responses and a simulation using a simple observer are given, showing that it is possible to model industrial mixing dynamics in a simple way and that this can be used for perturbation-based on-line estimation of the metabolic state of the system in regard to overflow metabolism. A frequency region where the model can be used has been identified, indicating which frequencies would be suitable for perturbation-based control in industrial fermentations.

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1. Introduction

In industrial fermentations, high volumetric productivity and product yield are highly desirable. To achieve a high productivity in a fed-batch fermentation, it is typically desirable to go from a low starting biomass concentration to a high biomass concentration in as short time as possible. However it is often not desirable to have high substrate concentrations even though this will lead to high specific substrate uptake and growth rates. This is due to the effects of a series of metabolic reactions labelled as overflow metabolism, caused by inhibition of the oxidative metabolism, which leads to the production of by-products at high substrate uptake rates. [Tempest and Neijssel, 1979] These by-products, such as ethanol, acetate and lactate, are commonly harmful to the organism; a well-known example is the inhibitory effects on growth caused by high levels of acetate [Luli and Strohl, 1990] but such compounds can not only inhibit growth but also reduce production of heterologous proteins [Jensen and Carlsen, 1990]. It has been shown that in several organisms overflow metabolism may not simply lead to production and excretion of certain by-products, but also have more extensive effects on cell metabolism. [Paczia et al., 2012]

Although overflow metabolism allows for fast short-term growth it is regarded as an undesirable trait both due to the inhibitory effects of the by-products and because substrate is diverted into producing these [El-Mansi, 2004]. Overflow metabolism occurs both in model organisms such as the bacterium *E.* coli [Neijssel et al., 1980] and the yeast *S.* cerevisiae [Sonnleitner and Käppeli, 1986] and in other commonly used industrial microorganisms such as *B.* licheniformis [Voigt et al., 2004].

In fed-batch fermentation processes, overflow metabolism can be avoided by maintaining a sufficiently low feed rate. The challenge in controlling the feed rate of such processes is therefore to keep the feed rate as high as possible to get a high volumetric productivity, while not exceeding the rate over which overflow metabolism occurs.

In a fed-batch fermentation, avoiding overflow metabolism will only limit the feed rate in the first phase of the process, before oxygen or heat transfer limitations occur. After this point, these factors will give a stricter limitation of the feed rate than the avoidance of overflow metabolism would. In this first phase the biomass concentration can increase exponentially and it will therefore be referred to as the exponential growth phase. It must be pointed out that although the exponential growth phase may only make up a small fraction of the duration of the process sequence, this phase can be critical for the entire process. Overflow metabolism or carbon starvation during this phase can have a large inhibiting effect on cell growth and product formation, potentially leading to a complete process failure in the latter phase of the process. Therefore, in order to ensure high productivity without risking pro-

cess failure, it is of high importance to ensure that the feed rate is set so that excessive overflow metabolism is avoided throughout the whole exponential growth phase.

There are several problems in achieving an optimal feed rate during the exponential growth phase. One is that as the optimal feed rate depends on the total amount of biomass, which increases exponentially, the feed rate must span a large range. The major difficulty is however one of sensing - concentrations of biomass, substrates and overflow metabolites can be difficult to measure in-situ, meaning that estimation of the metabolic state in regard to overflow metabolism becomes difficult. Although sensors for some of these exist and can be used in fermentations with defined media [Bittner et al., 1998; Rocha and Ferreira, 2002], the measuring problem can become significantly more difficult when using complex media as is typically the case in industrial production fermentations. Even in situations where biomass concentration can be measured or estimated in a precise manner, the maximum oxidative capacity depends on oxygen and overflow metabolite concentrations in the broth [Konstantinov et al., 1990] and can vary due to metabolic shifts [Swartz, 1996].

Several different approaches to the problem of finding the best feed rate while avoiding overflow metabolism exist today. One well-known approach to this type of problem, where the parameter to control cannot be measured directly, is to use observer-based regulators. Observers utilize software sensing, including a model of the system allowing on-line estimations of unmeasured states [Luttmann et al., 2012]; the accuracy of such estimations depends on the accuracy of the model used. Although many interesting methods for estimating the states of and controlling this type of system exist, they typically require measurements for which fast and reliable in-situ probes are not commonly available in industrial production processes today, such as concentrations of substrate and biomass in the fermentation broth or concentrations of oxygen and carbon dioxide in the outlet gas, and/or assumptions of constant yield parameters [Luttmann et al., 2012; Chéruy, 1997; Selişteanu et al., 2008; Dewasme and Vande Wouwer, 2008; Dewasme et al., 2011; Riesenberg and Guthke, 1999; Gnoth et al., 2008; Warth et al., 2010; Vargas et al., 2012]. In addition, in production processes with complex media, measurements of the principal substrate are not sufficient to determine the specific substrate uptake rate as several other substrates may be available for uptake.

Another approach is to use data-driven methods, such as artificial neural networks (ANNs). These can be used to achieve good control of bioprocesses, but require large amounts of historical process data for tuning them [Riesenberg and Guthke, 1999; Gnoth et al., 2008]. Although this needs not be a problem when using them to improve control in processes of which many batches have already been run, it makes implementation more difficult in processes which are rapidly developed and changed.

Our previous work in fermentation control has focused on utilizing periodic perturbations in the feed rate of fed-batch fermentations to determine its metabolic state in regard to overflow metabolism and to control the fermentation process based on this [Johnsson et al., 2013]. Using periodic perturbations to determine the state of a system and how to control it is known from e.g. perturbation-based extremum-seeking control [Dochain et al., 2011], from which algorithms have been developed intended for control of fed-batch processes where overflow metabolism is a concern [Dewasme et al., 2011; Vargas et al., 2012]. The work presented here has some similarity to such schemes but is, like our previous work, based on the principles utilized by for instance [Åkesson, 1999], [Velut, 2005] and [de Maré, 2006].

The aims of this work have been threefold. First, to determine whether periodic perturbations in the feed rate of a large industrial production fermentation could yield detectable variations in output signals without decreasing product yield significantly; it has been reported that variations in the feed rate of a laboratory-scale fed-batch process can decrease both its biomass and product yield [Lin and Neubauer, 2000]. Second, to develop a simple model of the relation between feed rate and dissolved oxygen level in the process, as the latter is a measurement for which on-line probes are available and can be found in typical industrial fermentors for aerobic processes [Alford, 2006] and modern dissolved oxygen probes allow for fast and robust measurements. Third, to utilize the model in an observer for on-line estimation of the system's metabolic state.

2. Theory

2.1 Nomenclature

Throughout this work, a number of different parameters are used to describe the system and the controller. Their descriptions and units can be found in Table 1.

2.2 Process dynamics

Considering the feed rate of liquid substrate (F) as the input signal and dissolved oxygen (DO) measurements as the output signal, the fed-batch fermentation process can be regarded as containing a series of dynamics as follows.

1. Substrate mixing.
2. Microbial uptake of substrate and oxygen.
3. Oxygen mixing.

Variable	Symbol	Unit
Biomass concentration	X	g / L
Broth volume	V	L
Feed rate	F	L / min
Specific substrate uptake rate	q_s	L / (min × g)
Critical specific substrate uptake rate	$q_{s,crit}$	L / (min × g)
Substrate uptake flux	ν_s	L / min
Critical substrate uptake flux	$\nu_{s,crit}$	L / min
Oxygen uptake rate	q_o	L / min
Desired oxygen uptake rate	q_o^*	L / min
Average dissolved oxygen in broth	DO_{avg}	mm Hg
Measured dissolved oxygen level	DO	mm Hg

Table 1. Nomenclature.

4. Dissolved oxygen (DO) probe dynamics.

Oxygen mixing dynamics can be regarded as divided into two parts, diffusion of oxygen from gas bubbles into the liquid phase and mixing of oxygen in the liquid phase to distribute it in the broth.

Ideally, not considering tank geometry and flow dynamics, mixing of feed and oxygen in the liquid phase can be regarded as simple first-order linear time-invariant (LTI) processes. It is known however that in large-scale mixed tanks, tank and impeller geometry can have significant effects on mixing dynamics which can require advanced models to describe [Paul et al., 2004]. Diffusion of oxygen from gas bubbles can be considered as a first-order LTI process [Villadsen et al., 2011] and typical dissolved oxygen probe dynamics have been showed to be near first-order [Glazer et al., 2004].

As the dynamics of microbial substrate and oxygen uptake are considerably faster than mixing dynamics in industrial-scale fermentors, microbial dynamics will be at pseudo-steady state in such implementations and can be approximated into their static form. Microbial uptake of substrate and oxygen, as well as production of overflow metabolites, has been studied to a large extent and biochemical models to describe this exist. Such models can contain a large number of parameters [Dauner and Sauer, 2001], meaning that fitting them to a certain strain can be difficult. However, models considering only the relations between uptake rates can be made considerably simpler.

The static relationship between substrate and oxygen uptake can be regarded as a saturation, where increasing substrate uptake rates lead to increased oxygen uptake rates until limitation of the oxidative metabolism sets in and production of overflow metabolites occur (the Crabtree effect)

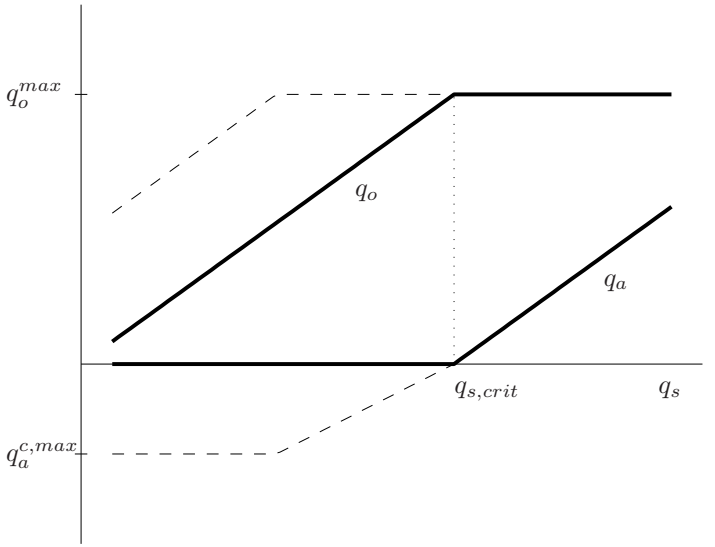
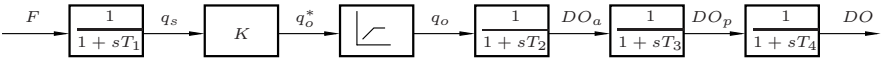
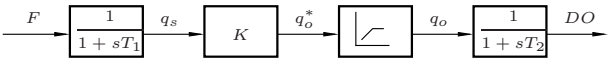


Figure 1. The relation between substrate uptake rate q_s and the rates of oxygen uptake q_o and acetate production q_a. This shows the saturation of oxidative metabolism at $q_{s,crit}$, which is marked with a dotted line. Dashed lines indicate uptake of acetate q_a^c and the correspondingly increased oxygen uptake, which can only occur if acetate is present and the substrate uptake rate sufficiently low. [Åkesson, 1999]

[Sonnleitner and Käppeli, 1986; Dewasme and Vande Wouwer, 2008], cf. Figure 1 for a case where acetate is the main overflow metabolite as for *B. licheniformis*. This limitation of the oxidative metabolism is connected to overflow metabolism, meaning that it can be exploited by a controller seeking to avoid accumulation of overflow metabolites in the fermentation broth. The types of overflow metabolites is not an important factor; this is of significance as the exact compounds produced in overflow metabolism are not always known [Voigt et al., 2004] and it has recently been shown that the products of overflow metabolism in a certain strain are not limited to a few compounds but rather consist of a wide array of different compounds whose production rates do not have constant relations [Paczia et al., 2012].



(a) The full process model, including a saturation and four first-order LTI processes.



(b) The simplified process model, including a saturation and two first-order LTI processes.

Figure 2. The full and simplified process models.

2.3 Process modelling

As outlined above, the system can ideally be regarded as a number of first-order LTI processes and a saturation. For modelling purposes the gain of one LTI process can be assumed to be equal to the gain of the whole process, so that other gains are equal to one. The input-output behaviour of the model will not be changed in any way when using this simplification, it is merely a way to allow estimations of meaningful parameters. This model of the system can be simplified by lumping together oxygen mixing in the liquid, oxygen diffusion and *DO* probe dynamics. As illustrated in figure 2, this gives a model with two first-order processes and a saturation yielding four model parameters in total.

2.4 Process robustness to perturbations

Ideally, F should be controlled so that the substrate concentration in the fermentor gives a substrate uptake rate q_s equal to the critical substrate uptake rate for overflow metabolism, $q_{s,crit}$ (cf. Figure 1). It is however not necessary to completely avoid production of overflow metabolites; for instance, acetate concentrations below 1 g/l appear to have little or no effect on *E. coli* growth in fed-batch fermentations [Luli and Strohl, 1990]. This means that a varying value of F, causing q_s to vary, could possibly be used to determine the system's metabolic state in regard to overflow metabolism without influencing the process yield negatively. This would require that variations in F small enough that process yield is unaffected can achieve variations in output signals which can be separated from noise.

3. Material and methods

3.1 Fermentation equipment

Experiments were performed in industrial production fermentors at the Novozymes A/S site in Kalundborg, Denmark. These are cylindrically shaped fermentors with a volume greater than 100 m^3, stirred by multible axial agitators. Feed is added through ports in the upper half, while air is inserted

through a sparger at the bottom. Dissolved oxygen in the broth is measured by an probe positioned near the bottom of the fermentor, in experiment 1 and 2 this was an electrode while in experiment 3 it was an optode.

The feed rate to a fermentor is controlled to its setpoint by an internal control loop measuring the feed rate and controlling the position of a valve in the feed stream. This internal loop was re-tuned to allow it to keep up with rapid variations in the feed rate setpoint.

3.2 Strain and growth conditions

The strain used in all experiments is a recombinant *B.* licheniformis strain used for industrial production of enzymes. Growth conditions were those typically used in the production process where this strain is used. For reasons of proprietarity, details for these cannot be provided.

3.3 Software

On-line control and measurement of the processes was performed with a sampling time of 6-12 seconds. As the process control system was unable to provide measurements with even sampling interval, interpolation between sampling points was necessary. Analysis of data was performed in MATLAB®, where the nonlinear grey-box model estimation methods in the System Identification Toolbox were used for fitting models to data and for creating a nonlinear estimator for the system.

3.4 Experiment design

In all experiments, sinusoidal waves were superimposed on F. Wave amplitude varied between 20 and 170 l/min and wave frequency varied between 2.5 and 20 mHz in order to determine for which settings a response in the dissolved oxygen signal could be achieved. The productivity and yield of all fermentations was evaluated to determine whether the oscillations in the feed rate would have a significant effect on these.

Two experiments were performed towards the end of fermentations, when biomass concentration is high and the feed uptake rate is far from the critical one, to allow for modelling of the system without interference from the saturation in oxidative metabolism. One experiment included step changes in F_{sp} and a change in agitator speed.

A third experiment was performed during the exponential growth phase at the start of a fermentation, as this phase is of greatest interest for avoiding overflow metabolism and the fermentation may be more sensitive to variations in F in this phase than in the later phase.

4. Results and discussion

The result analysis in this study focuses to a large extent on the relationship between F and DO. Mean values are removed from these to allow fitting of transfer-function models.

4.1 Perturbation responses and process robustness

In figure 3, F and DO are shown for all three experiments, giving an overview of input and output data. A detailed view of the last part of experiment 1 is shown in figure 4, illustrating very well that responses to perturbations are visible in the DO signal, significantly higher than the noise level.

The results of experiment 2 are of particular relevance when studying process robustness as it was performed at the start of a fermentation, when it can be expected to be more sensitive to disturbances due to the lower biomass concentration. Although perturbations in F during the exponential growth phase do not give rise to variations in DO, this can be attributed to the oxidative metabolism being saturated during this phase; immediately after the exponential increase in F is stopped at 5.5 h, clear responses in DO can be seen. If the appearance of a response in DO was due to increased biomass or feed rate in itself this response should be proportional to F, but as this is not the case saturation of the oxidative metabolism is the more likely explanation.

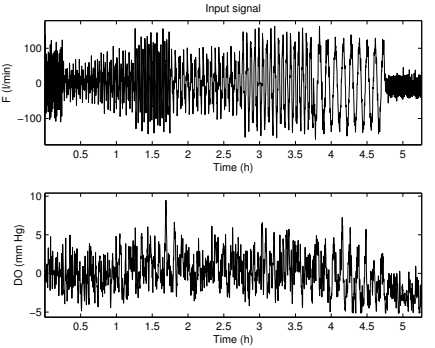
In all three fermentations, no significant decrease in productivity (defined as product activity at the end of the fermentation) was seen. This indicates that the robustness of the process to periodic variations in F is high and it is possible to achieve responses in DO without influencing productivity negatively.

4.2 Modelling

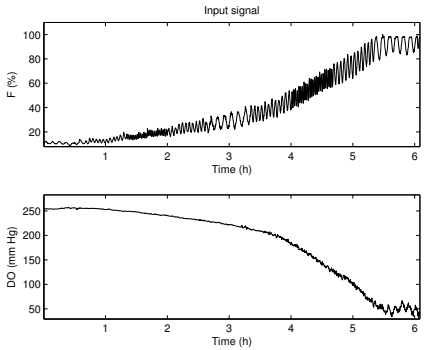
Although feed rate perturbations give rise to significant variations in DO, this is not necessarily sufficient to fit a simple model to data. Data from the two experiments performed at the end of fermentations were used so that the saturation would not have an effect on modelling. This means that using previous modelling assumptions, the model of the ideal system should be on the form given as a transfer function in (1).

$$DO(s) = \frac{K}{(1 + sT_1)(1 + sT_2)} F(s) \tag{1}$$

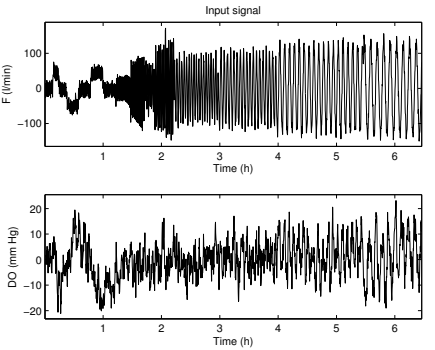
For determining the static gain K of the model, studying low-frequency behaviour and in particular the response to step changes is suitable. Fitting the model to data from the step changes of F_{sp} at the start of experiment 3, as illustrated in figure 5, suggests the value $K = -0.35$ for the equipment used in this experiment.



(a) Input and output for the first experiment. Mean values have been removed for reasons of confidentiality and to allow fitting of transfer function models.



(b) Input and output for the second experiment. F is normalized for reasons of confidentiality.



(c) Input and output for the third experiment. Mean values have been removed for reasons of confidentiality and to allow fitting of transfer function models.

Figure 3. Input F and output DO for the three experiments performed in industrial production scale.

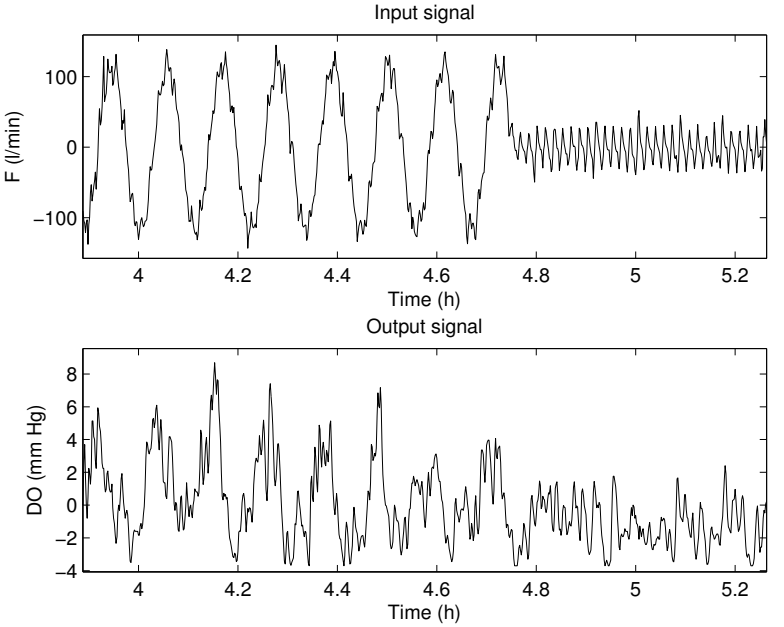


Figure 4. Input F and output DO for the last part of the first experiment. Mean values have been removed for reasons of confidentiality and to allow fitting of transfer function models. In the first region where F is perturbed this gives rise to significant variations in DO, while in the second region where F is near constant with only minor variations due to the internal feed rate control loop no such response is seen.

Step responses, acting on low frequencies, are not well suited for describing the dynamic behaviour of a process at higher frequencies. This means that although they can be relied on to provide a fairly accurate estimate of process gain, the estimations of the time constants T_1 and T_2 are not as reliable. Responses to sinusoidal perturbations such as those employed during most of experiments 1 and 3 are however suitable for determining system characteristics at their respective frequencies.

The model could not be well fitted to data over all perturbation frequencies (2.5-20 mHz), indicating that it could not be used to describe process behaviour in the whole frequency span. This is not surprising, as this would require ideal mixing. A set of common models were fitted to data: unstructured models of ARX, ARMAX, Output error and Box-Jenkins type [Madsen, 2008] (orders 1-8) and structured process models of order 1-3 on transfer function form with and without a zero and/or delay. None of these gave a

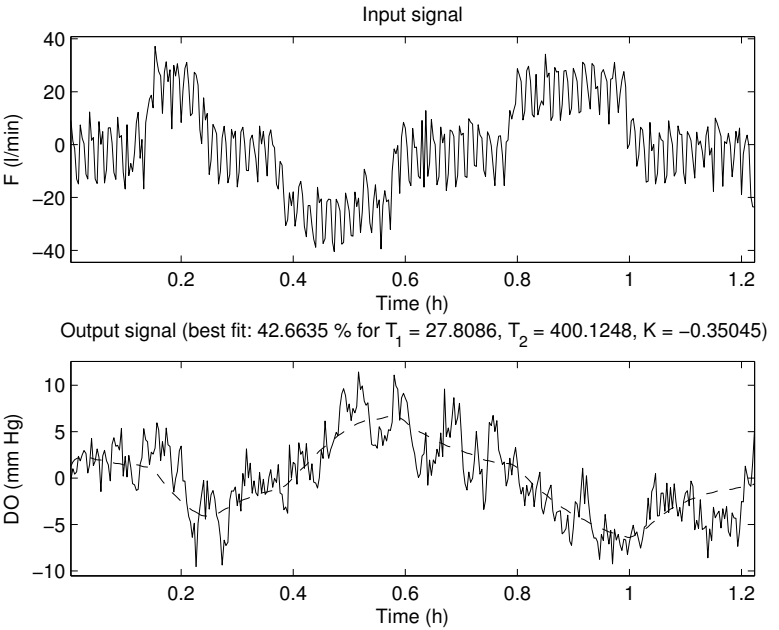


Figure 5. Fitting of the model to input-output data from step responses. Full lines are measured data, a dashed line indicates model output. Mean values have been removed to allow fitting of transfer-function models.

good fit to all data. As no model could describe process behaviour across all frequencies, the following areas were investigated:

1. Whether the model in (1) could be used within a limited frequency span, indicating that it could be used to describe the process response to perturbations of certain frequencies.

2. Whether other model structures could give a better fit to data within a limited frequency span or give a similar fit while being simpler, indicating that the model structure can be improved.

For each perturbation frequency, model fitting was performed for all model types in the set as well as for the model suggested in (1). For all structured models in experiment 3, the value of K was set to the value determined by the step response experiment when modelling based on data from this experiment. As a different probe was used in experiment 1, it was calibrated differently and the value of K found in experiment 3 could not be relied on in that case. Labelling for structured models is given in table 2,

Model structure	Symbol	Transfer function form
One pole	P	$\frac{K}{1+sT}$
Two independent poles	PP	$\frac{K}{(1+sT_1)(1+sT_2)}$
Three independent poles	PPP	$\frac{K}{(1+sT_1)(1+sT_2)(1+sT_3)}$
Double pole	2P	$\frac{K}{(1+sT)^2}$
Triple pole	3P	$\frac{K}{(1+sT)^3}$
Zero	Z	$(1+sT_z)$
Time delay	D	e^{-sL}

Table 2. Labelling for structured models.

the model in (1) is labelled as PP. A summary of model fitting results for data from experiments 1 and 3 can be seen in table 3 respectively 4, showing that for all model types the fit is low at perturbation frequencies of 5 mHz and higher. Several of the model types have significantly better fit to data at frequencies 3.33 and 2.5 mHz, indicating that they may describe system dynamics more accurately in this frequency span.

This is investigated further by fitting models to data corresponding to these input frequencies in the third experiment. Results of this model fitting are shown in table 5 and illustrated in figure 6. With the model in (1) as a starting point, the following conclusions can be drawn:

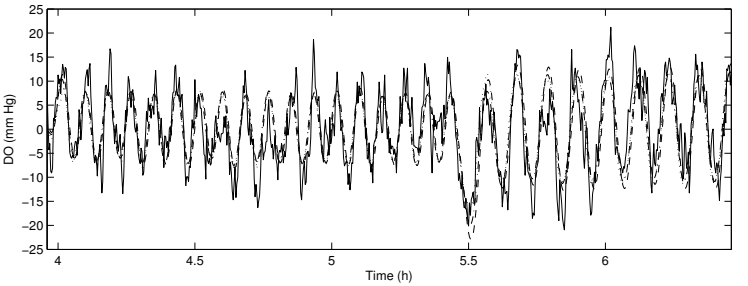
- Replacing one pole with a time delay gives approximately the same fit (PD).
- Removing one pole and adding a zero detoriates the fit somewhat (PZ).
- Structured models with a greater number of parameters achieve a somewhat better fit.
- Reducing the number of parameters by using a double pole detoriates the fit somewhat. Of the two-parameter models (P, 2P and 3P), this gives the by far best fit.
- High-order unstructured models can improve the fit somewhat.

	Perturbation frequency (mHz)			
	20	10	5	2.5
Model type	Fit to data (%)			
Unstructured				
ARX	11	3.7	4.9	35
ARMAX	16	4.5	4.9	41
Output error	19	4.5	5.5	41
Box-Jenkins	24	7.8	6.0	42
Structured				
P	8.3	0.58	2.6	27
PD	11	2.0	4.4	33
PZ	8.3	0.58	3.2	32
PDZ	11	2.0	4.4	33
2P	4.1	3.2	4.2	33
3P	11	3.5	4.2	33
PP	4.1	3.2	4.2	33
PPD	14	3.3	4.3	33
PPZ	11	3.3	4.3	33
PPDZ	14	3.3	4.3	33
PPP	11	3.5	4.2	33
PPPD	14	3.5	4.2	33
PPPZ	15	3.7	4.4	33
PPPDZ	15	3.7	4.4	33

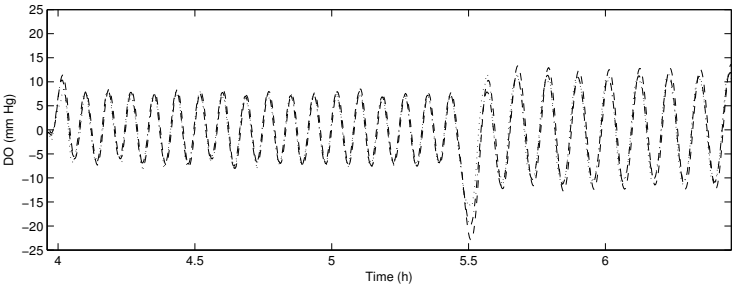
Table 3. Comparison of results for model fitting for experiment 1. Fit is defined as $100 \times \left(\frac{1-\|DO-DO_{model}\|_2}{\|DO-mean(DO)\|_2}\right)$ (%), a fit of 100 % would indicate that the model describes all of the variance within the dataset. For unstructured models, orders 1-8 were used and the best fit is shown. Labelling for structured models is given in table 2.

	Perturbation frequency (mHz)					
	16.7	10	6.67	5	3.33	2.5
Model type	Fit to data (%)					
Unstructured						
ARX	7.7	11	5.4	14	36	48
ARMAX	18	16	13	23	40	55
Output error	20	16	13	23	40	55
Box-Jenkins	21	17	13	24	40	57
Structured						
P	0.0	4.9	0.93	3.8	19	39
PD	2.4	9.6	1.7	10	31	47
PZ	0.0	8.2	1.6	11	35	47
PDZ	2.5	9.6	1.7	11	35	47
2P	5.8	9.0	2.7	11	34	42
3P	5.7	5.4	0.16	3.5	14	17
PP	8.7	9.2	2.7	11	35	47
PPD	11	9.6	2.9	11	35	47
PPZ	9.5	9.5	3.7	11	35	47
PPDZ	13	9.6	3.7	11	35	47
PPP	5.7	9.9	0.97	11	35	47
PPPD	5.9	10	1.7	11	35	47
PPPZ	5.9	9.9	1.7	11	36	47
PPPDZ	5.9	10	1.7	11	36	47

Table 4. Comparison of results for model fitting for experiment 3. Fit is defined as $100 \times \left(\frac{1-\|DO-DO_{model}\|_2}{\|DO-mean(DO)\|_2}\right)$ (%), a fit of 100 % would indicate that the model describes all of the variance within the dataset. For unstructured models, orders 1-8 were used and the best fit is shown. Labelling for structured models is given in table 2.



(a) Model output and measured output.



(b) Model output.

Figure 6. Illustration of model fit to output data for perturbation frequencies 3.33 and 2.5 mHz in experiment 3. A full line indicates measured data, a dashed line the model in (1), a dashed-dotted line a model with a double pole and a dotted line the best unstructured model (an 8th order Box-Jenkins model). Mean values have been removed to allow fitting of transfer-function models.

Figure 4.2 shows that it is possible to model the system response to perturbations in a satisfactory manner. Although there is significant high-frequency noise leading to a lower model fit, the oscillations caused by the perturbations are modelled well. Figure 4.2 shows that the outputs of the illustrated models are very similar. Therefore, as has been seen in table 5, it can be concluded that not much accuracy is lost when constraining the two-pole model to having a double pole.

4.3 Simulation

As the model in (1) is based on ideal mixing, it can be concluded that for input frequencies of 3.33 and 2.5 mHz, ideal mixing can be assumed in the fermentor. This strongly implies that the model holds in the whole spectrum between these frequencies, while the model can not be used to accurately describe the system response at higher input frequencies. It can also be con-

Model type	Fit to data (%)
Unstructured	
ARX	43
ARMAX	44
Output error	44
Box-Jenkins	44
Structured	
P	30
PD	42
PZ	41
PDZ	42
2P	38
3P	14
PP	42
PPD	42
PPZ	42
PPDZ	42
PPP	42
PPPD	42
PPPZ	42
PPPDZ	42

Table 5. Comparison of results for model fitting for data corresponding to input frequencies 3.33 and 2.5 mHz in experiment 3. Fit is defined as $100 \times \left(\frac{1 - \|DO - DO_{model}\|_2}{\|DO - mean(DO)\|_2} \right)$ (%), a fit of 100 % would indicate that the model describes all of the variance within the dataset. For unstructured models, orders 1-8 were used and the best fit is shown. Labelling for structured models is given in table 2.

cluded that although assuming similar time constants for feed and oxygen dynamics decreases the model's degree of freedom, it does not significantly decrease model fit to data.

Including the saturation in oxidative metabolism into the model as per figure 2.3 and defining the saturation as $q_o = min(q_s, q_{s,crit})$ gives a nonlinear model with one unknown parameter to estimate, the critical substrate uptake flux $\nu_{s,crit} = V \times X \times q_{s,crit}$. Fitting this model to on-line data gives a nonlinear observer and estimating ν_s in relation to $\nu_{s,crit}$ in this manner, a controller can be constructed for controlling F to give a desired value of ν_s.

No long-term accumulation of substrate will occur as long as the maximum substrate uptake rate $q_{s,max}$ is not exceeded. When this occurs, maximal overflow metabolism will also occur and for a controller which can avoid

long-term overflow metabolism it follows that long-term accumulation of substrate will also be avoided. Hence, it can be assumed that the relation between F and $q_s \times X \times V$ will only depend on mixing dynamics.

To illustrate how the model can be used for estimation of the metabolic state in regard to overflow metabolism, allowing control of the feed rate based on this estimation, we provide a simple example.

Using an *in* silico model of the system in figure 2.3, the system was perturbed using a sine wave with amplitude $A = 4$ l/min and frequency $\omega = 0.00333$ Hz. White noise with an amplitude equivalent to 10 % of the amplitude in unsaturated oscillations in DO was added to the system output to simulate process disturbances. An estimator using the same nonlinear grey-box model approach used for analysis of experimental data was employed to determine values for the saturation variable $\nu_{s,crit}$. The system used a sampling time of 10 s and a 600 s window for the estimator.

This simple approach has two drawbacks. First, it assumes a constant value of $\nu_{s,crit} - F$ throughout the estimation window, meaning that it will give an averaged value over the time period in the window although it is in fact the latest value which is of interest. Ideally, $\nu_{s,crit} - F$ should be allowed to vary within the window using recursive methods. Second, it gives rise to oscillations in the estimate following the oscillations in DO, requiring a filter which introduces an additional delay. However, high performance is not sought for here, merely a proof of concept.

Performance of the estimator when using a constant value for the unperturbed feed rate and a predefined ramp for the values of $\nu_{s,crit}$ are shown in figure 7. This shows a delay in estimations as expected but variations in $\nu_{s,crit}$ can be tracked. The maximal value of $|\nu_{s,crit} - F|$ which can be tracked is proportional to the amplitude of the perturbations in F. For perturbation amplitudes proportional to the current feed rate, $A = k_p \times F$, this relation becomes

$$|\nu_{s,crit} - F|_{max} = k_d \times k_p \times F$$

For time constants as given in this model $k_d = 0.45$ meaning that for $k_p = 0.3$, deviations up to 13.5 % of the current unperturbed feed rate can be tracked accurately. If $\nu_{s,crit}$ deviates from F by more than this the estimator can determine whether the value is above or below this interval, corresponding to no saturation effect and full saturation respectively, but not the actual value. This is a fundamental limitation in this type of perturbation-based approach.

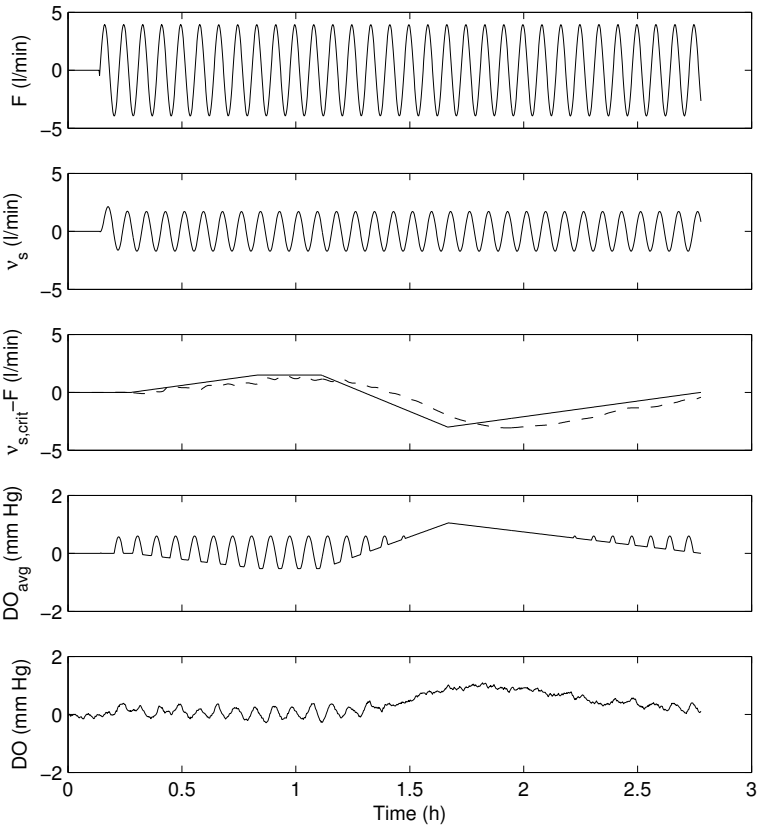


Figure 7. Outcome for the estimator. F is the input signal, ν_s is the substrate uptake rate, $\nu_{s,crit} - F$ is the remaining oxidative capacity (negative values indicate that the capacity is exceeded by the unperturbed feed rate) where a full line indicates the actual value and a dashed line the estimated value, DO_{avg} is an average dissolved oxygen level in the fermentor and DO is the measured dissolved oxygen level.

5. Conclusions

First, this work shows that it is possible to employ perturbations in the feed rate to achieve measurable responses in the dissolved oxygen signal of a large-scale (>100 m^3) industrial fed-batch fermentation without decreasing its productivity, showing that perturbation-based methods are viable in such processes.

Second, it shows that a simple model for mixing dynamics in the relation between feed rate and dissolved oxygen can give good fitting to experimental data from industrial-scale fermentations. Models of the suggested type can be incorporated in advanced biochemical models to simulate large-scale mixing effects and used in development of perturbation-based control strategies for this type of process. A suitable frequency region for perturbations to the input signal in the industrial scale fermentation process has been identified as well, which is of importance for the implementation of perturbation-based control strategies.

Third, a simple observer utilizing the suggested model is given to illustrate how it can be used for on-line process surveillance and control.

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