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Abstract

The recombinant DNA techniques have made it possible to produce many different proteins for a wide range of applications. The vector encoding for the recombinant protein is often inserted in the bacterium *E. coli* as it is a well studied and a well-known organism. To achieve a high productivity, it is important to reach a high cell density. This is obtained through fed-batch operation where the nutrient, usually glucose, is added continuously. The feeding strategy should be designed to avoid starvation and overfeeding. This is not an easy task as on-line measurements of key variables are normally not available. A probing feeding strategy using the measurements of the standard dissolved oxygen sensor is described in [Åkesson *et al.*, 2001a]. The key idea is to superimpose pulses on the feed-rate and make use of the responses in the dissolved oxygen in a feedback algorithm. It has been successfully implemented in cultivations with different *E. coli* strains and on different scales.

The probing feeding strategy is further developed in several aspects in this thesis. It is implemented with good results also in *V. cholerae* cultivations, which shows how general the strategy is. Also, a model of a bio-reactor operating in fed-batch mode is presented and verified. The effect on the tuning rules of the probing controller is investigated.

The probing feeding strategy is improved for a more efficient product synthesis. This new fermentation technique manipulates the temperature when the maximum oxygen transfer capacity of the reactor is reached. The strategy consists of a mid-ranging controller structure and a modified probing controller. It is analysed and evaluated in experiments and simulations.

Furthermore, some *E. coli* production strains need additions of amino acids or complex media besides the carbon nutrient to grow and produce the recombinant protein. The probing control concept is therefore extended in order to handle these situations. Feeding strategies for dual feeding of amino acids or complex media and glucose are developed.

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Contents

1

Introduction

Biotechnology offers a sustainable production of existing and novel products. The recombinant DNA techniques have made it possible to produce many different proteins, on a large scale, for a wide range of applications. The vector encoding for the recombinant protein is often inserted in the bacterium *E. coli* as it is a well studied and a well-known organism. To achieve a high productivity, it is important to reach a high cell density. It is obtained through fed-batch operation where the nutrient, usually glucose, is added continuously. The feeding strategy should be designed to avoid starvation and overfeeding. Overfeeding can lead to by-product formation through overflow metabolism. For example, when cultivating *E. coli*, acetic acid is produced when glucose is fed in excess, but it is also produced under anaerobic conditions. As acetic acid inhibits growth and recombinant protein production the design of a good feeding strategy is crucial to obtain good results. This is, however, not an easy task as on-line measurements of key variables (such as glucose concentration and acetic acid concentration) are normally not available.

A probing feeding strategy using the measurements of the standard dissolved oxygen sensor is described in [Åkesson *et al.*, 2001a]. When applying this feeding strategy, the acetic acid formation is avoided. The key idea is to superimpose pulses on the feed-rate and make use of the responses in the dissolved oxygen in a feedback algorithm. Also, to keep the reactor working under aerobic conditions the feed-rate is decreased when the maximum oxygen transfer capacity of the reactor is met. The probing feeding strategy maximises the feed-rate with respect to the limitation in the cell metabolism and the transfer capacity of the reactor. It has been successfully implemented in cultivations with different *E. coli* strains and on different scales.

In this thesis the probing feeding strategy is implemented also in *V. cholerae* cultivations, which shows how general the strategy is. Also, in [Åkesson *et al.*, 2001b] tuning rules of the probing feeding controller

are derived using a model of a bio-reactor. In this thesis the model is extended and verified, and the effect on the tuning rules is investigated.

The probing feeding strategy can also be improved for a more efficient product synthesis. Here we propose the use of the temperature instead of the feed-rate when the maximum oxygen transfer capacity of the reactor is met. There are many advantages of a lower temperature such as a higher production of soluble protein and less formation of inclusion bodies. The new fermentation technique is analysed and tuning rules are derived. It consists of a mid-ranging controller structure together with a modified probing feeding controller.

Furthermore, some bacteria need additions of amino acids or complex media besides the carbon nutrient to grow and produce the recombinant protein. The probing control concept is therefore extended in order to handle these situations. Feeding strategies for the dual feeding of amino acids or complex media and glucose are developed in this thesis.

1.1 Publications and Their Contributions

The thesis consists of five papers and an overview. The papers are listed below together with their main contributions:

- **Paper I**: de Maré, L., L. Andersson and P. Hagander (2003): "Probing Control of Glucose Feeding in *Vibrio cholerae* Cultivations." *Bioprocess and Biosystems Engineering*, **25**, pp. 221–228, © Springer 2003.
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In paper I, the probing feeding strategy is applied to *V. cholerae* cultivations with good results. This shows how general the feeding strategy is.

- **Paper II**: de Maré, L. and P. Hagander (2006): "Parameter Estimation of a Model Describing the Oxygen Dynamics in a Fed-batch *E. coli* Cultivation." Accepted to Reglermötet 2006.

In paper II, a linearised model describing a bio-reactor operating in fed-batch mode is extended and verified using experimental data. Its effect on the tuning rules of the probing feeding strategy is also investigated.

- **Paper III**: de Maré, L., S. Velut, E. Ledung, C. Cimander, B. Norrman, E. Nordberg-Karlsson, O. Holst and P. Hagander (2005): "A Cultivation Technique for *E. coli* Fed-batch Cultivations Operating Close to the Maximum Oxygen Transfer Capacity of the Reactor."

In paper III, a new cultivation technique, effective when operating at the maximum oxygen transfer capacity of the reactor, is applied to two different *E. coli* strains.

- **Paper IV**: Velut, S., L. de Maré and P Hagander (2006): "Bioreactor Control Using a Probing Feeding Strategy and Mid-ranging Control." Under revision for Control Engineering Practice.

In paper IV, analyses are done and tuning rules are derived for the new cultivation technique applied in paper III.

- **Paper V**: de Maré, L., C. Cimander, A. Elfwing and P. Hagander (2006): "Feeding Strategies for *E. coli* Cultivations Demanding an Enriched Environment". Submitted.

In paper V new feeding strategies, effective when more than one feed is to be employed, are developed and tested in simulations and cultivations.

Other Publications

Other publications that are related to the area are presented below. Early works on the new fermentation technique, effective when the maximum oxygen transfer capacity of the reactor is reached, are presented in [Velut *et al.*, 2004] and [de Maré *et al.*, 2004]. The use of the probing feeding control to investigate the influence of different induction times on the production is presented in [Ramchuran *et al.*, 2002]. Also, the probing control has been tested on a large scale application in [Velut *et al.*, 2002]. A study of another way to control the substrate feed, here with the use of acetic acid measurements, is presented in [de Maré and Hagander, 2002]. Often the flow rate can not be measured as peristaltic pumps are used, but it can be estimated and controlled by using an observer and balance measurements. This is the subject in [de Maré *et al.*, 2001].

1.2 Outline

In chapter 2 a background is given describing the cultivation procedures for production of recombinant proteins. This includes the rDNA techniques, high cell density cultivations, feeding strategies and modelling of bio-reactors. In chapter 3 the main contribution of paper I is described, where the probing feeding strategy is applied to *V. cholerae* cultivations.

In chapter 4 the experimental results from the new fermentation techniques developed in paper III and paper V are presented. In chapter 5 the modelling and control aspects of the probing feeding strategy (paper II) and the new fermentation techniques (paper IV and paper V) are covered.

2

Background

2.1 History

Microorganisms have been used for the processing of food for thousands of years. Fermentation of fruit to wine and milk to yogurt and cheese are examples of early applications of microorganisms. During the early 1940s the penicillin process was developed. The organism demanded rich medium at normal pH and large quantities of air. Thus methods for sterilisation of medium and air and efficient mixing and oxygen transfer had to be developed together with analytical methods and separation technology. Also techniques for strain improvement to obtain higher concentrations were developed. A large number of products became available after these technical breakthroughs.

Today, recombinant DNA techniques also allow the production of mammalian proteins using microorganisms such as *E. coli* in cultivations. Reviews of the technique are given in [Swartz, 1996] and [Makrides, 1996]. The products, obtained by use of biotechnology, range from pesticides used in agriculture, detergents and enzyme aided pulp bleaching to pharmaceuticals such as insulin. A survey treating the different areas where biotechnology is used is found in [Gavrilescu and Chisti, 2005].

When it comes to the control of pharmaceutical processes, the licensing procedure has imposed some constraints. The licensing process has favoured conservative strategies because they are well tried and well understood, [Bogle *et al.*, 1996]. Many manufacturing procedures have been treated as frozen as any subsequent changes have been extremely expensive. In 2004 the food and drug administration (FDA) launched a new initiative called process analytical technologies (PAT), see `http://www.fda.gov/cder/OPS/PAT.htm`. Its goal is to facilitate for the pharmaceutical industry to build quality into products through increased pro-

cess understanding. Thus the development of new analytical techniques that can be applied during the manufacturing process is favoured. PAT will lead to an increase in automation and information management and the need for sophisticated control strategies to improve productivity will increase. Therefore the scope of this thesis is right on time: feeding strategies based on probing control.

2.2 Recombinant DNA Techniques

Recombinant DNA techniques permit the creation of exactly known changes in the DNA structure. It has opened new possibilities to improve production strains and to produce new products. The technique is also used to manipulate the protein structures to give them better properties. To change the DNA structure, one uses e. g. restriction enzymes that hydrolyse DNA as well as other enzymes, ligases, that re-join the DNA. The new DNA is carried into the host cell by a vector. The vectors are bacterial phages or plasmids if the host cell is a bacterium. If the host cell is a higher organism virus is used as vector. The vectors consist of: an origin of replication to allow autonomous plasmid replication, an element providing selective pressure to allow cell transformation and plasmid retention, a convenient locus or loci for promoter and gene insertion. The choice of expression system for high-level production of recombinant proteins depends on many factors. These include cell growth characteristics, expression levels, intracellular and extracellular expression, post translational modifications and biological activity of the protein, [Jana and Deb, 2005].

Host

There are many different hosts used for the production of recombinant proteins. Reviews of hosts, used in the pharmaceutical industry, are given in [Schmidt, 2004], [Andersen and Krummen, 2002] and [Chu and Robinson, 2001]. Until the mid-90s, *E. coli* was the dominant host also for the production of protein pharmaceuticals. However, in recent years mammalian cell production is increasing and also advances are made using cell-free protein synthesis, [Swartz, 2001].

Escherichia coli There are many applications of recombinant protein production where *E. coli* is used as a host, examples are given in [Choi *et al.*, 2006]. There are many advantages of using *E. coli*. As *E. coli* has been extensively studied for decades, the bacterium is well characterised. Thus there is a large base of genetical and physiological knowledge, a

well-developed ability to quickly alter the organism and the ability to quickly assess the consequences of these alterations. Changes affecting transcription, translation, protein export [Choi and Lee, 2004], [Shokri *et al.*, 2003] and even protein folding [Baneyx and Mujacic, 2004] can be made.

The ability to secrete the recombinant protein to the periplasm or extracellular space is important in some cases. As a gram negative bacterium, *E. coli* has no natural protein secretion machinery for extracellular production. Extracellular production is often advantageous as it facilitates the isolation and purification procedures. *E. coli* consists of an inner and outer membrane that divides the organism into three compartments: the cytoplasm, the periplasm and the extracellular space. The decision to target recombinant proteins to one of the compartments rests on balancing the advantages and disadvantages of each compartment, reviewed in [Choi *et al.*, 2006].

A disadvantage of the foreign protein production taking place in the cytoplasm is that the stability of the proteins produced can be low due to proteolytic degradation. Proteases are responsible for the degradation of nonessential, nonfunctional proteins and thereby providing amino acids for the synthesis of new proteins, [Maurizi, 1992]. In the cytoplasm, high level protein production often leads to the formation of inclusion bodies. Inclusion bodies consist of folding intermediates, native and unfolded proteins. There are some advantages of inclusion body formation. They are easily purified, the protein is protected from proteases, and as the protein is inactive, toxic protein can accumulate in an inclusion body. Also, inclusion bodies can accumulate to much higher levels than soluble proteins. The disadvantage of inclusion body formation is the complicated and costly denaturation and refolding processes that are required to make the protein active. Factors that affect the folding of proteins are reviewed in [Baneyx and Mujacic, 2004]. Normally the formation of inclusion bodies is reduced when the cultivation temperature is lowered, [Schein and Noteborn, 1988]. Disadvantages of cytoplasmic production include the additional N-terminal methionine that is added to most recombinant proteins. Also, if the soluble protein is produced in the cytoplasm, the purification procedure is complex. Further, the low oxidation potential in cytoplasmic space does not promote the proper folding of most proteins with disulfide bonds, [Choi *et al.*, 2006]. Disulfide bonds are often present in complex mammalian proteins. The disadvantages of the cytoplasm have lead to the targeting of the recombinant protein to the periplasm. The secretion techniques are reviewed in [Choi and Lee, 2004].

In the secretion to the periplasm, the signal sequence is precisely removed and the proper N terminus is obtained. Also, the protease activity is lower than in the cytoplasm so the protein degradation takes place to a

smaller extent. The highly oxidised environment offers a greater chance for the correct formation of disulfide bonds [Makrides, 1996] and the purification procedure is simplified. A disadvantage is that secreted proteins may be deposited as periplasmic inclusion bodies. Thus the need to solubilise and fold aggregated proteins still exists. Improved technology has been developed for in vitro folding when required and it is an important contributor to the success of *E. coli* rDNA technology, [Swartz, 1996]. Also, the increased permeability of the cell membrane might lead to cell lysis.

One of the disadvantages of *E. coli* as a host has been that many post-translational modifications of complex eukaryotic proteins are not performed in a correct way, for example glycosylated proteins are produced without the sugars. New methods are being developed to handle this drawback, [Baneyx and Mujacic, 2004].

Another drawback is the *E. coli* ability to produce endotoxins, which could constitute a health hazard, [Johnston *et al.*, 1998]. Thus, if the product is to be used as a drug, purification procedures are necessary.

Vibrio cholerae One reason for cultivating *V. cholerae* is for the production of the vaccine against cholera. The cholera toxin consists of one A subunit and five B subunits. The B subunit of the cholera toxin is nontoxic and has been shown to possess high immunogenic activity. It can be used as a component in a cholera vaccine together with killed whole cells of *V. cholerae*. The vaccine has been shown to be effective in a clinical trial in Bangladesh, [Clemens *et al.*, 1986]. This vaccine is shown also to give a short-term protection against diarrhoea caused by the heat-labile enterotoxin produced by *E. coli*, [Peltola *et al.*, 1991] and [Clemens *et al.*, 1988]. CTB is further used as a component together with killed enterotoxigenic *E. coli* in a vaccine against ETEC (enterotoxigenic *E. coli*), [Svennerholm *et al.*, 1989]. A vaccine against ETEC is important as ETEC is one of the major causes of childhood diarrhoea in the developing countries [WorldHealthOrganisation, 1999] and the severe diarrhoea is a cause of death for children. The vaccine has been tested with good results on both adults and children, for example, in Egypt [Savarino *et al.*, 1999], [Savarino *et al.*, 1998] and in Bangladesh, [Qadri *et al.*, 2006], [Qadri *et al.*, 2003], [Qadri *et al.*, 2000]. Furthermore ETEC is a major cause of travellers' diarrhoea. Cholera toxin and the cholera toxin B subunit can be used as adjuvants, reviewed in [Holmgren *et al.*, 2005]. An example is given in [Bergerot *et al.*, 1997] where CTB is coupled to insulin in a vaccine against autoimmune diabetes. Therefore an efficient production of CTB is important.

Another reason for the cultivation of *V. cholerae* is the production of LTB [Panda *et al.*, 1995], the B subunit of the heat-labile enterotoxin (LT), formed in *E. coli*. The heat-labile enterotoxin (LT), partly responsible for causing ETEC, is similar to CT. LT activates the immune system and

could cause an immune response systemically and in the mucosal. Thus, the B subunit of LT is also produced for usage as a carrier and adjuvant.

To produce the B subunit efficiently without the production of the toxic A subunit, recombinant DNA technique is used. The ability to export the B subunit to the extracellular broth makes *Vibrio cholerae* a more attractive host than *E. coli* where CTB accumulates intracellular or in the periplasm, [Mendoza-Vega *et al.*, 1995]. Other expression systems used for CTB production are *Lactobacillus* [Slos *et al.*, 1998] and also recently reported is the silkworm, [Gong *et al.*, 2005]. When it comes to LTB production the yeast *P. pastoris* [Fingerut *et al.*, 2005] has been used as an expression system.

Other Hosts Other hosts used for the expression of proteins are the mammalian cell-culture systems with Chinese hamster ovary, Baby hamster kidney, and murine myeloma cell lines [Schmidt, 2004], [Chadd and Chamow, 2001], [Andersen and Krummen, 2002]. In some cases they are the only choices for the preparation of correctly modified proteins. They are also recognised as safe regarding infectious and pathogenic agents [Schmidt, 2004]. When it comes to yeast systems, it is *Saccharomyces cerevisiae* and *Pichia pastoris* that are most commonly used. Like *E. coli*, yeasts can be grown rapidly to high cell densities and the yeasts are not pathogenic. They are also able to secrete more efficiently, [Schmidt, 2004]. Also, the bacterium *Bacillus subtilis* has been used as many of its species naturally secrete large amounts of enzymes.

2.3 Cultivations

The objective during a cultivation is to maximise the volumetric productivity i. e. to obtain the highest possible amount of product in a given volume within a certain time. When using *E. coli* as a host, product isolation and purification costs can exceed fermentation costs by severalfold. The fermentation should thus be optimised for efficient and inexpensive downstream processing. Optimal isolation and purification methods depend on the initial disposition (secreted or cytoplasmic, soluble or inclusion body) of the product and the biochemical characteristics of both the product and the contaminants, [Swartz, 1996].

Equipment

There are different types of bio-reactors, the containment, in which the fermentation takes place. The one most commonly used, is the stirred tank reactor (STR), but there are also other types e. g. dialyse membrane

reactors. The advantage of dialyse membrane reactors is the continuous removal of inhibitory or toxic compounds without causing cell stress. To improve the oxygen transfer rate cyclone reactors and gas-lift reactors have also been designed.

For high cell density cultivations in industry, the STR under fed-batch operation is the reactor of choice because of its simplicity, its potential for high productivity, its suitability for robust fermentation and its wide distribution, [Riesenberg and Guthke, 1999].

Sensors Sensors are needed for process monitoring and control. Today the standard sensors used in a cultivation are: dissolved oxygen sensor, pH sensor, temperature sensor and a gas analyser which measures the CO_2 and O_2 content in the outlet air. Key variables such as cell mass and nutrient concentrations, product concentrations, by-product concentrations are not monitored on-line in general. An exception is the by-product ethanol produced in *Saccharomyces cerevisiae* cultivations. Ethanol is readily measured using a semiconductor gas sensor in combination with a permeable membrane, [Mandenius and Mattiasson, 1983]. Other substances have to be analysed off-line. The reason is that the sensors have to be sterilizable, reliable and robust.

However, the research has lead to the development of new sensors and analysing systems. Reviews are given in [Vojinovic *et al.*, 2006] and [Schugerl, 2001]. A few examples are given below. Software sensors, which are model based measurements, need a reliable process model to work well. The bio-sensors consist of a chemically specific receiver such as an enzyme or an antibody together with a transducer which gives an electrical current. The drawbacks of the bio-sensors are their heat sensitivity and the gradual activity loss of the biological receptors, [Schugerl, 2001]. Therefore they are not used in industry. One example of a promising biosensor is the Biacore sensor, which is based on surface plasmon resonance technology. Also, capacitance sensors can be used for biomass measurements, even though their sensitivity is low. The use of a conductivity sensor together with the base consumption can give estimates of the biomass and the by-product acetic acid, [Hoffmann *et al.*, 2000]. Other systems for measuring concentrations are flow injection analyses (FIA). As there is still a lack of reliable supervisory systems for on-line fault detection and correction, this is not used in industry either. Then there is also high performance liquid chromatography (HPLC) which has the drawback of long analysing times. For industrial application, optical measurement methods are more attractive because they are non-invasive and more robust. Examples are optical density, fluorescence measurements, near-infrared spectroscopy, [Riesenberg and Guthke, 1999], [Vojinovic *et al.*, 2006] and [Marose *et al.*, 1999]. These methods measure products, by-products and

substrate concentrations, but still these analysing equipments are far from being standard. An example of monitoring product concentration using fluorescence is given in [DeLisa *et al.*, 1999].

Operation Mode

The cultivation can be operated in three different modes.

- Batch: All nutrients required during one run are added before the cultivation is started, except for chemicals needed for pH adjustment as well as oxygen in an aerobic process. The final products are removed after the run.
- Continuous: In continuous processes all nutrients are continuously added to the bio-reactor and fractions of the medium are removed at the same flow rate as that of supplied nutrients to maintain a constant culture volume.
- Fed-batch: One or more nutrients are supplied to the bio-reactor during the cultivation and the products remain in the containment until the end of the run.

The fed-batch operation mode is effective for processes when varying concentrations of a nutrient affect the yield or productivity of the desired metabolite. It is desired when the nutrient concentration has to be kept low for different reasons. The reasons might be: substrate inhibition occurs, high substrate concentrations lead to by-product formation, e. g. overflow metabolism or catabolite repression occurs. Other reasons for fed-batch operations are: high cell concentration is to be achieved and the high initial concentration of the nutrient required becomes inhibitory, extension of operation time, replacement of water lost by evaporation, decreasing viscosity of broth, [Yamane and Shimizu, 1984].

Medium

It takes a lot of time to optimise a medium for growth and production. Sometimes calculations based on stoichiometry and inspection of the composition of the recombinant protein to be produced can give a hint. There are three different types of medium used in cultivations.

- Defined medium is chemically definable. The use of defined medium leads to the highest cell concentration obtained, since the nutrients are known and can be controlled.
- Complex medium. A complex medium contains nutrients which are not chemically definable such as yeast extract, tryptone, peptone and casaminoacids. A complex medium will usually support a higher specific growth rate, due to the presence of bio-synthetic intermediates

and growth factors which the cell no longer needs to synthesise, thus decreasing the metabolic burden, [Yee and Blanch, 1992]. Nutrients in complex media such as peptone and yeast extract can vary in composition and quality which makes fermentation with these complex media less reproducible.

- Semi-defined medium is a combination of the two.

Feed Composition In fed-batch cultivations, a feed with nutrients is added. The feed often consists of glucose and trace element solution. Sometimes it is necessary to add amino acids or yeast extract to the cultivation in order to enhance the cell growth and/or the product formation. The complex nitrogen additions also improve the product quality and decrease the proteolysis, [Swartz, 1996]. The additions can be included in the feed together with glucose and trace elements or they can be fed in a separate feed or added from the start.

Cultivation Property of the Host Organism

E. coli Protein production using *E. coli* can often be performed at a low cost. The low cost is based on the high volume obtained as it can be grown to high cell densities. Also the raw material costs are reasonable as *E. coli* does not require expensive medium components. The isolation and purification procedures are relatively inexpensive and the process performance is reliable. *E. coli* has a rapid growth rate and a corresponding ability to rapidly produce heterologous proteins.

A disadvantage with *E. coli* is the overflow metabolism. Acetate is produced when *E. coli* is grown under anaerobic or oxygen limitation conditions but also under aerobic conditions in the presence of excess glucose. Acetic acid inhibits growth [Luli and Strohl, 1990], [Shimizu *et al.*, 1988] and reduces recombinant protein production [Jensen and Carlsen, 1990], [Turner *et al.*, 1994], [MacDonald and Neway, 1990]. Acetate formation can be detected when the specific growth rate exceeds a certain critical growth rate. The critical growth rate is strain and medium dependent, [Meyer *et al.*, 1984].

V. cholerae *V. cholerae* has many similarities to *E. coli*. They are both gram-negative bacteria and there is evidence of acetic acid formation when cultivating *V. cholerae*, [Panda *et al.*, 1995].

High Cell Density Cultivation Conditions

Reviews on high cell density cultivations (HCDC) using *E. coli* are given in [Choi *et al.*, 2006], [Shiloach and Fass, 2005], [Jana and Deb, 2005],

[Riesenberg and Guthke, 1999], [Lee, 1996] and in [Yee and Blanch, 1992]. To improve productivity, it is important to achieve high cell densities. One has to minimise the problems encountered such as substrate and/or nutrient inhibition, inhibitory by-product formation, inhibitory product accumulation, product degradation and dissolved oxygen limitations in aerobic cultivations, [Yee and Blanch, 1992]. Furthermore, ammonia serves as the nitrogen source in many high cell density cultivations and is used to control pH. However, ammonia has to be kept at low concentrations since higher levels inhibits growth. On a large scale, the heat generation can become a problem as the cooling capacity of the reactor is limited.

In order to minimise the problems with HCDC, the following operation procedure is often used: phase one of the cultivation is a batch phase with maximum specific growth rate after which a fed-batch phase with reduced specific growth rate follows. It is necessary to prevent the accumulation of inhibitory metabolic by-products. Also, the fermentation processes are usually designed with separate growth and expression phases. By avoiding product expression during the initial growth period, product toxicities are avoided, rDNA protein expression does not compete for metabolic resources, and the duration of product exposure to modification reactions can be minimised, [Swartz, 1996]. The protein expression is started by induction. The induction can be, for example, a change in the cultivation temperature or the addition of a chemical agent. Optimal conditions for induction vary with the product, its disposition and the expression system. An important factor to consider when deciding on the induction time is the effect on the growth rate and the oxygen consumption in relation to the maximum oxygen transfer capacity of the reactor. A typical cultivation is outlined in figure 2.1.

To keep the desired pH, pressure, temperature and oxygen level in the reactor, PID control is well established. To manipulate the oxygen level, one uses the stirrer speed but also the air flow can be changed. When the maximum oxygen transfer capacity of the reactor is reached one can sparge with pure oxygen, reduce the temperature or increase the reactor pressure to avoid oxygen depletion.

One issue is to avoid inhibitory by-products formed from overflow metabolism. In the case of *E. coli*, it is the acetic acid formation that should be avoided. There are many ways:

- Manipulation of the temperature. By lowering the culture temperature, nutrient uptake and growth rate can be reduced, thus reducing the formation of toxic by-products. Also, decreasing the temperature reduces the formation of inclusion bodies [Schein and Noteborn, 1988].
- Manipulation of strain. Today strains can be improved to reduce or

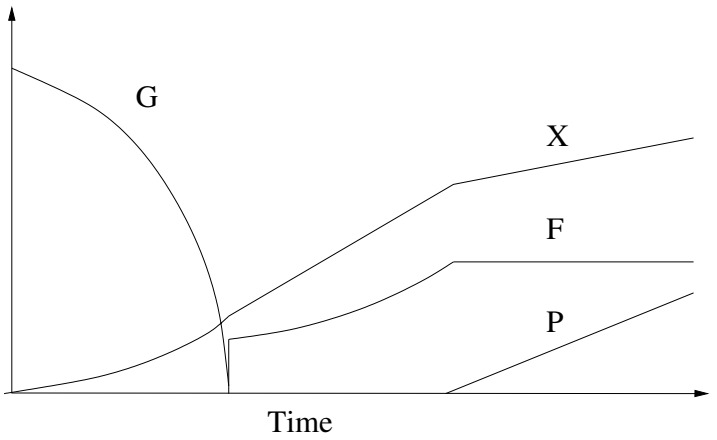

Figure 2.1 A typical cultivation. Phase 1 is a batch phase and the feed is started when the nutrients (here glucose) added initially are depleted. The protein production is started with induction when a satisfactory amount of cells is obtained. Notation: P product, F feed rate, G glucose concentration and X cell mass.

abolish detrimental by-product and/or its effects, [Riesenberg and Guthke, 1999], [Chou *et al.*, 1994]. An example is the acetate accumulation which occurs less in *E. coli* B strains than in K12 derivatives because of the activated glyoxylate shunt pathway in the former, [van de Walle and Shiloach, 1998].

- Manipulation of medium. An example is the use of glycerol as carbon source with which inhibitory by-products such as acetic acid are not produced as the growth rate is kept below the critical one, [Yee and Blanch, 1992]. Acetate forms in complex and defined media when the specific growth rate exceeds 0.2 h^{-1} or 0.35 h^{-1}, respectively, [Meyer *et al.*, 1984], [Paalme *et al.*, 1990]. The addition of some amino acids or yeast extract can alleviate the harmful effect of acetic acid, [Han *et al.*, 1993],[Koh *et al.*, 1992]. Also, the yeast extract lowers the glucose uptake rate and thereby leading to a lower acetic acid accumulation. [Han *et al.*, 1992], [Panda *et al.*, 1999], [Panda *et al.*, 2000].
- Removing the acetic acid concentration from the broth, for example by using a dialyse membrane reactor.
- Manipulation of feed. If the feed rate is controlled so that the critical growth rate is not exceeded, there is no accumulation of acetic acid, [Paalme *et al.*, 1990].

During the fed-batch cultivation, the feed rate is thus important. Besides avoiding overflow metabolism, one wants to avoid starvation as starvation might lead to proteolysis, which gives product degradation. Nutrient limitation can affect recombinant protein yields, plasmid copy number and plasmid stability.

As additions of complex components or amino acids often augment the protein production and/or cell growth, [Li *et al.*, 1998], [Panda *et al.*, 1999], [Panda *et al.*, 2000], [Rhee *et al.*, 1997] and [Takagi *et al.*, 1996], these components are sometimes supplied to the cultivation as well. Also, by feeding amino acids at a controlled rate, degradation of some recombinant proteins can be prevented, [Ramirez and Bentley, 1993], [Ramirez and Bentley, 1999], [Ramirez and Bentley, 1995], [Yoon *et al.*, 1994]. It is the elevated foreign protein expressions that usually result in activation of proteases. The protease activity is triggered by the low intracellular amino acid concentration, [Harcum and Bentley, 1999]. The shortage in intra-cellular amino acid pools is a consequence both of the transport and the availability of amino acids. The addition of high levels of supplemental amino acids may inhibit recombinant protein production due to feedback inhibition of critical bio-synthetic pathways. Thus the indiscriminate addition of amino acids has been shown to not result in optimal productivity, [Harcum *et al.*, 1992], and high concentrations are shown to inhibit cell growth, [Yee and Blanch, 1992], [Mizutani *et al.*, 1986], [Li *et al.*, 1998]. Thus a good feeding strategy is important also for the complex media or amino acid additions.

A block diagram of a bio-reactor is given in figure 2.2. One of the challenges during fed-batch operation is to design the feed controller.

2.4 Feeding Strategies

As described above, both overfeeding and underfeeding of the nutrient have a negative effect on the cell growth and the product formation. Development of a suitable feeding strategy is critical in fed-batch operation and reviews on the subject are given in [Choi *et al.*, 2006], [Riesenberg and Guthke, 1999], [Lee *et al.*, 1999], [Rani and Rao, 1999] and [Lee, 1996]. The control system development is not straightforward due to: lack of accurate models describing cell growth and product formation, the nonlinear and time-varying nature of the bio-process and the deficiency of the reliable on-line sensors for the quantification of key state variables, as mentioned above, [Shimizu, 1993].

On the industrial scale, open loop control is often used because of the lack of sensors. A predetermined feed profile is applied, often chosen to be exponential corresponding to a cell growth μ between 0.1-0.3 h^{-1}

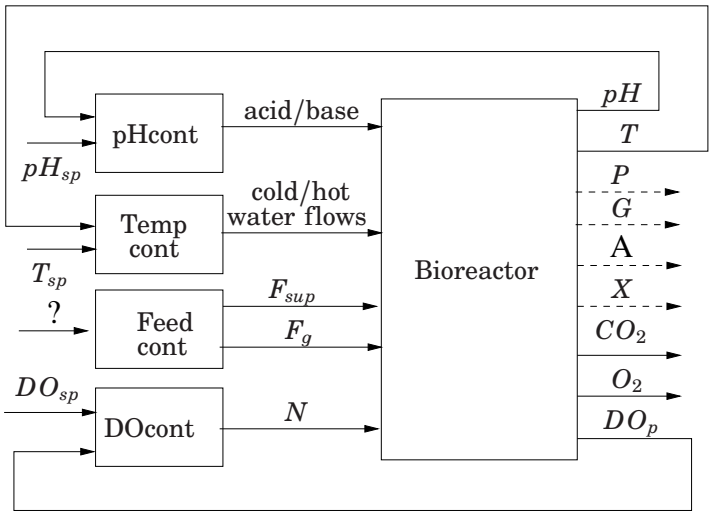

Figure 2.2 Block diagram of the process. One of the challenges when cultivating in fed-batch mode is to design a feed controller and which input or inputs to use. pH, T temperature, DO dissolved oxygen concentration, F_g glucose feed rate, F_{sup} feed rate of supplement, N stirrer speed, X cell mass concentration, G glucose concentration, P product concentration, A acetic acid concentration, O_2 oxygen concentration in the outlet air, CO_2 carbon dioxide concentration in the outlet air. The analyses normally done off-line are denoted dashed, cont = controller and sp = set-point.

to avoid acetate formation. Exponential profiles assume that cells grow exponentially with time. Process disturbances and system nonlinearities should be avoided, [Lee *et al.*, 1999].

If an accurate model of the system is available optimization procedures can be used to calculate the feeding strategy, [Lubbert and Jorgensen, 2001] and [Rani and Rao, 1999]. Lately the use of genetic algorithms for optimization has increased, an example is given in [Franco-Lara and Weuster-Botz, 2005]. In [Smets *et al.*, 2002] an optimal feed rate for a fed-batch bio-reactor is derived using the minimum principle of Pontryagin. When no feedback is applied, optimal feeding strategies are sensitive to disturbances and process model mismatches. In [Smets *et al.*, 2004] and [van Impe and Bastin, 1995] feedback is included through the use of adaptive controllers which are based on measurements of cell mass or substrate concentration, respectively.

To overcome the lack of sensors one can use indirect measurements of bio-activity patterns. Based on the DO measurement, a feeding strategy called DO stat is developed. It is based on the fact that when there is not enough nutrient, the oxygen consumption decreases and thereby

the dissolved oxygen level increases, [Konstantinov *et al.*, 1990]. Another feed controller makes use of the pH measurements and is called pH stat, [Suzuki *et al.*, 1990]. Also, measurements of the oxygen concentration, [Riesenberg *et al.*, 1991], and the carbon dioxide concentration, [Suzuki *et al.*, 1986], in the outlet gas can be used for model based feed control.

Another approach is to develop non-standard sensors that measure for example the substrate concentration, the cell concentration, the product or the by-product concentration, [Lee, 1996]. Feedback control is then applicable. Some examples are given below. In [Navratil *et al.*, 2005] near-infrared (NIR) spectroscopy is used to measure the by-product acetate, glucose and biomass in a *V. cholerae* culture. The biomass measurements are then used to control the feed-rate. In [Axelsson, 1989] and [Valentinotti *et al.*, 2003], *Saccharomyces cerevisiae* producing the by-product ethanol is cultivated. The ethanol concentration is measured using a semiconductor gas sensor (as described in chapter 2.3) and a mid-infrared spectrometer, respectively. In both cases a feed controller based on internal model control is derived which keeps the ethanol at a low concentration. Another example of model based control is given in [Chung *et al.*, 2006] where the substrate concentration, the dissolved oxygen concentration, the oxygen concentration in the outlet air are measured and used to manipulate the feed-rate, the stirrer speed and oxygen concentration in the inlet gas.

In [Svensson *et al.*, 2005] measurements of an on-line glucose analyser are used to manipulate the feed-rate in order to keep the glucose concentration at 0.2 g/l. Besides having access to an on-line glucose analyser, there is another difficulty when using this type of control. The critical glucose concentration is usually not known a priori and it is strain dependent. Also it might change during the cultivation. Thus the desired set-point of the controller is unknown. The fermentation technique applied is called temperature limited fed-batch (TLFB) and is first described in [Silfversparre *et al.*, 2002]. The glucose concentration in the reactor should be in excess. The temperature is used to control the dissolved oxygen concentration in the reactor and the temperature is the limiting factor of the cultivation. One advantage reported with the technique is a low formation of endotoxins. There are indications that the low endotoxins formation is a result of the avoidance of a severe glucose limitation, [Svensson *et al.*, 2005].

To overcome the lack of suitable models and unknown nonlinear relationships, artificial neural networks, fuzzy control and knowledge based supervision are used [Lee *et al.*, 1999]. It can be combined in hybrid control systems, see [Riesenberg and Guthke, 1999] and [Rani and Rao, 1999]. These are attempts to collect and use all the process knowledge available.

In the literature complex media or amino acids are often added proportionally to the glucose feed [Whiffin *et al.*, 2004], [Zawada and Swartz,

2005], [Ramchuran *et al.*, 2005], [Johnston *et al.*, 2003] ,[Panda *et al.*, 1999], [Panda *et al.*, 2000] and [Mizutani *et al.*, 1986], as a constant feed [Takagi *et al.*, 1996] or from the beginning, [Jensen and Carlsen, 1990]. The drawback when the supplement is added proportionally to the glucose feed is that the factor between the glucose feed and the complex media or amino acid additions must be known in advance. Sometimes the relation changes during the cultivation. For example the relation might change at the onset of the protein production.

A Probing Feeding Strategy

In order to gain knowledge of a process, one can expose the process to perturbations. This is used in extremum control, [Åström and Wittenmark, 1995]. In extremum control one wants to control a process with an unknown nonlinearity close to an optimum. This is done by correlating the effect on the output from a known perturbation on the input. The probing controller described below can be viewed as an extremum controller with the difference that it should be controlled close to a saturation instead of close to an optimum. In the biotechnology field, the method to deliberately expose the process to pulses and thereby control the feed rate has also been applied to an activated sludge system, [Yongacoglu *et al.*, 1982].

Under glucose-limited conditions, pulses superimposed on the glucose feed rate give rise to changes in the glucose uptake. These changes imply variations in the oxygen uptake that can be seen in the dissolved oxygen measurements. For glucose uptake, q_g, above a critical value, q_g^{crit}, the oxygen uptake is saturated and the oxygen response to feed pulses will change character. The pulse responses reveal if q_g is above or below q_g^{crit} and hence if acetate is being produced or not. In [Åkesson *et al.*, 2001a] a feedback algorithm using the information from the pulse responses has been developed to achieve a feeding strategy close to q_g^{crit}, see figure 2.3.

Dissolved oxygen is controlled by the manipulation of the stirrer speed, which ensures that dissolved oxygen is at the same level at the start of each pulse. During the feed pulses, the stirrer speed is fixed in order not to interfere with the pulse responses.

When the maximum transfer capacity of the reactor is reached (i. e. the maximum stirrer speed), a safety net decreases the feed rate in order to maintain aerobic conditions. This results in a feeding strategy that tends to optimise the performance with respect to the limitation in the cell metabolism and reactor capacity. A block diagram of the controller and the process is shown in figure 2.4. The strategy has been used successfully for different *E. coli* strains and on different scales, [Åkesson *et al.*, 2001a] and [Velut *et al.*, 2002]. An example of a cultivation with the used strategy is shown in figure 2.5. One may notice that the feed rate is decreased by

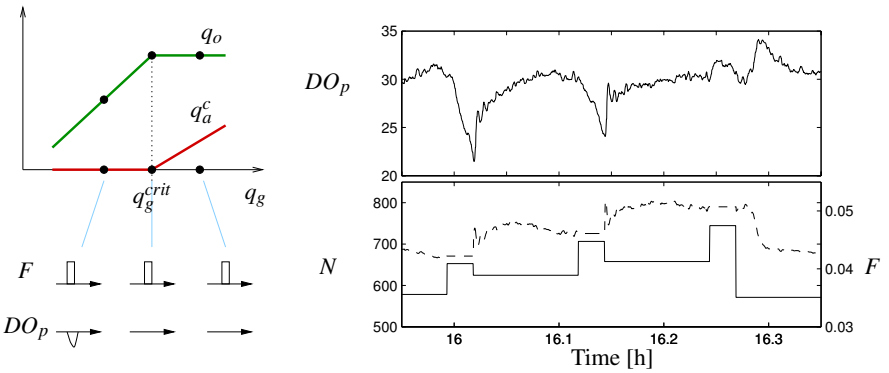

Figure 2.3 Principles of the probing technique (left) and application to a fed-batch experiment (right). Acetate formation ($q_a^c > 0$) occurs when the specific oxygen uptake rate q_o saturates, which can be detected in the oxygen signal DO_p by superimposing pulses in the feed rate F. When a pulse response is visible in DO_p, the feed rate is increased at the end of the pulse. In the experiment, the absence of a response to the third pulse indicates overfeeding and it leads to a feed decrement. The stirrer speed N is frozen during a probing pulse and regulates DO_p at 30% between two successive pulses.

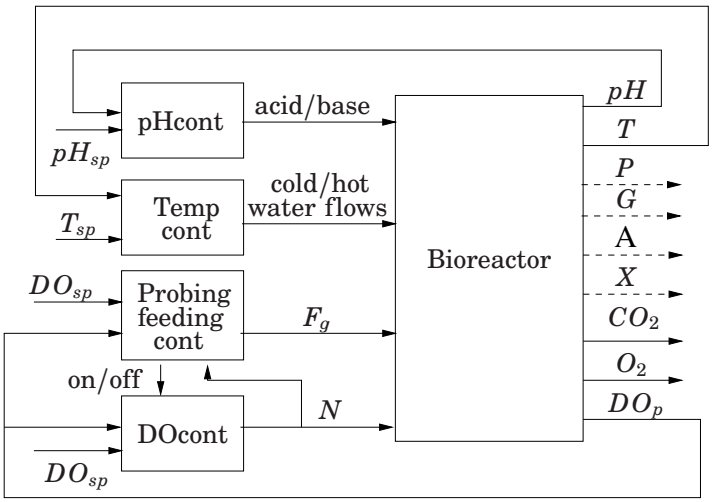

Figure 2.4 Block diagram showing the probing feeding strategy. pH, T temperature, F_g glucose feed rate, N stirrer speed, X cell mass concentration, G glucose concentration, A acetic acid concentration, P product concentration, DO_p dissolved oxygen concentration, O_2 oxygen concentration in the outlet air, CO_2 carbon dioxide concentration in the outlet air. The analyses normally done offline are denoted dashed, cont = controller and sp = set-point.

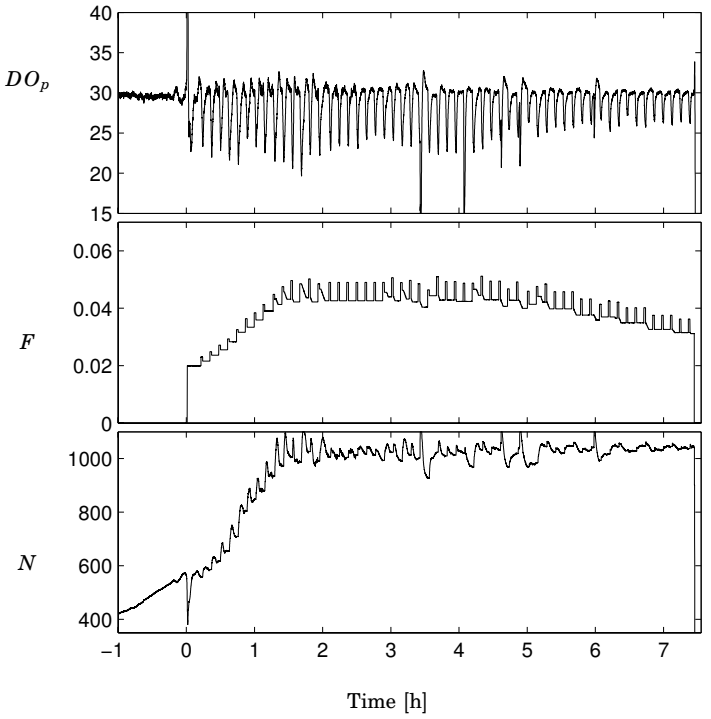

Figure 2.5 A cultivation using the probing feeding strategy with *E. coli* BL21(DE3). The fed-batch part of the cultivation is shown. From the top: DO_p [%], dissolved oxygen, F [l/h] glucose feed rate, N [rpm] stirrer speed. At t = 3.4 h and t = 4.1 anti-foam is added which has a large impact on the dissolved oxygen.

30 % during the phase where the limited oxygen transfer of the reactor is reached. The small changes in the reactor glucose concentration and dissolved oxygen resulting from the feed pulses do not affect the cells. In [Neubauer *et al.*, 1995] very large changes were made in the glucose concentration and that did not affect the cell growth, even though there were implications on a higher cell stress. In [Taherzadeh *et al.*, 2000] another area for the pulse technique is presented: fermentation of lignocellulosic hydrolyzates by *Saccharmyces cerevisiae*. Pulses are superimposed on the feed rate and the responses in the carbon dioxide concentration in the outlet gas are used in the feed control.

Tuning Rules for a Probing Feeding Strategy When using a proportional probing feed controller, the increase in the feed is decided by:

$$\Delta F(k) = \kappa \frac{DO_{pulse}(k) - y_r}{DO^* - DO_{sp}} F \tag{2.1}$$

where F is the glucose feed rate, DO_{pulse} is the size of the pulse response, DO^* and DO_{sp} are the saturated dissolved oxygen concentration and the set-point of the dissolved oxygen, respectively. Furthermore, the controller parameters are the controller gain, κ, and the desired pulse response, y_r. There are several more parameters that need to be chosen such as the pulse duration T_{pulse}, the length between the pulses $T_{control}$ and the pulse height γ_p. For the tuning of the feed controller and practical issues such as the handling of measurement noise, see [Åkesson *et al.*, 2001b] and [Velut, 2005]. A linearised version of the model presented below, in chapter 2.5, is used to derive tuning rules for the probing feeding controller in [Åkesson *et al.*, 2001b]. The values of the controller gain κ and the pulse height γ_p are linked to the process gain. The values of the pulse duration T_{pulse} and the time between the pulse $T_{control}$ are linked to a lumped time constant of the process.

2.5 Modelling

In order to find a good cultivation technique and to analyse, design and tune the controllers needed, it is essential to know and understand the system. There are many approaches taken to describe cell metabolism: simple unstructured models that consider the growth of the entire culture or structured model on the cellular level where the biomass is compartmentalised. Due to the complexity and size of the structured models their use for bio-reactor design and on-line control is limited. A lab-scale reactor is usually considered well mixed with a homogeneous culture and the modelling procedure is thereby simplified. The dynamics are usually described by mass balance differential equations. These equations combine two elements: a reaction network and a set of kinetic functions. The reaction network describes the biological reactions that are assumed to occur in the system. The kinetic functions describe the velocity of the reactions. The most difficult problem lies in the modelling of the kinetic functions. Two major obstacles are: the selection of the biological and physiochemical factors that are supposed to influence the kinetics and must be incorporated in the model. Once this selection has been made, one has to choose an appropriate analytical description of each kinetic function. It is possible to implement a two-step procedure for identifying separately the reaction

structure and the kinetic structure for a general class of dynamical models of bio-processes, as described in [Chen and Bastin, 1996] and references therein. One can also use artificial neural networks to describe the unknown nonlinear reaction rates and combine them with mass balances to give a hybrid model, [Chen *et al.*, 2000].

A Model of a Bio-reactor Operating in Fed-batch Mode

In [Åkesson *et al.*, 2001b] the following model of a well mixed bio-reactor operating in fed-batch mode is presented. The mass balances are given by:

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

The expressions for the growth rate μ, the acetate consumption q_a, the oxygen consumption q_o and the glucose uptake q_g are given in the appendix. The expressions are inspired by [Xu *et al.*, 1999]. For notation and parameter explanation, see table 2.1 and table A.1 in the appendix. Henry's law is used to give the dissolved oxygen concentration DO in %:

$$DO = HC_o$$

The dissolved oxygen sensor dynamics is approximated as:

$$T_p \frac{dDO_p}{dt} + DO_p = DO$$

Parameter Estimation

The estimation and validation procedure of parameters of a model is important. Reviews of identification and parameter estimation in continuous-time systems are given in [Unbehauen and Rao, 1998], [Unbehauen and Rao, 1990] and [Young, 1981]. In [Bohlin and Graebe, 1995] a method for stochastic grey-box identification is reviewed and the technique offers

Table 2.1 Variables in the model presented in chapter 2.5.

Symbol	Description
V	reactor medium volume
F	glucose feed into the reactor
N	stirrer speed
G	glucose concentration
A	acetic acid concentration
X	cell mass concentration
C_o	dissolved oxygen concentration

several advantages at the expense of a longer computational time and advanced algorithms. The identification of bio-technical models from experimental input/output data is not an easy task because of the complexity and nonlinearity of the underlying systems. There are two basic methods for parameter estimation: off-line processing (batch) or on-line (recursive). In general, although recursive algorithms yield less efficient parameter estimates, they have the advantage of on-line operation, [Young, 1981]. Thus on-line techniques should be employed if the model is to be used for on-line control purposes (e. g. adaptive controllers).

Often kinetic parameters are estimated by either determining one parameter at a time from a specific experiment or by calculating all parameters simultaneously using least square or maximum likelihood criterion. These procedures are possible if the number of parameters to be determined is limited. Also, sufficient experimental data have to be available to perform a robust estimation of each parameter. The estimation problem is non-linear and most likely contains multiple local minima. Multiple minima might also be a consequence of limited data. The solution is the global minimum which hopefully also provides the biologically most reasonable parameters. One can check how robust the minimum is by rerunning the estimation routine with the new set of parameters as initial guesses. Also the robustness can be evaluated by starting from different but also biologically reasonable parameters. If a large number of kinetic parameters are to be estimated, a procedure using weighted least squares and Tikhonov regularisation is outlined in [Lei and Jorgensen, 2001]. Also for validation purposes, the estimated parameters should be checked against a data set not used in the estimation procedure, i. e. cross validation.

2.6 Challenges

In a fed-batch cultivation, one of the most important factors is the feeding strategy, as mentioned above. The further development of the probing feeding strategy is a challenging and important task.

One issue is how general the probing feeding strategy is and if it is applicable to other organisms than *E. coli*. Further, to define tuning rules for the probing feeding controller, a model is used, see chapter 2.4. There is a need to verify that model using experimental data. Another issue is the handling of the limited oxygen transfer capacity of the reactor. In the probing feeding strategy, the feed rate is decreased which results in a low residual glucose concentration in the reactor, see figure 2.5. This imposes stress on the cells which might lead to e. g. proteolysis. In some applications there is a need for additions of amino acids or complex medium to enhance the growth and/or production. How to feed this supplementary substrate in an effective manner is also an important issue.

The aim of the thesis is to extend the probing feeding strategy to meet these challenges. The probing feeding strategy is applied to *V. cholerae* cultivations in order to investigate how general the strategy is. The model presented in section 2.5 is extended to describe the influence of the changing oxygen concentration in the outlet gas as well. Experimental data is used to verify a linearised version of the model and the effect on the tuning rules is investigated. To handle the limited oxygen transfer capacity of the reactor, the use of the temperature is proposed. A lower temperature is favorable in many aspects, for example the inclusion body formation is reduced and the produced protein is soluble to a larger extent, [Schein and Noteborn, 1988]. Furthermore, the probing control concept is extended to handle dual feeding of amino acids or complex medium as well as glucose.

3

Probing Control Applied to a New Organism

The probing feeding strategy, as described in chapter 2.4, is now employed on *V. cholerae* to examine how general the technique is. The results are given in paper I. Earlier, the technique was tested on different *E. coli* strains on different scales [Velut *et al.*, 2002], [Åkesson *et al.*, 2001a].

The production of *V. cholerae* and CTB is important, as mentioned in chapter 2. They are used as components in a cholera vaccine, [Clemens *et al.*, 1986]. CTB is further used in a vaccine against ETEC (enterotoxigenic *Escherichia coli*) [Svennerholm *et al.*, 1989]. Also, the cholera toxin and the cholera toxin B subunit can be used as adjuvants, reviewed in [Holmgren *et al.*, 2005].

When cultivating *V. cholerae*, there is evidence of acetic acid formation, [Panda *et al.*, 1995]. It is probable that the acetic acid has the same negative effect on cell growth and recombinant protein production as reported for *E. coli*.

Experimental Results

In an initial experiment it is tested if the dissolved oxygen signal can be used to indicate acetic acid formation. As this is confirmed, see figure 3.1, the probing feeding strategy is implemented and five cultivations are done.

During these cultivations defined medium is used, therefore no CTB is produced. There is a need for complex media or amino acid additions to boost the production [van de Walle *et al.*, 1990]. However, as the amount of cells is correlated to the amount of product produced, [van de Walle *et al.*, 1990], the produced cell mass is used as a measurement of how effective the feeding strategy is. Reproducible results are obtained, which shows the strength of the probing feeding strategy. The strategy avoids acetic

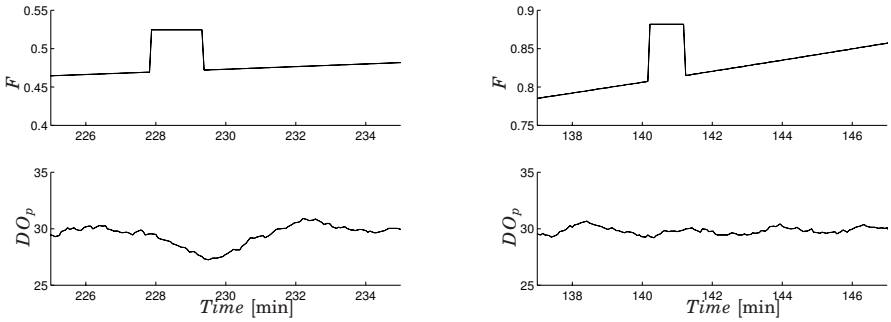

Figure 3.1 Confirmation of the use of the dissolved oxygen sensor measurements in order to detect acetic acid formation. In a. there is a response in dissolved oxygen DO_p to a pulse superimposed on the feed rate F. There is no acetic acid present. The feed rate corresponds to a μ of 0.2 h^{-1}. In b. there is no response in dissolved oxygen DO_p to a pulse superimposed on the feed rate F. There is acetic acid present. The feed rate corresponds to a μ of 0.5 h^{-1}.

acid accumulation and at the same time a growth rate of about 0.4 h^{-1} is obtained during the fed-batch part, resulting in a cell mass of 20-23 g/l after 12 hours of feeding.

Acetic acid is accumulated during the batch phase to around 2 g/l. Therefore the change in metabolism after the depletion of the acetic acid during the fed-batch phase is clearly seen, see figure 3.2. The pulse responses to superimposed pulses on the glucose feed are larger after the depletion of the acetic acid, thus the cells are now capable of growing at a higher growth rate. This indicates that the acetic acid has a negative effect on the cell growth, just as in the case with *E. coli*. The larger pulse responses lead to increased feed rate and thus the increasing demand of the cells is being satisfied. This shows the importance of having feedback included in the feeding strategy. It would be hard to predict when the acetic acid is depleted. An alternative would be to wait until also the acetic acid is depleted before the feed is started. This would probably not be as effective, as acetic acid is consumed faster in the presence of some glucose, at least for *E. coli*, [Xu *et al.*, 1999].

Thus good results are obtained when using the probing feeding strategy and not much a priori knowledge of the microorganism is needed. This shows how effective the strategy is and that it can be used in many situations, for example in the process development phase.

To further explore the applicability of the probing feeding strategy, it has also been applied to cultivations of *Bacillus subtilis*, [Karlsson, 2005] and *Saccharomyces cerevisiae*, [Fredriksson, 2001].

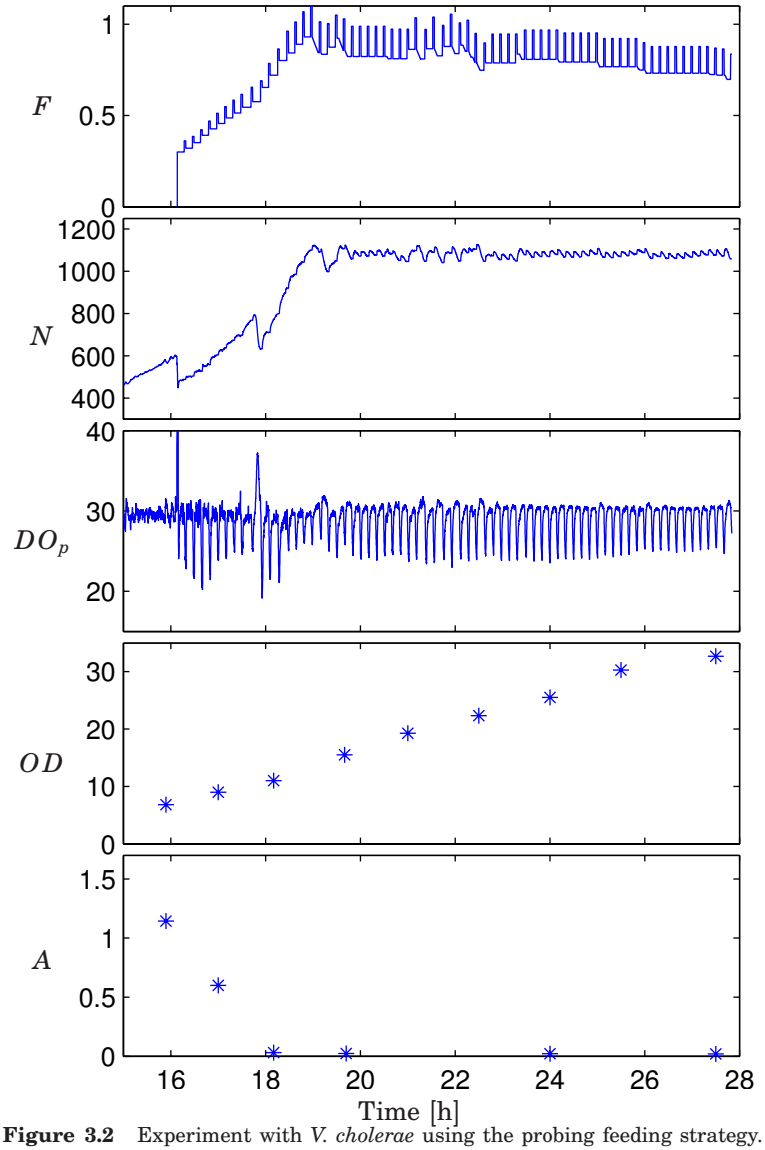

Figure 3.2 Experiment with *V. cholerae* using the probing feeding strategy. The acetic acid accumulated after the batch phase is depleted around 1.5 hours after feed-start. At that point the pulse responses in the dissolved oxygen tension to feed pulses superimposed on the glucose feed are increasing because the cells are now capable of growing at a higher growth rate. From the top: feed-rate F [g/min], stirrer speed N [rpm], dissolved oxygen tension DO_p [%], cellmass (optical density) OD and acetic acid A [g/l].

4

New Fermentation Techniques

Two challenges are discussed in this chapter. How the probing feeding strategy is to be extended in order to meet these challenges is treated as well.

One issue is the handling of the limited oxygen transfer capacity of the reactor. In the probing feeding strategy, the feed rate is decreased which results in a low residual glucose concentration in the reactor, see figure 2.5. This imposes stress on the cells which might lead to proteolysis. There are indications that a severe glucose limitation triggers the release of endotoxins [Svensson *et al.*, 2005]. The release of endotoxins complicates the purification procedure and the product quality. Also, endotoxins in the exhaust gas aerosols may constitute a health hazard, [Johnston *et al.*, 1998]. We propose the use of the temperature instead of lowering the feed rate, details are given in paper III. The idea of lowering the temperature in order to decrease the oxygen consumption is not new, [Bauer and White, 1976]. As mentioned in chapter 2, a lower temperature is favorable in many aspects. For example, the inclusion body formation is reduced and the produced protein is soluble to a larger extent [Schein and Noteborn, 1988]. Also, the proteolysis of the recombinant protein is reduced.

In some applications there is a need for additions of amino acids or complex medium to enhance the growth and/or production. One example is the cultivation of *V. cholerae* for cholera vaccine production, [van de Walle *et al.*, 1990]. How this supplementary substance should be fed when there is almost no process knowledge available is an important issue. A fast way to find a good feeding strategy would shorten the process development phase considerably. Here we make use of the pulsing technique to control the additional feed. Details are given in paper V.

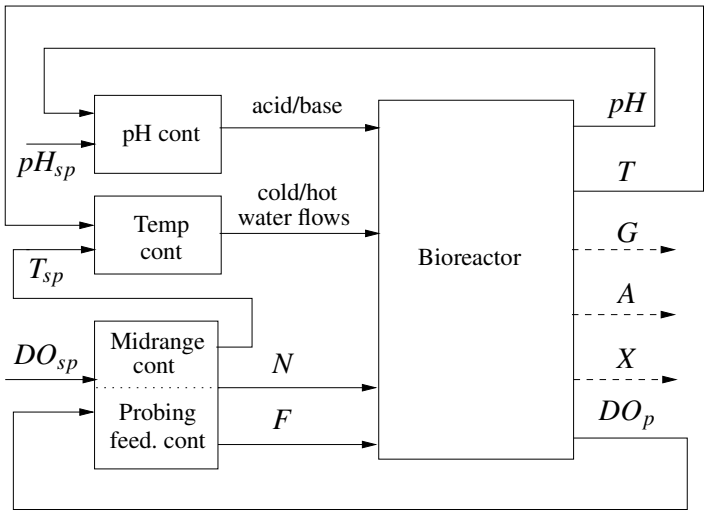

Figure 4.1 A block diagram of the process showing the new fermentation technique effective when the maximum transfer capacity is reached. The variables are: pH, temperature T, dissolved oxygen DO_p, acetic acid concentration A (off-line), cell mass concentration X (off-line), glucose concentration G (off-line), glucose feed rate F and stirrer speed N, cont = controller, sp = set-point.

4.1 Handling of the Limited Oxygen Transfer Capacity of the Reactor

We propose the use of the temperature once the maximum oxygen transfer capacity is reached instead of decreasing the feed. The derived controller structure is a mid-ranging configuration, where both the stirrer speed and the temperature are manipulated simultaneously. The model used for the design and tuning of the controllers is given in paper IV and it is also discussed in chapter 5. A block diagram showing the new process configuration is given in figure 4.1. The strategy is as follows: the probing feeding strategy is used until the stirrer speed passes N_{sp}. Then the temperature part of the mid-ranging controller is activated. Also, down-pulses instead of up-pulses are superimposed on the glucose feed. The reason is that a slightly higher residual glucose concentration is then obtained in the reactor. This is advantageous as the temperature should be the limiting factor during this part of the cultivation and not the low glucose concentration in the reactor. If this is the case, the advantage of a low endotoxin formation, reported when using the TLFB technique [Silfversparre *et al.*, 2002]

and [Svensson *et al.*, 2005], is applicable. Glucose excess and temperature limitation can be achieved at various temperature values. Operation at very low temperatures implies low stirrer speeds and feed rates, and results in a poor utilization of the reactor. Our objective is to exploit the full capacity of the reactor. Thus the feed rate and the stirrer speed are to be maximised with respect to the constraints, while the temperature is kept as close to 37°C as possible. The new technique is designed to lower the temperature only to compensate for the limitation on oxygen transfer. If the oxygen consumption does not increase after the maximum oxygen transfer capacity of the reactor is reached, there is no need for a temperature decrease or a decrease in the feed. In this case, the difference between the probing feeding strategy (described in chapter 2.4) and the new fermentation technique is small. An example of a cultivation with the new strategy is shown in figure 4.2.

Experimental Results

The strategy is tested on two different *E. coli* strains: *E. coli* W3110 producing the protein ZZT2 and *E. coli* BL21(DE3) producing xylanase. When cultivating *E. coli* BL21(DE3) using the new technique, a higher cell mass is obtained and more active protein is produced than with the probing feeding strategy. The resulting glucose concentration in the reactor is higher (1.2 g/l compared to 0.15 g/l), thus the temperature and not the low glucose concentration is limiting the cultivation. Also, the glucose excess obtained does not lead to a larger acetic acid accumulation (around 0.3 g/l).

When it comes to *E. coli* W3110, the need for a temperature decrease to lower the oxygen demand is much smaller than for *E. coli* BL21. The cell mass during the production phase is not increasing much and also the protein production does not seem to cause any major changes in the oxygen demand of the cells. As the new technique is flexible, it adapts to the characteristics of the system and therefore the resulting temperature decrease is small. The difference between the new technique and the probing feeding strategy (described in chapter 2.4), is thus small for *E. coli* W3110.

The experiments show the flexibility and how effective the new fermentation technique is. The temperature is only decreased if there is need for it. In the case of *E. coli* BL21, there is a need for a temperature decrease and the goals are fulfilled: the cultivations are temperature limited and there is a controlled glucose excess in the reactor. These conditions lead to a higher soluble protein production and a higher biomass. Also, a strength of the strategy is that a controlled excess of glucose is obtained without any a priori information of the critical glucose concentration.

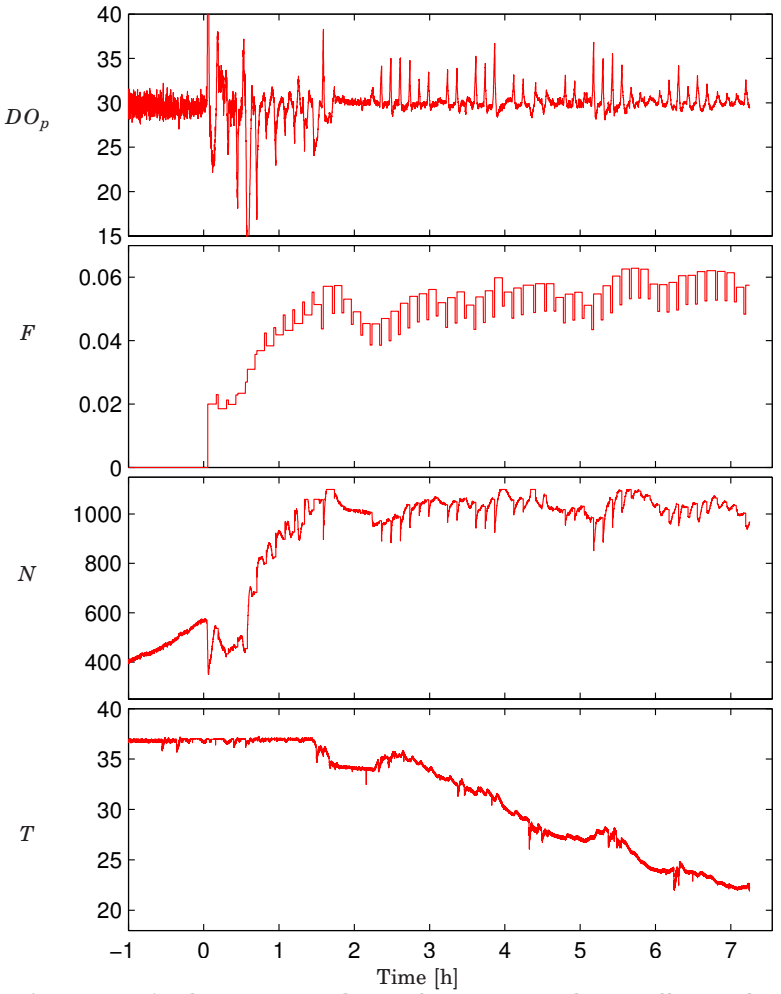

Figure 4.2 A cultivation using the new fermentation technique effective when the maximum oxygen transfer capacity of the reactor is met with *E. coli* BL21(DE3). The fed-batch part of the cultivation is shown. From the top: DO_p [%], dissolved oxygen, F [l/h] glucose feed rate, N [rpm] stirrer speed and T [^{o}C] temperature.

4.2 Dual Feeding of Amino Acids or Complex Media and Glucose

As mentioned above, it is sometimes necessary to add supplementary amino acids or complex media. Some industrial strains are auxotrophic and thus need one or more amino acids in order to grow at all. As it is

common to add complex media or amino acids to a fermentation in the industry it is a relevant question how the supplements should be added in the most effective manner. Some amino acids are more readily used as energy sources, while others are used directly as building blocks in the protein production, [Gschaedler and Boudrant, 1994]. Also, the amount of amino acids used as energy sources depends on the amount of glucose present. For an efficient growth the complex components or added amino acids should be used primarily as building blocks and the glucose should be used as an energy source.

Currently the addition of complex compounds or amino acids are mostly done proportionally to the glucose feed [Whiffin *et al.*, 2004], [Zawada and Swartz, 2005], [Johnston *et al.*, 2003] ,[Panda *et al.*, 1999], [Panda *et al.*, 2000]. The drawback when the supplement is added proportionally to the glucose feed is that the factor between the glucose feed and the complex media or amino acid additions must be known in advance. Sometimes the relation changes during the cultivation. For example the relation might change at the onset of the protein production. Here we suggest one strategy for the addition of a known amino acid and another strategy for the addition of complex media. In both the strategies, a predetermined exponential profile is followed by the glucose feed and pulses are superimposed on the feed. When the maximum oxygen transfer capacity of the reactor is reached, i. e. maximum stirrer speed, the feed rate is kept constant. One might consider to lower the temperature in order to keep the reactor working under aerobic conditions, as in the above strategy. The feeding strategies presented can be powerful tools in the process development phase, as they can be used when there is almost no process knowledge available. A block diagram describing the process configuration is shown in figure 4.3.

A Feeding Strategy for Known Amino Acids

The following feeding strategy for the amino acid feed is proposed. Pulses are superimposed on the amino acid feed and the feed is manipulated by the responses in the dissolved oxygen to the pulses. The idea is that the cells consume oxygen when they use and take up the 'extra' amino acid added in the pulse. If there is already enough of the amino acid, the cells do not increase their oxygen demand. Therefore the amino acid feed is only increased when there is a pulse response. If there is no response to the feed pulse, the feed rate is kept constant. The reason for the superimposed pulses on the glucose feed is to detect if glucose is accumulating.

An experiment with an *E. coli* strain that is lysine auxotroph using the proposed feeding strategy is shown in figure 4.4. Here the controller is designed to give a response to every other pulse, which also succeeded. According to the pulse responses, there is no glucose accumulating, thus

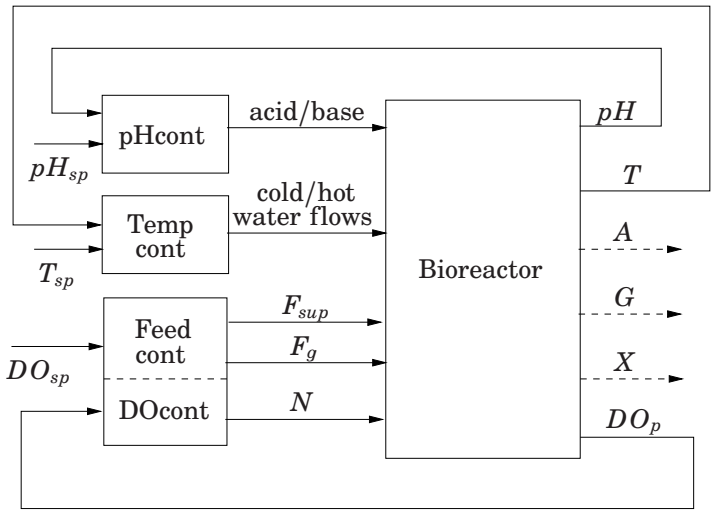

Figure 4.3 A block diagram of the process showing the configuration of dual feeding. The variables are: pH, temperature T, dissolved oxygen DO_p, acetic acid concentration A (off-line), cell mass concentration X (off-line), glucose concentration G (off-line), glucose feed F_g, feed with the needed supplement F_{sup} and stirrer speed N, cont = controller, sp = set-point.

enough amino acid is added in order to consume the glucose. The cell growth follows the predetermined exponential profile set in the glucose feed, $\mu = 0.15$ h^{-1}. Also the amino acid does not seem to be used as an energy source, as there is base added during the cultivation. The base is added to keep the pH at 7 during the cultivation. There is actually evidence in the literature that the amino acid lysine is not readily used as an energy source, [Gschaedler and Boudrant, 1994]. This fact might contribute to the success of the feeding strategy. To implement the feeding strategy, one should know which amino acid to add and also an approximate value of the yield coefficient $Y_{x/aa}$. Here it is obtained from a batch experiment.

A Feeding Strategy for Complex Media

Bacteria readily use complex media for energy purposes and building blocks. Thus there is a difference when compared to amino acid additions, as several amino acids are mostly incorporated in the bacterial proteins, [Gschaedler and Boudrant, 1994]. Therefore a different feeding strategy is needed for the complex media feed. The goal is to cultivate the cells as effectively as possible. For an efficient growth, the complex components should be used primarily as building blocks and the glucose should be used

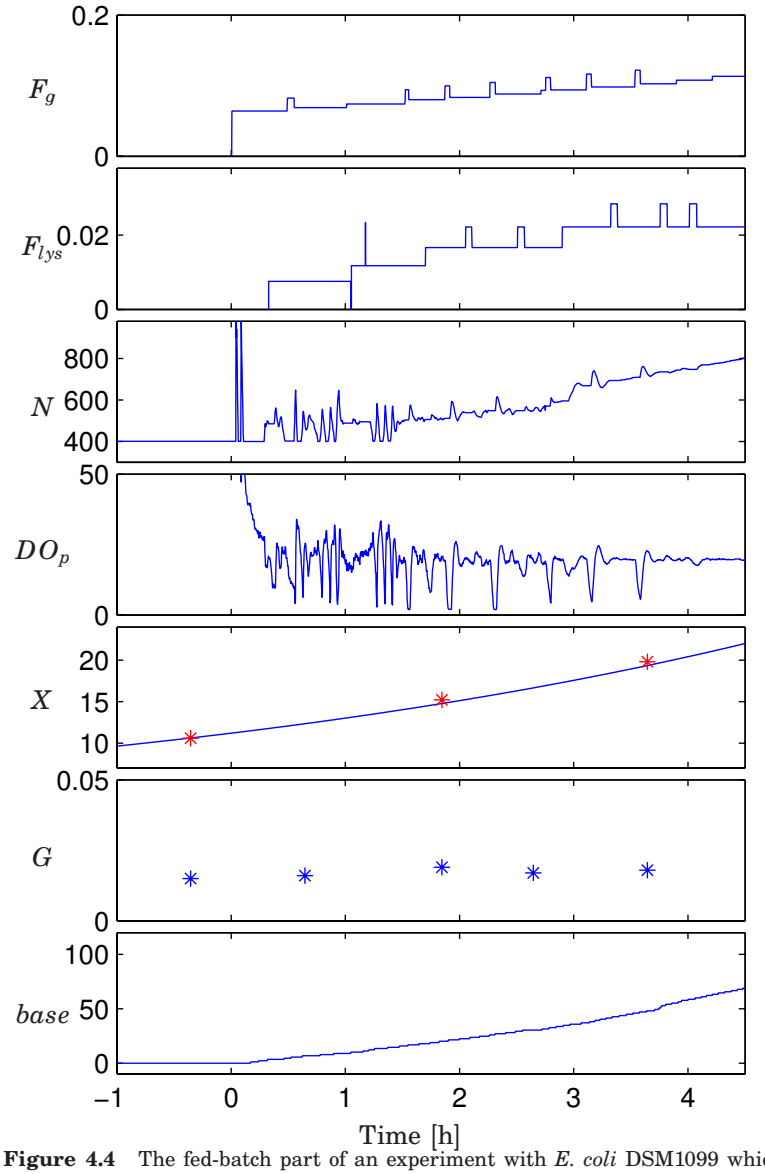

Figure 4.4 The fed-batch part of an experiment with *E. coli* DSM1099 which is lysine auxotroph. The glucose feed is chosen to be exponential, corresponding to a $\mu = 0.15$ h^{-1} and with superimposed pulses. A cell growth corresponding to a growth rate of $\mu = 0.15$ h^{-1} is denoted solid in the figure. Lysine is manipulated by the response in the dissolved oxygen to superimposed pulses on the lysine feed. From the top: F_g [l/h] glucose feed, F_{lys} [l/h] lysine feed, N [RPM] stirrer speed, DO_p [%] dissolved oxygen, X [g/l] cell mass, G [g/l] glucose concentration, *base* [g] added base.

as an energy source. Feedback in the feeding strategy is important in order to obtain reproducible results, as the composition of complex media differs from lot to lot [Iding *et al.*, 2001]. The following feeding strategy is proposed. The complex media feed is manipulated by the pulse responses in the dissolved oxygen to the pulses superimposed on the glucose feed. The principle behind the strategy is that if the substance is missing, the cells can not grow. Thus the cells can not take up the extra glucose given in the pulse. Therefore the complex media feed is increased when the pulse response is small and decreased when the response is large.

An experiment with the proposed strategy is shown in figure 4.5. During the experiment glucose is not accumulating. Thus there is enough complex medium in order to take up the glucose. In the case of overfeeding of the complex medium, the amount of base added for pH control is expected to be low. This is due to the release of ammonia when complex components are used for cellular metabolism. Not much base is added during the later part of the cultivation, thus maybe a part of the complex medium is used for energy purposes. However, there are indications that the two amino acids threonine and leucine, to which the bacterium is auxotroph, are not accumulating during the cultivation (results not shown). In that perspective, too much complex medium is not added and an adequate feed rate is obtained. Further, a high cell growth is obtained, $\mu \approx 0.45\ h^{-1}$. More studies should be carried out in order to confirm the results, but even so the strategy seems promising.

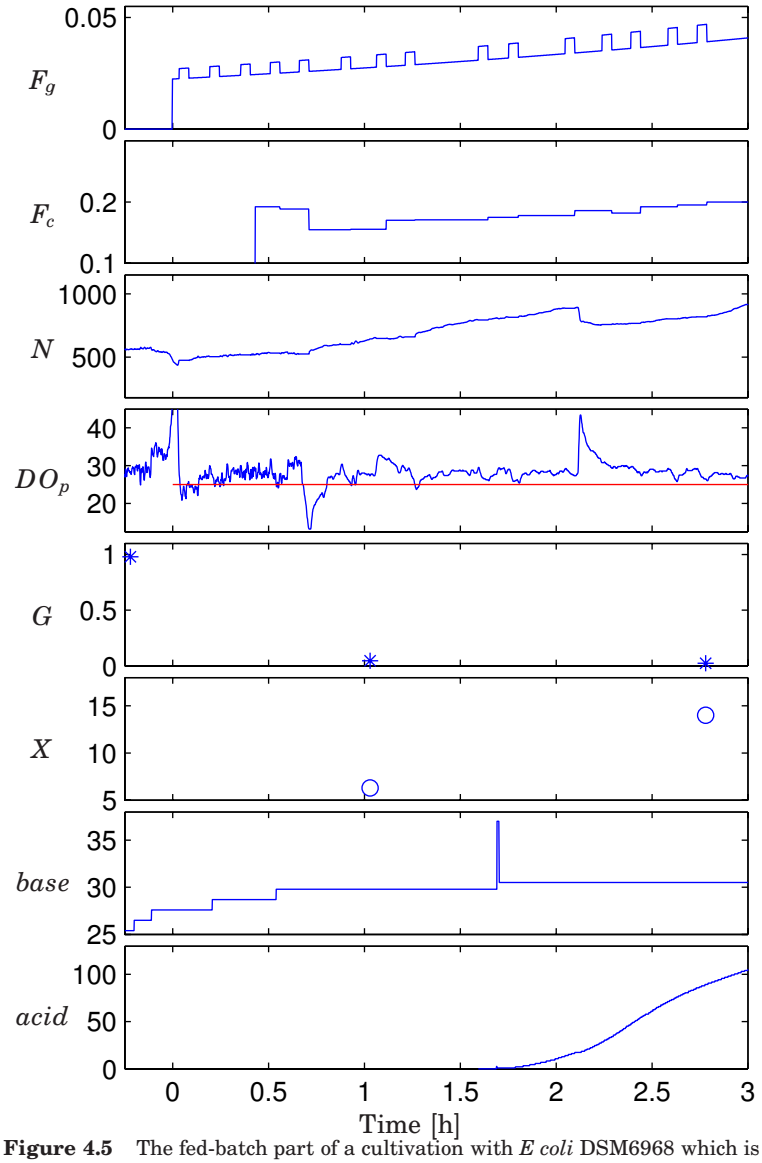

Figure 4.5 The fed-batch part of a cultivation with *E coli* DSM6968 which is auxotroph to leucine and threonine is shown. An exponential glucose feed corresponding to a $\mu = 0.2$ h^{-1} is chosen. The peak in the dissolved oxygen tension at 2.1 h is the result of a change in the airflow. The airflow is changed when the CO_2 concentration in the outlet air is high. From the top: F_g [l/h] glucose feed, F_c [l/h] complex media feed, N [RPM] stirrer speed, DO_p [%] dissolved oxygen, G [g/l] glucose concentration, X [g/l] cell mass, *base* [g] added base, *acid* [g] added acid. Also the set-point of the pulse responses $y_r = 5$ is denoted solid.

5

Modelling and Control Design

In chapter 2.5 a model of a bio-reactor working in fed-batch mode is given, originating from [Åkesson *et al.*, 2001b]. The linearised version is used in order to derive tuning rules for the probing feeding strategy.

In this chapter, the model is extended with an equation describing the effect of the changing oxygen concentration in the outlet gas. The model parameters are estimated and verified with the use of experimental data. The influences of the extended model on the tuning rules derived in [Åkesson *et al.*, 2001b] are investigated. Details are given in paper II.

The effect of the temperature is included into the model in order to design the new fermentation technique, effective for operation at the maximum oxygen transfer capacity of the reactor. The model is used in the controller tuning procedure. Details are given in paper IV.

A model is presented describing the influence of an amino acid without which an auxotroph bacterium is not able not grow. The model is used in the development of a suitable feeding strategy. Details are given in paper V. In the same paper, a model describing the influences of complex media on an auxotroph bacterium is given and used in the design of a feed controller.

5.1 Model Describing the Oxygen Dynamics

An equation describing the changing concentration in the outlet air of the bio-reactor is added to the model in chapter 2.5 describing a bio-reactor operating in fed-batch mode. The gas analyser is described by a first order system and a time delay. The resulting model is then linearised and presented in figure 5.1. The inclusion of the changing oxygen concentration in the outlet gas leads to a positive feedback connection in the model

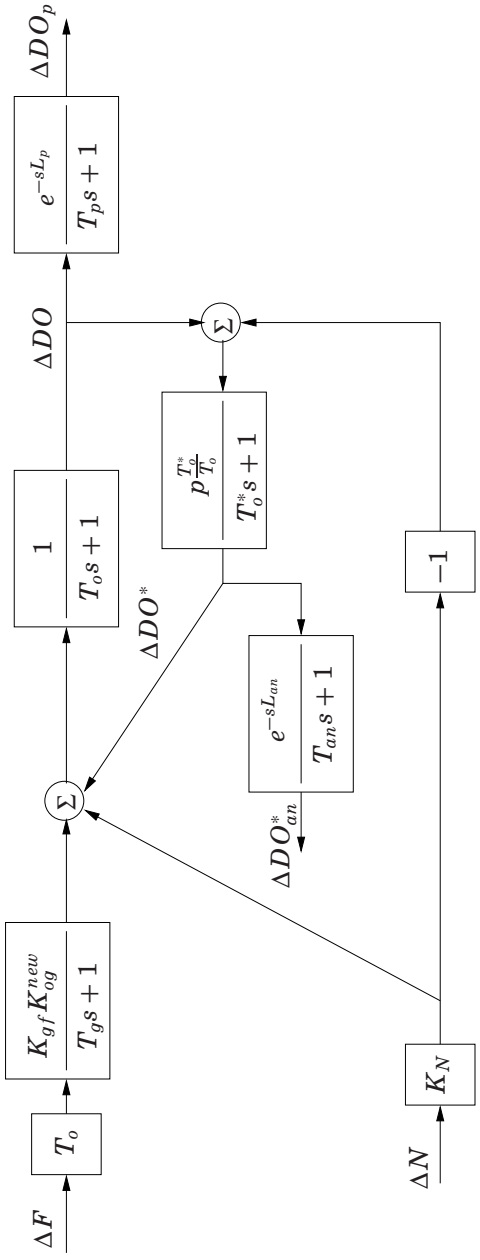

Figure 5.1 Block diagram of the linearised process describing a bio-reactor working in fed-batch mode. Details are given in paper II.

structure. The extended model results in a slower and smaller response in the dissolved oxygen to pulses superimposed on the glucose feed. Some of the model parameters are time-varying which makes the estimation procedure somewhat complicated. Four of the parameters, $K_{gf}K_{og}^{new}$, T_g, p, T_o^*, are estimated with good results. The data used in the identification procedure is obtained from experiments using the probing feeding strategy.

Tuning Rules of the Probing Feeding Controller

The tuning rules derived in [Åkesson *et al.*, 2001b] are based on the process gain and the process dynamics. As these are changed as a consequence of the extended model, the tuning rules are modified. T_{pulse}, the pulse duration, should be chosen slightly longer as the introduced feedback connection leads to slower process dynamics. The requirement on the controller gain κ to ensure stability is: $\kappa < 1 - pT_o^*/T_o$ which is lower than the bound $\kappa < 1$ obtained in [Åkesson *et al.*, 2001b]. Also, when examining the feed controller described in equation (2.1) DO^* is included. In Åkesson's work DO^* is assumed to be constant. It will lead to a lower closed loop gain. To reduce the effect on the stationary error one can add the integral part to the feed controller, as described in [Åkesson *et al.*, 2001b]. An alternative is to make use of the measurements of the gas analyser, which are proportional to DO^*, as a gain scheduling variable.

5.2 Model Describing the Temperature Influence

The influence of the temperature on the model is incorporated in the uptake rates, and also in the maintenance coefficient, using Arrhenius relation. A simulation of the resulting model together with experimental data is shown in figure 5.2. Here the equations describing the changing oxygen concentration in the outlet air are also included, see [de Maré *et al.*, 2004]. The model is used in the development of the strategy, which is effective when the maximum oxygen transfer capacity of the reactor is reached. As mentioned in chapter 4, two requirements should be fulfilled to benefit from the advantage of the temperature limited fed-batch technique of a lower endotoxin formation. There should be a slight excess of glucose in the reactor and the temperature should be the limiting factor of the cultivation.

Glucose Excess by Means of a Probing Feeding Controller

To achieve a controlled excess of glucose in the reactor, down-pulses instead of up-pulses are superimposed on the glucose feed, see figure 5.3. An

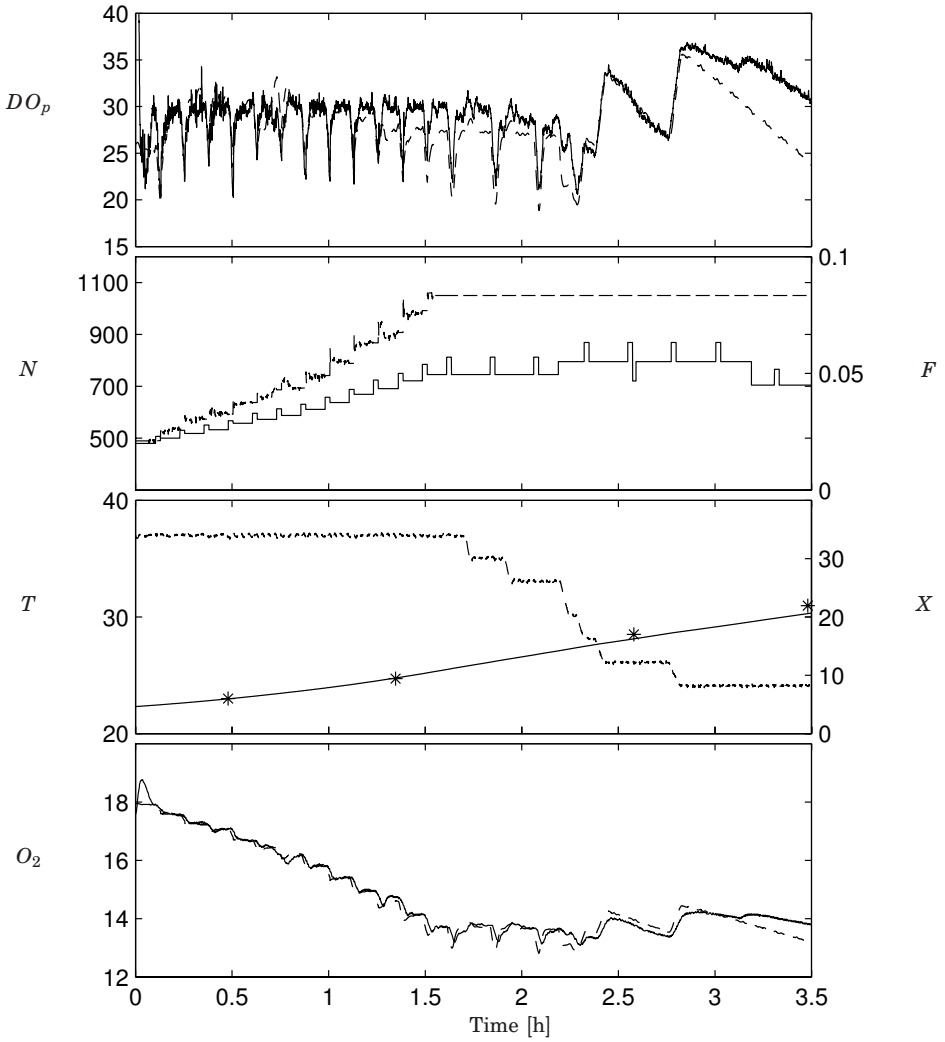

Figure 5.2 Simulation of the model describing the temperature influence together with experimental data. From the top: dissolved oxygen DO_p (simulation dashed), stirrer speed (dashed) N and feed F, temperature T (dashed) and cell mass X (experimental data '*'), oxygen concentration in the outlet gas O_2 (simulation dashed).

analysis is made which shows that it is possible to achieve a stationarity above q_g^{crit}. This is not obvious, as acetic acid is produced when $q_g > q_g^{crit}$ and is consumed during the down-pulse. The balance between the acetate production and acetate consumption can be achieved with a proper choice of the controller parameters. This gives an upper bound on the choice of

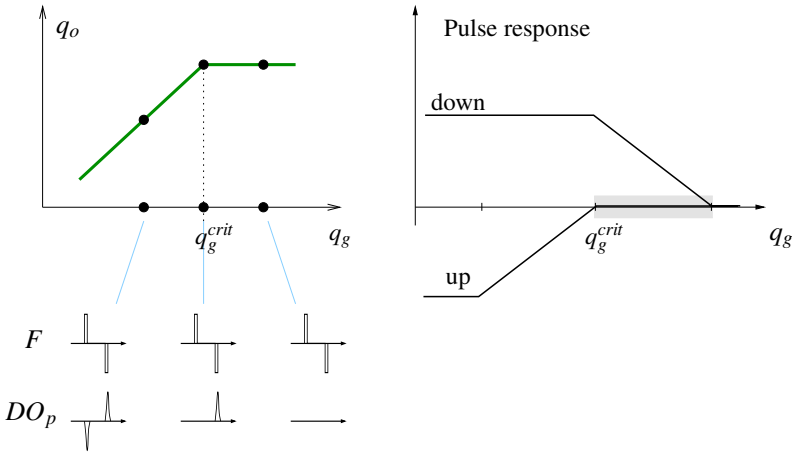

Figure 5.3 The amplitude of the pulse responses in dissolved oxygen as a function of q_g, in the absence of acetate. The shaded area indicates the region where the detection method registers responses to down pulses but not to up pulses.

the probing feeding controller parameter $T_{control}$, the duration between the pulses.

Dissolved Oxygen Control Using Mid-Ranging

The temperature is used to reduce the oxygen uptake and thereby maintaining a constant oxygen level. Manipulation of the temperature for the feedback control of the oxygen concentration is however not a simple task. There are limitations on the achievable performance of the loop. Compared to the agitation system, the cooling process is much slower. Furthermore, there often exist constraints on the rate of change, strongly related to the temperature of the incoming cooling flow.

Given these limitations, dissolved oxygen control based on the sole manipulation of temperature would not be sufficient for a proper application of the probing strategy. The stirrer speed is therefore used simultaneously with the temperature to achieve a satisfactory performance. A mid-ranging controller is used, see [Allison and Isaksson, 1998]. Mid-ranging control solves an allocation problem in the presence of saturations. When properly tuned, the controller maintains the fast control signal below its saturation. In this configuration, shown in Figure 5.4, two SISO controllers are connected in cascade. The first controller C_1 manipulates the stirrer speed N and is tuned to handle the fast disturbances. The objective of C_2 is to keep the control signal N in its operating range. It takes

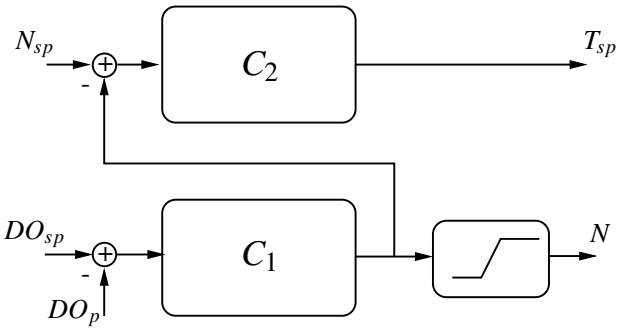

Figure 5.4 Block-diagram over the mid-ranging control scheme. C_1 is a controller manipulating N to take care of the fast disturbances on the output. C_2 manipulates T to maintain N around N_{sp}, below the saturation.

care of slow disturbances. In the strategy the first controller C_1 is used alone until the control signal N reaches the saturation. A benefit of having two SISO controllers concerns the design procedure: the controllers are tuned one at a time just like in conventional cascade control. The fast controller C_1 is designed separately to get a satisfactory performance before the activation of the second loop. The second controller C_2 is tuned in such a way that it does not interfere with the initial control loop at high frequencies.

5.3 Model Describing the Amino Acid Influence

The influences of the needed amino acid (here lysine) on auxotroph bacteria are described using double Monod kinetics. Then glucose can not be taken up and consumed without the needed amino acid. Also no cell growth takes place on the needed amino acid solely. If available, the needed amino acid is taken up proportionally to the glucose. The model is given in paper V. It is used primarily to investigate the behaviour of the system and illustrate the principle of the feeding strategy.

Development of a Suitable Feeding Strategy

A simulation with the model and the feeding strategy described in chapter 4 is shown in figure 5.5 where the bacteria are auxotroph to the amino acid lysine. As mentioned there, the glucose feed follows a predetermined exponential profile with pulses superimposed on it. The amino acid feed F_{aa} is controlled by the responses in the dissolved oxygen to pulses super-

imposed on it as follows:

$$\Delta F_{aa} = \begin{cases} \gamma_p F_{aa} & \text{if } DO_{pulse} > DO_{reac} \\ 0 & \text{otherwise} \end{cases}$$

where γ_p is the pulse height and DO_{reac} the detection limit for a pulse response DO_{pulse}. An approximate value of the yield coefficient $Y_{x/aa}$ is needed in order to choose the controller parameters. The approximate value can be obtained from the literature or by doing a batch experiment, as here. The controller for the amino acid feed is of on/off configuration which leads to the oscillations in the glucose concentrations, see figure 5.5. The use of a proportional controller would lead to a better performance considering the oscillating glucose concentration.

5.4 Model Describing the Complex Media Influence

The difference between the model describing the amino acid utilization and the complex media utilization is that the bacteria are able to grow solely on complex media. Also the complex media might be used as an energy source when there is not enough glucose available.

The model describing the influences of the complex media on the bacteria is thus based on the following assumptions: primarily, the bacteria use the glucose as an energy source and the complex media as building blocks in the cell metabolism. If there is not enough glucose available for energy purposes, some of the complex medium is used as energy. If there is no complex medium available the bacteria can not take up glucose. The cells can take up and grow on complex media, even if there is no glucose available. Also the complex medium is described as one component, thus the accumulation of complex components that are not used in the cell metabolism is not taken into account. The primary use of the model is also in this case to investigate the behaviour of the system in order to develop a suitable feeding strategy. The model is also used to illustrate the principle of the feeding strategy and to derive some tuning guidelines.

Development of a Suitable Feeding Strategy

A simulation with the feeding strategy proposed in chapter 4 is shown in figure 5.6. The following feeding strategy is proposed: an exponential glucose feed during the growth phase as well as a complex feed which is regulated by the responses in the dissolved oxygen to pulses superimposed on the glucose feed. A proportional pulse controller is used to adjust the

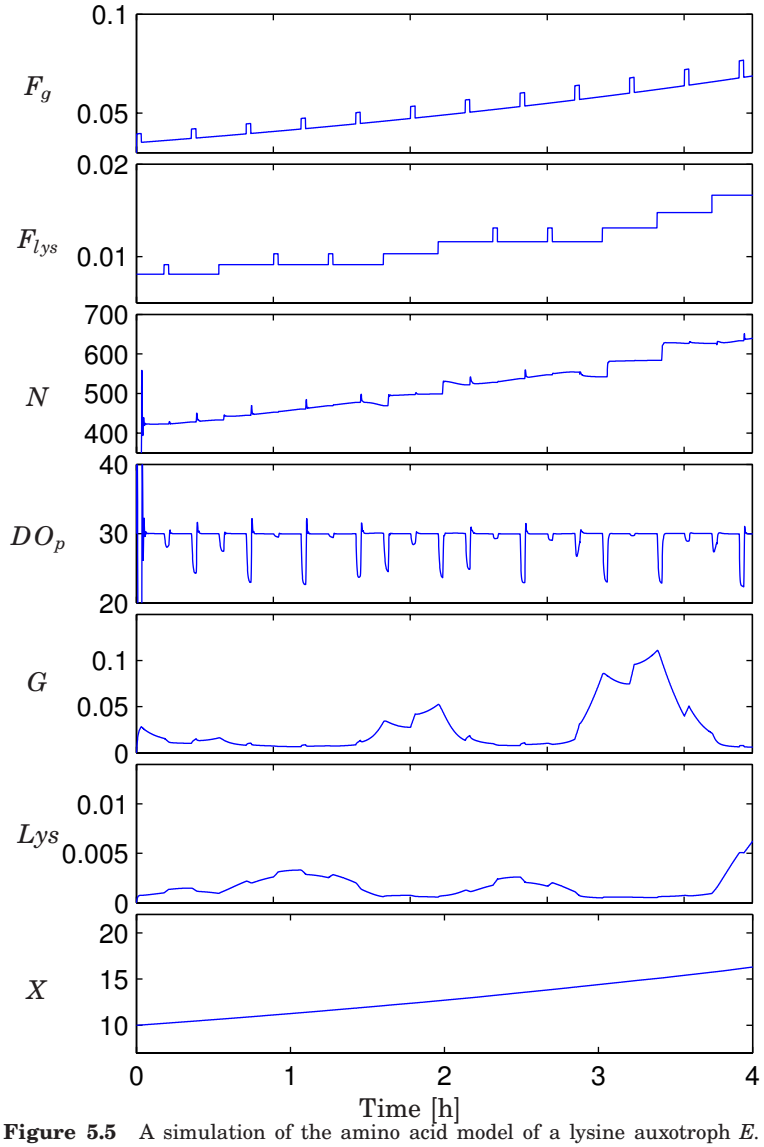

Figure 5.5 A simulation of the amino acid model of a lysine auxotroph *E. coli*. Here the glucose feed is chosen to be exponential corresponding to a $\mu = 0.15$ h^{-1} and the amino acid (here lysine) feed is regulated by the responses in the dissolved oxygen tension to the superimposed pulses on the amino acid feed. From the top: F_g [l/h] glucose feed, F_{lys} [l/h] lysine feed, N [RPM] stirrer speed, DO_p [%] dissolved oxygen, G [g/l] glucose concentration, Lys [g/l] lysine concentration.

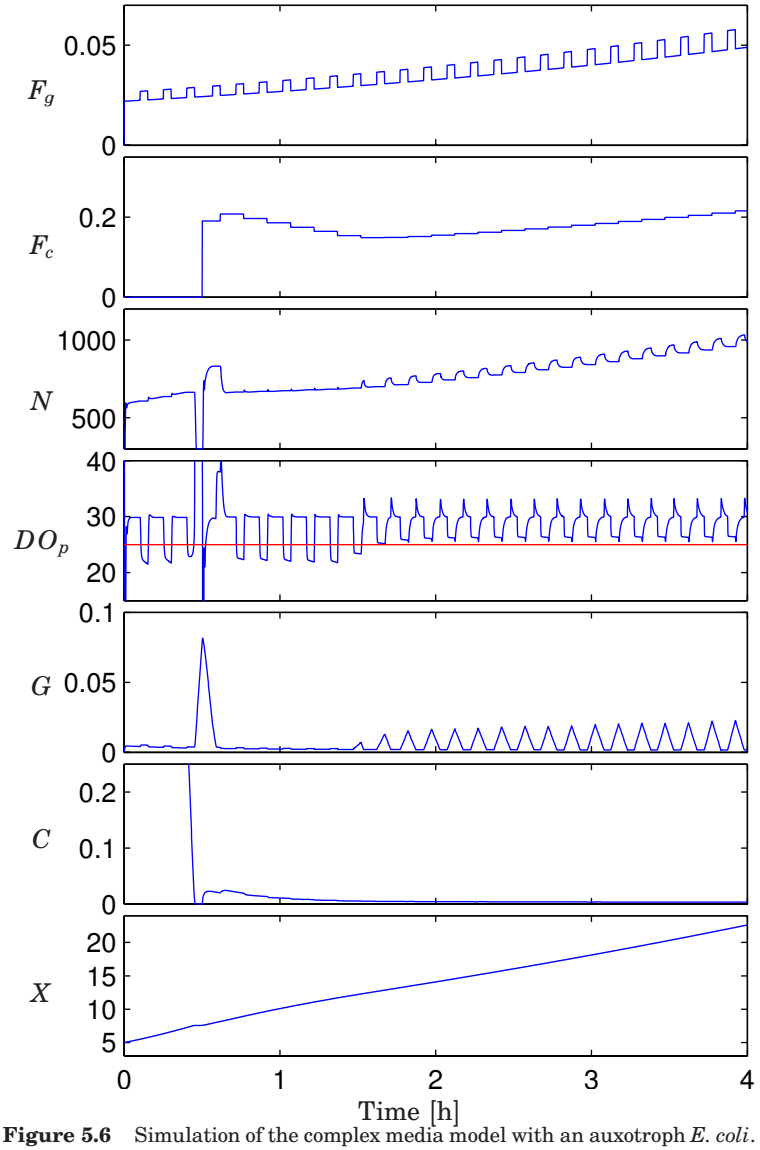

Figure 5.6 Simulation of the complex media model with an auxotroph *E. coli*. Here an exponential glucose feed is chosen corresponding to $\mu = 0.2\ \mathrm{h}^{-1}$. The complex feed is regulated by the responses in the dissolved oxygen tension to superimposed pulses on the glucose feed. From the top: F_g [l/h] glucose feed, F_c [l/h] complex media feed, N [RPM] stirrer speed, DO_p [%] dissolved oxygen, G [g/l] glucose concentration, C [g/l] complex media concentration, X [g/l] cell mass.

complex media feed F_c.

$$\Delta F_c = \frac{\kappa(y_r - DO_{pulse})}{DO^* - DO_{sp}} F_c \tag{5.1}$$

where DO_{pulse}, DO_{sp}, DO^* are the pulse response, the set-point and the saturated dissolved oxygen concentration, respectively. When choosing the parameters of the controller, κ and y_r, there are some guidelines: the desired pulse response y_r should be chosen to be smaller than an upper bound. If it is chosen too small, it is difficult to add enough complex media to support the exponential growth. This is also the case if the controller gain κ is chosen very small. Boundaries of κ to assure a stable system are also derived.

6

Conclusion

The probing feeding strategy as described in [Åkesson *et al.*, 2001a] is further developed in several aspects in this thesis.

The probing feeding strategy is applied to cultivations with *V. cholerae* leading to good results. Earlier it had been tested solely on different *E. coli* strains. This shows how useful the technique is and that it can be applied to different organisms resulting in a good performance without much a priori information.

The derived tuning rules for the probing feeding strategy in [Åkesson *et al.*, 2001b] are based on a bio-reactor model. This model is here extended to include the changing oxygen concentration in the outlet air. It is verified by using experimental data from cultivations to which the probing feeding strategy is applied. The effect of the extended model on the tuning rules is also investigated. The most important results are that a lower bound on the probing controller gain κ has to be fulfilled to ensure stability. Also a slightly longer pulse duration T_{pulse} is necessary, as the process dynamics is slightly slower than postulated in [Åkesson *et al.*, 2001b]. Finally in Åkesson's work DO^* is assumed to be constant. This will lead to lower closed loop gain. To prevent this, one can add the integral part to the feed controller, as described in [Åkesson *et al.*, 2001b]. An alternative is to make use of the measurements of the gas analyser, as a gain scheduling variable.

A new fermentation technique, effective when the maximum transfer capacity is reached, is developed and is based on probing control. The dissolved oxygen is controlled by using a mid-ranging controller configuration manipulating the stirrer speed and the temperature. The new fermentation technique is evaluated in experiments using two different sets of reactors and *E. coli* strains. The temperature is lowered once the maximum oxygen transfer capacity of the reactor is reached. Good results are obtained, as the resulting low temperature promotes the production of soluble, active protein. Based on a model describing the temperature

influence, the technique is analysed and tuning guidelines are presented. An upper bound on the control phase $T_{control}$ is given, to assure that no acetic acid is accumulating. The mid-ranging configuration consists of two SISO controllers which can be tuned one at a time just like in a conventional cascade controller.

The probing feeding strategy is further developed in order to handle the case where more than one feed are to be added. It is necessary for the production strains of *E. coli* which demand amino acids or complex media to grow and/or produce the recombinant protein. Two feeding strategies are presented. In both the strategies the glucose feed follows an exponential profile until the maximum oxygen transfer capacity of the reactor is reached and pulses are superimposed on the feed-rate. When the needed amino acid is known, the following strategy is proposed: the amino acid feed is regulated by the response in the dissolved oxygen to pulses superimposed on the known amino acid feed. The strategy is tested in an experiment with an auxotroph *E. coli* with good results. In the development of the strategy, a model is used and simulations are shown to illustrate the principles. When complex media are to be added, the following strategy is proposed: the complex media feed is manipulated by the response in the dissolved oxygen to the pulses superimposed on the glucose feed. The strategy is tested in an experiment and good results are obtained, even though more investigations will be needed. Simulations are done to illustrate the principles and the model is used to derive some tuning guidelines. As almost no process knowledge is needed in order to use these feeding strategies, they can be used to shorten the process development phase considerably.

Finally, the strategies developed are built on concepts that are easy to understand and standard sensors are used. Hopefully this will make the techniques attractive. To facilitate the implementation procedure of the strategies a control system software is needed which has easy access to signals, is flexible and user-friendly.

7

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A

Appendix

A.1 Uptake Rates

The glucose uptake rate q_g is given by:

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

The glucose used for maintenance purposes is given by:

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

The glucose used for growth uptake is thus:

$$q_{gg} = q_g - q_m$$

Splitting into an oxidative flow and a fermentative flow gives:

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

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

Specific acetate production q_a^p is given by:

$$q_{ap} = q_{gg}^{fe} Y_{ag}$$

Specific acetate consumption q_a^c:

$$q_{ac}^{pot} = q_{ac}^{max} A/(k_a + A)$$

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

Table A.1 Parameters in the model presented in chapter 2.5.

Symbol	Description
G_{in}	glucose concentration in feed
H	Henrys constant
k_{sg}	saturation constant for glucose uptake
C_o^*	saturation concentration diss. oxygen
q_g^{max}	maximum specific glucose uptake
q_o^{max}	maximum specific oxygen uptake
q_{mc}	maintenance coefficient
Y_{oa}	oxygen/acetate yield
Y_{og}^{ox}	oxygen/glucose yield for growth
Y^{om}	oxygen/glucose yield for maintenance
Y_{xa}	biomass/acetate yield
Y_{xg}^{ox}	oxidative biomass/glucose yield
Y_{xg}^{fe}	fermentative biomass/glucose yield

The resulting acetate formation rate q_a:

$$q_a = q_{ap} - q_{ac}$$

Specific growth rate μ:

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

Specific oxygen uptake rate q_o is given by:

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

I

Original paper

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Probing control of glucose feeding in Vibrio cholerae cultivations

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Abstract Infection with *Vibrio cholerae* is a significant problem in many developing countries. Cultivation of *V. cholerae* is used in production of cholera toxin B subunit, which is a component in a cholera vaccine. Fed-batch cultivations with *V. cholerae* in defined media have been conducted and reproducible results were obtained. A probing feeding strategy developed by Åkesson for *Escherichia coli* cultivations has been tested. The strategy is working as well for *V. cholerae* as for *E. coli* in minimizing the amount of acetic acid formed and avoiding anaerobic conditions. At 2 h after the feed start most of the acetic acid accumulated during the batch phase is consumed. The resulting feed rate tends to be the highest possible with respect to the constraints from cell metabolism and mass transfer, thus maximizing productivity in terms of biomass. A cell dry weight of 20–23 g/l is obtained after 12 h of feeding.

Keywords *V. Cholerae*, Probing feeding strategy, Fed-batch cultivations

1 Introduction

Cholera is a significant problem in many developing countries. The cholera toxin (CT) responsible for the diarrhea consists of one A subunit (CTA) and five B subunits (CTB). *Vibrio cholerae* is for example cultivated for production of CTB. The B subunit of CT is non-toxic but has been shown to possess high immunogenic activity and can be used as a component in a cholera vaccine [1]. This vaccine also protects against diarrhea caused by the heat-label enterotoxin of *Escherichia coli* [2]. It is also possible to produce the CTB by cultivating *E. coli* [3], even though the protein then will accumulate in the intracellular fraction. Another way to produce cholera vaccine is to use a live and attenuated strain as described in [4] and [5].

Another reason for cultivating *V. cholerae* might be for the production of the heat-label enterotoxin of *E. coli* [6]. *V. cholerae* growth rate, similarities to *E. coli*, and the ability to export protein to the extracellular broth make it an attractive host for production and recovery of non-glycosylated proteins. *V. cholerae* is a gram-negative microorganism and the techniques of fermentation generally applied to recombinant *E. coli* should work.

It is reported in [7] that toxin is produced only when cultivating *V. cholerae* in defined media enriched with a combination of amino acids or complex media. Different media for producing the toxin are described in [7, 8, 9].

In the literature, there is evidence of acetate formation when cultivating *V. cholerae* [10] and it is probable that the acetate has the same negative consequences for *V. cholerae* as for *E. coli*. During cultivations with *E. coli* the accumulation of the by-product acetate is a problem. Acetate production reduces growth and recombinant protein formation [11, 12]. Formation of acetate in *E. coli* cultures occurs under anaerobic conditions, but also under fully aerobic conditions in situations with excess carbon source [13, 14]. High specific growth rate, high specific glucose uptake, bottlenecks in the Krebs cycle, limited respiratory capacity, or a combination of any of the above has been suggested to trigger the acetate overflow metabolism [15, 16, 17]. The accumulation of acetate can be reduced by manipulation of strains, media, and cultivation conditions. Different strategies are reviewed in [18] and [19]. In fed-batch cultures, the feed rate of the carbon source can be manipulated to restrict the formation of acetate.

Mats Åkesson has developed a feeding strategy using standard sensors. A probing feedback algorithm is used to adjust the feed rate to avoid overflow metabolism while maintaining a high growth rate. The aim is to use the strategy to control the glucose feed in *V. cholerae* cultivations. While avoiding acetic acid formation and thereby increasing the cell productivity, it should improve the cultivation results. Defined media without additions of amino acids are used and no toxin production is obtained. Experiments using complex media and a *V. cholerae* strain encoding for CTB production have also been carried out successfully (results not shown).

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2
Probing feeding strategy

Excess glucose feeding inhibits cell growth because of organic acid production, and cell growth is also seriously inhibited by starvation [20], therefore it is important to have a good feeding strategy.

Under glucose-limited conditions pulses superimposed to the glucose feed rate give rise to changes in the glucose uptake. These changes imply variations in the oxygen uptake that can be seen in the dissolved oxygen measurements. For glucose uptake, q_g, above a critical value, q_g^{crit}, the oxygen uptake is saturated and the oxygen response to feed pulses will change character. The pulse responses reveal whether q_g is above or below q_g^{crit}, and hence if acetate is being produced or not.

In [21], a simple feedback algorithm using the information from the pulse responses was developed to achieve feeding around q_g^{crit}, that is, the highest glucose uptake without acetate formation. Dissolved oxygen is controlled by manipulation of the stirrer speed, which ensures that dissolved oxygen is at the same level at the start of each pulse. During the feed pulses, the stirrer speed is fixed so as not to interfere with the detection algorithm. A safety net to avoid limitations in the oxygen transfer is also described. No feed increment is allowed when the stirrer speed approaches its maximum, as the oxygen demand then would exceed the oxygen transfer capacity. Between pulses, when the stirrer speed is controlling the dissolved oxygen, the feed rate is decreased as long as the stirrer speed is at or close to its maximum.

In [22], a proportional-integral feed controller is described, which means that the feed is changed with an amount related to the size of the pulse response. A reference value for the size of the dissolved oxygen response to a feed pulse is introduced. The reference value makes it possible to keep q_g below q_g^{crit}. The feed is decreased with an amount relative to the pulse size when there is no response to a pulse in the feed. There is also a safety net that delays the feed pulse until the setpoint in the dissolved oxygen is reached.

3
Materials and methods

A preliminary experiment is carried out to verify that the dissolved oxygen signal can be used to detect acetate production also in *V. cholerae* cultivations. Five experiments with the probing feeding control are conducted.

3.1
Microorganisms

V. cholerae JS1569 is the microorganism cultivated in the experiments. It is a rifampicin-resistant, *ctx*A-deleted derivative of 569B, classical Inaba, [23].

3.2
Bioreactor equipment and cultivation conditions

3.2.1
Preparations and media

V. cholerae is day 1 spread over an LB plate. It is incubated at 36°C over night (16–24 h) or at 20°C for three nights. The bacteria growth is dissolved in a 10-ml shake-flask media on day 2. The optical density (OD) is measured at 600 nm and the solution is diluted. The shake-flask is inoculated and incubated at 36°C, 160 rpm during 24 h. Depending on the desired length of the batch phase, the fermenter is inoculated with a volume of around 100 ml or 4 ml of a solution from the shake-flask with OD around 4 or 1, respectively. Table 1 lists the shake-flask media and the media in the bioreactor. The initial amount of glucose in the bioreactor is 10 g/l.

3.2.2
Control

Two different 6-l reactors are used (Belach Bioteknik AB, Stockholm, Sweden). Data logging and controlling strategy are implemented and handled by Genesis Control Series software (Iconics Inc., Foxborough, USA). pH is kept at 7.5 with 25% NH_3. The airflow is set to 0.5 vvm and the temperature setpoint is 36°C.

Table 1. Shake-flask media, bioreactor media, precultivation media, cultivation media, trace element solution, and 60% glucose solution

Media component	Volume [ml]	Media component	Weight [g]
Shake-flask media		**Cultivation media**	
Precult media	400 ml	$(NH_4)_2SO_4$ (2.5 g/l)	20 g
Trace elements	0.4 ml	K_2HPO_4 (10.1 g/l)	80.8 g
$MgSO_4$ 1.2 M	0.8 ml	KH_2PO_4 (1.57 g/l)	12.56 g
60% Glucose	7.3 ml	NH_4Cl (0.5 g/l)	4 g
Inoculum volume	1, 0.5, 2 ml	Na-citrat (0.5 g/l)	4 g
Bioreactor media		Dissolved in dH_2O	1 l
Cultivation media	500 ml	**Trace elements**	
Distilled H_2O	3600 ml	$FeCl_3$*$6H_2O$ (8.1 g/l)	1.62 g
$MgSO_4$ 1.2 M	12 ml	$ZnSO_4$*$7H_2O$ (0.36 g/l)	0.072 g
Adecanol	0.5 ml	$CuSO_4$*$5H_2O$ (0.31 g/l)	0.062 g
Trace elements	4 ml	Na_2-EDTA*$2H_2O$ (1 g/l)	1.0 g
60% Glucose	76 ml	$CoCl_2$*$6H_2O$ (0.36 g/l)	0.072 g
Inoc. Vol., OD 1/OD 4	4 ml/100 ml	$MnSO_4$*H_2O (0.3 g/l)	0.06 g
Precultivation media		$CaCl_2$*$2H_2O$ (1 g/l)	0.2 g
$(NH_4)_2SO_4$ (2.5 g/l)	3.75 g	Dissolved in dH_2O	200 ml
K_2HPO_4 (10.1 g/l)	15.15 g	**60% Glucose feed**	
KH_2PO_4 (1.57 g/l)	2.35 g	Glucose * H_2O	2640 g
Na-citrat (0.5 g/l)	0.75 g	Dissolved in 1.8 l hot dH_2O	Final vol 4 l
Dissolved in dH_20	1.5 l		

3.2.3
RPM–pO_2 controller

For the feeding strategy, it is important that a well-tuned controller is available for the dissolved oxygen. The dissolved oxygen tension is kept at 30% using an RPM–pO_2 controller with three different sets of proportional integral (PI) parameters. Since oxygen dynamics may vary significantly during cultivation in batch and fed-batch mode, there may be tuning problems when a controller with fixed parameters is used and high performance is desired. Therefore, a control strategy based on PI control and gain scheduling from the stirrer speed is proposed in [24] and used here. Starting stirrer speed is 300 RPM.

3.2.4
Feed-rate profiles

When there is a peak in dissolved oxygen tension, the glucose feed is started at a value of 0.3 g/min. Its composition is listed in Table 1. When OD 10, 20 and 30 are reached, 8 or 12 ml $MgSO_4$ 1.2 M and 4 ml of trace element solution are added into the bioreactor. In the experiments, the feeding strategy described in Sect. 2 is used to control the glucose feed rate. A proportional feed controller is used, which means that the amount with which the feed is changed is proportional to the size of the dissolved oxygen response minus a reference value. The reference value for the dissolved oxygen response to a feed pulse is 1.5%. Theoretically, the highest feed rate that could be obtained with the parameter values of the feed controller used here corresponds to a μ of 0.6 h^{-1}. For a detailed description of the feed controller and its parameters, see [21].

3.3
Analyses

3.3.1
Sample treatment

The samples are withdrawn through a sampling port. Samples are mainly collected for acetic-acid analysis and they are centrifuged (10,000 g) for 10 min at 4°C. The supernatants are sterile filtered and kept frozen at –20°C. Prior to the analyses, the samples are heated for 20 min at 80°C.

OD is measured at 600 nm. The amount of glucose is estimated using glucose sticks (Clinistix 2844, Bayer Corporation USA). Acetic acid concentrations are determined enzymatically using test kits number 148261 (Boehringer, Mannheim, Germany). Cell dry weight is measured as follows. Triple samples of 5 ml are collected and then centrifuged (10,000 g) for 6 min at 4°C. The pellets are dissolved in 5 ml of phosphate buffer. The samples are centrifuged (10,000 g) again for 6 min at 4°C and the supernatants are removed. The samples are dried over night at 105°C.

4
Results

4.1
Pulse experiments

In a preliminary experiment, periods with and without acetate production are achieved by manipulating the feed rate, as described in [25]. Pulses are added to the feed rate and it is confirmed that the dissolved oxygen signal can be used for detection of acetic acid (see Figs. 1 and 2a).

4.2
Experiments with the pulse program

In Fig. 2b, an example of a feed pulse during one of the experiments is shown. The amount with which the feed is changed is proportional to the size of the amplitude of the dissolved oxygen response minus a reference value, as described in Sect. 3.2.4. The reference value is the desired value of the dissolved oxygen response to a pulse in the glucose feed.

An overview of the fed-batch part of one of the cultivations is shown in Fig. 3a. A lot of acetate is produced during the batch phase and a total concentration around 1.5 g/l is accumulated before the fed-batch part is started.

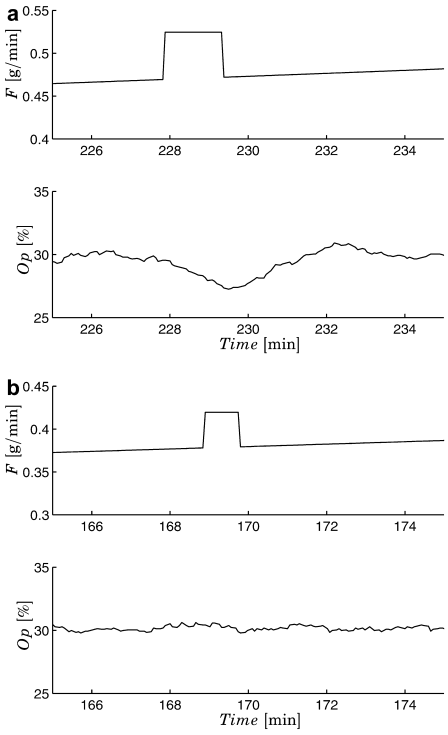

Fig. 1. **a** There is a response in dissolved oxygen *Op* to a pulse in feed rate *F*. There is no acetic acid present. The feed rate corresponds to a μ of 0.2 h^{-1}. The specific glucose uptake is below q_g^{crit}, therefore no production of acetic acid occurs. **b** There is no response in dissolved oxygen *Op* to a pulse in feed rate *F*. There is acetic acid present. The feed rate corresponds to a μ of 0.2 h^{-1}. The specific oxygen uptake is below q_g^{crit} and acetic acid consumption occurs. Time after feed start

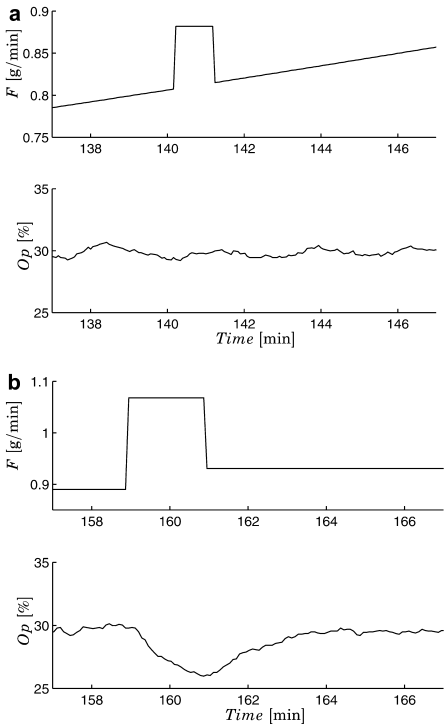

Fig. 2. **a** There is no response in dissolved oxygen *Op* to a pulse in feed rate *F*. There is acetic acid present. The feed rate corresponds to a μ of 0.5 h^{-1}. The specific glucose uptake is above q_s^{crit} and production of acetic acid occurs. **b** There is a response in dissolved oxygen *Op* to a pulse in feed rate *F*. The feed is increased with an amount that is proportional to the size of the oxygen response. Time after feed start

The accumulated amount of acetic acid decreases the pulse responses in the dissolved oxygen tension. When the acetic acid is depleted there is an increase in pulse responses, which results in a feed rate increase. The initial amount of acetate is consumed approximately 2 h after the feed start and there is a peak in the dissolved oxygen tension at the moment of depletion. The maximum oxygen transfer capacity of the reactor is reached 3 h after the feed start.

4.2.1
Equipment limitations

In one of the experiments, the pump limit was reached. In this case, there was no safety net implemented. The feed was decreased a couple of times since the pulse responses were small. The small pulse responses depended on that the real size of the pulses was small since the maximum pump speed was reached. An overview of a part of the fed-batch part in this experiment is shown in Fig. 3b.

4.3
Reproducibility

4.3.1
Feed and stirrer profiles

The feed profiles and the stirrer profiles of the five experiments are shown in Fig. 4. In Fig. 4a, two of the three experiments shown are remarkably similar in the profiles.

In Fig. 4b, the solid line is the experiment where the pump limit is reached. During the experiment denoted with a dashed line, there was a problem with the airflow during the first 4 h after the feed start. This resulted in a dip in the feed profile between 2 and 4 h and a dip in the stirrer profile at 4 h. Otherwise, the feed profiles and the stirrer profiles in Fig. 4b are very similar to each other and to the ones in Fig. 4a.

4.3.2
Cell production and acetate consumption

The cell growth for the five experiments is shown in Fig. 5a. At 8 h after the feed start, the OD obtained was between 20 and 30, which means that the amount of cells produced slightly differs. In Table 2, the cell dry weight and the overall cell yield are shown. An average of the specific growth rate after the batch phase until the maximum oxygen transfer capacity or the pump limit was reached is shown in Table 3. The μ obtained was less than 0.6 h^{-1}, which theoretically is the maximal μ achieved with these controller parameters (see Sect. 3.2.4). Hence, the feeding strategy does not limit the cell growth. Also shown in Table 3 is the μ_{max} obtained during the batch phase. In Fig. 5b, the acetic-acid concentrations for the five experiments are shown. The amount of acetate produced during the batch phase was around 1.5 g/l for three of the experiments. For two of the experiments, 2.5 g/l was produced during the batch phase. Nevertheless, the acetate was consumed 2 h after the feed start for all the experiments except one (denoted ∇), where it took more than 3 h. This was the experiment with the airflow problem.

5
Discussion

5.1
Evaluation of the pulse program

To avoid misinterpretations of the pulse responses in the dissolved oxygen tension a well-tuned RPM–pO_2 controller was used with good results, as seen in Fig. 3a. The oxygen responses to feed pulses are seen clearly and are easily interpreted. In the preliminary experiment, the slope of the dissolved oxygen response at the end of the feed pulse does not show a tendency to decrease (see Fig. 1a). The length of the feed pulses was therefore prolonged to 2 min in all the experiments. An example of a longer pulse is shown in Fig. 2b and the slope of the pulse response decreases at the end of the feed pulse. To make the pulse responses in the dissolved oxygen tension clearer, the height of the feed pulses was also increased (compare Figs. 1a and 2b).

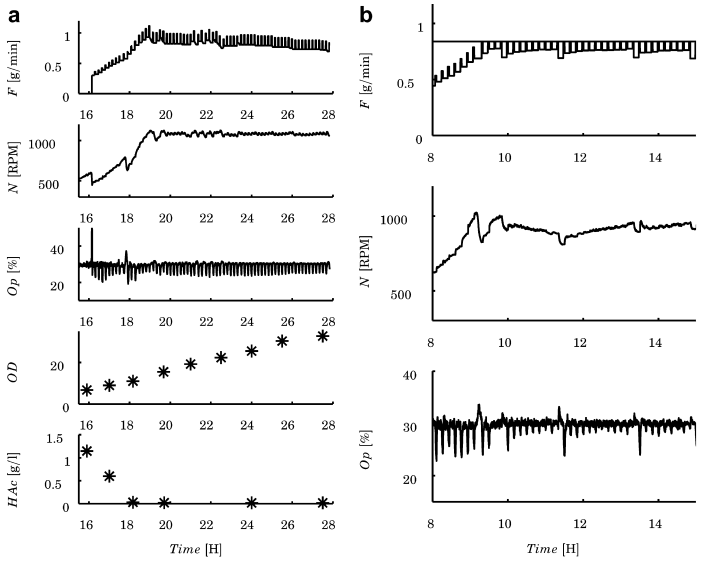

Fig. 3. **a** A fed-batch part of a cultivation with *V. cholerae* is shown. **b** A part of the fed-batch part of a cultivation with *V. cholerae* limited by the pump is shown. From top to bottom: glucose feed rate *F*, stirrer speed *N*, dissolved oxygen tension *Op*, cell concentration *OD* and acetate concentration *HAc*

5.1.1
Acetic-acid consumption period

The acetate accumulated after the batch phase affects the size of the pulse responses in the dissolved oxygen (see Fig. 3a). With less acetate present the glucose feed would have increased faster. If the glucose feed had increased faster, it would have taken longer to consume the amount of acetate present. On the other hand, if the glucose feed had decreased too much, it would probably have taken longer to consume the acetic acid. According to [26] the consumption is faster in the presence of some glucose than in its absence in *E.coli* cultivations. The program worked well in consuming the amount of acetate accumulated during the batch phase in all the cultivations (see Fig. 5b).

5.1.2
Avoiding overflow metabolism

After the amount of acetic acid present after the batch phase is consumed the pulse program is working for 1.5 h in avoiding overflow metabolism, see Fig. 3a. No indications of overflow metabolism could be observed, which is in agreement with the low glucose concentration, estimated from sticks, and acetate concentrations from the off-line analysis. The feed resulting from the feeding strategy makes it possible to keep q_g slightly below q_g^{crit}. Usually, q_g^{crit} is not known and its value may change in the course of the cultivation. It is therefore valuable to use feedback as in the probing feeding strategy (see Sect. 2). The program is thus working well in avoiding overflow metabolism.

5.1.3
Avoiding anaerobic conditions

When the oxygen transfer capacity was reached 3 h after the feed start, the pulse program was avoiding anaerobic conditions by decreasing the feed rate when necessary and not allowing feed increments. The dissolved oxygen was kept at 30% (see Fig. 3a). The program was thus working well in avoiding anaerobic conditions. Other measures might be taken to avoid anaerobic conditions, such as decreasing the temperature, [27].

5.1.4
Equipment limitations

To deal with pump limitations, one could make the pulses smaller as the maximum capacity of the pump is approached. Or one simply uses the real pulse size in the computations for the feed. Here, no safety net was implemented, but it seems as if the program handled it in the right way (see Fig. 3b). The cell growth, denoted o in Fig. 5a, was not lower than for the other experiments, even though it was the pump that limited the cultivation and not the oxygen transfer capacity.

5.2
Reproducibility

5.2.1
Feed and stirrer profiles

The dashed profile in Fig. 4a is different, which might be due to problems with the oxygen sensor or/and with the

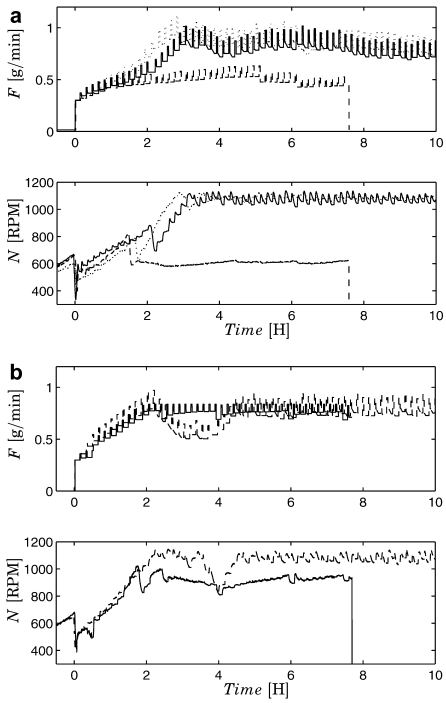

Fig. 4. **a** The feed profiles and stirrer profiles for the three experiments conducted in bioreactor 1 are shown. Notation: *solid* corresponds to x, *dotted* corresponds to * and *dashed* corresponds to + in Fig. 5. **b** The feed profiles and stirrer profiles for the two experiments conducted in bioreactor 2 are shown. The pump limit is reached in one of the cultivations (*solid*) and there were problems with the airflow the first 4 h in the other cultivation (*dashed*). Notation: *solid* corresponds to o and *dashed* corresponds to ∇ in Fig. 5. Time after feed start

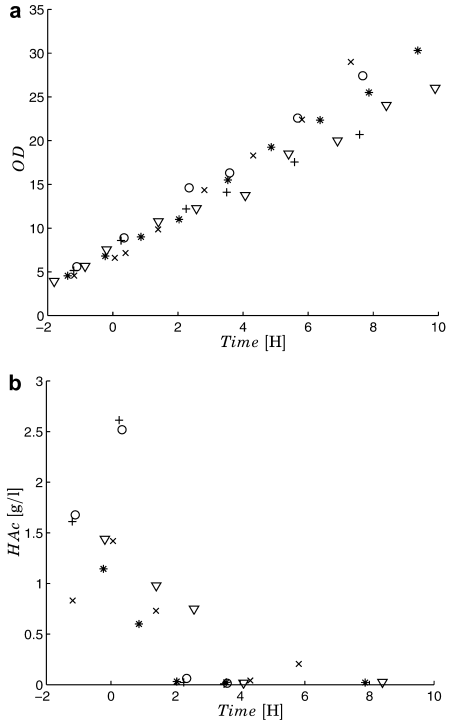

Fig. 5. **a** The cell growth during the five experiments is shown. **b** The acetic acid concentration during the five experiments is shown. Time after feed start

mixing in the reactor which may have affected the cells. Under stress conditions the *V. cholerae* can convert to viable but non-cultivable form that cannot be recovered or/and undergo morphological changes [28]. Even though we do not know if it happened, it could be the explanation for the difference in the behavior. The cell growth and the specific growth rate was not as good in this experiment, denoted + in Fig. 5a and in Table 3, when comparing it to the others. Otherwise, the profiles in Fig. 4 look similar.

5.2.2
Cell production

When producing CTB for cholera vaccine the amount of cells produced is interesting since that is correlated to the amount of protein produced [7,8]. It has been reported when cultivating *V. cholerae* for CTB production and for production of B subunit of *E. coli* enterotoxin that the

Table 2. Cell dry weight [g/l] and overall cell yield $Y_{x/s}$ [g/g] after the batch phase, 7 h after feed start, and 12 h after feed start

Experiment	'x'	'o'	'+'	'*'	'∇'
CDW after batch phase	3.7	4.8	4.8	4.4	4.3
CDW 7 h after feed start		18.6	14.6		
$Y_{x/s}$ 7 h after feed start		0.35	0.37		
CDW 12 h after feed start				22.9	20.1
$Y_{x/s}$ 12 h after feed start				0.25	0.28

Table 3. Maximum specific growth rate μ_{max} [h^{-1}] obtained during the batch phase and the average of specific growth rate μ [h^{-1}] from the feed start until the maximum oxygen transfer capacity or the pump limit is reached. Cultivations x, +, and * were carried out in bioreactor 1 and cultivations ∇ and o were carried out in bioreactor 2

Experiment	'x'	'o'	'+'	'*'	'∇'
μ_{max} [h^{-1}]	0.58	0.54	0.53		
μ [h^{-1}] after feed start	0.33	0.44	0.23	0.35	0.44

maximum expression of the product is obtained in the late exponential and stationary phase [7, 8, 10].

The cell dry weight shown in Table 2 can be compared to that obtained by the pulse program using defined media cultivating *E. coli* BL21 with plasmid pBRMX14 encoding a xylanase [21]. Around 4 g CDW/l was produced from *V. cholerae* after the batch phase, which is approximately the same as for *E. coli* BL21. This is expected since the amount of initial glucose is the same (10 g/l). At 7 h after the feed start, around 14.5–18.5 g CDW/l was produced from *V. cholerae* compared to more than 40 g CDW/l for *E. coli* BL21. For *V. cholerae*, a μ_{max} of around 0.6 h^{-1} was obtained during the batch phase (see Table 3). This is lower than the μ_{max} of 0.8 h^{-1} obtained for *E. coli* BL21.

E. coli BL21 produces a very small amount of acetate, which also could be a reason for the faster cell growth. In the presence of acetic acid, the specific growth rate of *V. cholerae* was lower (result not shown). This theory was confirmed from the feed profile in Fig. 3a. Here the slope of the feed profile increased when the acetate was depleted. The negative consequences of acetate was further noticed in one of the cultivations, where it took longer to consume the acetic acid produced during the batch phase (▽ in Fig. 5b). The cell growth seemed to be affected by the acetic acid present. One can distinguish a small dip in OD measurements at 2–4 h (Fig. 5a).

The μ_{max} values obtained in these experiments for *V. cholerae* are presented in Table 3 and can be compared to other values reported. A μ_{max} of 1.2 h^{-1} and 1.34 h^{-1} for recombinant *V. cholerae* encoding for the B subunit of *E. coli* enterotoxin cultivated in complex media were noted in [10] and [6], respectively. The reason for the difference is the media used. In most situations a higher specific growth rate is achieved when complex media are used since there are more precursors available for the metabolism. The μ_{max} reported for a *ctx*A-deleted strain of *V. cholerae* in defined media enriched with amino acids ranged from 0.37–0.84 h^{-1}, depending on the cultivation temperature and pH. For a pH of 8 and a temperature of 37°C, a μ_{max} of 0.75 h^{-1} has been noted [7]. This is quite close to the μ_{max} around 0.6 h^{-1} (pH 7.5, temperature 36°C) obtained here on defined media without addition of aminoacids.

The overall cell yields $Y_{x/s}$ shown in Table 2 decreased in the course of the experiments, the reason being that more and more of the glucose was required for maintenance purposes. In [10], *V. cholerae* was cultivated in fed-batch mode for production of B subunit of *E. coli* enterotoxin. Complex media were used and the overall cell yield $Y_{x/s}$ was 0.4 g/g without induction for protein production after 9 h of cultivation (7 h of feeding). With sequential IPTG induction the overall cell yield was decreased to 0.32 g/g. These results are similar to the ones obtained here (0.35 g/g), even though there are significant differences in cultivation conditions, i.e., different media, feeding strategies, and strains.

6 Conclusion

Fed-batch cultivations with *V. cholerae* in defined media were conducted and reproducible results were obtained. A probing feeding strategy developed by Åkesson for *E. coli* cultivations was tested. The strategy works well for *V. cholerae* in minimizing the amount of acetic acid formed and avoiding anaerobic conditions. Most of the acetic acid accumulated during the batch phase had been consumed two hours after the feed start. The resulting feed rate seems to be the highest possible with respect to the constraints from cell metabolism and mass transfer, thus maximizing productivity in terms of biomass. The amount of cells produced was 20–23 g/l after 12 h of feeding.

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II

PARAMETER ESTIMATION OF A MODEL DESCRIBING THE OXYGEN DYNAMICS IN A FED-BATCH *E. COLI* CULTIVATION

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Abstract A model describing the oxygen dynamics in an *E. coli* fed-batch cultivation is presented. In a linearised version the parameters are estimated and validated with good results. The model is used to discuss the guidelines for feed controller tuning derived in (Åkesson *et al.*, 2001b).

Key words Bio-reactor model, parameter estimation, *E. coli* fermentation.

1. INTRODUCTION

Today many proteins are produced by genetically modified microorganisms. One of the host organisms used is the bacterium *Escherichia coli*. To achieve a good productivity, high cell concentration and high cell productivity are desired and this is usually obtained through fed-batch cultivations. Much work is done on how to determine the addition of the growth-limiting carbon, often glucose, (Riesenberg and Guthke, 1999), (Lee, 1996). This is important as underfeeding will lead to productivity loss and starvation. Overfeeding leads to carbon nutrient accumulation or by-product formation, such as acetate. Acetate production reduces growth and recombinant protein production, (Luli and Strohl, 1990). In (Åkesson *et al.*, 2001a) a probing feeding strategy is presented. By superimposing short pulses on the substrate feed, on-line detection of acetate formation is made using the dissolved oxygen sensor. A feedback algorithm is used to adjust the feed rate to avoid overflow metabolism and thereby acetate formation while maintaining a high growth. To derive guidelines on the tuning of the feed controller, a linearised model is used in (Åkesson *et al.*, 2001b). Here the model is extended and verified. As the model is based on physical principles it is a continuous-time system. Reviews on identification and parameter estimation in continuous time are given in (Unbehauen and Rao, 1998), (Unbehauen and Rao, 1990) and (Young, 1981). The model is not used for on-line control and therefore the parameter estimation is done off-line. Also, the effect of the extended model on the tuning rules is investigated.

2. PROCESS DESCRIPTION

The process is a bio-reactor operating in fed-batch mode. Here we consider the case with two inputs: the stirrer speed N and the feed rate F, and three on-line outputs: the oxygen concentration in the airflow O_2 which is measured using a gas analyser, the dissolved oxygen concentration in the medium DO and the reactor medium volume V. The cell mass is measured off-line, see figure 1. A full non-linear model of the bio-reactor is presented together with a linear version. In this model also the changing oxygen concentration in the outlet air is included, equation (6), which is not the case in (Åkesson *et al.*, 2001b).

2.1 *Full model*

Mass balances of a fed-batch bio-reactor are

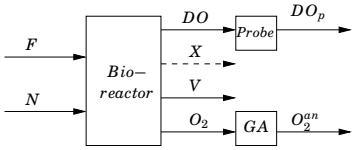

Fig. 1 Block diagram of the process. DO dissolved oxygen concentration, F feed rate, N stirrer speed, V the reactor medium volume, X cell mass concentration, GA = gas analyser, probe = dissolved oxygen probe.

given by:

$$\frac{dV}{dt} = F \tag{1}$$

$$\frac{d(VG)}{dt} = FG_{in} - q_g(G)VX \tag{2}$$

$$\frac{d(VA)}{dt} = q_a(G,A)VX \tag{3}$$

$$\frac{d(VX)}{dt} = \mu(G)VX \tag{4}$$

$$\frac{d(VC_o)}{dt} = K_L a(N)V(C_o^* - C_o) - q_o(G)VX \tag{5}$$

$$\frac{d(V_g O_2)}{dt} = Q(O_2^{in} - O_2) - \frac{RTK_L a(N)V}{PM}(C_o^* - C_o) \tag{6}$$

The expressions for the growth rate μ, the acetate consumption q_a, the oxygen consumption q_o and the glucose uptake q_g are given in the appendix. For notation and parameter values, see table 1 and table 3. The gas volume in the reactor V_g is given by

$$V_g = V_{tot} - V$$

where V_{tot} is the reactor volume. Henry's law gives the dissolved oxygen concentration DO in %:

$$DO = HC_o$$

Oxygen concentration in the outlet air O_2 is related to oxygen concentration in equilibrium with the gas bubbles in the reactor, C_o^* and DO^*, as

$$O_2 = \frac{HC_o^* O_2^{in}}{100} = \frac{DO^* O_2^{in}}{100} \tag{7}$$

This is based on the assumption that the gas bubbles are well mixed in a small stirred reactor (Enfors and Häggström, 1994). The dissolved oxygen sensor dynamics is approximated as:

$$T_p \frac{dDO_p}{dt} + DO_p = DO \tag{8}$$

together with a time delay denoted L_p. The gas analyser is described by:

$$T_{an} \frac{dO_2^{an}}{dt} + O_2^{an} = O_2 \tag{9}$$

together with a time delay denoted L_{an}.

Table 1 Variables in the model.

Symbol	Description
V	reactor medium volume
O_2	oxygen conc in outlet air
F	glucose feed into the reactor
N	stirrer speed
G	glucose concentration
A	acetic acid concentration
X	cell mass concentration
$C_o^{(*)}$	(sat.) dissolved oxygen conc.

2.2 *Linearised model*

Linearised versions of equations (2), (5) and (6) with respect to F, N, q_g, DO and DO^*, when $q_o < q_o^{max}$ and no acetic acid is present, are presented. Also the relations in equation (7) and equation (12) in appendix are used. The influences of the deviations $\Delta X = X - X^o$ and $\Delta V = V - V^o$ are assumed to be small and are therefore neglected.

$$T_g \frac{d\Delta q_g}{dt} + \Delta q_g = K_{gf}\Delta F$$

$$T_o \frac{d\Delta DO}{dt} + \Delta DO = K_{og}\Delta q_g + K_N \Delta N + \Delta DO^*$$

$$T_o^* \frac{d\Delta DO^*}{dt} + \Delta DO^* = K_{o^*o}\Delta DO + K_{o^*N}\Delta N$$

The linearised parameters are given in the appendix. After the introduction of $p = \frac{100RTV^o}{O_2^{in}HMPV_g^o}$, $K_{gf}K_{og}^{new} = \frac{K_{gf}K_{og}}{T_o}$ and the dynamics of the sensors, equations (8) and (9), the following block diagram is obtained, see figure 2. A feedback connection is introduced through (6).

3. EXPERIMENTAL DATA

Data from two experiments using the probing feeding strategy described in (Åkesson *et al.*, 2001a) are used in the parameter estimation. For medium composition and equipment used, see (de Maré *et al.*, 2005). One of the experiments is shown in figure 3. As seen in the figure, linearisation around a stationary trajectory is necessary.

3.1 *Trajectories*

First the trajectories of the inputs are determined. Stationarity is assumed in the beginning of each pulse and F^o and N^o are adapted in a least-squares sense to these points. As the output DO_p is controlled to 30 % between the superimposed feed-pulses, $DO^o = 30$ %. To be able to calculate the trajectory of the output O_2^{an}, V^o,

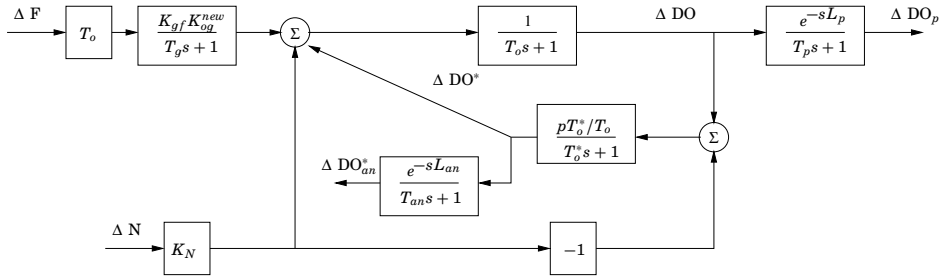

Fig. 2 Block diagram of the linearised process. The parameters are given in the appendix.

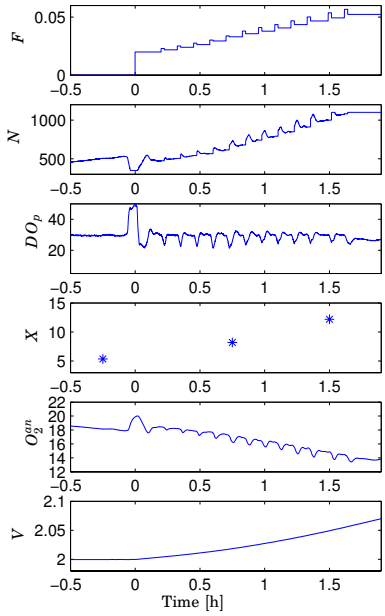

Fig. 3 Experimental data from cultivation 1. The region used for identification is from 0.2 h to 1.6 h where $q_o < q_o^{max}$ and no acetic acid is present. From the top: feed rate F [l/h], stirrer speed N [rpm], dissolved oxygen DO_p [%], cell mass concentration X [g/l], oxygen concentration in the outlet air O_2^{an} [%], reactor medium volum V [g/l]. Time after feed-start.

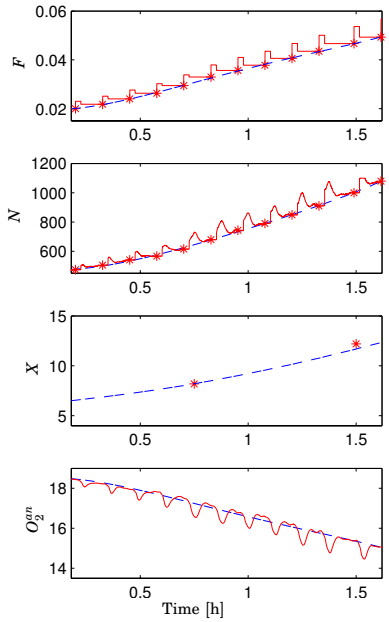

Fig. 4 Cultivation 1. The trajectories: feed rate F^o [l/h], stirrer speed N^o [rpm], cell mass concentration X^o [g/l] and oxygen concentration O_2^o [%] (dashed) together with experimental data (solid) are shown.

X^o and q_g^o are needed. The change in volume is small as seen in figure 3 and $V^o = V$. X^o is calculated from (4) where Y_{xg}^{ox}, Y_{og}^{ox}, q_m, Y^{om} are taken from (Xu *et al.*, 1999), see table 3. q_g^o is calculated from (2). The trajectory O_2^o is then determined using (5) and (6):

$$O_2^o = O_2^{in} - \frac{X^o RTV^o}{PQM}((q_g^o - q_m)Y_{og}^{ox} + q_m Y^{om})$$

Recalibration of the gas analyser is necessary in order to correlate O_2^{an} to O_2^o. In figure 4 and figure 5 F, N, X and O_2^{an} together with their trajectories are shown for cultivation 1 and cultivation 2, respectively.

4. PARAMETER ESTIMATION

As is seen in figure 2, there are 6 parameters to estimate, $K_{gf}K_{og}^{new}$, T_g, T_o, K_N, p, T_o^* from the bio-reactor and 4 parameters from the measurement equipment T_p, L_p, T_{an}, L_{an}. As this is not possible using the two sets of data available, we have to make some assumptions. Here we assume that the oxygen probe dynamics and the gas analyser dynamics are known. When examining the 6 parameters left we suspect T_o and

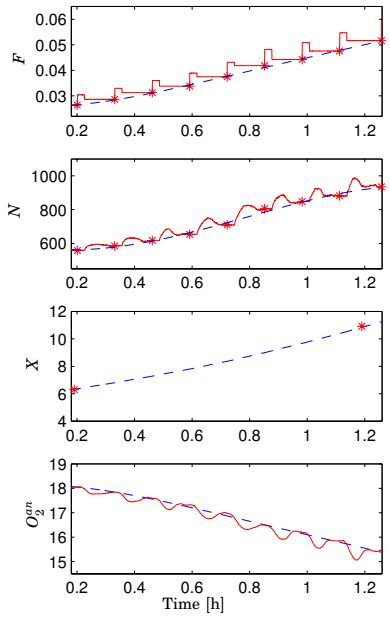

Fig. 5 Cultivation 2. The trajectories: feed rate F^o [l/h], stirrer speed N^o [rpm], cell mass concentration X^o [g/l] and oxygen concentration O_2^o [%] (dashed) together with experimental data (solid) are shown.

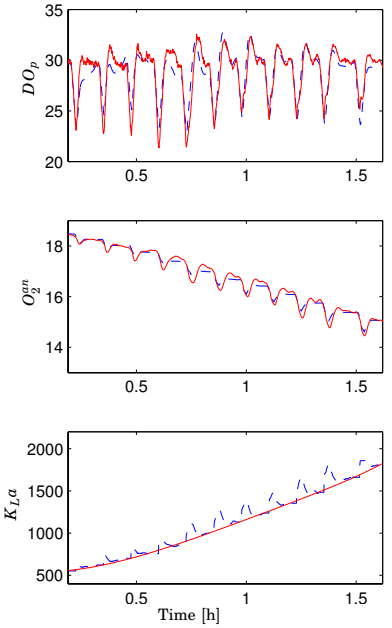

Fig. 6 Cultivation 1, simulation of the full model. From the top: dissolved oxygen DO_p^{sim} [%], oxygen concentration $O_{2,an}^{sim}$ [%], oxygen transfer $K_La(N)^{sim}$ [h^{-1}] (dashed) together with experimental data (solid) and K_La^o (solid).

K_N to vary a lot. They depend on K_La^o and $\frac{\partial K_La}{\partial N}$, respectively, which change much during a cultivation. Therefore we calculate K_La^o using (6)

$$K_La^o = \frac{QPMH}{V^oRT}\frac{O_2^{in} - O_2^o}{DO^{*o} - DO^o}$$

and then $K_La(N)$ is calculated using K_La^o and N_o, see below. Thus we can determine $T_o = \frac{1}{K_La^o}$ and K_N. There are now four parameters left to identify which seems possible with the data available.

4.1 *Determination of K_N using $K_La(N)$*

To determine $K_La(N)$, a third order polynomial is chosen and its coefficients are adapted in a least-squares sense using N^o and K_La^o. The relation $K_La(N)$ is given by:

$$K_La(N) = \alpha_1 N^3 + \alpha_2 N^2 + \alpha_3 N + \alpha_4$$

The values of α are given in table 3 and their values differ for cultivation 1 and cultivation 2. In order to evaluate the expressions for $K_La(N)$, simulations with the non-linear model are shown in figure 6 and figure 7. The parameter values used are given in table 3.

4.2 *Adaptation*

The parameters left for estimation are: $K_{gf}K_{og}^{new}$, T_g, T_o^* and p. The minimisation criterion chosen is the cost-function V_{min}

$$\begin{aligned} V_{min} &= V_1 + V_2 \\ &= \Sigma(DO_p^{sim} - DO_p^{exp})^2 \\ &\quad + \Sigma(DO_{an}^{*,sim} - DO_{an}^{*,exp})^2 \end{aligned} \quad (10)$$

and the algorithm used is the Nelder-Mead simplex method. For adaptation, data from cultivation 1 are used. The starting values of the parameters are calculated from table 3 and are given in table 2 together with the obtained result from the minimization. For comparison purposes also the cost function for the non-linear simulation in figure 6 is presented. DO_p^{sim} and $DO_{sim}^{*,an}$ are shown together with the experimental data in figure 8.

4.3 *Validation*

For validation, data from cultivation 2 are used and the resulting DO_p^{sim} and $DO_{sim}^{*,an}$ obtained are shown in figure 9. The cost function for the validation and for the non-linear simulation in figure 7 is presented in table 2. To investigate

Table 2 Result of the parameter estimation. The data are sampled every 5 s. 1026 data points of cultivation 1 are used for adaptation and 907 data points of cultivation 2 are used for validation.

Data	Model	purpose	$K_{gf}K_{og}^{new}$	T_g [s]	T_o^* [s]	p	V_1	V_2	V_{min}
cult. 1	full model	-	-	-	-	-	1050	450	1500
cult. 1	linear mod.	start. values	$-1.45\cdot10^6$	12.6	20.4	0.0524	1505	585	2090
cult. 1	linear mod.	adaptation	$-1.43\cdot10^6$	13.4	17.5	0.0516	1290	490	1780
cult. 2	full model	-	-	-	-	-	865	55	920
cult. 2	linear mod.	validation	$-1.43\cdot10^6$	13.4	17.5	0.0516	840	120	960

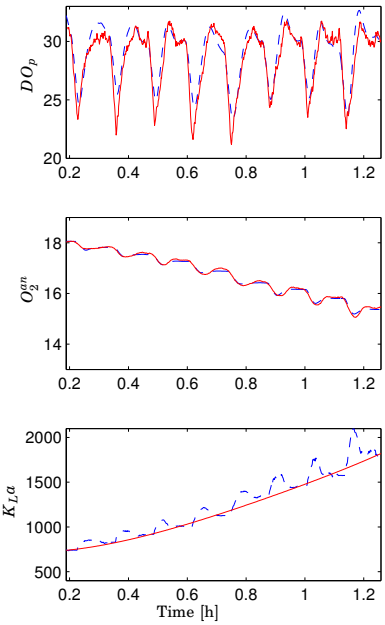

Fig. 7 Cultivation 2, simulation of the full model. From the top: dissolved oxygen DO_p^{sim} [%], oxygen concentration $O_{2,an}^{sim}$ [%], oxygen transfer $K_La(N)^{sim}$ [h^{-1}] (dashed) are shown together with experimental data (solid) and K_La^o (solid)

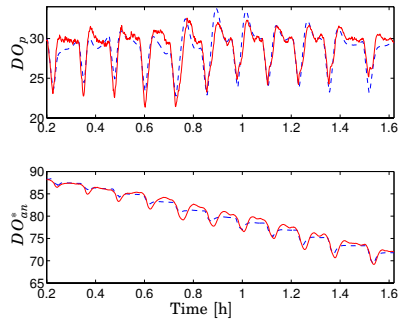

Fig. 8 Cultivation 1, adaptation of the linearised model. From the top: dissolved oxygen DO_p^{sim} [%], dissolved oxygen concentration in equilibrium with the outlet air DO_{an}^{*sim} [%] are shown (dashed) together with experimental data (solid).

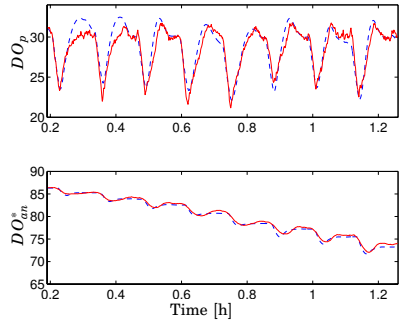

Fig. 9 Cultivation 2, validation of the linearised model. From the top: dissolved oxygen DO_p^{sim} [%], dissolved oxygen concentration in equilibrium with the outlet air DO_{an}^{*sim} [%] are shown (dashed) together with experimental data (solid).

the robustness of the obtained result more simulations are done with different parameter values. In these studies a strong correlation between p and T_o^* is noticed. As the value of p should not deviate much from the starting value as it contains well known physical parameters, we believe that the right minimum is found.

5. REVISED TUNING OF THE PROBING FEED CONTROLLER

When using a proportional probing feed controller the increase in the feed F is decided by

$$\Delta F(k) = \kappa \frac{DO_{pulse}(k) - y_r}{DO^* - DO^o} F \qquad (11)$$

where DO_{pulse} is the pulse response, y_r is the desired pulse response and κ the controller gain. There are several more parameters that need to be chosen such as the pulse duration T_{pulse}, the length between the pulses $T_{control}$ and the pulse height γ_p. In (Åkesson *et al.*, 2001b) some tuning rules are derived which we will examine here and modify if necessary. In our model the changing oxygen concentration in the outlet air is included which leads to additional dynamics

and a changed process gain.

The choice of T_{pulse} and $T_{control}$ depends on the process dynamics. In (Åkesson *et al.*, 2001b) T_{pulse} is chosen as a lumped time constant $T_{max} = T_p + T_o^{max} + T_g^{max}$ and $T_{control}$ is chosen to $4T_{pulse}$. Here a pulse response DO_{pulse} to a feed pulse F_p is given by

$$DO_{pulse} = \frac{K_{gf}|K_{og}|(T_o^* s+1)e^{-sL_p}}{((T_o^* s+1)(T_o s+1) - p\frac{T_o^*}{T_o})(T_g s+1)(T_p s+1)} F_p$$

Thus as long as $p\frac{T_o^*}{T_o} \ll 1$ the guideline above still applies. Here $p\frac{T_o^*}{T_o}$ varies between 0.1-0.5, see table 2. Also, $T_o = \frac{1}{K_L a^o}$ and $K_L a^o$ is presented in figure 6 and figure 7. Considering the variation in $p\frac{T_o^*}{T_o}$ we suggest the use of $2T_o^*$ as the maximal lumped time constant for

$$\frac{T_o^* + 1}{(T_o^* s+1)(T_o s+1) - p\frac{T_o^*}{T_o}}$$

This gives a $T_{max} = T_p + T_g^{max} + 2T_o^* + L_p$ of approximately 110 s.

The choice of γ_p and κ depends on the process gain. When choosing γ_p it must be assured that the oxygen level does not become too low during a pulse which gives the upper limit. In steady state the amplitude of the oxygen response away from DO^o is given by:

$$DO_{pulse} = \frac{K_{gf}|K_{og}|}{1 - p\frac{T_o^*}{T_o}} F_p \leq \frac{DO^* - DO^o}{(1 - p\frac{T_o^*}{T_o})} \gamma_p$$

where $F_p = \gamma_p F$. Thus the upper limit for the value of γ_p is lower than in (Åkesson *et al.*, 2001b) where

$$DO_{pulse} \leq (DO^* - DO^o)\gamma_p$$

In (Åkesson *et al.*, 2001b) the controller gain $\kappa < 1$ ensures stability but in our case the corresponding requirement on κ is: $\kappa < 1 - p\frac{T_o^*}{T_o}$ which leads to a lower value of κ.

6. DISCUSSION

As is seen in figure 8 and figure 9, the linearised model seems to capture the behaviour well. Deviations are seen around 1 hour for cultivation 1 and in the beginning of cultivation 2. DO_{an}^* seems more difficult to adapt in cultivation 1. Also, note that different time constants T_{an} are used for the two cultivations, which can be explained by the fact that the behaviour of the gas analyser changes over time.

The time-variation in all the parameters T_g, T_o^*, $K_{gf}K_{og}^{new}$ and p is neglected, but even so there are not big differences in the results obtained when using the full model, see table 2. One explanation is that the time-variation in $K_{gf}K_{og}^{new}$ and p depends on the variation in V^o and V_g which is small, see figure 3. An investigation where the variations in T_g, depending on X and q_g^o, and in T_o^*, depending on T_o, are taken into account has been made, but the results are similar. Therefore the model with constant parameters seems suitable to use in the investigation of the feed controller tuning.

When it comes to the controller tuning, the equation describing the changing oxygen concentration in the outlet air makes a difference. A tighter upper bound on controller gain κ has to be satisfied to ensure stability. Also, when examining the feed controller described in equation (11) DO^* is included. In Åkesson's work DO^* is assumed to be constant. It will lead to a larger stationary error in the pulse responses than expected. To prevent this one can add the integral part to the feed controller, as is described in (Åkesson *et al.*, 2001b). An alternative is to make use of the measurements of the gas analyser, which are proportional to DO^*, as a gain scheduling variable.

In summary, a model describing the oxygen dynamics in a *E. coli* fed-batch cultivation is presented. In a linearised version the parameters are estimated and validated with good results. The model is used to discuss the guidelines on the feed controller tuning, derived in (Åkesson *et al.*, 2001b).

7. ACKNOWLEDGEMENT

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A. APPENDIX

A.1 *Uptake rates*

The glucose uptake rate q_g is given by:

$$q_g = \frac{q_g^{max} G}{k_s + G} \tag{12}$$

The glucose used for maintenance purposes is given by:

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

The glucose used for growth uptake is thus:

$$q_{gg} = q_g - q_m$$

Splitting into an oxidative flow and a fermentative flow gives:

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

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

Specific acetate production q_a^p is given by:

$$q_{ap} = q_{gg}^{fe} Y_{ag}$$

Specific acetate consumption q_a^c :

$$q_{ac}^{pot} = q_{ac}^{max} A/(k_a + A)$$

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

The resulting acetate formation rate q_a:

$$q_a = q_{ap} - q_{ac}$$

Specific growth rate μ:

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

Specific oxygen uptake rate q_o is given by:

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

A.2 *Linearised model*

The linearised parameters:

$$K_{og} = -\frac{HX^o}{K_L a^o} Y_{og}^{ox} \qquad T_g = (\frac{\partial q_g^o}{\partial G} X^o)^{-1}$$

$$K_{gf} = \frac{G_{in}}{V^o X^o} \qquad T_o = (K_L a^o)^{-1}$$

$$K_{gf} K_{og}^{new} = \frac{K_{gf} K_{og}}{T_o} = \frac{H Y_{og}^{ox} G_{in}}{V^o}$$

$$T_o^* = \frac{V_g^o}{V^o RT 100 K_L a^o + QMHPO_2^{in}}$$

$$K_N = \frac{DO^{*o} - DO^o}{K_L a^o} \frac{\partial K_L a}{\partial N}$$

$$K_{o^* o} = \frac{100 V^o RT K_L a^o}{V^o RT 100 K_L a^o + QMHPO_2^{in}} = \frac{pT_o^*}{T_o}$$

$$K_{o^* N} = -\frac{(DO^{*o} - DO^o) 100 V^o RT}{V^o RT 100 K_L a + QMHPO_2^{in}} \frac{\partial K_L a}{\partial N}$$

$$= -\frac{K_N p T_o^*}{T_o}$$

where $p = \frac{V^o RT 100}{PMHO_2^{in} V_g^o}$.

Table 3 Parameters in the model.

Symbol	Value	Unit	Description
$Vtot$	3	l	total reactor volume
R	8.314	J/(mol K)	ideal gas constant
T	22	^{o}C	air flow temperature
P	101.3	kPa	pressure
M	32	g/mol	oxygen molar mass
O_2^{in}	20.9	%	oxyg. conc. in the inlet air
G_{in}	500	g/l	glucose conc. in feed
H	14000	%l/g	Henrys const.
k_{sg}	0.01	g/l	saturation const. for glucose uptake
q_g^{max}	1.6	g/gh	max. spec. glucose uptake
q_o^{max}	0.6	g/gh	max. spec. oxygen uptake
q_{mc}	0.06	g/gh	maintenance coefficient
Y_{oa}	0.55	g/g	oxygen/acetate yield
Y_{og}^{ox}	0.414	g/g	oxygen/glucose yield for growth
Y^{om}	1.07	g/g	oxygen/glucose yield for maintenance
Y_{xa}	0.4	g/g	biomass/acetate yield
Y_{xg}^{ox}	0.51	g/g	oxidative biomass/glucose
Y_{xg}^{fe}	0.15	g/g	fermentative biomass/glucose
L_p	5	s	time delay dissolved oxygen sensor
T_p	60	s	time const. dissolved oxygen sensor
L_{an}	65	s	time delay gas analyser
T_{an}	0, 15	s	time const. gas analyser (cult. 1, cult 2)
Q	147, 161	l/h	air flow (cult. 1, cult. 2)
α_1	$5.9\cdot 10^{-8}$		oxygen transfer parameter (cult. 1)
α_2	$-3.7\cdot 10^{-4}$		oxygen transfer parameter (cult. 1)
α_3	2.6		oxygen transfer parameter (cult. 1)
α_4	-581		oxygen transfer parameter (cult. 1)
α_1	$1.9\cdot 10^{-5}$		oxygen transfer parameter (cult. 2)
α_2	-0.041		oxygen transfer parameter (cult. 2)
α_3	31		oxygen transfer parameter (cult. 2)
α_4	-7160		oxygen transfer parameter (cult. 2)

III

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A cultivation technique for *E. coli* fed-batch cultivations operating close to the maximum oxygen transfer capacity of the reactor

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Key words: *Escherichia coli*, fed-batch cultivation, feeding strategy, fermentation process

Abstract

A cultivation strategy combining the advantages of temperature-limited fed-batch and probing feeding control is presented. The technique was evaluated in fed-batch cultivations with *E. coli* BL21(DE3) producing xylanase in a 3 liter bioreactor. A 20% increase in cell mass was achieved and the usual decrease in specific enzyme activity normally observed during the late production phase was diminished with the new technique. The method was further tested by growing *E. coli* W3110 in a larger bioreactor (50 l). It is a suitable cultivation technique when the O_2 transfer capacity of the reactor is reached and it is desired to continue to produce the recombinant protein.

Introduction

Today many proteins are produced by genetically modified microorganisms. One of the host organisms used is *Escherichia coli*. During its cultivation the accumulation of the by-product acetate is a problem, especially during high cell density cultivations. High cell density cultivations are often used since it is one of the most effective techniques for maximizing both cell mass and foreign protein concentration, leading to a high productivity.

It is well known that acetate production reduces growth and recombinant protein formation (Luli & Strohl 1990, Turner *et al.* 1994). Formation of acetate in *E. coli* cultures occurs under anaerobic conditions but also under fully aerobic conditions in situations with excess carbon source (Konstantinov *et al.* 1990, Pan *et al.* 1987). In fed-batch cultivations the feed-rate of the carbon source can be manipulated to restrict the formation of acetate.

The probing feeding strategy described in Åkesson *et al.* (2001) can be used to avoid acetate accumulation. By superimposing short pulses in the substrate feed-rate, on-line detection of acetate formation can be made using the dissolved oxygen sensor (Åkesson *et al.* 1999). However a cultivation technique based on the manipulation of the feed might be improved by also using the temperature. The temperature limited fed-batch technique is described in Silfversparre *et al.* (2002). The basic idea is to decrease the cultivation temperature to control O_2 consumption rate and thereby avoiding O_2 limitation. Also, the substrate has to be fed in excess in order to prevent substrate limitation. Use of this technique has, in previous trials, led to minimization of released endotoxins (Silfversparre *et al.* 2002), as well as to lowered

proteolysis rate, when compared to substrate-limited fed-batch (Rozkov 2001). The main obstacle with the temperature-limited, fed-batch technique is to achieve a non-growth limiting glucose concentration in the reactor without accumulating acetic acid, since an on-line glucose sensor is usually not available. Also the critical glucose concentration is not known and it changes during the cultivation.

In this paper a cultivation technique combining the advantages of temperature-limited fed-batch and probing feeding control is presented. The temperature is decreased to lower the O_2 demand and the growth rate. A mid-ranging controller structure is used to manipulate the temperature and the stirrer speed to control the dissolved O_2 tension. The probing feeding strategy is changed when the maximum stirrer speed is reached to obtain a slight excess of glucose. This is achieved by adding down-pulses instead of up-pulses to the feed. The resulting strategy thus limits the growth rate without the risk of acetate accumulation. The technique is evaluated in experiments.

Materials and methods

Two different *E. coli* strains were cultivated: *E. coli* BL21(DE3) and *E. coli* W3110, in two different sets of reactors.

E. coli BL21(DE3)

E. coli BL21(DE3) was cultivated in defined media producing xylanase cloned in pET22b (Nordberg Karlsson *et al.* 1998). The enzyme production was induced by adding IPTG 1−1.5 h after feed-start. Inoculum (1 ml) from frozen stocks were incubated at 30 °C over night in 100 ml medium, containing (per liter) 10 g glucose, 2 g $(NH_4)_2SO_4$, 14.6 g K_2HPO_4, 3.6 g $NaH_2PO_4 \cdot H_2O$, 0.5 g $(NH_4)_2K$ citrate, 2 ml 1 M $MgSO_4 \cdot 7(H_2O)$, 0.1 g ampicillin and 2 ml trace elements solution (Holme *et al.* 1970). The cultivation was performed in a 3 l bioreactor, (Belach bioteknik AB, Stockholm, Sweden) with a final volume of 2 l. The pH was kept at 7 by titration with 6.7 M NH_4OH. The temperature was 37 °C unless otherwise stated and the aeration was 2 l/min. The feed was started when the dissolved O_2 tension increased abruptly, and it consisted of (per liter) 500 g glucose, 50 ml 1 M $MgSO_4$ and 10 ml trace element solution. The dissolved O_2 sensor used was a polarographic electrode and its temperature dependence was low. Data logging, dissolved O_2 control and the feeding strategy were implemented on a standard PC using the industrial control system SattLine (ABB Automation, Malmö, Sweden) or Industrial IT (ABB Automation, Malmö, Sweden).

Analyses

Samples were withdrawn through a sampling port at the bottom of the reactor, using 25 ml pre-sterilized metal-capped glass tubes.

Cell dry weight determination

Cell dry weight (CDW) was determined after centrifuging (1400 *g*, 15 min, at room temperature) triplicate samples (4 ml) in pre-weighed glass tubes. After centrifugation, cell-pellets were dried over night (105 °C) and subsequently weighed to determine CDW.

Glucose and acetic acid analysis

Samples for glucose and acetic acid determinations were collected in tubes half filled with 0.132 M perchloric acid (ice cold) and centrifuged (1400 *g*, 10 min, at room temperature). The supernatant was dispensed (1 ml portions) into eppendorf tubes and neutralized with 3.6 M K_2CO_3 and kept frozen (−20 °C). The samples were then analyzed with a high performance liquid chromatography (HPLC) system (Agilent 1100 series). An organic acid and alcohol analysis ion-exclusion column (Micro-Guard precolumn cation H-cartridge (30 × 4.6 mm) followed by an Aminex HPX-87-H (300 × 7.8 mm), Bio-Rad, Hercules, USA) was used at 50 °C with 80 mM H_2SO_4 as mobile phase at 0.6 ml/min followed by refractive index detection.

Enzyme activity

Xylanase activity was determined using the 3,5-dinitrosalicylic acid (DNS) method (Bailey *et al.* 1992). The activity was determined under the conditions described in Nordberg Karlsson

et al. (1998) using xylose as standard (2–10 mM). Enzyme blanks were prepared for each sample by incubating the substrate, 1% (w/v) birch xylan (Birch 7500, Roth, Karlsruhe, Germany), at 65 °C for 5 min, then adding the DNS reagent and immediately thereafter the enzyme. The xylanase activity was expressed in units (U) defined as the amount catalyzing the transformation of 1 μmol substrate per min under standard conditions.

E. coli W3110

E. coli W3110 was cultivated in defined media producing the protein ZZT2 which was cloned in pRIT44T2. The protein production was induced by adding indole 3-acrylic acid 3.5 h after the feed-start. Colonies from an agar plate were used to inoculate shake-flasks with defined media containing (per liter) 10 g glucose, 0.1 g tryptophan, 0.1 g thiamin, 2.64 g $(NH_4)_2SO_4$, 2 g Na_2SO_4, 14.6 g K_2HPO_4, 3.6 g $NaH_2PO_4 \cdot (H_2O)$, 1 g $(NH_4)_2$H-citrate, 2 ml 1 M $MgSO_4 \cdot 7(H_2O)$, 0.1 g ampicillin and 3 ml trace elements solution (Holme *et al*. 1970). When the OD reached 5, the 100 l stainless steel bioreactor containing 50 l of the media mentioned above was inoculated. pH was regulated at 7.0 by addition of 25% NH_4OH. The temperature was 35 °C–32 °C and the airflow was 1.5 vvm. The feed was started when the dissolved O_2 increased abruptly and it contained (per liter) 500 g glucose, 0.1 g tryptophan, 0.1 g thiamin, 5.36 g $(NH_4)_2SO_4$, 4 g Na_2SO_4, 29.2 g K_2HPO_4, 7.2 g $NaH_2PO_4 \cdot (H_2O)$, 2 g $(NH_4)_2$H citrate, 2 ml 1 M $MgSO_4 \cdot 7(H_2O)$, 0.1 g ampicillin and 3 ml trace elements solution. The dissolved O_2 sensor used was a polarographic electrode. A combination of two control systems was used to control the cultivation, BioPhantom (Belach bioteknik AB, Stockholm, Sweden) and SattLine (ABB Automation, Malmö, Sweden).

Analyses

Samples were taken through a sample port at the bottom of the tank into a 100 ml Pyrex bottle.

Cell dry weight determination

Cell dry weight (CDW) was determined after centrifuging (3000 *g*, 10 min, at room temperature) triplicate samples (5 ml) in pre-weighed glass tubes. After centrifugation, cell-pellets were dried for 24 h (105 °C) and subsequently weighed to determine CDW.

Glucose analysis

After each sampling, 2 ml for glucose determination was immediately transferred to a tube filled with 2 ml 0.132 M perchloric acid (cold) and centrifuged (for 10 min at 3000 *g*). 3.5 milliliter of supernatant was transferred to another tube with 75 μl 3.6 M K_2CO_3 and kept frozen (−20 °C) in aliquots. Glucose concentrations were estimated from thawed preserved samples according to the enzymatic D-glucose UV-method from Boehringer Mannheim.

Product analysis

For product determination, disintegrated cell samples were analysed using SDS-PAGE and immunoblotting using a rabbit anti protein-A antibody as primary antibody.

Feeding strategies

The original probing feeding strategy

The original probing strategy, described in Åkesson *et al*. (2001) is used in the reference cultivation. The strategy has been successfully used for *E. coli* cultivations on 3 l up to 12 m^3 reactors (Ramchuran *et al*. 2002, Velut *et al*. 2002). It has also been tested on other microorganisms presenting similar overflow metabolism phenomena (de Maré *et al*. 2003).

Feed control

Pulses are superimposed to the feed-rate and they give rise to responses in the dissolved O_2 tension. The responses in the dissolved O_2 tension are used in a feedback algorithm that adjusts the feed-rate. It works as follows:

- When the response in the dissolved O_2 is large enough, the feed-rate is increased proportionally to the size of the pulse response.
- When there is no visible response in the dissolved O_2, the feed-rate is decreased with a fixed proportion.

When the maximum O_2 transfer capacity of the reactor is reached i.e. the maximum stirrer speed, the probing feeding strategy decreases the feed-rate to keep the reactor working under aerobic conditions. The decrease is done at a constant rate until the stirrer speed has left its saturation region.

Dissolved oxygen control

The dissolved O_2 is controlled between the pulses using the stirrer speed. A gain-scheduled PID with respect to the stirrer speed is used and a set-point value of 30% is chosen. During a feed pulse the stirrer speed is frozen.

The modified feeding strategy

In this strategy the temperature instead of the feed is decreased to lower the oxygen demand when a value slightly below the maximum stirrer speed is reached. The temperature is kept as high as possible considering the O_2 limitation of the reactor. A slight glucose excess in the reactor is achieved by shifting the up-pulses superimposed to the feed to down-pulses.

Feed control

The same feed controller as described in the original probing control strategy is used until the maximum stirrer speed is reached. Then the up-pulses are shifted to down-pulses. When a down-pulse is made the dissolved O_2 signal will increase instead of decrease if the cultivation is glucose limited. The feed-rate is adjusted as before depending on the size of the response in the dissolved O_2. With down-pulses a slight glucose excess in the reactor can be achieved. At a given feed-rate a down-pulse may indeed lead to a response in the dissolved O_2 when an up-pulse would not. The slight glucose excess is important since the goal in this part of the cultivation is to let the temperature be the limiting factor and not the glucose concentration.

Dissolved oxygen control

Aerobic conditions should be maintained during the entire cultivation. For that purpose three control variables can be manipulated between the pulses: the stirrer speed N, the feed-rate F and the temperature T. Here a so-called mid-ranging controller structure, (Allison & Isaksson 1998), is chosen to control the dissolved O_2 to 30% (Figure 1). The first controller R_1 manipulates the stirrer speed N and it is tuned to handle the fast disturbances. The second controller R_2 manipulates the temperature to keep the stirrer speed at N_{ref}, below $N_{\max}$. The second controller R_2 is much slower than R_1 and typically takes care of slow disturbances like cell growth. The second loop involving the temperature is activated once the stirrer speed has reached N_{ref}. For design and tuning of the controller structure see Velut *et al.* (2005), article in preparation. The feed-rate is used to control the dissolved O_2 only when both the stirrer speed and the temperature are saturated.

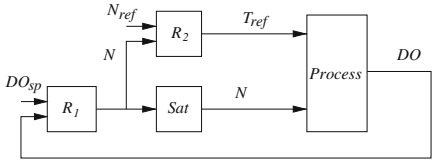

Fig. 1. A block-diagram of the mid-ranging controller. Sat stands for saturation in the stirrer speed i.e. stirrer speed N saturates when reaching N_{max}. R_1 is a gain scheduled PID used also when 'only' N is controlling the dissolved O_2. R_2 is a PID controller. DO_{sp} is the set-point of the dissolved O_2 tension, 30%. N_{ref} and T_{ref} are the reference values of the stirrer speed and the temperature respectively.

Temperature control

A well tuned temperature controller is important when the temperature is to be changed. Here the temperature control is performed by a proportional pulse-width modulation of the cold and hot water flows.

Results

Three cultivations with *E. coli* BL21(DE3) are presented and two cultivations with *E. coli* W3110. These results were obtained at a first attempt.

E. coli BL21(DE3)

The reference cultivation where the original probing feeding strategy is used is shown in Figure 2. During the later part of the cultivation

the controller lowers the feed to keep the reactor working in aerobic conditions. In Figure 3 the other two cultivations are shown where the modified feeding strategy is used. During the later part of the cultivation the temperature is here decreasing to a final value of 22 °C. Figure 4 shows the cell mass, acetic acid, glucose and product activity. The cell mass obtained in the reference cultivation is 34 g/l. With the modified feeding strategy the cell mass produced is a little greater, around 41 g/l. In the reference cultivation the final glucose and acetic acid concentrations in the reactor are 0.1 g/l. With the modified feeding strategy the final glucose concentration is 1.2 g/l and the final acetic acid concentration is 0.3 g/l. The specific enzyme activity starts to decrease 3.5–4.5 h after feed-start in all three cultivations. With the modified feeding strategy, the apparent decrease in specific activity is caused by a ceased production of active enzyme (and unchanged activity in U/l during the last hour of the production phase). In the reference cultivation, both the activity (U/l) and specific activity decreased (the latter from 7200 U/g CDW to 2600 U/g CDW). In addition a significant increase in activity in the cell broth was monitored in the reference cultivation (indicating cell-lysis) (Figure 4), and despite combining cell broth and intracellular activity the total activity was reduced at the end of the cultivation.

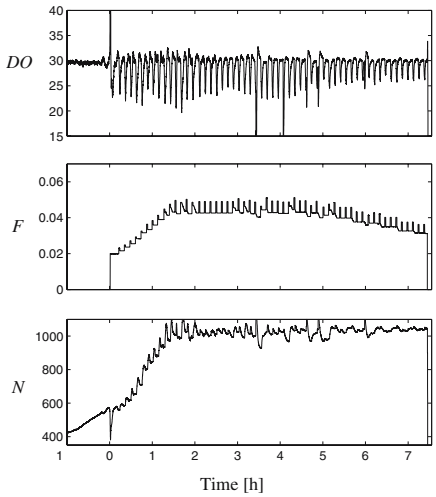

Fig. 2. A cultivation with *E. coli* BL21(DE3) where the original probing feeding strategy is used. The fed-batch part of the cultivation is shown. From top: *DO* [%] dissolved O_2, *F* [l/h] feed and *N* [rpm] stirrer speed. At $t = 3.4$ h and $t = 4.1$ h antifoam is added which has a large impact on the dissolved O_2. Time after feed-start.

E. coli *W3110*

A cultivation where the modified feeding strategy is used is shown in Figure 5. The temperature is first decreased to 32 °C and then increased again to 35 °C to keep the stirrer working at its maximum. The final cell mass achieved is around 27 g/l. The glucose concentration of the reactor was around 25 mg/l during the fed-batch part of the cultivation. During the course of the cultivation,

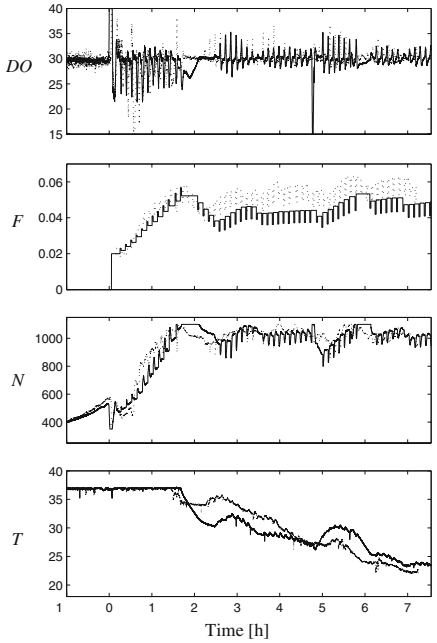

Fig. 3. Experiments with *E. coli* BL21(DE3) using the modified probing feeding strategy. The fed-batch part of the cultivation is shown. From top: *DO* [%] dissolved O_2, *F* [l/h] feed, *N* [rpm] stirrer speed, *T* [°C] temperature. At $t = 4.9$ h a filter in the outlet gas is replaced in the cultivation denoted solid. Reference value $N_{ref} = 1000$ rpm and maximum value $N_{max} = 1100$ rpm. Time after feed-start.

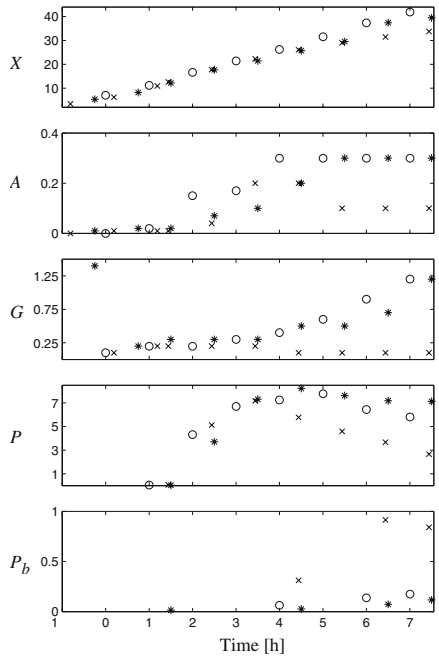

Fig. 4. From top: *X* [g/l] cell mass, *A* [g/l] acetic acid, *G* [g/l] glucose concentration, *P* [U/mg CDW] specific product activity in the cells and *Pb* [U/mg CDW] specific product activity in the broth for the three experiments with *E. coli* BL21(DE3). The reference cultivation (×), the other two where the modified feeding strategy is used are denoted ○ (dashed in Figure 3) and * (solid in Figure 3). Induction took place at $t = 1$ h(○) and t = 1.5 h(*, ×) respectively. Time after feed-start.

after the induction, an increasing amount of the protein ZZT2 is produced (data not shown).

For comparision purposes, a reference cultivation was done where the original probing feeding strategy is used. Here the glucose concentration of the reactor was around 50 mg/l during the fed-batch part and the final cell mass obtained 12.4 h after feed-start was 29 g/l. The protein ZZT2 is produced after induction (data not shown).

Discussion

The goal is to utilize the reactor as effective as possible with respect to the production of the recombinant protein. When producing xylanase using the original probing feeding strategy, a decrease in enzyme activity was observed approximately 2 h after induction (Figure 4, Ramchuran *et al.* 2002). This may be due to the low glucose concentration in the reactor which could lead to stress for the bacteria. The low glucose concentration is the result of the limitation in the oxygen transfer capacity of the reactor. To keep the reactor working under aerobic conditions when the maximum stirrer speed is reached the feed-rate is decreased as described previously.

Another way to handle the limitation in the O_2 transfer is to lower the temperature, which is done in the modified feeding strategy. As seen in Figures 3 and 4 the strategy leads to reproducible results. A higher glucose concentration is achieved, around 1 g/l, without significant acetic acid accumulation [Figure 4 (○ and ×)]. Avoiding accumulation of acetic acid is important since high levels inhibit growth and production.

A lower temperature and a higher glucose concentration seem to be less stressful for the bacteria and the decrease in xylanase activity (U/l) during the late production phase can be avoided [Figure 4 (○ and (*)]. One reason for the higher xylanase activity is that the proteolysis of the produced enzyme occurs to lesser extent when using the temperature limited fed-batch technique in comparison to the substrate limited fed-batch (Rozkov 2001). In an attempt to verify if the cells are less stressed, the amount of endotoxins was analyzed but the results were inconclusive. During the reference cultivation more foaming was observed during the last hours, indicating cell-lysis. The larger amount of active xylanase in the broth [Figure 4(×)] further supports this idea. Thus in this case, with *E. coli* BL21(DE3) producing xylanase, the modified feeding strategy is the preferred technique.

In Figure 5 describing *E. coli* W3110 the temperature is not decreasing as much as in the cultivations with *E. coli* BL21(DE3) shown in Figure 3. At 3 h (Figure 5) the stirrer speed passes $N_{\text{ref,}}$ leading to a shift in the strategy (section feeding strategies). The shift from up-pulses to down-pulses leads to an overshoot in the O_2 demand resulting in the initial decrease in the temperature. In this region the induction also takes place, but the start of the protein production does not seem to cause any major changes

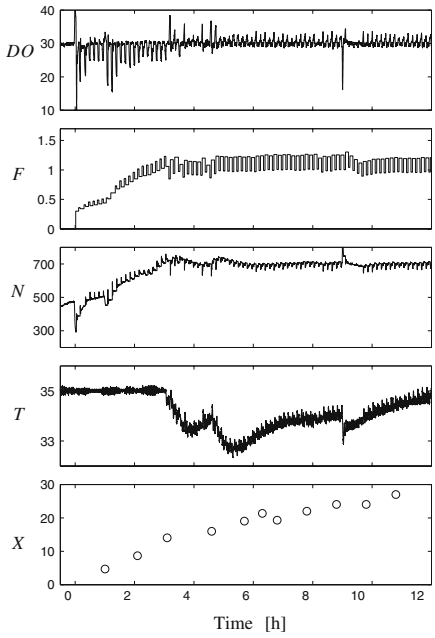

Fig. 5. Experiment with *E. coli* W3110 using the modified probing feeding strategy. The fed-batch part of the cultivation is shown. From top: *DO* [%] dissolved O_2, *F* [l/h] feed, *N* [rpm] stirrer speed, *T* [°C] temperature and *X* [g/l] cell mass. At t = 9 h anti-foam is added. Reference value $N_{ref} = 700$ rpm and maximum value $N_{max} = 800$ rpm. Induction took place at $t = 3.5$ h. Time after feed-start.

in the O_2 demand of the cells. The cell mass during the production-phase is not increasing much when compared to *E. coli* BL21(DE3). For *E. coli* W3110 it increases from 20 to 27 g/l (8 h of production) and for *E. coli* BL21(DE3) it increases from 12 to 40 g/l (6 h of production). Thus the need for a temperature decrease to lower the O_2 demand is much smaller for *E. coli* W3110 than for the *E. coli* BL21(DE3) construct. This demonstrates the flexibility of the modified feeding strategy. It adapts to the characteristics of the system. For a maximal utilization of the reactor, the temperature is only decreased to compensate for the limitation in the O_2 transfer. The proposed technique is thus designed to behave as the original probing feeding strategy when the total O_2 consumption does not increase significantly during the production-phase. In the case with *E. coli* W3110 the difference between using the probing feeding strategy and the modified feeding strategy is then small. This is supported by the similar final cell masses obtained with the two strategies (27 and 29 g/l, respectively) and the similar low residual glucose concentrations in the reactor (around 25 and 50 mg/l, respectively).

Figure 5 further demonstrates that the modified feeding strategy is useful in pilot-scale applications. When the culture volume is large, there are limitations on how fast the temperature of the culture can be changed. This imposes constraints on the achievable performance of a dissolved O_2 control loop if using only the temperature. The use of both the stirrer speed and the temperature is important in order to rapidly control the dissolved O_2 to the set-point between the feed-pulses. In the modified feeding strategy the mid-ranging controller structure is chosen (section feeding strategies). As shown in Figures 5 and 2 the control of the dissolved O_2 works as expected. The stirrer speed regulates the dissolved oxygen quickly between the feed-pulses while the temperature varies slowly in trying to keep the stirrer speed N at a value N_{ref}, slightly below its saturation N_{max}.

The margin between N_{ref} and N_{max} is important as it keeps some authority to control the fast disturbances that are introduced for instance by the feed-pulses. A ramping O_2 demand results in a stationary deviation from N_{ref}. A slow temperature loop results in a larger deviation requiring a larger margin between N_{ref} and N_{max}. A larger margin is thus required in industrial scale with a slow temperature loop.

Conclusions

A cultivation strategy combining the advantages of temperature-limited fed-batch and probing feeding control has been presented. It is the preferred cultivation technique when the O_2 transfer capacity of the reactor is reached and it is desired to continue to produce the recombinant protein. The technique is evaluated in cultivations with *E. coli* BL21(DE3) producing xylanase in a 3 l bioreactor. A 20% larger cell mass is obtained and the decrease in the specific enzyme

activity in the late production-phase was reduced when comparing the new technique to the probing feeding strategy. The flexibility of the modified strategy is further demonstrated in a cultivation with *E. coli* W3110 conducted in a larger bioreactor (50 l).

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IV

Paper IV

Bioreactor Control Using a Probing Feeding Strategy and Mid-Ranging Control

S. Velut L. de Maré P. Hagander

Abstract

The paper presents a fed-batch fermentation technique for bioreactors operating close to their maximum oxygen transfer capacity. The method combines the advantages of the probing feeding strategy and the temperature limited fed-batch technique. When the maximum oxygen transfer capacity of the reactor is reached, the temperature is decreased to lower the oxygen demand. To achieve a good control of the dissolved oxygen a mid-ranging controller manipulating the stirrer speed and the temperature is used. The feeding strategy is analysed and it is also illustrated by simulations and an experiment.

Key words Fermentation processes, feeding strategy, probing, dissolved oxygen control, mid-ranging, temperature

Under revision for Control Engineering Practice.

1. Introduction

E. coli is a common host organism for production of recombinant proteins. It is a well-known bacterium that can be quickly grown to high cell density. The main problem encountered when cultivating *E. coli* is the accumulation of the by-product acetate. Formation of acetate occurs in two situations: under anaerobic conditions or under fully aerobic conditions by overflow metabolism, that is when the carbon source in the medium is in excess. To limit the carbon source in the reactor it is usually fed continuously. When the carbon source is the limiting factor, the technique is called substrate limited fed-batch. One feeding strategy that can be used to dose the substrate feed and that avoids acetate accumulation is the probing feeding strategy described in [Åkesson, 1999].

The probing feeding strategy is optimal in the sense that it tends to maximize the feed rate with respect to the constraints from the cells and the reactor. High cell density can therefore be achieved in short time, without accumulation of the inhibitive by-product acetate. As far as the recombinant product is concerned, the probing technique leads generally to a good productivity. For an optimized product synthesis, the technique could however be further developed.

When the cell density is high, the maximum oxygen transfer capacity of the reactor may be reached. To prevent a rapid decrease of the dissolved oxygen to zero, the feed rate should not be further increased. The common approaches to handle the limited oxygen transfer rate are to freeze the feed rate or to manipulate it to control the oxygen level. The latter strategy, which is often referred as DO-stat, [Konstantinov *et al.*, 1990], is integrated in the probing strategy. A safety net does not allow feed increments when the stirrer speed is close to its maximum. If the oxygen consumption increases further or the oxygen transfer deteriorates, the oxygen level is maintained constant by decreasing the glucose supply rate. The resulting low feed rate may have negative consequences on the protein production. Release of undesirable products into the media, degradation of the recombinant protein (proteolysis) and foaming are other examples of complications due to such cell stress, see [Rozkov, 2001] and [Han, 2002]. An efficient cultivation technique should therefore avoid the severe glucose limitation that can happen in the late fed-batch phase.

An alternative way to lower the oxygen demand is to decrease the temperature of the culture, see [Bauer and White, 1976]. When compared to substrate limited fed-batch, the temperature limited fed-batch (TLFB) technique as presented in [Silfversparre *et al.*, 2002] seems to minimize the release of endotoxins, as well as the proteolysis rate, [Rozkov, 2001]. The main obstacle with the temperature limited fed-batch technique is to achieve a non-growth limiting glucose concentration in the reactor without

accumulating acetic acid, [Han, 2002]. An on-line glucose sensor is usually not available and the critical glucose level is not known in advance and it may change during a cultivation.

The main contribution of the paper is an automated strategy that provides suitable cultivation conditions for operation at the maximum oxygen transfer rate. The novel fermentation technique addresses the challenging problem of avoiding starvation while limiting acetate formation at that cultivation stage. The principles of the technique, which combines the probing strategy and the TLFB technique, are first explained and illustrated using a bioreactor model. A simple analysis of the oxygen and glucose control loops is then performed. Design considerations that help the user to tune the controllers are also presented. The strategy is finally evaluated with simulations and the results from a fed-batch experiment in a laboratory-scale bioreactor with *E. coli* are shown.

2. Process description

To provide suitable conditions for growth and production it is common to monitor and control the essential cultivation variables, see figure 1. The dissolved oxygen, pH and temperature are the variables that normally are measured on-line. The acetic acid concentration, the cell mass and the glucose concentrations are usually analysed off-line. In the novel fermentation technique the dissolved oxygen signal is shared by the mid-ranging controller and the probing controller to manipulate the stirrer speed, the glucose feed rate and the temperature.

2.1 A bioreactor model

In this section, a mathematical model describing a bioreactor running in fed-batch mode is presented. For notation and parameter values, see table 1 and table 3. The mass balance equations for the media volume V, the glucose concentration G, the acetic acid concentration A, the cell mass X

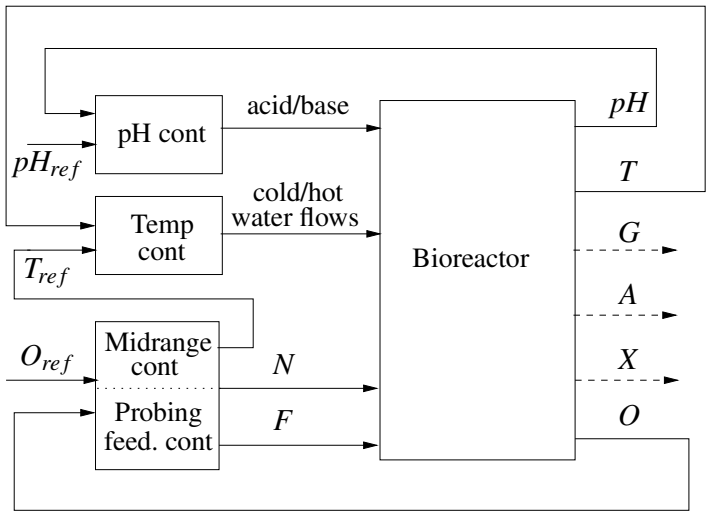

Figure 1. Block diagram of the bioreactor with the main control loops. The measured variables are: pH, temperature T, dissolved oxygen O, acetic acid concentration A (off-line), cell mass concentration X (off-line), glucose concentration G (off-line), glucose feed rate F and stirrer speed N, cont = controller, ref = reference value

and the oxygen concentration C_o are:

$$\begin{aligned}
\frac{dV}{dt} &= F \\
\frac{d(VG)}{dt} &= FG_{in} - q_g(G,T)VX \\
\frac{d(VA)}{dt} &= q_a(G,A,T)VX \\
\frac{d(VX)}{dt} &= \mu(G,A,T)VX \\
\frac{d(VC_o)}{dt} &= K_L a(N)V(C_o^* - C_o) - q_o(G,A,T)VX
\end{aligned} \tag{1}$$

The cell metabolism is described by the glucose uptake rate q_g, the oxygen uptake rate q_o, the acetate production rate q_a and the growth rate μ. Acetate is produced when the oxygen uptake rate saturates ($q_o = q_o^{max}$), *i.e.* when the glucose uptake rate exceeds a critical value q_g^{crit}. If acetate is present in the media, it may also be consumed when $q_o < q_o^{max}$. The metabolic expressions are similar to the ones presented in [Åkesson *et al.*, 2001b], and they are given in Appendix A.1. The oxygen transfer coefficient

K_La is assumed to be a function of the agitation speed N. The glucose concentration of the feed is denoted by G_{in} and C_o^* is the dissolved oxygen concentration in equilibrium with the oxygen in the gas bubbles.

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

$$q_g^{max}(T) = q_{g,37}^{max} f(T), \quad f(T) = e^{-50(\frac{1}{T}-\frac{1}{37})} \tag{2}$$

The acetate and oxygen uptake rates q_a and q_o are modified in a similar fashion and also the maintenance coefficient q_{mc}, [Esener *et al.*, 1983]. It should be noticed that the temperature dependence is almost linear in the range 37^{o}C to 25^{o}C. We strive for a model that is as simple as possible, so the influence of the temperature on K_La and the solubility of oxygen is neglected. The resulting effect on the oxygen transfer appears anyway to be relatively small in the range 20 ^{o}C to 40^{o}C, as pointed out in [Enfors and Häggström, 1994].

Most sensors measure the dissolved oxygen tension O instead of the dissolved oxygen concentration C_o. They are related by Henry's law

$$O = HC_o \tag{3}$$

The dynamics in the oxygen probe and in the temperature control system should also be taken into account. They are modeled by first order systems with time constants T_p and T_t, respectively.

3. A probing feeding strategy

In this section, the principles of the probing strategy as described in [Åkesson, 1999] are briefly presented and some needs for improvement are then pointed out.

3.1 Principles

To prevent anaerobic conditions, the dissolved oxygen (DO) concentration can be maintained at a constant level. This requires a balance between the oxygen uptake rate (OUR) and the oxygen transfer rate (OTR). According to the mass balance equation for oxygen in (1), one should have

$$0 = \underbrace{K_La(N)(O^* - O_{ref})}_{OTR} - \underbrace{q_o(G,A,T)HX}_{OUR} \tag{4}$$

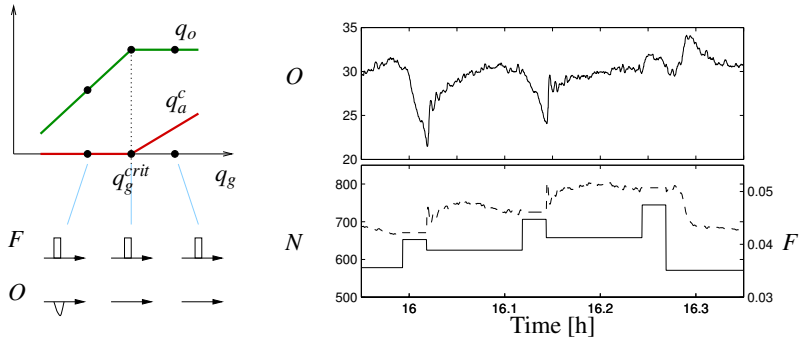

Figure 2. Principles of the probing technique (left) and application on a fed-batch experiment (right). Acetate formation ($q_a^c > 0$) occurs when the specific oxygen uptake rate q_o saturates, which can be detected in the oxygen signal O by superimposing pulses in the feed rate F. When a pulse response is visible in O, the feed rate is increased at the end of the pulse. In the experiment, the absence of response to the third pulse indicates overfeeding and it leads to a feed decrement. The stirrer speed N is frozen during a probing pulse and regulates O at 30% between two successive pulses.

where O_{ref} denotes the desired the dissolved oxygen level. Feedback control can be used to achieve this balance throughout the cultivation. The probing approach solves the problem by periodically manipulating OTR and OUR. The basic control sequence consists of two steps:

- Probing: superimpose a short pulse on the feed rate and evaluate the response in the dissolved oxygen signal. If a pulse response is visible, the respiratory capacity of the cells is not exceeded ($q_o < q_o^{max}$) and the feed rate is increased at the end of the pulse. When overfeeding is detected (no pulse response) the feed rate is decreased.
- Regulation: retrieve the balance between oxygen uptake and supply by acting on OTR, *i.e.* by manipulating the stirrer speed N.

The probing technique does not only achieve the oxygen balance, but it also maximizes the oxygen uptake rate while avoiding overflow metabolism. Figure 2 illustrates the principles of the probing technique and shows a part of an experiment where the probing method is applied. The control law updating the feed rate after a probing pulse is

$$F(k+1) = \begin{cases} F(k) - F_{dec} & \text{if } O_{pulse} < O_{reac} \\ F(k) + \kappa(O_{pulse}(k) - y_r) & \text{otherwise} \end{cases} \tag{5}$$

where $O_{pulse}(k)$, y_r and κ are the size of the kth pulse response, the desired pulse response size and the probing gain, respectively. O_{reac} is the

detection limit of a pulse response. The controller parameters are collected in table 2. When a pulse response is registered, the feed is incremented proportionally to the response amplitude. When no clear pulse response is visible, the feed rate is decreased by F_{dec} to avoid overfeeding. The complete probing algorithm includes additional logic for practical issues such as measurement noise or wait for stationarity before a probing pulse. Further details can be found in [Åkesson, 1999] where simple tuning rules are also given.

3.2 Operation at the maximum oxygen transfer

When the cell density is high, the oxygen transfer capacity of the reactor may be reached and the feed rate can no longer be increased. If the oxygen consumption increases due to growth, or if the oxygen transfer deteriorates, the oxygen uptake rate should be lowered to guarantee aerobic conditions. In the probing approach, the sequence of control actions is modified as follows

- No feed increment after a pulse is allowed if $N > N_{high}$
- Decrease F between the pulses as long as $N > N_{high2} > N_{high}$

The threshold N_{high2} is normally chosen to be below the maximum stirrer speed to let a sufficient margin to the stirrer speed controller for disturbance rejection. In [Åkesson *et al.*, 2001b] it is proposed to lower the feed supply by

$$\frac{dF}{dt} = -\gamma F \tag{6}$$

where γ is some positive constant. For better control performance in presence of quick disturbances, feedback from dissolved oxygen can be used between the pulses, see [Velut, 1999]. A feeding strategy that manipulates the feed rate to control the dissolved oxygen concentration is often referred to as DO-stat, see [Konstantinov *et al.*, 1990]. It should be pointed out that, once the stirrer speed has reached its maximum, operation at q_o^{max} is no longer possible and the late fed-batch phase can be characterized by a severe glucose limitation.

Figure 3 shows an experiment performed in a 3 l reactor where the probing feeding strategy is used. The feed rate is rapidly increased to meet the glucose demand from the growing biomass. The maximum stirrer speed is reached 2 hours after feed start. Thereafter, because of cell growth, the feed is gradually lowered during 6 hours, leading to a decrease of 27%. The low feed rate resulting from a long operation at the maximum oxygen transfer rate can lead to cell stress and starvation, which is detrimental for product synthesis. Manipulation of the culture temperature

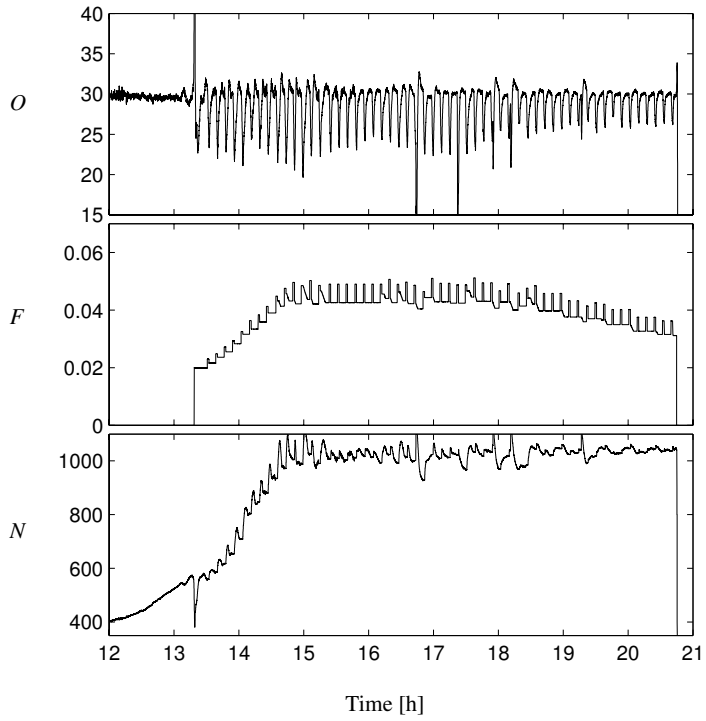

Figure 3. A cultivation where the original probing feeding strategy is used. The fed-batch part of the cultivation is shown. From top: dissolved oxygen O ([%]), feed rate F ([l/h]) and stirrer speed N ([rpm]). At t = 16.7 h and t = 17.5 h antifoam is added which has a large impact on the dissolved oxygen.

might be a better way to regulate the oxygen demand once the maximum oxygen transfer capacity of the reactor has been attained.

4. Control Problem

Combining the probing strategy and the temperature-limited technique is a non-trivial control problem. Figure 4 is an illustration of the control configuration at high oxygen transfer rates. To achieve the dissolved oxygen balance, three control variables are available: the feed rate F, the stirrer speed N and the temperature T. The feed rate passes through the oxygen consumption bottleneck, which has a temperature dependent size. The stirrer speed operates close to its saturation.

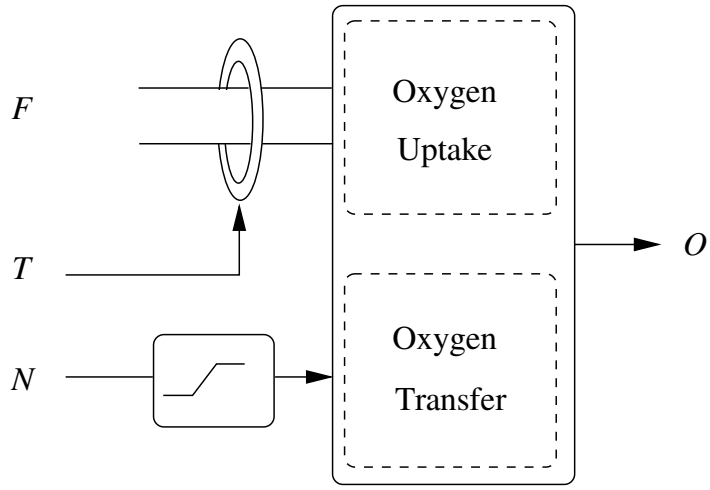

Figure 4. Schematic view of the control problem. To achieve a balance between oxygen uptake and transfer three control variables are available: the feed rate F, the stirrer speed N and the temperature T. The feed rate passes through the oxygen consumption bottleneck of temperature dependent size. The stirrer speed operates at N_{ref}, close to saturation.

For an efficient application of the TLFB technique the following cultivation conditions are required: temperature should be the limiting factor and glucose should be in slight excess.

4.1 Temperature-based DO control.

Temperature is used to reduce the oxygen uptake and thereby maintaining a constant oxygen level. Manipulation of the temperature for feedback control of the oxygen concentration is however not a simple task. There are limitations on the achievable performance of the loop. Compared to the agitation system the cooling process is much slower. Furthermore, there often exist constraints on the rate of change, strongly related to the temperature of the incoming cooling flow. Apart from the possibly slow cooling system, the uncertainty in the temperature influence on the cells represents a strong limitation. As the dynamic is not accurately known, it is not recommended to design a DO controller with a high bandwidth.

4.2 Glucose excess.

Achieving glucose excess implies a significant risk for acetate accumulation to inhibitive levels. The main obstacle with the TLFB technique is to achieve a non growth-limiting substrate concentration without acetate

accumulation. Feedback appears to be necessary, but the difficulty is that no on-line measurement of neither glucose nor acetate is generally available. Another complication is the temperature dependence of q_o^{max} and of q_g^{crit}.

4.3 Efficient utilization of the reactor.

Glucose excess and temperature limitation can be achieved at various temperature values. Operation at very low temperatures would imply low stirrer speeds and feed rates, and results in a poor utilization of the reactor. Our objective will be to exploit the full capacity of the reactor. This implies that the feed rate and the stirrer speed should be maximized with respect to the constraints, while the temperature should be as close to 37^oC as possible.

5. A combined feeding strategy

A feeding strategy that combines the advantages of the TLFB technique and probing control will now be described. The early fed-batch phase is run under glucose limited conditions, using the original probing strategy described in [Åkesson *et al.*, 2001a]. When the maximum oxygen transfer capacity is approached, that is when the stirrer speed N reaches N_{ref} close to the maximum value N_{max}, the temperature limitation mode is activated. The probing approach can be used to achieve a controlled excess of glucose without acetate accumulation. An efficient control strategy for the regulation of dissolved oxygen is also included.

5.1 Feed control

The probing feeding strategy has proved to be an efficient tool for maximizing the feed rate while minimizing acetate production.

Contrary to the original probing feeding technique, increments in the feed rate at this stage of the cultivation are allowed for a good control around q_g^{crit}.

To achieve a slight glucose excess, down-pulses instead of up-pulses could be applied. When a down-pulse is made the dissolved oxygen signal will increase if $q_o < q_o^{max}$. The feed rate is adjusted as before depending on the size of the response in the dissolved oxygen. Figure 5 illustrates the benefit of using down-pulses: pulse responses are visible for higher q_g values. Operation at a q_g value slightly above q_g^{crit} results in a low concentration of acetic acid, which is non-inhibitive to growth or protein production.

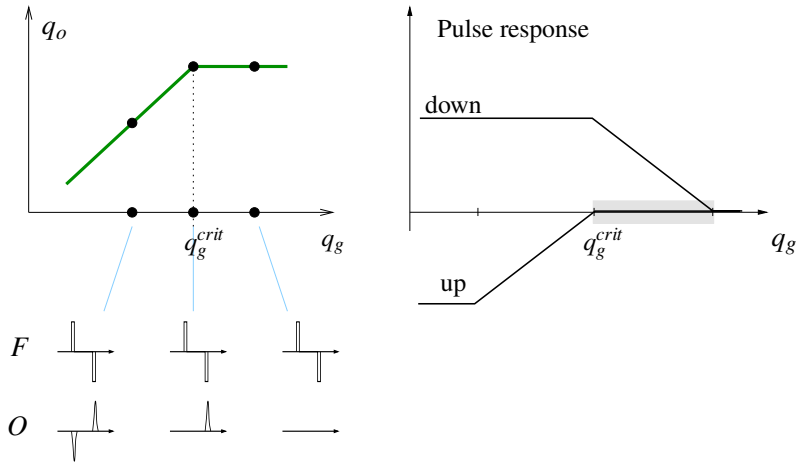

Figure 5. The amplitude of the pulse responses in dissolved oxygen as a function of q_g, in the absence of acetate. The shaded area indicates the region where the detection method registers responses to down pulses but not to up pulses.

5.2 Dissolved oxygen control

Faster DO-control allows frequent probing, which results in a better overall control performance. Given the limitations mentioned in Section 4, dissolved oxygen control based on the sole manipulation of temperature would not be sufficient for a proper application of the probing strategy. The stirrer speed is therefore used simultaneously with the temperature to achieve a satisfactory performance. Dissolved oxygen control can be viewed as a control allocation problem. To allocate the control signals taking into consideration the static and dynamic constraints, the so-called mid-ranging controller is used, see [Allison and Isaksson, 1998]. In this configuration, shown in Figure 6, two SISO controllers are connected in cascade. The first controller C_1 manipulates the stirrer speed N and it is tuned to handle the fast disturbances. The objective of C_2 with input $N - N^{ref}$ is to keep the control signal N in its operating range. It should take care of slow disturbances such as cell growth. The advantage of this structure is its simplicity. Moreover, the first controller C_1 can be used alone until the control signal N reaches the saturation.

5.3 Proposed cultivation technique

In summary, when N reaches N_{ref}

- the up-pulses superimposed to the feed are shifted to down pulses and increments to the feed are still allowed.

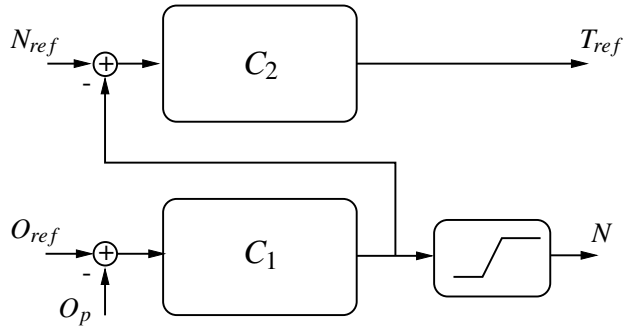

Figure 6. Block-diagram over the mid-ranging control scheme. C_1 is a controller manipulating N to take care of the fast disturbances on the output. C_2 manipulates T to maintain N around N_{ref}, below the saturation.

- the temperature loop is activated to keep the stirrer speed close to N_{ref}

The fed-batch technique should result in a maximal utilization of the reactor. Cells are fed at their maximal uptake capacity ($q_o = q_o^{max}(T)$) and the stirrer speed is kept close to its maximum. The feed rate is not used to control DO unless the maximum cooling capacity of the reactor is reached.

6. Analysis and tuning

Due to the temperature dependence of the maintenance requirements, temperature-based DO control is preferable to feed-based DO control to avoid starvation. This is demonstrated in Appendix B using the process model previously described.

The proposed cultivation technique is based on two separate control loops. The objective of the first loop, involving the feed flow rate, is to achieve a slight glucose excess. The second loop aims at maintaining a constant dissolved oxygen level by manipulation of temperature and stirrer speed. If the DO controller is appropriately tuned, the cross-coupling between the two loops is weak. The cultivation technique will be analysed in two steps by examining the control loops separately.

6.1 Feed control

The original probing feeding strategy leads to a stationary specific uptake rate q_g that is below q_g^{crit}. At steady state, some acetate is produced during

the probing pulse, but it is rapidly consumed during the following oxygen control phase. According to Figure 5, it seems possible to achieve a stationary state above q_g^{crit} by making down-pulses. Since acetate is continuously produced when $q_g > q_g^{crit}$, it is not obvious that such a steady state can be achieved. The acetate should be consumed during the down-pulse. The model equations can be used to show the feasibility of the approach and provide some help in the design procedure. The parameters of the feed controller are collected in table 2.

Stability analysis For simplicity reasons the glucose and oxygen dynamics in Equation (1) will be neglected in the analysis. The following static equations then relate together with (39) the feed rate to the dissolved oxygen concentration:

$$\Delta q_g = K_g \Delta F \tag{7}$$

$$\Delta O = K_o \Delta q_o \tag{8}$$

Figure 7 (left) shows the phase portrait describing the acetate dynamic in the plane (q_g, A). The state space is divided into 3 regions and the acetate dynamics is linearized in each region, see (38)-(41). In the region Ω_3 acetate is produced while it is consumed in Ω_1 and Ω_2. In Ω_2 the rate of consumption is limited by the respiratory capacity of the cells ($q_o = q_o^{max}$). The objective is to operate at stationarity in the region Ω_3. By stationary state it is meant that the feed rate computed by the probing controller is, at steady state, a constant corresponding to a q_g value above q_g^{crit}. Since the controller periodically performs probing pulses on top of the feed rate, a stationary state should be interpreted as a closed trajectory in the plane (q_g, A), see Figure 7 (left):

- The acetate accumulated during the control phase should be totally consumed during the probing phase
- The small pulse response should not lead to any feed adjustment

For a pulse response to be visible, the specific oxygen uptake rate should not be saturated during the entire pulse. It is therefore necessary that $\chi_4 \in \Omega_1$. The search for steady state solutions $\chi(t)$ will be restricted to satisfy

$$\chi(t) = \begin{bmatrix} q_g(t) \\ A(t) \end{bmatrix} \in \begin{cases} \Omega_3 & \text{for } t \in (0, T_c) \\ \Omega_1 & \text{for } t \in (T_c, T) \end{cases} \tag{9}$$

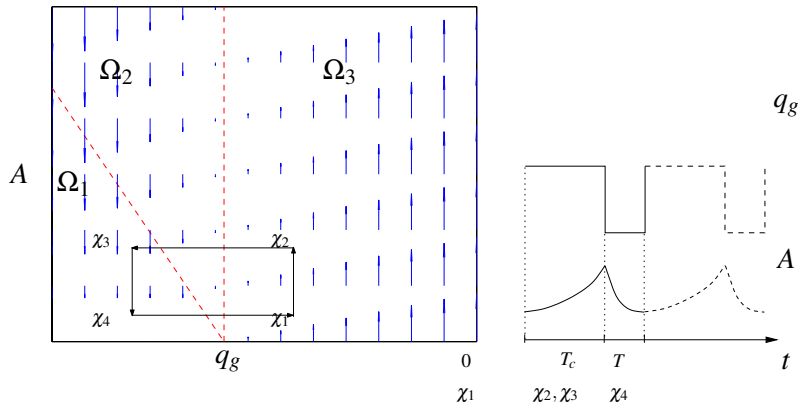

Figure 7. Left: phase portrait of the acetate dynamics in the (q_g, A) plane. A stationary trajectory is shown in the phase plane and as a function of time (right). The control phase with length T_c starts at $\chi_1 \in \Omega_3$: the feed rate is constant and acetate is continuously produced. The probing pulse, performed downwards, results in a jump from χ_2 to $\chi_3 \in \Omega_1$, where acetate is consumed. At the end of the pulse, the feed rate recovers its initial level if the change in the oxygen uptake rate from χ_2 to χ_3 gives a pulse response $O_{pulse} = y_r$.

According to the linearized equations (41), the acetate dynamic becomes

$$\frac{dA}{dt} = \begin{cases} Y_{ag}(q_g - q_g^{crit} - q_m)X & \text{for } t \in [0, T_c) \\ -\dfrac{A}{\tau}X & \text{for } t \in [T_c, T) \end{cases} \tag{10}$$

The specific glucose uptake rate, related to the feed rate through (8) is given by

$$q_g(t) = \begin{cases} q_g(0) & \text{for } t \in [0, T_c) \\ q_g(0) - q_g^p & \text{for } t \in [T_c, T) \end{cases} \tag{11}$$

and at the end of the probing period, it is adjusted by the probing controller as in (5) depending on the pulse response size O_{pulse}. Using (7) it becomes:

$$q_g(T) = q_g(0) + \kappa K_g(O_{pulse}(T) - y_r) \tag{12}$$

where q_g^p is the amplitude of the variation in q_g due to the probing pulse. The size $O_{pulse}(T)$ of a pulse is proportional to the change in the specific oxygen uptake rate after a pulse:

$$O_{pulse}(T) = K_o\left(q_o(T) - q_o(T_c)\right) \tag{13}$$

where q_o is a function of q_g and A given by (39).

By integrating the acetate dynamic along the probing cycle, we get the following condition for (q_g, A) to be a stationary solution:

$$\begin{bmatrix} q_g \\ A \end{bmatrix}(T) = A_e \begin{bmatrix} q_g \\ A \end{bmatrix}(0) + a_e \tag{14}$$

where A_e is given in Appendix C and a_e is a constant vector. If the probing gain κ and the control phase duration T_c satisfy

$$0 < \kappa \frac{K_o K_g Y_{og}}{2} < 1 \tag{15}$$

$$0 < T_c < \frac{\tau(1 + e^{T_{pulse}X/\tau})}{Y_{oa} Y_{ag} X} \left(\frac{2}{\kappa K_o K_g Y_{og}} - 1\right) \tag{16}$$

then we have a stable stationary point (q_g, A) in the region Ω_3. The stability analysis indicates that, with a proper choice of the control parameters, a balance between acetate production and uptake can be achieved in spite of the regular operation above q_g^{crit}.

Help for tuning Inequality (15) that limits the probing gain is similar to the conditions derived in [Åkesson, 1999] and [Velut and Hagander, 2004]. The choice $\kappa^{-1} = K_o K_g Y_{og}$ leads to a dead-beat control and requires an estimate of the process gain from feed rate to dissolved oxygen concentration, see Appendix D for more details.

Inequality (16) is a direct consequence of the operation above q_g^{crit} between the probing pulses. The acetate dynamic and the pulse length affect the upper-bound on T_c. Short probing pulses and a slow acetate consumption require short control phases. A good dissolved oxygen controller is thus critical when performing down pulses. Choosing dead-beat control and the numerical values listed in Appendix D, the constraint (16) becomes $0 < T_c < 13$ min.

When the glucose and oxygen dynamics cannot be neglected, the stability conditions (15)–(16) should be adjusted. Slow dynamics require longer control phases and allow higher gains, as it is shown in [Velut, 2005].

6.2 Dissolved oxygen control using mid-ranging

Mid-ranging control solves an allocation problem in presence of saturations. When properly tuned, the controller maintains the fast control signal below its saturation. It will therefore be assumed in the analysis that the stirrer speed operates in the linear region. Some nonlinear considerations taking the saturation into account will be later presented. The parameters of the mid-ranging controller structure are collected in table 2.

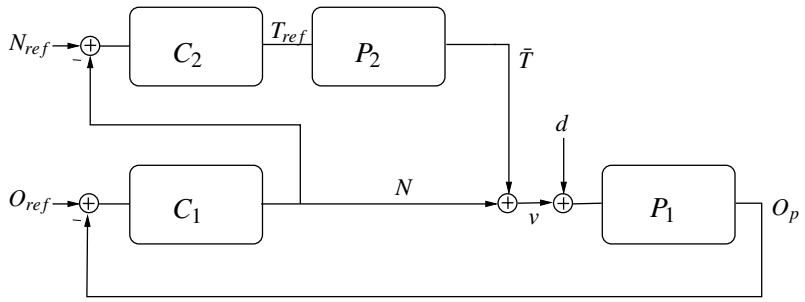

Figure 8. Oxygen control using a mid-ranging configuration. Disturbances like feed changes or cell growth are modeled by the signal d.

Design procedure. Mid-ranging is a simple control structure that solves the allocation problem. A benefit of having two SISO controllers concerns the design procedure: the controllers can be tuned one at a time just like in conventional cascade control. Mid-ranging and conventional cascade control are actually closely related. The mid-ranging control problem

$$\begin{aligned} y &= P_1 \begin{bmatrix} 1 & P_2 \end{bmatrix} u \\ u &= \begin{bmatrix} 1 & C_2 \end{bmatrix}^T C_1 y \end{aligned}$$

can be viewed as the dual problem of the cascade control problem

$$\begin{aligned} y &= \begin{bmatrix} 1 & P_2 \end{bmatrix}^T P_1 u \\ u &= C_1 \begin{bmatrix} 1 & C_2 \end{bmatrix} y \end{aligned}$$

A dual relationship can also be demonstrated using the method presented in [Bernhardsson and Sternad, 1993]. The close correspondence between mid-ranging control and cascade control strengthens the design procedure in two steps. The fast controller C_1 can be designed separately to get satisfactory performance before the activation of the second loop. The second controller C_2 should be tuned in such a way that it does not interfere with the initial control loop at high frequencies. It is assumed that both controllers are PI controllers and that C_1 has already been designed. Some guidelines for the design of C_2 can now be presented.

The closed-loop system for dissolved oxygen control is represented in Figure 8. Disturbances on the dissolved oxygen representing growth or feed changes are modeling by the signal d.

The role of C_2 is to compensate for the saturation in N by increasing the control authority in the low frequency range. An integrator in C_2

is thus necessary to get $N = N_{ref}$ at stationarity in spite of constant disturbances d.

In order to understand how the control allocation is influenced by C_2, one can write the total control effort v as:

$$v = \bar{T} + N = (1 - P_2C_2)N + P_2C_2N_{ref}$$

At those frequencies where P_2C_2 is small compared to 1, the temperature controller is not active compared to the stirrer speed. The choice

$$C_2 = \frac{k_i}{s} \tag{17}$$

gives a low-pass behavior. The value of the integrator gain k_i is a compromise between robustness and performance.

Robustness. The uncertainty in the temperature dynamic P_2 should be considered when designing C_2. The norm $||S||_\infty$ of the sensitivity function defined by

$$S = \frac{1}{1 + G_{N,\bar{T}}P_2C_2} \tag{18}$$

can be used as a robustness measure. $G_{N,\bar{T}} = \frac{P_1C_1}{1+P_1C_1}$ denotes the inner loop transfer function. A low value for $||S||_\infty$ will ensure a well-damped behavior, which is of primary importance to avoid overreaction of the temperature.

Performance. The disturbance d includes a ramp function modeling the cell growth. This leads to a stationary error $N_\infty - N_{ref}$ depending on the integrator gain:

$$N_\infty - N_{ref} = \frac{1}{k_iP_2(0)}\Delta d \tag{19}$$

The ramp disturbance reduces the operating range of the stirrer speed for a good rejection of fast disturbances. The integrator gain should therefore be chosen large enough for a sufficient margin to the saturation.

Numerical example. The design procedure can be applied on the bioreactor example using numerical values corresponding to a laboratory-scale reactor, see Appendix D. The oxygen dynamics, described by P_1 and P_2 is obtained after linearization of the reactor model, see Apppendix A.2.

$$\begin{aligned} P_1 &= \frac{K_N}{(T_os + 1)(T_ps + 1)} \\ P_2 &= \frac{\frac{K_T}{K_N}}{T_ts + 1} \end{aligned} \tag{20}$$

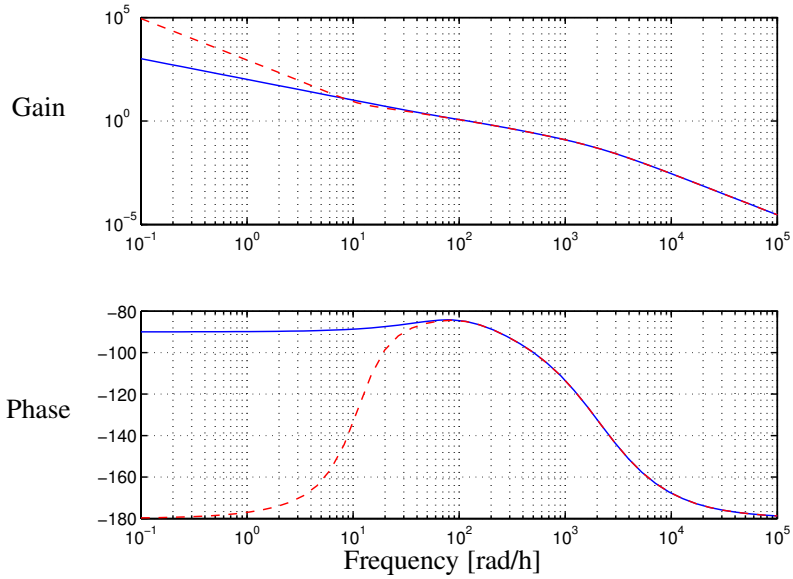

Figure 9. Bode plots for the open-loop system P_1C_1 without the temperature controller (solid) and $P_1C_1(1-P_2C_2)$ with the temperature controller (dashed).

The PI controller manipulating the stirrer speed has gain $K = 18$ and integral time $T_i = 40\ s$. This gives good performance when operating at $N \approx 1000\ rpm$, see [Åkesson and Hagander, 1999]. The maximal stirrer speed is $N_{max} = 1100\ rpm$ and a growth of 6 $g/(lh)$ is considered. To guarantee $M_s < 1.5$ and a static error less than 50 rpm in presence of the load disturbance d, we should choose k_i in the range $0.17 - 0.5$. Taking $k_i = 0.3$ leads to a M_s value of 1.3 and a stationary error $N_\infty - N_{ref} =$ 29 rpm.

Figure 9 shows a bode diagram for the loop transfer with and without the extra loop involving the temperature. The controller C_2 contributes to a larger gain in the low frequency region and prevents the stirrer speed from saturating. Its influence around the cross-over frequency $\omega_c \approx 100\ rad/h$ is negligible. A step response simulation is shown in Figure 10. The amplitude of the step corresponds to a feed change as large as a pulse. The disturbance results in a peak in the agitation which is approximately equal to the available margin to the saturation (100 rpm). Note that the disturbance rejection at the output is almost unchanged when using the additional loop.

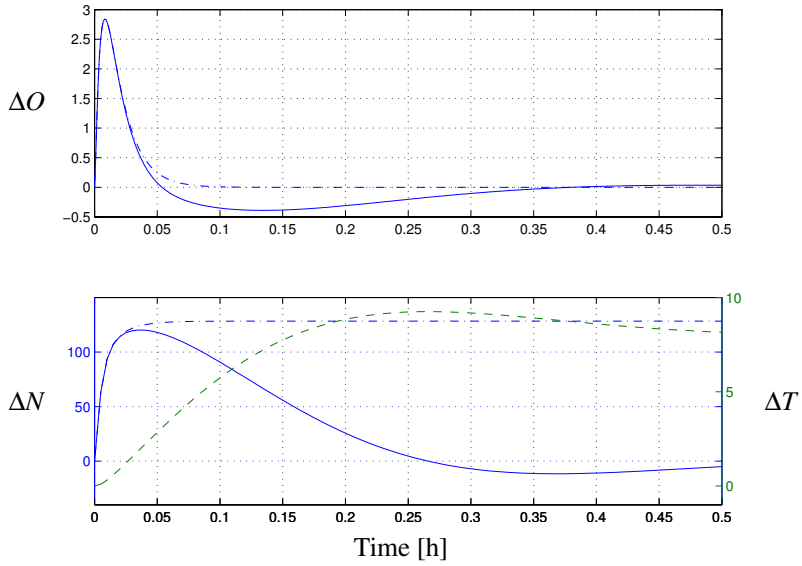

Figure 10. Step disturbance simulation of the dissolved oxygen control loop. Top: dissolved oxygen O ([%]). Bottom: stirrer speed N ([rpm]) in solid and temperature T ([^{o}C]) in dashed. The simulation result shown in dash-dotted is without the temperature control loop. If no dissolved oxygen control takes place, the pulse response size is 8.3 % (not shown).

Nonlinear considerations. The design procedure was based on linear analysis. When large disturbances like antifoam addition perturb the process, the agitation speed will inevitably saturate. To avoid wind-up phenomena, an anti-windup scheme is often implemented to keep the control signal close to saturation $N \approx N_{max}$. If the reference value N_{ref} is chosen close to N_{max}, the input to the temperature controller C_2 will be constant and small during saturation of N. The temperature loop will then be broken and ineffective. For reasonable performance during saturation, the agitation speed should not be quickly reset by the anti-windup controller. An alternative would be to use $sat(N) - N$ as an extra input to the temperature controller. It could be used to increase the control authority of the temperature controller when the agitation saturates.

7. Evaluation of the control strategy

The fermentation technique previously described will now be evaluated using simulations and experiment.

7.1 Simulation

The full nonlinear model given in Section 2.1 is used together with the numerical values listed in Appendix D to simulate a bioreactor running in fed-batch mode. Figure 11 shows the result of a simulation.

When the stirrer speed has reached $N_{ref} = 1000\ rpm$ at $t \approx 3\ h$, the second controller is activated and the temperature starts to decrease. The acetate accumulation after activation of the temperature controller is rapidly consumed. At $t \approx 3.7\ h$ a stationary state is achieved as predicted by the simple analysis from Section 6.1. The pulse responses are of desired size and no feed adjustment is done. The acetate pattern is as it was expected: short accumulation phases are followed by consumption phases and the resulting acetate concentration is in average small. When the control phase is long ($T_c = 15\ min$), no stationary state is achieved and acetate follows another pattern with large accumulations (Figure 11, dotted line). This is predicted by (16).

Temperature decreases slowly to compensate for the increasing oxygen demand from the growing biomass. At $t \approx 4.4\ h$, a step disturbance modeling a degradation of the oxygen transfer is introduced. The agitation reacts rapidly to control the oxygen level and the next pulse is not delayed. The disturbance in $K_L a$ leads also to a decrease in the feed rate. The stationary error in the stirrer speed due to cell growth is 25 rpm, which is close to the value derived by linear analysis.The objectives described in Section 4 are achieved: the temperature is slowly lowering the oxygen demand and the feed rate is adjusted to operate close to $q_o^{max}(T)$. The stirrer speed is kept close to the saturation level and takes care of the fast disturbances.

7.2 Experiment

The new fermentation technique was implemented and tested on a 3 l bioreactor. Figure 12 shows the fed-batch part of an experiment with *E. coli* Tuner (DE3). The material and methods used for the cultivation can be found in [de Maré *et al.*, 2005].

Temperature control was performed using pulse-width modulation of the cold and hot water flows. At $t \approx 15\ h$ the initial glucose amount from the batch phase is totally consumed and the glucose starts to be fed into the reactor. After 1.5 h of feeding the stirrer speed reaches $N_{ref} = 1000\ rpm$ and the temperature starts to decrease. The initial decrease in the temperature does not seem to influence the dissolved oxygen concentration. This

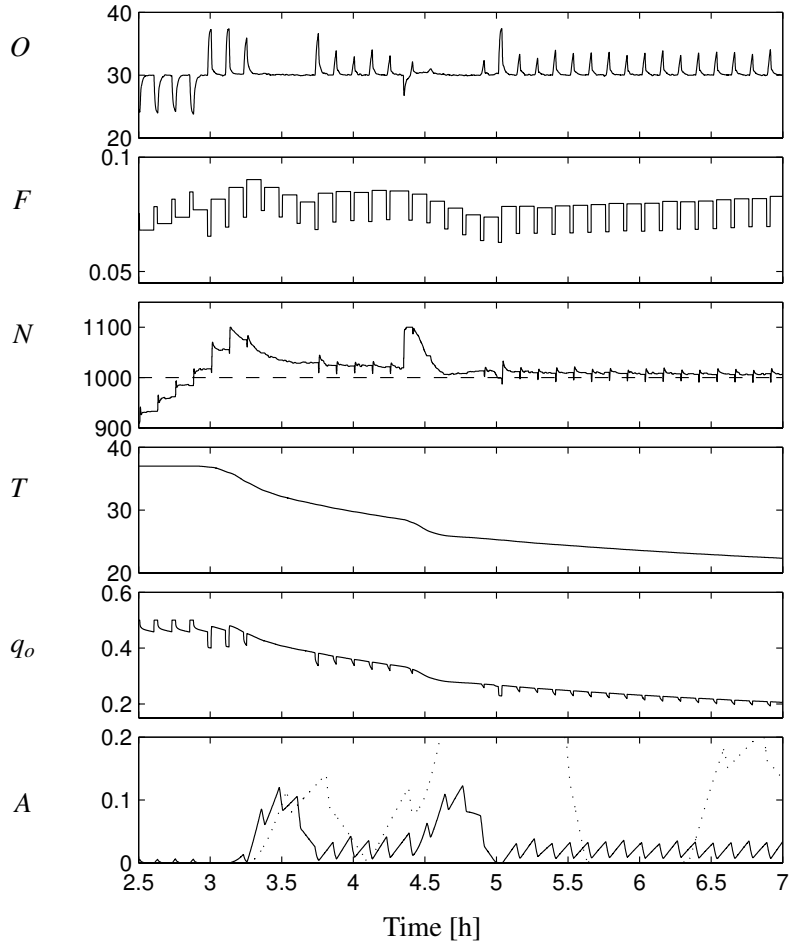

Figure 11. Simulation of the nonlinear model using the modified probing strategy ($\kappa = 1.4\ 10^{-3},\ y_r = 3,\ T_c = 6\ min$). From top: dissolved oxygen O([%]), feed rate F([l/h]), stirrer speed N([rpm]), temperature T([^{o}C]), specific oxygen uptake rate q_o([1/h]), acetate A([g/l]). At $t \approx 3\ h$ up-pulses are shifted to down-pulses and the temperature starts to decrease. At $t \approx 4.4\ h$ a decrease in $K_L a$ modeling a degradation of the oxygen transfer is introduced. The acetate in dotted line is the result of a control phase with length $T_c = 15\ min$.

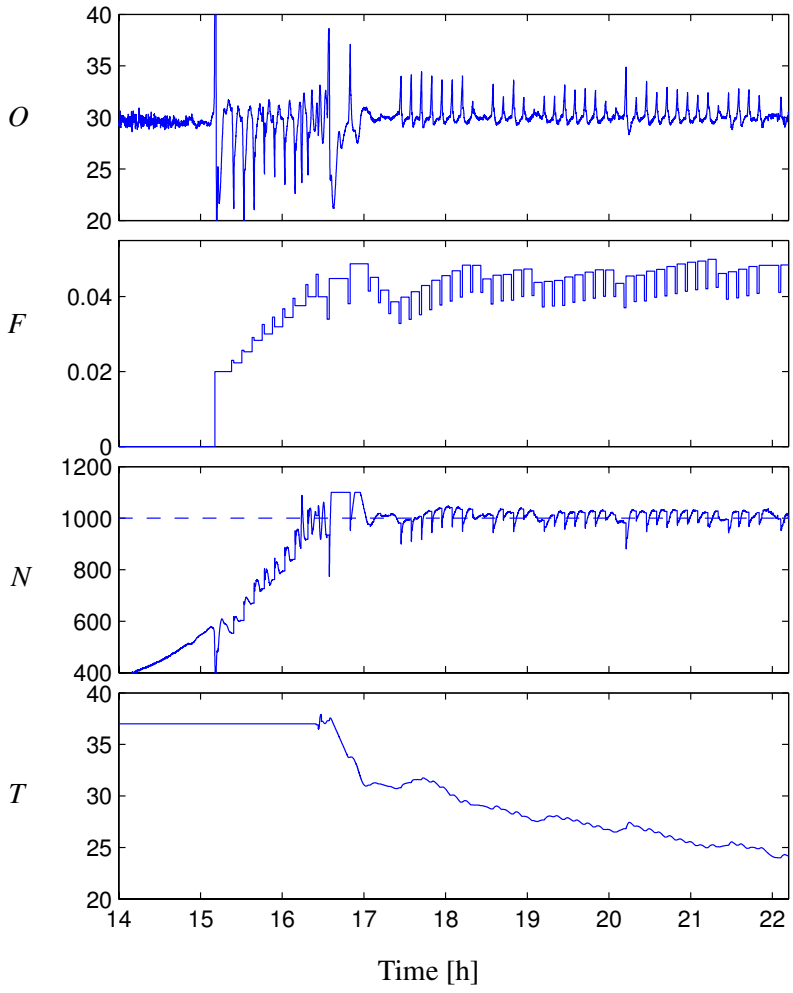

Figure 12. Fed-batch part of an experiment using the novel fermentation technique ($\kappa = 1.4\ 10^{-3}$, $y_r = 3$, $T_c = 6\ min$). From top: O ([%]) dissolved oxygen, F ([l/h]) feed, N ([rpm]) stirrer speed, T ([^{o}C]) temperature. N_{ref} is dashed.

can be explained by the model which predicts a lack of authority when the glucose is not in excess. The stationary gain from temperature to dissolved oxygen obtained by linearization of the process equations when $q_o < q_o^{max}$, is actually zero. The lack of temperature authority may also be due to the weaker influence of the temperature around 37 ^oC compared to the model. At $t = 17\ h$ no pulse response is visible in the oxygen signal, which indicates that glucose is in excess. The feed is consequently decreased to avoid large acetate accumulations. As far as the dissolved oxygen control is concerned, good performance is achieved. Dissolved oxygen is rapidly brought back to the set-point after every pulse. The agitation speed takes care of the fast disturbances and it operates around N_{ref}. The static error is about 40 rpm, which leads to a 60 rpm margin to the saturation. Since no large disturbances such as antifoam addition act on DO, the stirrer speed never saturates. Temperature decreases slowly from 37 ^{o}C to 25 ^{o}C to compensate for the increasing oxygen demand due to growth and protein production. The decrease in the oxygen transfer rate that is often observed in the late cultivation phase contributes also to the temperature decrease. An evaluation of the novel technique with respect to glucose, acetate and protein concentrations is presented in [de Maré *et al.*, 2005]. Compared to the original probing technique, the new strategy leads to a higher glucose level, no inhibitive acetate accumulation, a higher cell mass and a better protein production.

Unlike the original probing technique, over-feeding can easily occur when the maximum stirrer speed has been reached. Indications of glucose excess from the probing pulses led to a decrease in the feed at four occasions. This shows the importance of the glucose feeding in temperature limited fed-batch cultivations. It is interesting to compare the feed profiles from Figures 3 and 12. In the first experiment, a 30% decrease in the feed was necessary to keep the reactor working in aerobic conditions. In the second experiment, the temperature was instead lowered and it was never necessary to decrease the feed to control the dissolved oxygen.

8. Summary

A fermentation technique for bioreactors operating close to their maximum oxygen transfer capacity has been designed and analysed. It combines the advantages of probing control and of the temperature limited fed-batch technique. By performing down-pulses in the feed rate, a controlled excess in glucose can be achieved and starvation is thereby avoided. The analysis of the glucose control loop demonstrates the feasibility of the strategy: in spite of the regular operation above the critical glucose concentration, acetate does not accumulate. The analysis points also out the

necessity of an efficient dissolved oxygen control, i.e. of a short control phase. This is achieved by manipulating temperature and agitation in a mid-ranging configuration. Tuning guidelines are derived from the analysis to facilitate the design of the mid-ranging controller. The efficiency of the cultivation technique was demonstrated by simulations and an experiment.

9. Acknowledgement

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A. Reactor model with temperature dependence

A.1 Metabolic rates

The temperature influence on all maximal specific uptake rates is modelled by a multiplicative factor $f(T)$ defined by

$$f(T) = e^{-50(\frac{1}{T}-\frac{1}{37})} \tag{21}$$

The maximal uptake rates $q_a^{c,max}(T)$, $q_g^{max}(T)$, $q_o^{max}(T)$ and the maintenance coefficient $q_{mc}(T)$ can thus be written as

$$q_a^{c,max}(T) = q_{a,37}^{c,max} f(T) \tag{22}$$
$$q_g^{max}(T) = q_{g,37}^{max} f(T) \tag{23}$$
$$q_o^{max}(T) = q_{o,37}^{max} f(T) \tag{24}$$
$$q_{mc}(T) = q_{mc,37} f(T) \tag{25}$$

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

$$q_a^{c,pot}(A,T) = q_a^{c,max}(T)\frac{A}{k_a + A} \tag{26}$$
$$q_g(G,T) = q_g^{max}(T)\frac{G}{k_s + G} \tag{27}$$

Part of the glucose is used for maintenance:

$$q_m(T) = \min(q_g(G,T), q_{mc}(T)) \tag{28}$$

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

$$q_a = q_a^p - q_a^c \tag{29}$$
$$q_{gg} = q_g - q_m \tag{30}$$

where q_a^p is the production of acetic acid and q_a^c stands for the acetic acid consumption.

Splitting into an oxidative flow and a fermentative flow gives:

$$q_{gg}^{ox} = \min((q_o^{max} - q_m Y_{om})/Y_{og}, q_{gg}) \tag{31}$$
$$q_{gg}^{fe} = q_{gg} - q_{gg}^{ox} \tag{32}$$

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

$$q_a^p = q_{gg}^{fe} Y_{ag} \tag{33}$$

$$q_a^c = \min(q_a^{c,pot}, (q_o^{max} - q_{gg}^{ox} Y_{og} - q_m Y_{om})/Y_{oa}) \tag{34}$$

$$\mu = q_{gg}^{ox} Y_{xg}^{ox} + q_{gg}^{fe} Y_{xg}^{fe} + q_{ac} Y_{xa} \tag{35}$$

$$q_o = q_{gg}^{ox} Y_{og} + q_m Y_{om} + q_{ac} Y_{oa} \tag{36}$$

q_o^{max} and q_g^{crit} are related through the following equation

$$q_o^{max} = (q_g^{crit} - q_m) Y_{og} + q_m Y_{om} \tag{37}$$

A consequence of the model assumptions is that the saturation in the oxygen uptake rate occurs for the same values of G and A independently of T.

A.2 Linearized model

Acetate dynamics. Acetate is produced when the glucose uptake rate exceeds a critical value q_g^{crit}, corresponding to the maximal respiratory capacity of the cells. The rate of acetate production is proportional to the glucose excess:

$$q_a^p = Y_{ag}(q_g - q_g^{crit} - q_m), \quad q_g - q_m > q_g^{crit} \tag{38}$$

If acetate is present in the media, it may also be consumed. The consumption requires oxygen and it is therefore limited by the available oxidative capacity of the cells:

$$q_o = \min(q_o^{max}, Y_{og}(q_g - q_m) + Y_{om} q_m + Y_{oa} q_a^c) \tag{39}$$

where the consumption rate q_a^c follows a Monod-type law, see (26). For low acetate concentrations we can make the linear approximation

$$q_a^c \approx \frac{A}{\tau}, \quad \text{with } \tau = \frac{k_a}{q_a^{c,max}} \tag{40}$$

The different regimes for the acetate dynamics can finally be approximated by

$$\frac{dA}{dt} = \begin{cases} -\dfrac{A}{\tau} X, & \text{if } Y_{oa}\dfrac{A}{\tau} + Y_{og}(q_g - q_m) + Y_{om} q_m < q_o^{max} \\ Y_{ag}(q_g - q_g^{crit}) X, & \text{if } Y_{og}(q_g - q_m) + Y_{om} q_m > q_o^{max} \\ -\dfrac{1}{Y_{oa}}(q_o^{max} - Y_{og} q_g) X, & \text{otherwise} \end{cases} \tag{41}$$

Oxygen dynamics. During short periods of time, the volume V and the biomass X are approximately constant. Assuming that glucose is present in excess ($q_o = q_o^{max}$), the dissolved oxygen dynamics is decoupled from the glucose one and it can be approximated by

$$T_o \frac{d\Delta O}{dt} + \Delta O = K_T \Delta T + K_N \Delta N + d \tag{42}$$

where

$$\begin{aligned} K_T &= \frac{\partial q_o}{\partial T} HX (K_L a)^{-1} \qquad & T_o &= (K_L a)^{-1} \\ K_N &= \frac{\partial K_L a}{\partial N} (O^* - O)(K_L a)^{-1} \end{aligned} \tag{43}$$

The signal d models all disturbances acting on the oxygen concentration. When d represents the influence of cell growth, it can be written as:

$$d = K_x X \qquad K_x = q_o H (K_L a)^{-1} \tag{44}$$

B. Temperature- versus feed-based DO control

The growth uptake q_{gg} in (30) is the amount of glucose per cell and per unit of time, that is not used for maintenance purposes. Starvation can be characterized by a low q_{gg} value. A constant dissolved oxygen concentration requires from (4) that

$$0 = OTR - q_o(T, q_g) HX \tag{45}$$

From (30), (36) and (45) it follows that

$$q_{gg} = \frac{OTR^{max}}{HXY_{og}} - \frac{Y_{og}^m}{Y_{og}} q_m(T)$$

As

$$q_m(T) = q_{m,37} f(T)$$

and from (21) we know that $f(T) < 1$ for $T < 37$, we can conclude that the growth uptake q_{gg} is higher at low temperature. This implies that the temperature-based DO control is preferable when the maximum oxygen transfer of the reactor is reached.

C. Steady state with down-pulses

Integration of the acetate dynamics in the regions Ω_1 and Ω_3 leads to

$$A(T_c) = A(0) + Y_{ag}(q_g - q_g^{crit} - q_m)T_c X \tag{46}$$

$$A(T) = A(T_c)e^{-\frac{T_{pulse}X}{\tau}} \tag{47}$$

$$= (A(0) + Y_{ag}(q_g - q_g^{crit} - q_m)T_c X)e^{-\frac{T_{pulse}X}{\tau}}$$

The specific oxygen uptake rate at $t = T_c$ and $t = T$ is given by

$$q_o(T_c) = q_o^{max} \tag{48}$$

$$q_o(T) = Y_{og}(q_g - q_g^p - q_m) + \frac{Y_{oa}}{\tau}A(T) + Y_{om}q_m \tag{49}$$

The change in the specific glucose uptake rate after the pulse becomes

$$q_g(T) = q_g(0) + \kappa K_g(K_o(q_o(T) - q_o(T_c)) - y_r) \tag{50}$$

The condition

$$q_g(T) = q_g(0) \tag{51}$$

$$A(T_c) = A(0) \tag{52}$$

can be written in matrix form

$$\begin{bmatrix} q_g(T) \\ A(T) \end{bmatrix} = A_e \begin{bmatrix} q_g(0) \\ A(0) \end{bmatrix} + a_e \tag{53}$$

where A_e is given by:

$$A_e = \begin{bmatrix} 1 - \kappa K(Y_{og} + Y_{oa}Y_{ag}\frac{T_c X}{\tau}e^{-\frac{XT_{pulse}}{\tau}}) & -\frac{\kappa}{\tau}Y_{oa}Ke^{-\frac{XT_{pulse}}{\tau}} \\ XT_cY_{ag}e^{-\frac{XT_{pulse}}{\tau}} & e^{-\frac{XT_{pulse}}{\tau}} \end{bmatrix} \tag{54}$$

Jury's stability test can be applied to get a condition for A_e to be stable. This leads to equations (15) and (16).

D. Operating point and numerical values

The operating point that is chosen for the analysis is

$$OTR = OTR^{ref} = K_L a(N_{ref})(O^* - O_{ref}) \tag{55}$$

$$q_o = q_o^{max} \tag{56}$$

with $K_L a = \alpha(N - N_0)$. The cell density X becomes

$$X = \frac{OTR^{ref}}{q_o^{max} H} \tag{57}$$

The gains K_o and K_g defined in (8) are given by

$$K_o = \frac{HX}{K_L a} \approx \frac{O^* - O_{ref}}{q_o^{max}} \tag{58}$$

$$K_g = \frac{G_i n}{VX} = \frac{G_{in} q_o^{max} H}{OTR^{ref} V} \tag{59}$$

The approximation is based on the stationary equations (1). The numerical values used in the simulations and in the analysis are given in Table 3.

Table 1. Variables in the model

Symbol	Description
G	glucose concentration
A	acetic acid concentration
X	cell mass concentration
C_o	dissolved oxygen concentration
V	reactor volume
F	glucose feed rate
N	stirrer speed
T	temperature
O	dissolved oxygen concentration in %
q_g	specific glucose uptake rate
q_m	glucose used in maintenance
q_a	specific acetic acid uptake
q_o	specific oxygen consumption rate
μ	cell growth rate
$K_L a$	volumetric oxygen transfer coefficient

Table 2. Parameters of the probing feeding controller (PFC) and the mid-ranging controller (C_1 and C_2) together with their numerical values.

controller	Symbol	Value	Unit	Description
PFC	O_{pulse}	-	-	size of pulse response, input
PFC	y_r	1.5	%	desired pulse response
PFC	κ	1.4	$\cdot 10^{-3}$ l/(h%)	gain
PFC	T_c	6	min	control phase duration
PFC	T_{pulse}	1.5	min	pulse duration
PFC	T	7.5	min	pulse+control phase duration
PFC	O_{reac}	1	%	detection of pulse response
PFC, C_2	N_{ref}	1000	rpm	ref. value stirrer speed, N
PFC	N_{max}	1100	rpm	max. stirrer speed, N
C_1	K	18	rpm/%	gain
C_1	T_i	40	s	integral time
C_1	O_{ref}	30	%	dissolved oxygen ref. value
C_2	k_i	0.3	^{o}C/(rpm s)	integral gain

Table 3. Numerical values of corresponding to a laboratory-scale bioreactor with *E. coli*

Symbol	Value	Unit	Description
G_{in}	500	g/l	glucose conc. in feed
H	14000	(l%)/g	Henrys const.
O^*	100	%	oxygen sat. constant
C_o^*	$100/H$	g/l	oxygen sat. constant
k_s	0.01	g/l	sat. const. for gluc. uptake
k_a	0.05	g/l	sat. const. for acet. uptake
α	3	$(\text{h rpm})^{-1}$	oxygen transf. const.
N_0	289	rpm	oxygen transf. const.
$q_{a,37}^{c,max}$	0.2	g/gh	max. spec. acet. uptake
$q_{g,37}^{max}$	1.5	g/gh	max. spec. glucose uptake
$q_{g,37}^{crit}$	1.25	g/gh	crit. glucose uptake
$q_{mc,37}$	0.15	g/gh	maintenance coefficient
$q_{o,37}^{max}$	0.66	g/gh	max. spec. oxygen uptake
Y_{ag}	0.55	g/g	acetate/glucose yield
Y_{oa}	0.55	g/g	oxyg./acet. yield
Y_{og}	0.50	g/g	oxyg./gluc. yield for growth
Y_{om}	1.07	g/g	oxyg./gluc. yield for maint.
Y_{xa}	0.4	g/g	biomass/acet. yield
Y_{xg}^{ox}	0.51	g/g	oxidative biomass/gluc.
Y_{xg}^{fe}	0.15	g/g	fermentative biomass/gluc.
V	2	l	reactor volume
T_p	30	s	oxyg. probe time cst
T_t	4	min	time cst of temp. dyn.
O_{ref}	30	%	ref. value for DO

V

Paper V

Feeding Strategies for *E. coli* Fermentations Demanding an Enriched Environment

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Abstract

The addition of a carbon nutrient feed to a fed-batch cultivation is often not enough to obtain satisfactory growth and/or production. In some cases, an additional feed with for example supplementary amino acids or complex media is required. This work presents the development of feeding strategies where more than one feed is required and the process knowledge is low. Simulations and cultivations with *E. coli* using the proposed feed controllers are shown and the strategies work well. The feeding strategies can be used to shorten the process development phase considerably.

Key words Fermentation process, feeding strategy, fed-batch cultivation, *Escherichia coli*, complex medium, amino acid.

Submitted.

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1. Introduction

Today many proteins are produced by genetically modified microorganisms. One of the host organisms used is the bacterium *Escherichia coli*. To achieve a good productivity, high cell concentration and high cell productivity are desired and this is usually obtained from fed-batch cultivations. Much work is done on how to determine the addition of the growth-limiting carbon, often glucose, [Riesenberg and Guthke, 1999], [Lee, 1996]. This is important as underfeeding will lead to some productivity loss and starvation. Overfeeding leads to carbon nutrient accumulation or by-product formation, as acetate. Acetate production reduces growth and recombinant protein production, [Luli and Strohl, 1990].

Sometimes it is not enough to add a carbon nutrient feed in order to obtain a satisfactory growth and/or production, e. g. due to auxotrophic production strains. In some cases additions of supplementary amino acids or complex media (containing for example yeast extract) are needed to enhance productivity and/or growth rates, [Panda *et al.*, 2000], [Zawada and Swartz, 2005], [Yoon *et al.*, 1994]. Also, adding yeast extract is shown to help in reducing the acetic acid formation and its inhibitory effect, [Panda *et al.*, 2000], [Han *et al.*, 1992], [Koh *et al.*, 1992].

There are some drawbacks when adding all of the needed supplements from the beginning of the cultivation, [Yamane and Shimizu, 1984]. Examples of these are:

- The supplement can be used for other purposes than intended, i. e. by-product formation.
- The composition of for example yeast extract differs from lot to lot [Iding *et al.*, 2001], i. e. different amounts should be added to each cultivation in order to allow for reproducible operation.
- If the process knowledge is low, it is difficult to know in advance exactly how much to add of the needed supplement.
- A high concentration of the needed supplement might be inhibitory.

Furthermore the need of the supplement might change during the course of the cultivation, e. g. in the transition from growth to production. Therefore the focus of this paper is on how to feed this supplementary substance when there is almost no knowledge available of the process.

Studies have been done where the needed supplement is added proportionally to the carbon nutrient feed [Zawada and Swartz, 2005], [Johnston *et al.*, 2003], [Whiffin *et al.*, 2004], [Panda *et al.*, 2000]. In order to handle disturbances in cultivation conditions, uncertainties in yield coefficients and changes in the requirements, the feeding strategy should include feedback to adjust the relation between the two feeds.

In [Åkesson *et al.*, 2001a], a feeding strategy is presented where the feed used consists mainly of glucose and trace elements. By superimposing

short pulses to the substrate feed, on-line detection of acetate formation can be made using the dissolved oxygen sensor. A feedback algorithm is used to adjust the feed rate to avoid overflow metabolism and thereby acetate formation while maintaining a high growth rate. Here the pulsing technique is used to control two feeds simultaneously: The feed of known amino acids or complex media are adjusted by a feedback algorithm using the responses of the dissolved oxygen to superimposed pulses, while the carbon nutrient feed follows a predetermined profile. The presented feeding strategies work well, and as almost no process knowledge is required they can be used to shorten the process development phase considerably. To illustrate the feeding strategies simulations and experiments are shown.

2. Materials and method

Two different *E. coli* strains, DSM1099 and DSM6968, were cultivated. *E. coli* strain DSM1099 demands the amino acid lysine for growth (lysine auxotroph). When lysine is lacking diamino pimelic acid (DAP) is formed. *E. coli* strain DSM6968 demands the amino acids leucine and threonine for growth. The cultivations were performed in an 18 liter Belach bioreactor with a 7 liter working volume equipped with the control and logging system Phantom. Inoculum (2 ml) was incubated around 8 h at 37^o C in 100 ml medium, containing (per liter) 5 g NH_4Cl, 0.8 g K_2SO_4, 3 g KH_2PO_4, 6 g Na_2HPO_4, 5.6 g Tri-Na-Citrate$\cdot 2H_2O$, 20 mg $CaCl_2\cdot 2H_2O$, 0.8 g $MgCl_2\cdot 6H_2O$, 60 mg $CoCl_2\cdot 6H_2O$, 0.1 g $FeCl_3\cdot 6H_2O$, 0.25 g Thiamin-HCl, 0.14 g L-Lysine (experiments 1 and 2) and 4.6 g yeast extract (experiment 3), 5.3 g Glucose$\cdot H_2O$ and 1 ml trace element solution containing (per liter) 0.65 g $(NH_4)_6Mo_7O_{24}$ cot$4H_2O$, 1.38 g $CuSO_4\cdot 5H_2O$, 5.4 g $MnCl_2\cdot 4H_2O$, 6.6 g $ZnSO_4\cdot 7H_2O$, 1.42 g $CoCl_2\cdot 6H_2O$. The medium in the reactor contained (per liter) 4.2 g H_3PO_4, 3.6 g H_2SO_4, 1 g KOH, 2 g NaOH, 0.5 g $MgSO_4\cdot 7H_2O$, 3.5 g NH_4SO_4, 0.4 g K_2SO_4, 20 mg $FeSO_4\cdot 7H_2O$, 22 g (experiments 1 and 2) 7.5 g (experiment 3) Glucose$\cdot H_2O$, 6 ml trace elements solution, 77 mg citric acid$\cdot H_2O$, 10 mg Thiamin-HCl, 0.05 g Struktol, 0.6 g L-Lysine (experiments 1 and 2) and altogether during the batch-phase 22.5 g yeast extract (experiment 3). The pH was regulated by a 25 % NH_3 solution and a 2 M H_2SO_4 solution. The aeration was controlled to 7 L/min, unless otherwise stated, the temperature to 37 ^{o}C, pH to 7 and the reactor pressure to 1.1 bar.

E. coli DSM1099 Two cultivations were done with this strain and experiment 1 was a batch cultivation. Experiment 2 was a fed-batch cultivation where the glucose feed consisted of (per liter) 660 g Glucose$\cdot H_2O$ and

17.14 g $MgSO_4 \cdot 7H_2O$. The glucose feed was started when the dissolved oxygen increased abruptly, indicating the depletion of the initial glucose. The lysine feed consisted of 80 g/Liter L-Lysine. It was started around 30 min after the feed-start of the glucose feed. The starting values of the glucose feed and the lysine feed were chosen to 0.064 L/h and 0.008 L/h, respectively. For the feeding strategy, see section 4.

E. coli DSM6968 Experiment 3 was a fed-batch cultivation where the glucose feed was started when the dissolved oxygen increased abruptly, indicating the depletion of the initial glucose. It consisted of (per liter) 660 g Glucose$\cdot H_2O$ and 17.14 g $MgSO_4 \cdot 7H_2O$. The complex feed, consisting of 200 g yeast extract/liter, was started when there was no visible pulse response after a pulse superimposed on the glucose feed, indicating the depletion of the initial complex medium. During the batch-phase approximately 3 times as much complex medium as glucose was added in order to consume the glucose. This information was used in the calculation of the starting values of the feeds. The starting values were chosen to, 0.022 L/h and 0.19 L/h, respectively. For the feeding strategy, see section 5. The dissolved oxygen controller and feed controller were implemented and regulated from a computer using Matlab.

Dissolved oxygen control The control of the dissolved oxygen is important when using the probing feeding strategy, [Åkesson *et al.*, 2001b]. Here a PI controller manipulating the stirrer speed was used and it kept the dissolved oxygen at 20 % (experiments 1 and 2) and at 30 % (experiment 3). During the fed-batch experiments the stirrer speed was frozen during the pulses to facilitate the interpretation of the pulse response in the dissolved oxygen tension.

Analysis Analyses of glucose and acetate were performed simultaneously on a HPLC (Agilent 1100) using 2 AMINEX-87H column in series. As a mobile phase 80 mM H_2SO_4 was used. Glucose was detected using refractive index and acetate using a UV260 nm sensor. Cell dry weight (CDW) determination was conducted as follows. 10 g of mesh was spun down at 2600 g for 10 minutes, the supernatant was decanted. The pellet was washed once and was left to dry for 24 h at 105 ^oC.

3. Problem formulation

During a cultivation there are not many on-line measurements available, see figure 1. The dissolved oxygen, pH and temperature are the variables that are normally available. The product, the cell mass, the limiting

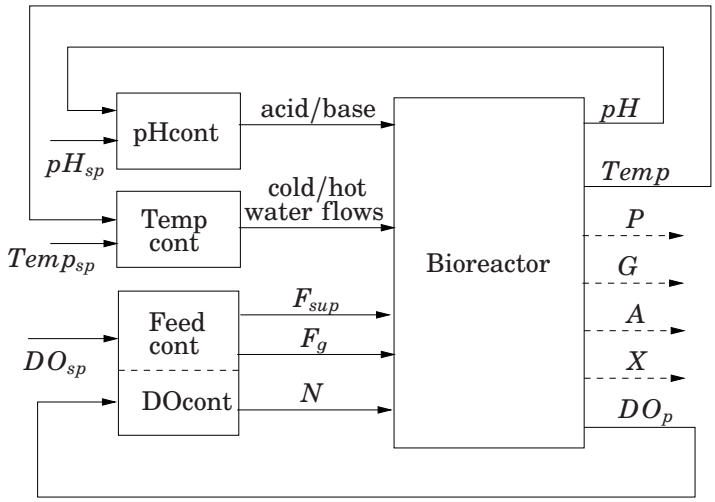

Figure 1. A block diagram of the process. The variables are: pH, temperature $Temp$, dissolved oxygen DO_p, product concentration P (off-line), acetic acid concentration A (off-line), cell mass concentration X (off-line), glucose concentration G (off-line), glucose feed F_g, feed with the needed supplement F_{sup} and stirrer speed N, cont = controller, sp = set-point.

carbon nutrient (here glucose), the by-product (here acetic acid) concentrations are usually analysed off-line. In this paper two different feed controllers are developed, which both make use of the dissolved oxygen signal to adjust the feed with the needed supplement F_{sup}. In both the feeding strategies a predetermined exponential profile is applied to the glucose feed F_g. In feeding strategy 1, where the needed supplement is an amino acid, F_{sup} is regulated by the responses in the dissolved oxygen to pulses superimposed on F_{sup}. In feeding strategy 2, where complex medium is the added supplement, F_{sup} is regulated by the responses in the dissolved oxygen to pulses superimposed on F_g. The reason for two different feeding strategies is that the bacteria readily use complex medium also for energy purposes while several amino acids are mostly incorporated in the bacterial proteins, [Gschaedler and Boudrant, 1994]. For an efficient growth the complex components or added amino acids should be used primarily as building blocks and the glucose should be used as energy source.

4. Feeding strategy 1

The amino acid that is needed for growth and/or production must be known in advance in order to use feeding strategy 1. In our case it is the amino acid lysine. The amount of lysine added should make it possible for the cell growth to follow the profile assumed in the glucose feed. Inhibition of the glucose uptake rate is assumed to take place when the concentration of lysine is low. In order to illustrate and develop the ideas behind the feeding strategy, a model is used, see appendix A.1.

4.1 Simulations

In figure 2 a simulation of the model of *E. coli* DSM1099 with an exponential glucose feed corresponding to $\mu = 0.15\ \mathrm{h}^{-1}$ and with superimposed pulses is shown. The lysine feed is chosen to be the simplest possible i. e. constant (dashed). In the beginning there is too much lysine present and it is the amount of glucose that limits the cultivation. Later on it is the amount of lysine that is limiting and glucose is accumulating. Note that there are no responses in the dissolved oxygen tension to superimposed pulses on the glucose feed during this period.

In figure 2 a simulation where the lysine feed is chosen to be proportional to the glucose feed is also shown (solid). This works fine if the exact amount that should be added is known, but this amount could change during the different regimes of the cultivation. In the simulation an error of 1 % in the yield coefficient is introduced and this leads to an accumulation of glucose (0.2 g/L after 4.5 hours).

Strategy 1 When the missing substance is known in advance, the following strategy is proposed: the glucose feed follows an exponential profile during the growth-phase until the maximum oxygen transfer capacity is met with superimposed pulses for detection of glucose accumulation. The lysine feed is regulated by the responses in the dissolved oxygen to the superimposed pulses on the lysine feed. If there is a response in the dissolved oxygen the lysine feed is increased by the size of the pulse. If there is no response, the lysine feed is not changed. The lysine feed F_{lys} is thus adjusted as follows:

$$\Delta F_{lys} = \begin{cases} \gamma_p F_{lys} & \text{if } \Delta DO > DO_{reac} \\ 0 & \text{otherwise} \end{cases} \tag{1}$$

Here γ_p is the pulse height and DO_{reac} is the detection limit for a pulse response, ΔDO.

The idea is that the cells consume oxygen when they use and take up the lysine added in the pulse. If there is already enough lysine, the cells do not increase their oxygen demand. Therefore the lysine feed is only increased if there is a response in the dissolved oxygen tension. In figure 3 a simulation with the proposed strategy is shown.

Choice of controller parameters The controller is given in equation 1. When this type of on/off controller is chosen it is difficult to avoid oscillations in the glucose concentration, see figure 3. The pulse height γ_p and the pulsing frequency are chosen so that almost every other pulse superimposed on the lysine feed should give a response in the dissolved oxygen tension. In order to choose this, an approximate value of the yield coefficient for lysine is needed. The estimate of the yield coefficient does not have to be very accurate since the controller will compensate. It can be obtained from a batch experiment, as in this paper. The time between the pulses also depends on the dissolved oxygen controller and should be chosen so that the dissolved oxygen tension is well back at its set-point. If it is chosen too long it is difficult to follow the exponential growth. Here $\gamma_p = 13$ % and $DO_{reac} = 1$. The duration between the lysine pulses is chosen to 24 min and the pulse length is chosen to around 2 min.

4.2 Experimental verification

Experiment 1 is a batch cultivation in order to find an approximate yield coefficient for lysine, $Y_{x/lys}$. It is estimated to 17 g cells/g lysine. Experiment 2 is a fed-batch experiment. An exponential glucose feed corresponding to $\mu = 0.15$ h^{-1} with superimposed pulses as well as a lysine feed regulated according to strategy 1 are added, see figure 4 and figure 5.

Feed control There are responses in the dissolved oxygen to almost every other pulse superimposed on the lysine feed, just as predicted. The control leads to a cell growth following the profile corresponding to the glucose feed, $\mu = 0.15$ h^{-1}, denoted solid in figure 4. Also base is added during the cultivation indicating that lysine is not used as a carbon source, i. e. overfed. If lysine is used as an energy source ammonia is released and therefore the need for adding base is low. Too little lysine would give an accumulation of glucose and pulse responses to pulses superimposed on the glucose feed would not be visible. Both the pulse responses and the off-line glucose analysis show that this is not the case. Thus, enough lysine is added to support the growth. The yield coefficient, estimated from the fed-batch experiment is close to the estimation done during the batch experiment: around 16.5 and 17 g cells/g lysine, respectively. This also indicates that the feed control gives a good lysine feed rate. The

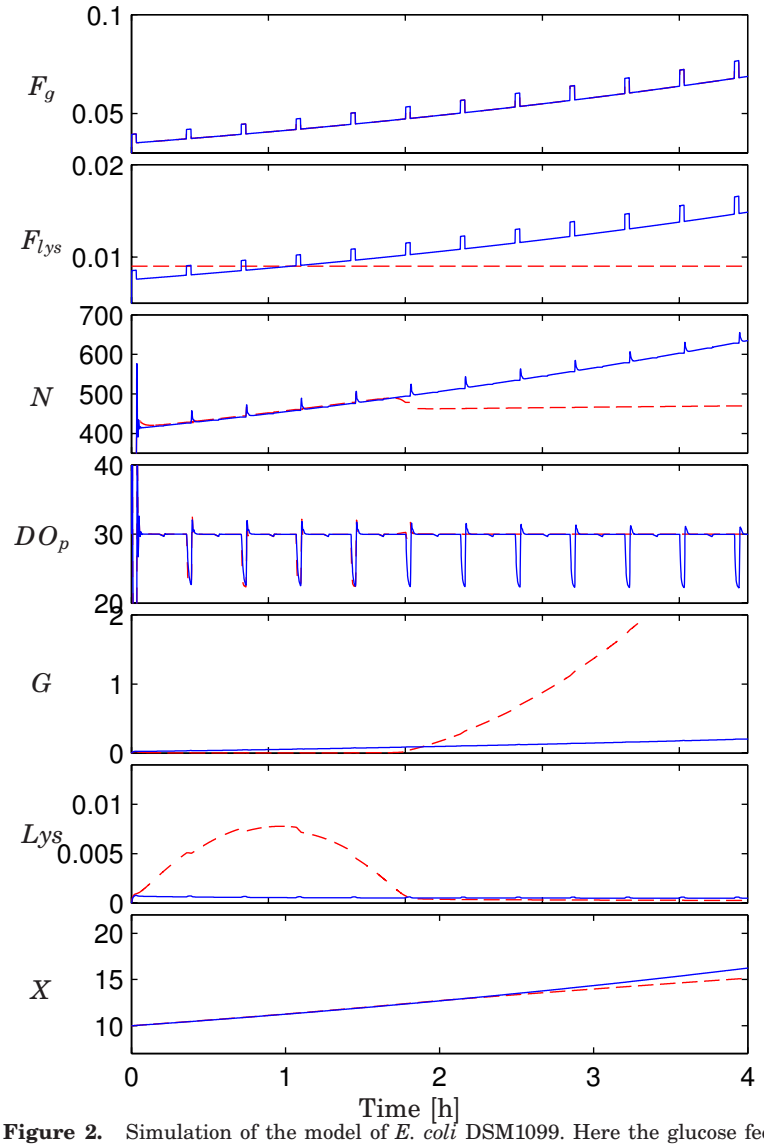

Figure 2. Simulation of the model of *E. coli* DSM1099. Here the glucose feed is exponential, corresponding to a $\mu = 0.15$ h^{-1}. In the simulation (denoted dashed), where the lysine feed is chosen to be constant, glucose is accumulating towards the end. In the simulation (denoted solid), the lysine feed is chosen to be proportional to the glucose feed. An error of 1 % in the yield coefficient is introduced leading to an accumulation of glucose 0.2 g/L after 4.5 hours. From the top: F_g [L/h] glucose feed, F_{lys} [L/h] lysine feed, N [RPM] stirrer speed, DO_p [%] dissolved oxygen, G [g/L] glucose concentration, Lys [g/L] lysine concentration, X [g/L] cell mass.

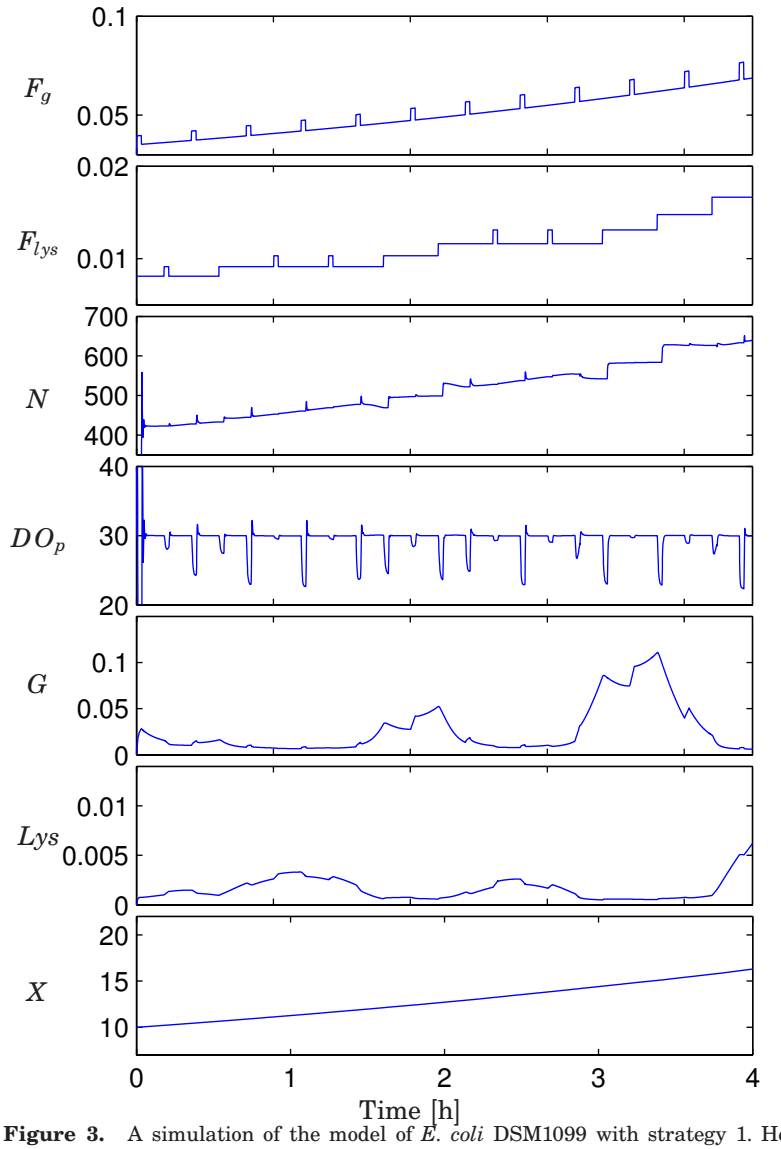

Figure 3. A simulation of the model of *E. coli* DSM1099 with strategy 1. Here the glucose feed is chosen to be exponential corresponding to a $\mu = 0.15$ h^{-1} and the lysine feed is regulated by the responses in the dissolved oxygen tension to the superimposed pulses on the lysine feed. From the top: F_g [L/h] glucose feed, F_{lys} [L/h] lysine feed, N [RPM] stirrer speed, DO_p [%] dissolved oxygen, G [g/L] glucose concentration, Lys [g/L] lysine concentration, X [g/L] cell mass.

controller parameters are given above. One factor that might contribute to the success of the feeding strategy is that lysine is not readily used as an energy source by the bacteria, [Gschaedler and Boudrant, 1994].

Dissolved oxygen control During the first part the DO-stirrer controller is tuned, thereafter the pulse responses are clearly visible, see figure 5.

Model vs experimental data When comparing the model of *E. coli* DSM1099 to the experimental data, it seems as if the model captures the behaviour well. There are pulse responses to almost every other pulse superimposed on the lysine feed, indicating that the estimation of the yield coefficient is accurate. The oscillations in the glucose concentration, shown in the model are not confirmed by the experimental data, as the offline glucose measurements are collected too seldom.

5. Feeding strategy 2

When using strategy 2 it is assumed that the substance or substances needed for growth and/or production are not known and therefore a complex medium is added. Here the glucose fed should be used as the energy-source and the complex medium fed should be used as building blocks in the growth and/or production, [Ingraham *et al.*, 1983]. In order to illustrate and develop the ideas behind the feeding strategy, a model is used, see appendix A.2.

5.1 Simulations

A simulation of the model of *E. coli* DSM6968 is shown in figure 6. An exponential glucose feed is chosen corresponding to a $\mu = 0.2\ \mathrm{h}^{-1}$ and pulses are superimposed on it. The feed of complex medium is constant. This leads to an accumulation of complex compounds and later to a lack thereof.

Strategy 2 When the substance or substances missing for enhanced growth are not known, the following strategy is proposed: an exponential glucose feed during the growth phase and a complex feed which is regulated by the responses in the dissolved oxygen to pulses superimposed on the glucose feed. If the response from a pulse is larger than a reference value y_r, the complex feed is decreased. If the response is smaller than y_r, the complex feed is increased. Thus the feed containing complex medium

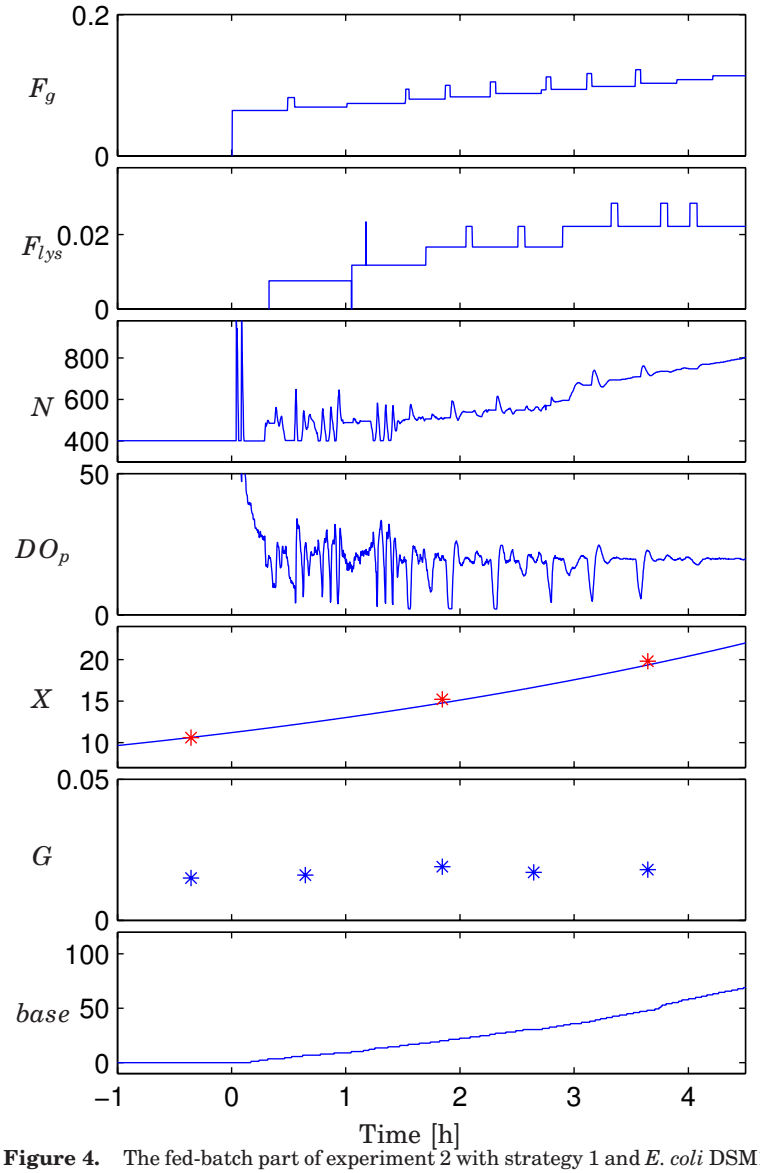

Figure 4. The fed-batch part of experiment 2 with strategy 1 and *E. coli* DSM1099. The glucose feed is chosen to be exponential, corresponding to a $\mu = 0.15$ h^{-1} and with superimposed pulses. From the top: F_g [L/h] glucose feed, F_{lys} [L/h] lysine feed, N [RPM] stirrer speed, DO_p [%] dissolved oxygen, X [g/L] cell mass, G [g/L] glucose concentration, *base* [g] added base.

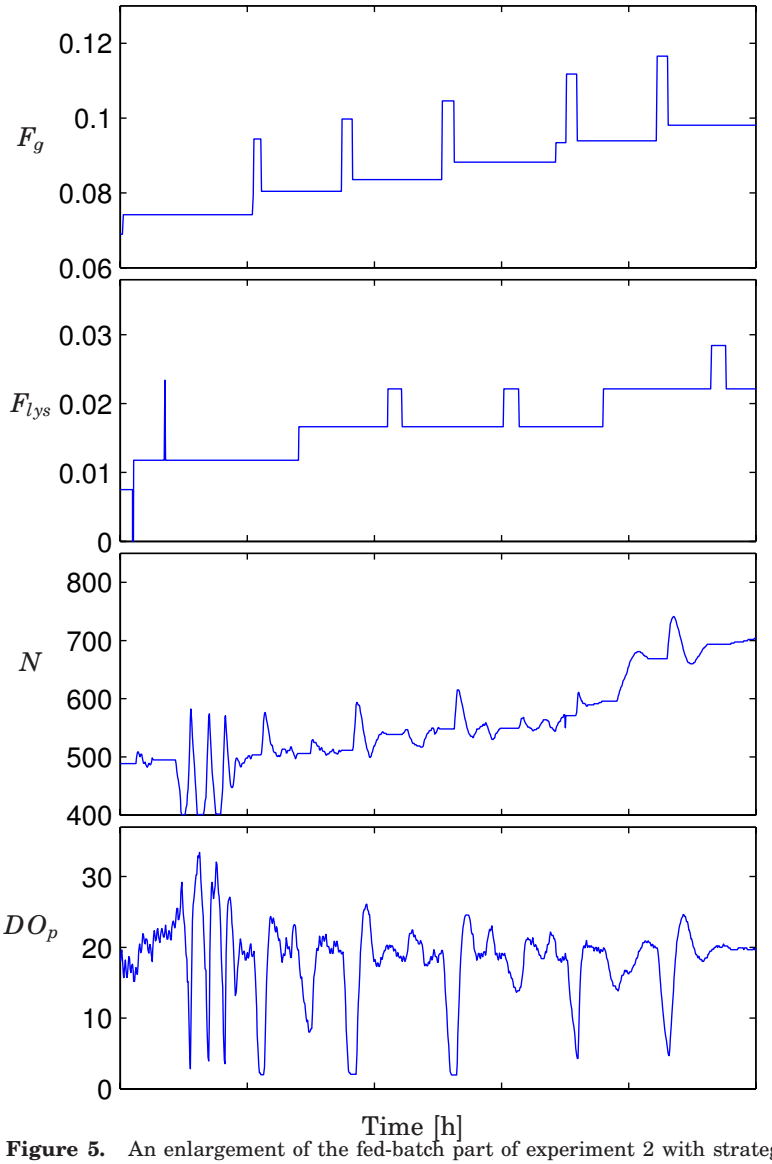

Figure 5. An enlargement of the fed-batch part of experiment 2 with strategy 1 and *E. coli* DSM1099. The glucose feed is chosen to be exponential, corresponding to a $\mu = 0.15$ h^{-1} and with superimposed pulses. From the top: F_g [L/h] glucose feed, F_{lys} [L/h] lysine feed, N [RPM] stirrer speed, DO_p [%] dissolved oxygen.

F_c is adjusted as:

$$\Delta F_c = \frac{\kappa(y_r - \Delta DO)}{DO^* - DO_{sp}} F_c \tag{2}$$

where κ is the controller gain and y_r is the desired pulse response. ΔDO, DO_{sp} and DO^* are the pulse response, the set-point and the saturated dissolved oxygen concentration, respectively.

The principle behind the strategy is that if the substance is missing, the cells can not grow. Thus the cells can not take up the extra glucose given in the pulse. Therefore, the response in the dissolved oxygen becomes small and the complex feed should increase.

The reason for not using pulses in the complex feed is that it is difficult to interpret the pulse response. A situation with no pulse response would probably be a situation where too much complex medium is added.

In figure 7 a simulation of the proposed strategy is shown. It is assumed that twice as much complex medium as glucose is needed to be fed, i. e. $F_c \approx \frac{2F_g G_{in}}{Comp_{in}}$ (for notation see table 1). The strategy is chosen to be such that a growth of $\mu = 0.2$ h^{-1} is obtained. The resulting growth rate is higher initially as high starting values of the feeds are chosen, but declines towards $\mu = 0.2$ h^{-1}.

Choice of controller parameters The controller is given by equation (2). When choosing the parameters of the controller there are some guidelines: the desired pulse response y_r should be chosen to be smaller than the maximum pulse response (calculated to ≈ 8 in appendix A.3). Here y_r is chosen to 5. If y_r is chosen larger than the maximum pulse response, too much complex medium will be added. If it is chosen too small, it is difficult to add enough complex medium to support the exponential growth. This is also the case if the controller gain κ is chosen very small. If κ is chosen large it will lead to an oscillating complex medium feed. The boundaries of κ to assure a stable system are $0<\kappa<2(1+\eta\beta)$, where β is a function of the yields $Y^*_{o/c}$ and $Y_{o/g}$, (see appendix A.3 and table 1 where also η is described). Here κ is chosen to 1. In appendix it is also shown that the influence of the increasing cell mass helps to avoid overfeeding of the complex feed. The choice of the pulse frequency depends on the dissolved oxygen controller and should be chosen to be such that the dissolved oxygen tension is well back at its set-point when it is time for the next pulse. If the frequency is chosen too low, it will be difficult to add enough complex medium to support the exponential growth. Here the time between the pulses is chosen to 6 min. The pulse length should be chosen so that the dissolved oxygen sensor dynamics and the glucose

dynamics are negligible in comparison. Here the pulse duration is chosen to 3 min.

5.2 Experimental verification

A batch cultivation is made (results not shown) and the principle behind the strategy is fulfilled: i. e. the cells can not take up the extra glucose given in a pulse when there is a lack of the limiting substances. Experiment 3 is a fed-batch experiment shown in figure 8 where pulses are superimposed to an exponential glucose feed corresponding to a $\mu = 0.2$ h^{-1}. The feeding strategy 2 is used together with a safety net, see below, to adjust the complex feed.

Feed control The starting value of the complex feed, calculated in the material and method section, is somewhat too high. The calculation is based on that three times as much complex medium as glucose was needed during the batch phase. During the fed-batch part a factor 2 between the glucose feed and the complex medium feed seems more likely when looking at data in figure 8, (compare to $F_c \approx \frac{2F_g G_{in}}{Comp_{in}}$). One explanation for the changed factor is that during the batch part the complex medium is used also as an energy source and not only as building blocks. The changed factor shows how important it is to use feedback control. The high starting value leads to large pulse responses in the dissolved oxygen. As a result the complex feed is decreased. The pulse responses are then decreasing and therefore the complex feed starts to increase. When looking at the off-line glucose measurements, there is no accumulation and thus enough complex media are fed during the experiment. The feed controller and the model do not take into consideration the complex components that are not used by the bacteria. These complex components might accumulate to inhibitive concentrations, even though this does not seem to be the case here. The feeding strategy focuses on applying the needed substance or substances after the demand of the bacteria. The controller parameters are given above.

The resulting cell growth in this experiment is higher than expected: $\mu = 0.45$ h^{-1} according to CDW measurements (two measurements). This growth rate is further supported by calculations using the carbon dioxide evolution rate CER, (results not shown). If the growth follows the profile assumed in the glucose feed, it should be 0.2 h^{-1}. A cell growth above 0.2 h^{-1} is reported to lead to acetic acid accumulation when complex medium is used [Meyer *et al.*, 1984]. During the fed-batch part of the experiment three samples are analysed for acetic acid. The highest concentration, 5.5 g/L, is reached 1 hour after the feed-start. As the addition of yeast extract reduces the inhibiting effect of acetic acid, [Koh *et al.*, 1992], the

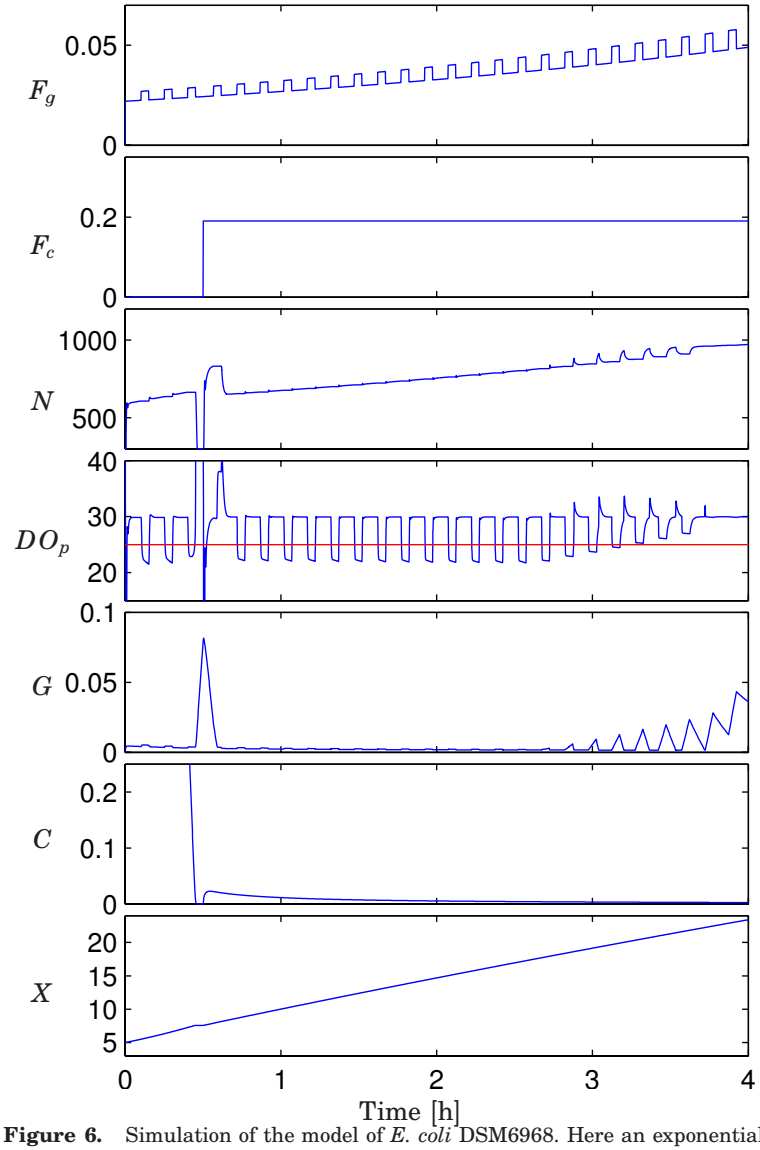

Figure 6. Simulation of the model of *E. coli* DSM6968. Here an exponential glucose feed is chosen corresponding to $\mu = 0.2$ h^{-1}. The complex feed is chosen to be constant which leads to glucose accumulation towards the end of the simulation. From the top: F_g [L/h] glucose feed, F_c [L/h] complex medium feed, N [RPM] stirrer speed, DO_p [%] dissolved oxygen, G [g/L] glucose concentration, C [g/L] complex medium concentration, X [g/L] cell mass.

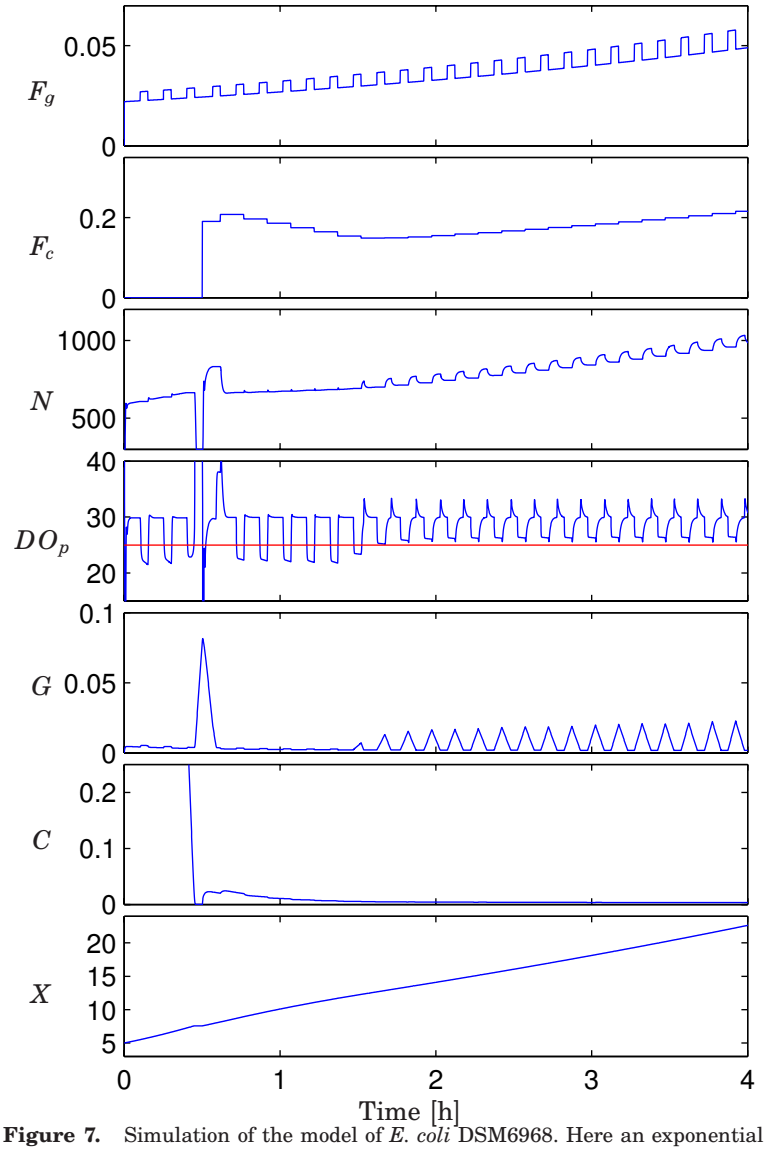

Figure 7. Simulation of the model of *E. coli* DSM6968. Here an exponential glucose feed is chosen corresponding to $\mu = 0.2$ h^{-1}. The complex feed is regulated by the responses in the dissolved oxygen tension to the superimposed pulses on the glucose feed, i. e. strategy 2. Here the glucose accumulation is avoided. High starting values of the glucose feed and the complex medium feed lead to a high cell-growth during the first 1.5 hours. From the top: F_g [L/h] glucose feed, F_c [L/h] complex medium feed, N [RPM] stirrer speed, DO_p [%] dissolved oxygen, G [g/L] glucose concentration, C [g/L] complex medium concentration, X [g/L] cell mass.

accumulated acetic acid has probably not a large effect on the cultivation result.

In the case of overfeeding of the complex medium, the amount of base added for pH control is expected to be low. This is due to the release of ammonia, when a complex nitrogenous source is used for cellular metabolism. In this experiment it is likely that a part of the complex medium is used for energy purposes as even some acid is added during the later part of the cultivation to control the pH. However, there are indications that the two amino acids leucine and threonine, to which the bacterium is auxotroph, are not accumulating during the cultivation (results not shown). In that perspective, too much complex medium is not added and an adequate feed rate is obtained.

Safety net The task of the safety net is to avoid the overfeeding of the complex medium and it makes use of the adjustments in the complex medium indirectly: if the stirrer speed has not increased between two pulses superimposed on the glucose feed after an increase in the complex medium feed, the complex medium feed is decreased. This is the reason for the decrement in the complex feed at 2.2 hours.

6. Future works

6.1 Improvements of the strategies

To improve strategy 1, one can introduce a proportional controller, i. e. the lysine feed is adjusted proportionally to the size of the pulse responses. This would maybe decrease the oscillations seen in the glucose concentration in figure 3. One can also replace the up-pulses with down-pulses. This might lead to a slightly higher lysine concentration in the reactor.

In both strategies one should also change the glucose profile once the maximum stirrer speed is reached in order to avoid oxygen depletion. Another way to prevent oxygen depletion is to lower the temperature, [de Maré *et al.*, 2005]

6.2 Another feeding strategy

Another feeding strategy one might use is to adjust the glucose feed through the original pulse programme and to assume a profile in the complex feed, see the simulation in figure 9. To find the stability boundaries of the controller gain κ the analysis done in appendix A.3 was repeated (keeping q_c constant) leading to $0<\kappa<2(1+\eta\beta)$. The influence of the cell mass helps to avoid overfeeding also in this case.

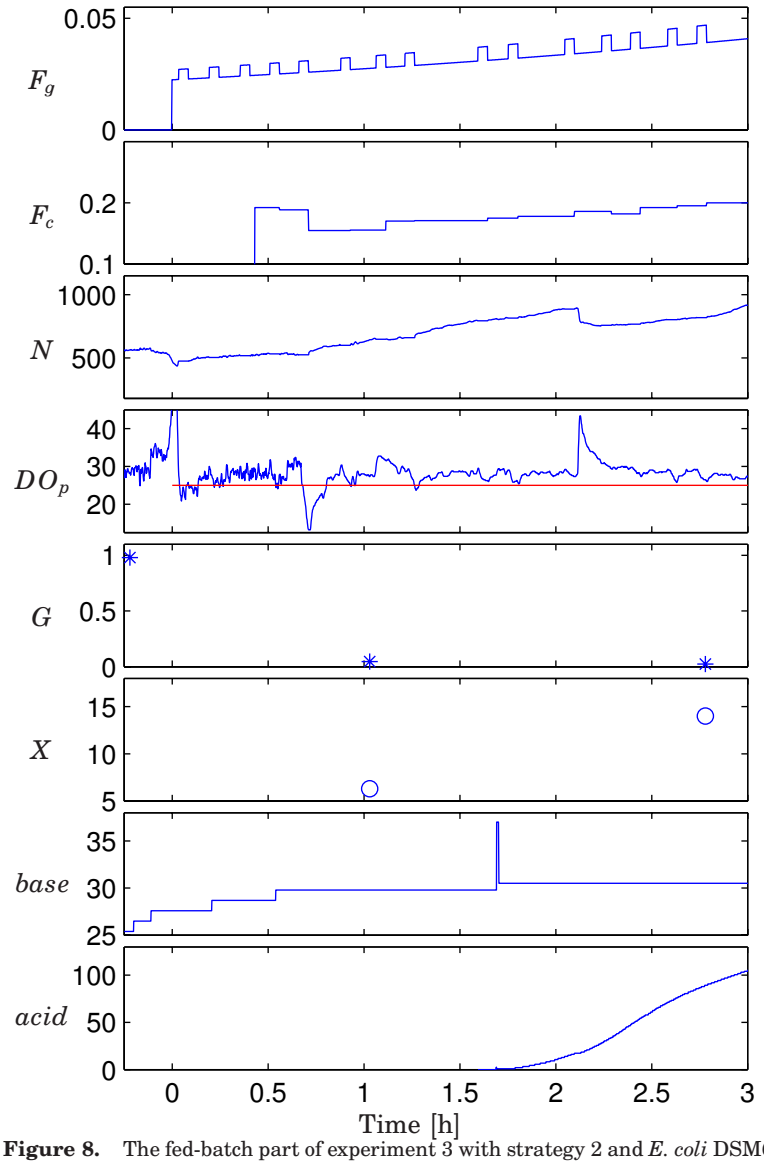

Figure 8. The fed-batch part of experiment 3 with strategy 2 and *E. coli* DSM6968 auxotroph to threonine and leucine. An exponential glucose feed corresponding to a $\mu = 0.2$ h^{-1} is chosen. The peak in the dissolved oxygen tension at 2.1 h is a result of an increase in the airflow. This is done as the CO_2 concentration in the outlet air was high. From the top: F_g [L/h] glucose feed, F_c [L/h] complex medium feed, N [RPM] stirrer speed, DO_p [%] dissolved oxygen, G [g/L] glucose concentration, X [g/L] cell mass, *base* [g] added base, *acid* [g] added acid.

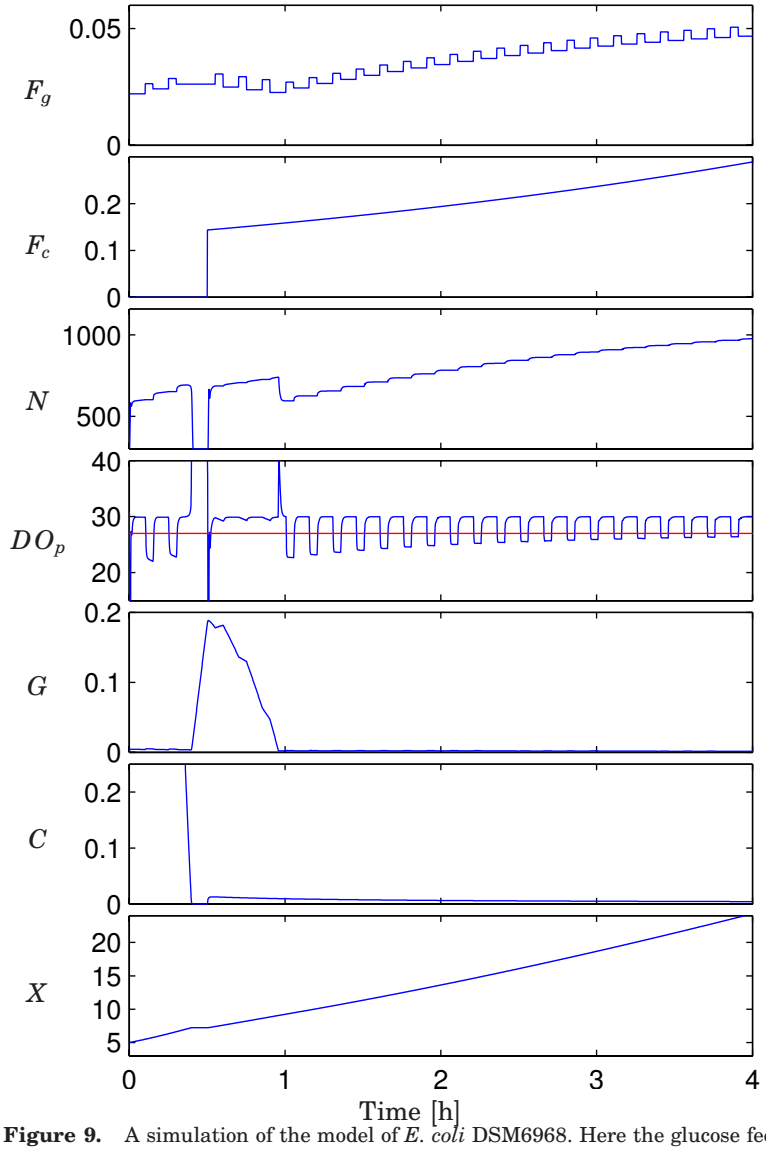

Figure 9. A simulation of the model of *E. coli* DSM6968. Here the glucose feed is adjusted according to the original probing feeding strategy with a y_r chosen to be 3. The complex feed is an exponential one corresponding to a $\mu = 0.2$ h^{-1}. From the top: F_g [L/h] glucose feed, F_c [L/h] complex medium feed, N [RPM] stirrer speed, DO_p [%] dissolved oxygen, G [g/L] glucose concentration, C [g/L] complex medium concentration, X [g/L] cell mass.

7. Conclusions

Sometimes it is not enough to add a glucose feed to a fed-batch cultivation in order to obtain a satisfactory growth and/or production, e. g. due to auxotrophic production strains. Then an additional feed is required with supplementary amino acids or complex medium. How this supplementary substance is to be added in an effective manner is a challenging issue. Here two feeding strategies, based on probing control, are presented and evaluated. In both the strategies the glucose feed rate follows a predetermined exponential profile and pulses are superimposed on it.

When the amino acid that is needed to enhance the growth and/or the production is known the following feeding strategy is proposed for the amino acid feed: the feed is regulated by the responses in the dissolved oxygen to superimposed pulses on the limiting amino acid feed. The strategy was successfully tested in a cultivation where the limiting amino acid was lysine. A cell growth that corresponded to the exponential profile in the glucose feed was obtained and no glucose accumulation took place i. e. there was no lack of lysine.

When the substance needed is not known the following feeding strategy is proposed for the complex medium feed: the feed is regulated by the responses in the dissolved oxygen to the superimposed pulses on the glucose feed. The strategy was tested in a cultivation where the limiting substances were two different amino acids. In our case there are indications that some of the complex medium was used as an energy source. However, there are also indications that the two amino acids, to which the bacterium is auxotroph, are not accumulating during the cultivation. Therefore an adequate feed rate was probably obtained. More studies should be carried out in order to confirm the results, but even so the strategy seems promising.

The methods could allow for two advantages for production of proteins:
- As almost no process knowledge is required in order to apply the feeding strategies, they can be used to shorten the process development phase considerably.
- In regard of the FDA PAT guideline (`http://www.fda.gov/cder/OPS/PAT.htm`) for improved control of a pharmaceutical production process, the methods could allow for improved reproducibility of a production process and possibly of the protein quality as well.

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A. Appendix

Two models are presented. The feeding strategies are designed to operate around a μ which is chosen below the critical growth rate where acetate is accumulating. Therefore the overflow metabolism is not modeled. It can be included by introducing a maximum oxygen uptake rate and letting the glucose react to acetic acid when this threshold is passed. In both models the following relations are used: The relation between K_La and the stirrer speed N is given by:

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

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

$$DO = HC_o$$

The dissolved oxygen sensor dynamics is approximated as:

$$T_p \frac{dDO_p}{dt} + DO_p = DO$$

Mass balances of a fed-batch bio-reactor are given by:

$$\frac{dV}{dt} = F_g + F_{sup} \tag{3}$$

$$\frac{d(VG)}{dt} = F_g G_{in} - q_g(G, sup)VX \tag{4}$$

$$\frac{d(VX)}{dt} = \mu(G, sup)VX \tag{5}$$

$$\frac{d(VC_o)}{dt} = K_L a(N)V(C_o^* - C_o) - q_o(G, sup)VX \tag{6}$$

where sup is the added needed supplement, in our case either lysine or complex medium. For notation and values of the parameters, see table 1.

A.1 Model of *E. coli* DSM1099

The assumptions made in the model are:

- The cells can not take up glucose without lysine.
- No growth takes place on lysine solely.

The mass balance of lysine (Lys) is given by:

$$\frac{d(VLys)}{dt} = F_{lys} Lys_{in} - q_{lys}(G, Lys)VX$$

The other mass balances are given by equations (1)-(4), where the supplement (sup) is lysine. The cells can not take up glucose without lysine, thus $q_g(G, Lys)$ can be described by double Michaelis-Menten:

$$q_g(G, Lys) = \frac{q_g^{max} G Lys}{(k_{sg} + G)(k_{slys} + Lys)}$$

The amount lysine that is consumed together with glucose is described by:

$$q_{lys}(G, Lys) = q_g(G, Lys) Y_{lys/g}$$

The yield coefficient $Y_{lys/g}$ is calculated from the experimental $Y_{x/lys}$ as $Y_{lys/g} = \frac{Y_{x/g}}{Y_{x/lys}}$. A part of the carbon source is used for maintenance:

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

The glucose flow can be divided into two: q_g^{en} that is used for energy purposes and q_g^{an} that is used in the anabolism.

$$q_g^{an} = (q_g - q_m) Y_{x/g} \frac{C_x}{C_g}$$

$$q_g^{en} = q_g - q_g^{an}$$

Growth μ and oxygen consumption q_o are described by:

$$\mu = (q_g - q_m) Y_{x/g}$$
$$q_o = q_g^{en} Y_{o/g}$$

A.2 Model of *E. coli* DSM6968

The following assumptions are made in the model:

- The glucose uptake q_g is only used for energy purposes.
- Growth can take place on complex medium.
- The complex uptake q_c is independent of the glucose uptake.
- The complex medium is used as building blocks firstly and as energy source secondly.

The mass balance of the complex medium (C) is:

$$\frac{d(VC)}{dt} = F_c Comp_{in} - q_c(C) V X$$

The other mass balances are given by the equations (1)-(4), where the supplement (sup) is complex medium. The glucose uptake rate when there is enough complex medium present is given by:

$$q_g^{norm}(G) = \frac{q_g^{max} G}{(k_{sg} + G)}$$

The complex uptake rate is described by:

$$q_c(C) = \frac{q_c^{max} C}{(k_{scomp} + C)}$$

The complex medium flow into the cells is divided into two: q_c^{an} that describes the amount of complex medium that is used as building blocks in the cell growth, and q_c^{en} that describes the amount of complex medium used for energy purposes. The complex medium goes to q_c^{en} if there is not enough glucose to handle the energy requirements. This leads to that a fraction, denoted ϕ, of q_c goes to cell growth. It is also assumed that twice (η=2) as much complex medium as glucose is demanded for an efficient cell growth, i. e. glucose is used as energy and the complex medium is used as building blocks. After introduction of $q_g^{limit} = \frac{q_c}{\eta} + q_{mc}$, the model is given by:
If $q_g^{norm} > q_g^{limit}$

$$\begin{aligned} q_c^{an*} &= q_c \\ q_c^{en} &= 0 \\ q_g &= q_g^{limit} \end{aligned}$$

else if $q_{mc} < q_g^{norm} < q_g^{limit}$

$$\begin{aligned} q_g &= q_g^{norm} \\ q_c^{an,1*} &= \eta(q_g^{norm} - q_{mc}) \\ q_c^{an,2*} &= (q_c - \eta(q_g^{norm} - q_{mc}))\phi \\ q_c^{an*} &= q_c^{an,1*} + q_c^{an,2*} \\ q_c^{an,2} &= q_c^{an,2*} Y_{x/c} \frac{C_x}{C_c} \\ q_c^{en} &= q_c - \eta(q_g^{norm} - q_{mc}) - q_c^{an,2} \end{aligned}$$

else $q_g^{norm} < q_{mc}$

$$\begin{aligned} q_g &= q_g^{norm} \\ q_c^{an*} &= max(0, (q_c + \eta(q_g^{norm} - q_{mc}))\phi) \\ q_c^{an} &= q_c^{an*} Y_{x/c} \frac{C_x}{C_c} \\ q_c^{en} &= q_c - q_c^{an} \end{aligned}$$

The cell growth μ is described by:

$$\mu = q_c^{an*} Y_{x/c}$$

The specific oxygen consumption is described by q_o, where $q_g^{en} = q_g$

$$q_o = q_g^{en} Y_{o/g} + q_c^{en} Y_{o/c}$$

A.3 Linearised model, pulse response and stability analysis of *E. coli* DSM6968

Linearised model Linearised equations when $q_{mc} < q_g < q_g^{limit}$.

$$
\begin{aligned}
T_o \frac{d\Delta DO}{dt} + \Delta DO &= K_{og}\Delta q_g + K_N \Delta N + K_{oc}\Delta q_c \\
T_g \frac{d\Delta q_g}{dt} + \Delta q_g &= K_{gf}\Delta F_g \\
T_c \frac{d\Delta q_c}{dt} + \Delta q_c &= K_{cf}\Delta F_c \\
T_p \frac{d\Delta DO_p}{dt} + \Delta DO_p &= \Delta DO
\end{aligned}
$$

$$
\begin{aligned}
K_{og} &= -\frac{HX}{K_L a}(Y_{o/g} - \eta Y^*_{o/c}) & K_{oc} &= -\frac{HX}{K_L a} Y^*_{o/c} \\
K_N &= \frac{DO^* - DO}{K_L a}\frac{\partial K_L a}{\partial N} & K_{gf} &= \frac{G_{in}}{VX} \\
K_{cf} &= \frac{Comp_{in}}{VX} & T_g &= (\frac{\partial q_g}{\partial G}X)^{-1} \\
T_o &= (K_L a)^{-1} & T_c &= (\frac{\partial q_c}{\partial C}X)^{-1}
\end{aligned}
$$

where $Y^*_{o/c} = (1 - Y_{x/c}\phi\frac{C_x}{C_c})Y_{o/c}$ is introduced.

The pulse response and stability analysis The pulse response is given by 3 equations when $q_g > q_{mc}$:

$$
\Delta DO = \begin{cases} |K_{og}|q_g^{pulse} & \text{if } q_g < q_g^{limit} - q_g^{pulse} \\ |K_{og}|(q_g^{limit} - q_g) & \text{otherwise} \\ 0 & \text{if } q_g > q_g^{limit} \end{cases}
$$

where $q_g^{pulse} = K_{gf}\Delta F_g^{pulse}$. The glucose dynamics ($T_g$), the complex medium dynamics (T_c), the oxygen dynamics (T_o) and the dissolved oxygen sensor dynamics (T_p) are assumed to be fast compared to the pulse length and control phase. This gives the following response when $q_{mc} < q_g < q_g^{limit} - q_g^{pulse}$:

$$
\begin{aligned}
\Delta DO = |K_{og}|K_{gf}\Delta F_g &= \frac{HG_{in}}{VK_L a}(Y_{o/g} - \eta Y^*_{o/c})\Delta F_g \\
&= \frac{DO^* - DO_{sp}}{1 + \frac{q_c + \eta q_{mc}}{q_g}\frac{Y^*_{o/c}}{Y_{o/g} - \eta Y^*_{o/c}}}\Delta F_g \approx 8
\end{aligned}
$$

when $DO^* = 80$, $DO_{sp} = 30$ and $q_g \approx q_g^{limit}$. This is the maximum pulse response that can be obtained with these values of the parameters.

For $q_g^{limit} - q_g^{pulse} < q_g < q_g^{limit}$, the response is given by:

$$\Delta DO(k+1) = \Delta DO(k) + \frac{|K_{og}|}{\eta}(q_c(k+1) - q_c(k))$$

when q_g is assumed to be constant (F_g is an exponential feed corresponding to the exponential cell growth) and $q_c(k+1) = q_c(k) + K_{cf}\Delta F_c - \epsilon$. ϵ describes the influence of the changing cell mass VX. With the controller, equation (2), the closed loop response is:

$$\begin{aligned}\Delta DO(k+1) = (1 - \frac{|K_{og}|K_{cf}\kappa F_c}{\eta(DO^* - DO_{sp})})\Delta DO(k) \\ + \frac{|K_{og}|K_{cf}\kappa F_c}{\eta(DO^* - DO_{sp})} y_r - |K_{og}|\epsilon\end{aligned}$$

As

$$|K_{og}|K_{cf} = \frac{DO^* - DO_{sp}}{F_c\frac{q_g}{q_c} + \frac{q_c + \eta q_{mc}}{q_c}\frac{Y_{o/c^*}}{Y_{o/g} - \eta Y^*_{o/c}}} \approx \frac{DO^* - DO_{sp}}{F_c(\frac{1}{\eta} + \beta)}$$

with $\frac{q_g}{q_c} \approx \frac{1}{\eta}$, $\frac{\eta q_{mc} + q_c}{q_c} \approx 1$ and $\frac{Y^*_{o/c}}{Y_{o/g} - \eta Y^*_{o/c}} = \beta$. This gives:

$$\begin{aligned}\Delta DO(k+1) = (1 - \frac{\kappa}{1 + \eta\beta})\Delta DO(k) \\ + \frac{\kappa}{1 + \eta\beta} y_r - |K_{og}|\epsilon\end{aligned}$$

The stability boundaries of κ are then given by: $0 < \kappa < 2(1 + \eta\beta)$ and the convergence point y^* is given by:

$$y^* = y_r - \frac{(1 + \eta\beta)|K_{og}|\epsilon}{\kappa}$$

The influence from ϵ helps to reduce the risk of overfeeding the complex medium.

Table 1. Parameters and variables in the models. [†1] In our case the complex medium consist of yeast extract, [†2] $Y^*_{o/c} = (1 - \phi Y_{x/c} \frac{C_x}{C_c}) Y_{o/c}$

Symbol	Value	unit	Description
G_{in}	600	g/L	glucose conc. in feed
$Comp_{in}$	200	g/L	complex conc. in feed
Lys_{in}	80	g/L	lysine conc. in feed
H	14000	%L/g	Henrys constant
k_{sg}	0.01	g/L	sat. const. for glucose uptake
k_{slys}	0.01	g/L	sat. const. for lysine uptake
k_{scomp}	0.01	g/L	sat. const. for complex uptake
α	1.4	$(\text{hRPM})^{-1}$	oxygen transfer const.
N_0	140	RPM	oxygen transfer const.
q_g^{max}	1.2	g/gh	max. spec. glucose uptake
q_{lys}^{max}	1	g/gh	max. spec. lysine uptake
q_c^{max}	1	g/gh	max. spec. complex uptake
q_{mc}	0.06	g/gh	maintenance coefficient
η	2		relation for efficient growth $\frac{q_c}{q_g - q_{mc}}$
ϕ	0.86		fraction to cell growth when $q_g < q_g^{limit}$
$Y_{o/c}$	0.73	g/g	oxygen/complex yield [†1]
$Y^*_{o/c}$	0.1	g/g	oxygen/complex yield [†2]
$Y_{x/c}$	1	g/g	cell/complex yield
$Y_{x/g}$	0.5	g/g	cell/glucose yield
$Y_{x/lys}$	17	g/g	lysine/glucose yield
$Y_{o/g}$	1.07	g/g	oxygen/glucose yield
C_g	1/30 mol	C/g	carbon content in moles/g glucose
C_x	0.04 mol	C/g	carbon content in moles/g cells
C_c	0.04 mol	C/g	carbon content in moles/g complex [†1]
V	7	L	reactor volume
F_g		L/h	glucose feed into the reactor
F_c		L/h	complex feed into the reactor
F_{lys}		L/h	lysine feed into the reactor
G		g/L	glucose concentration
$Lys, (C)$		g/L	lysine, (complex) concentration
X		g/L	cell mass concentration
$C_o^{(*)}$	(80/H)	g/L	(sat.) dissolved oxygen conc.