KTH Biotechnology

Analytical tools for monitoring and control of fermentation processes

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Stockholm 2007

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Printed at Universitetsservice US-AB
Box 700 44
SE-100 44 Stockholm
Sweden

ISBN 978-91-7178-794-1

ABSTRACT

The overall objective of this work has been to adopt new developments and techniques in the area of measurement, modelling and control of fermentation processes. Flow cytometry and software sensors are techniques which were considered ready for application and the focus was set on developing tools for research aiming at understanding the relationship between measured variables and process quality parameters. In this study fed-batch cultivations have been performed with two different strains of *Escherichia coli* (*E.coli*) K12 W3110 with and without a gene for the recombinant protein promegapoietin.

Inclusion body formation was followed during the process with flow cytometric detection by labelling the inclusion bodies with first an antibody against the protein promegapoietin and then a second fluorescent anti-antibody. The approach to label inclusion bodies directly in disintegrated and diluted cell slurry could be adopted as a method to follow protein production during the process, although the labelling procedure with incubation times and washings was somewhat time-consuming (1.5 h). The labelling of inclusion bodies inside the cells to follow protein production was feasible to perform, although an unexplained decrease in the relative fluorescence intensity occurred late in process. However, it is difficult to translate this qualitative measurement into a quantitative one, since a quantitative protein analysis should give data proportional to the volume, while the labelling of the spheric inclusion bodies gives a signal corresponding to the area of the body, and calibration is not possible. The methods were shown to be useful for monitoring inclusion body formation, but it seems difficult to get quantitative information from the analysis.

Population heterogeneity analysis was performed, by using flow cytometry, on a cell population, which lost 80-90% viability according to viable count analysis. It was possible to show that the apparent cell death was due to cells incapable of dividing on agar plates after induction. These cells continued to produce the induced recombinant protein. It was shown that almost all cells in the population (≈97%) contained PMP, and furthermore total protein analysis of the medium indicated that only about 1% of the population had lysed. This confirms that the "non-viable" cells according to viable count by cfu analysis produced product.

The software sensors X_{NH3} and μ_{NH3}, which utilises base titration data to estimate biomass and specific growth rate was shown to correlate well with the off-line analyses during cultivation of *E. coli* W3110 using minimal medium. In rich medium the μ_{NH3} sensor was shown to give a signal that may be used as a fingerprint of the process, at least from the time of induction. The software sensor $K_La C^*$ was shown to respond to foaming in culture that probably was caused by increased air bubble dispersion. The $R_{O/S}$ coefficient, which describes the oxygen to substrate consumption, was shown to give a distinct response to stress caused by lowered pH and addition of the inducing agent IPTG.

The software sensor for biomass was applied to a highly automated 6-unit multi-bioreactor system intended for fast process development. In this way also specific rates of substrate and oxygen consumption became available without manual sampling.

Keywords: *Escherichia coli*, flow cytometry, software sensors, viability, inclusion bodies, biomass, specific growth rate, stress, population heterogeneity, process analytical technology.

LIST OF PUBLICATIONS

This thesis is based on the work performed to produce the following papers and manuscripts, referred to in the text by their Roman numerals.

I. Sundström, Heléne, Wållberg, Fredrik, Ledung, Erika, Norrman, Bo, Hewitt, Christopher J and Enfors, Sven-Olof. (2004) Segregation to non-dividing cells in recombinant *Escherichia coli* fed-batch fermentation processes. Biotechnology Letters 26(19), 1533-1539.

II. Wållberg, Fredrik, Sundström, Heléne, Ledung, Erika, Hewitt, Christopher J and Enfors, Sven-Olof. (2005) Monitoring and quantification of inclusion body formation in *Escherichia coli* by multi-parameter flow cytometry. Biotechnology Letters 27, 919-926.

III. Sundström, Heléne and Enfors, Sven-Olof. (2007) Software sensors for fermentation processes. Bioprocess and Biosystems Engineering, DOI 10.1007/s00449-007-0157-5.

IV. Sundström, Heléne and Enfors, Sven-Olof. (2007) A bioreactor system for high throughput process development. Manuscript.

TABLE OF CONTENTS

1 INTRODUCTION

1.1 AIM OF STUDY

The overall objective of this work has been to adopt new developments and techniques in the area of measurement, modelling and control of fermentation processes. Flow cytometry and software sensors are techniques, which were considered ready for application and the focus was set on developing tools for research aiming at understanding the relationship between measured variables and process quality parameters.

The first goal was to use flow cytometry to monitor cell viability and concentration of inclusion bodies in the cells. By proper permeabilisation of the cells the inclusion bodies in the cells may be stained with fluorescent antibodies and detected by flow cytometry. A loss of viability, according to viable count analysis on agar plates, is often seen in high cell density cultures producing recombinant proteins, but the impact of this has not been well understood since these cells may still produce product. A unique property of flow cytometry is the possibility to do population heterogeneity analysis by analysing each cell in a population of thousands of cells. The inclusion body analysis was therefore used to study the production of a recombinant protein and the membrane integrity in each cell when the viable count analysis indicated a dying population.

Software sensors are continuously derived data obtained from mathematical models with measured standard variables as inputs. One great advantage of software sensors is the low cost, there is no need for investments in instrumentation. However, the application requires models and knowledge for interpretation of the data. In this investigation the sensors were used off-line, but they can also be integrated in the process control software. Based on pH control data, two software sensors were developed for calculating biomass concentration and the specific growth rate. Oxygen mass balances were also used to measure the oxygen transfer capacity and the ratio of oxygen per glucose consumption ($R_{O/S}$). The $R_{O/S}$ sensor was studied as a potential sensor for physiological stress.

Fermentation processes include a large number of parameters, which can be varied to reach an optimal process design and the use of mini-bioreactors for multi-parallel cultivations is a trend to speed up process design. This development puts an increasing demand on the reduction of off-line analysis and for this purpose software sensors for biomass and growth rate were integrated in a 6-

unit mini-reactor system with a large degree of automation. When such a system is used the number of logged variables becomes very large and to facilitate the data handling also a MATLAB based toolbox was developed for efficient off-line calculations and data presentation. The whole system is intended as a tool for process optimisation using multivariate data analysis.

In this study fed-batch cultivations have been performed with two different strains of *Escherichia coli* (*E.coli*) K12 W3110 with and without a gene for the recombinant protein promegapoietin.

1.2 Background PAT

Process analytical technology (PAT) has been described as a new framework for innovative pharmaceutical manufacturing. The traditional focus on documentation and control was to be replaced by a new approach based on a thorough understanding of the manufacturing process.

In a “Guidance to the industry” FDA describes their intention to create a regulatory framework that will encourage development and innovative manufacturing and quality assurance in the pharmaceutical industry. This guidance, Process Analytical Technology, was intended to help the manufacturing industry to develop and implement new efficient analytical tools to improve the whole manufacturing process and at least maintain the current level of product quality assurance.(FDA 2004a)

But why? According to FDA's analysis of the so called pipeline problem (i.e. the recent decrease, instead of the expected increase, in drugs released to the market) the number of new drugs submitted to FDA has declined significantly during the last several years. Contrary to this fact, there has been an extensive increase of product development costs during the last ten years. In the report, called "Innovation/Stagnation Challenge and Opportunity on the Critical Path to New Medical Products" (FDA 2004b), FDA expresses fear of the national health if this inability of forwarding basic discoveries to the market continues. The report identifies three main areas where explanations can be found and proper actions should be taken. Firstly, the immense advances in the basic sciences have left applied sciences behind, since medical production development technology hasn't been able to keep pace with discovery technology. Secondly, a majority of products entering clinical trial fails, in many cases due to lack of new tools that can be used to describe the safety and effectiveness of the new product. Finally, the path to market is long, costly

and inefficient, in large depending on the current lack of convenient evaluation methods. (FDA 2004b)

What are the incentives for PAT in a process industry? Increased know-how about the process will of course raise its value. Another major driving force would be to increase the operation efficiency. As described by Rick E Cooley from Eli Lilly & Co, faster and more accurate process-steps can be taken if off-line analysis is eliminated. Minimized variability can be accomplished by using on-line measurements, e.g. by enabling feedback control of critical process parameters and automatic sequencing of a process (Cooley 2003). Other benefits can be reduction of the risk of processing errors, e.g. by decreasing the manual handling of samples. Also, a reduced risk of product contamination can be achieved using in- or on-line measurements since the system can be closed throughout the process.

Process control is one of the most important tasks in the chemical industry and Kueppers and Haider states that as the pressure to cut costs increases, the use of process analytical tools also increases (Kueppers and Haider 2003). The authors outline benefits of integrated process analysis and control to be: constant update of process status, rapid feedback at failure, process quality proof, risk minimizing and analysis error reduction. Advantages from an economical point of view would, according to the same authors, be: faster action at process failure, automated sample preparation, increased production rate and product yield, and decreased by-product formation.

PAT is not a new technology, process analysis has been applied and developed in process industry way back. Rather, "PAT is one tool in a process improvement toolbox also including, for instance, a strategy plan, training, process automation and process control" (Cooley 2003). In a thorough review on process analytical chemistry (Workman et al. 2001), built on a series of earlier publications (Beebe et al. 1993; Blaser et al. 1995; Workman et al. 1999), the authors point out that the data from various techniques used in process analyses nowadays also is recognised to have an impact beyond the production phase. Real time measurements are vital in discovery, development and in evaluation of product performance. In process analysis there is a demand for on-line analytical techniques that predict product performance, in contrast to techniques that mainly establish the specifications of the product. Today, the motivation to strive towards continuous production processes, where on-line and in-line techniques more commonly are applied, is high according to Kueppers and Haider. These techniques have the advantages of being significant time savers, more robust and more user-friendly (Kueppers and Haider 2003).

In biotechnological processes some analysis techniques are generally regarded as a minimum standard. Among these are measurement and control of pH, DOT (dissolved oxygen tension), temperature, pressure, exhaust oxygen and carbon dioxide, liquid- and air-flow rates, stirrer speed and broth weight/volume. In an extensive review Bernhard Sonnleitner treats this part of bioprocess analysis (Sonnleitner 2000). Many of the routine measurements can be further exploited by the use of software sensors. A software sensor is a model-based calculation of a new variable from the logged data.

Most analytical tools developed for measuring and understanding the bioprocess result in an average data for the whole population of cells in the reactor. However, bioreactors are heterogeneous with respect to cells, which are both live and dead, producing and non-producing, dividing and non-dividing, due to segregation. Measurements of single cells or populations of cells in culture, can give valuable information for process understanding and development. On-line flow cytometry with automated sampling from bioreactors is not yet common, but at-line analysis, as applied in this work, can become a routine analytical tool for fermentation processes. Flow cytometry offers the possibility of analysing up to 1500 cells (or particles) per second and the information obtained can be correlated with different cell characteristics and cell components.

1.3 The fed-batch cultivation technique

There are three main cultivation techniques for microbial processes: batch, fed-batch and continuous cultivation. In batch processes all nutrients and substrates are added from start. Growth will not be substrate limited, and the cells will therefore grow at their maximum rate (μ_{max}) until process design limits are reached, i.e. when oxygen transfer or cooling capacities are exceeded, or until nutrients are depleted or by-products reach inhibitory levels. A continuous process has an inlet and an outlet flow of complete medium with the same rates, resulting in a constant volume and steady state conditions. This technique is often suitable for investigating cell physiology, but not commonly used in industrial processes.

In this study the fed-batch technique has been applied. In a fed-batch cultivation a short initial batch phase is often applied and an inlet feed of concentrated solution of the energy substrate is started when the initial substrate (often glucose) is depleted. By using substrate limitation cell growth is limited to a rate where cultivation can be performed without reaching, or exceeding, the limits of cooling and oxygen transfer capacity. Furthermore, substrate limitation has an impact on

the metabolism in the sense that overflow metabolism and catabolite repression can be avoided. Growth limitation can also be achieved by lowering the temperature (Jahic et al. 2003; Svensson et al. 2005).

An example of a fed-batch process is shown in figure 1. In this simulation of an *E. coli* culture on minimal medium, the process was started with a batch phase, where the cells grow unrestricted at maximum rate, μ_{max}. A feed is started when the carbon source (glucose) is depleted. The first exponential stage of the feed is designed to keep the growth rate constant at a value below μ_{max}. In the following constant feed phase, the cells will experience an increasing degree of limitation of substrate and the growth rate declines.

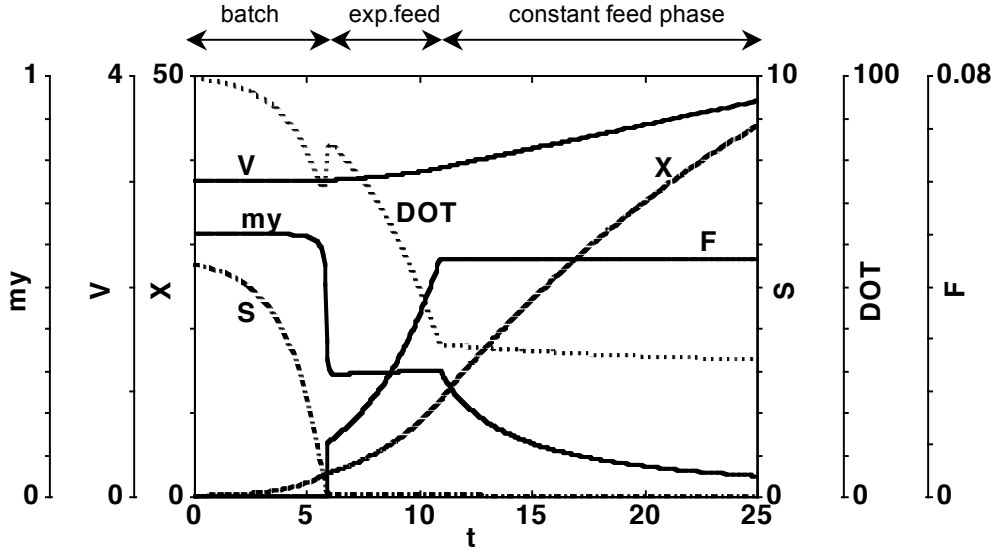


Fig. 1. Simulation of a fed-batch process with initial values: X= 0,07 g/L; S=5,5 g/L; V=3 L; F0=0,01 L/h; Fmax=0,045 L/h; Si=500g/L (X=biomass, S=substrate, V= volume, F0=initial feed rate, Fmax=maximum and constant feed rate, Si=concentration of substrate in feed solution, my=specific growth rate (μ), DOT=dissolved oxygen tension)

To be able to cope with sudden changes in the environment microorganisms have evolved adaptive regulations. The response to stress inducing conditions like starvation, heat, or sudden changes in pH, osmolarity or oxygen levels is regulated on transcriptional level. In bioprocesses the two most common events causing these adaptive stress responses are changes in temperature and nutrient depletion/deficiency. The reactions on temperature stress are categorised as heat shock, envelope stress or cold shock, and nutrient stress causes general stress response or stringent response. (Wick and Egli 2004)

Several conditions in a fed-batch culture may induce physiological stress of different types. The higher the cell density, the lower becomes the specific growth rate (fig. 1). This is due to the gradually increasing degree of nutrient limitation. It is often observed that the level of guanosine tetraphosphate (ppGpp) suddenly increases when the specific growth rate approaches 0.05 h^{-1} in *E. coli* fed-batch processes (Andersson et al. 1996). This reaction may indicate induction of the stringent response, which is associated with high levels of ppGpp (Wick and Egli 2004). The high cell density reached in fed-batch cultures, typically above 30 g per litre dry cell weight (dcw) in an *E. coli* process, results in concentration gradients with respect to the limiting substrate and dissolved oxygen. These gradients and also pH gradients are amplified in large bioreactors and have been shown to cause both stress responses and metabolic responses (Enfors et al. 2001). Also the expression of recombinant proteins may induce stress for several reasons. One mechanism is that the protein, if not correctly folded, binds chaperones like DnaK, which induce the heat-shock response (Goff and Goldberg 1985; Bukau 1993; Fredriksson et al. 2006). Another mechanism may be the action of the inducer itself (Kosinski et al. 1992).

The bioreactors used in these studies are standard stirred tank reactors coupled to devices for monitoring and control. An *E. coli* cultivation requires, as a minimum, to be controlled by stirrer speed, temperature, pH and aeration rate. A schematic drawing of the common stirred tank reactor (STR) and the standard on-line measured variable is shown in figure 2.

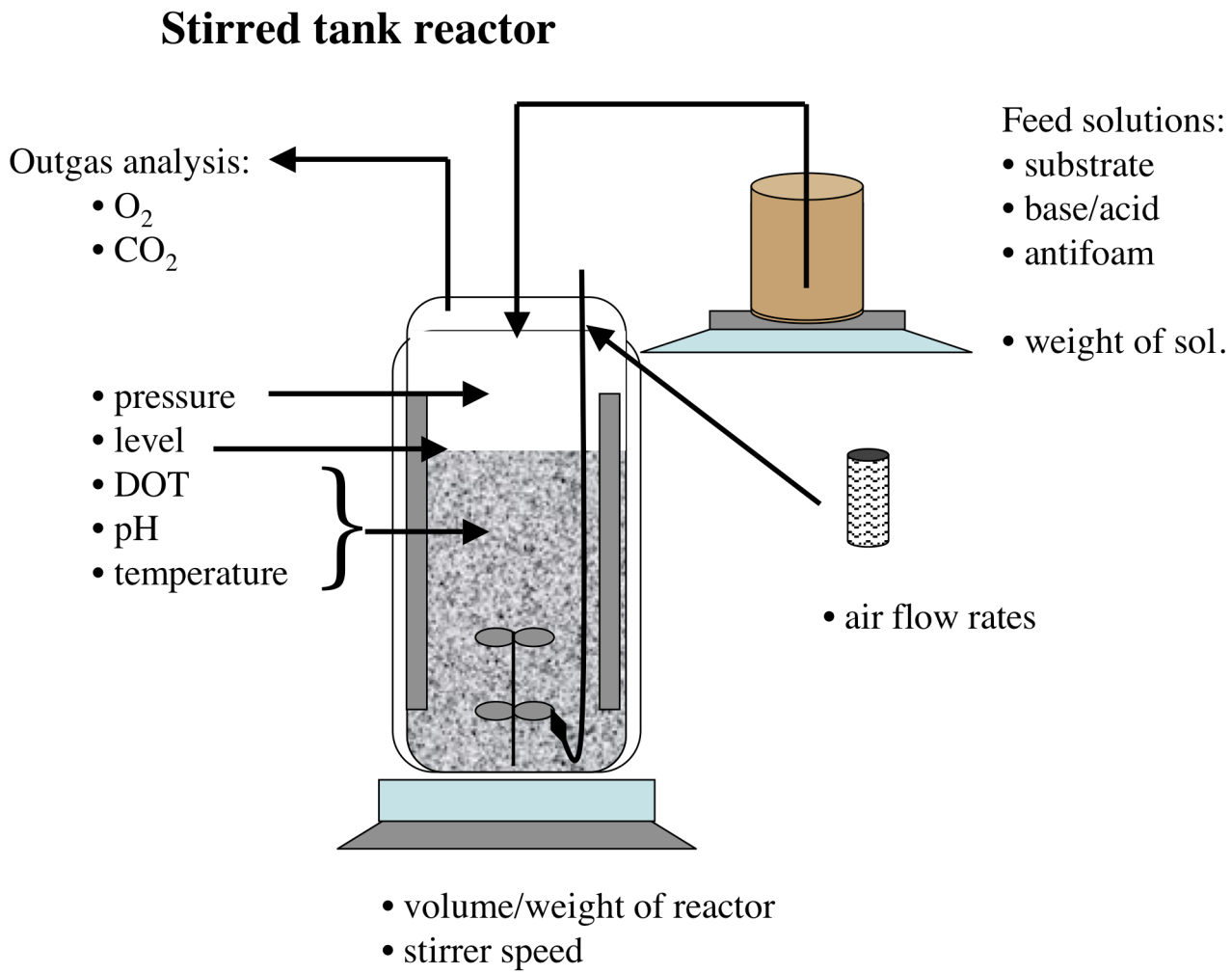


Fig. 2. Instrumentation and monitoring of a bioreactor.

1.4 Flow cytometry

Flow cytometry is a technique for discimination and counting of cells, or cellular particles, in liquid suspension. The technique is a powerful tool for analysing heterogeneity in cell populations, since properties are measured on an individual basis, rather than as a population average. Many applications of flow cytometry are based on the detection of cell-bound fluorescent labels attached to the molecules or organelles of interest. Cell sorting equipment that allows selection of cells is available.

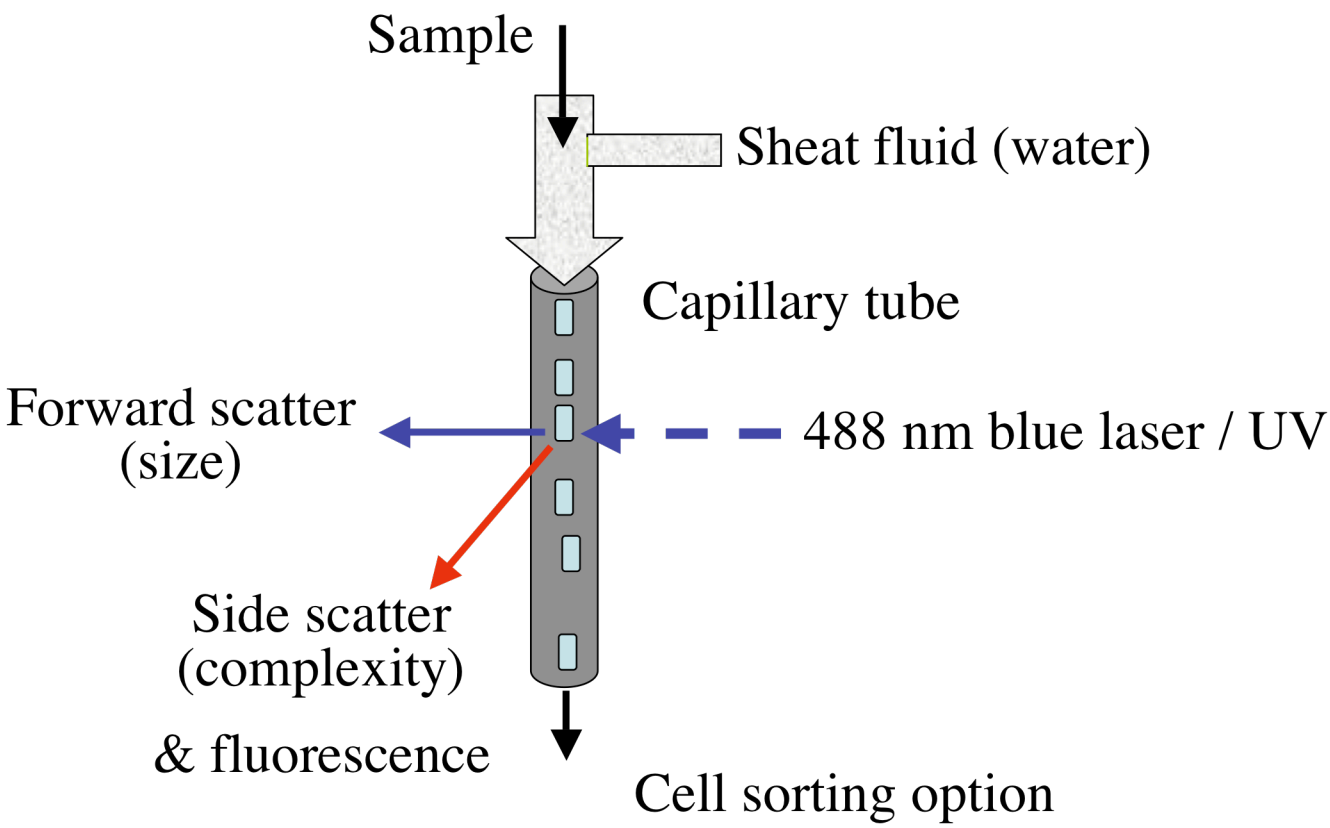


Fig.3. The principle of flow cytometry. Cells in suspension pass a cuvette in a very narrow stream. The focused beam of the light source hits particles in the stream, creating scattered light (an optical signal). The signal is detected, converted into an electronic signal and displayed for analysis.

Figure 3 shows the principle of a flow cytometer. Cells flow in a very narrow stream of sheath fluid passing a light beam of a laser or a mercury lamp. The interaction of light with cells gives information of fluorescence, light scattering and absorbing properties that can be correlated with different cell characteristics and cell components. Depending on the physical properties of the illuminated particle, light will scatter differently. In flow cytometry, the scattered light is measured at small angles, forward angle light scatter (FALS), and at 90^{o} from the incoming light, right angle light scatter (RALS, also often termed side angle light scatter, SALS). The size of the cell will mostly be reflected in the FALS measurement, while internal components of the cells can affect the RALS readings. Fluorescence is measured at 90 degrees from the incoming light.

The use of flow cytometry has been more widespread for animal cell cultivations, for instance to perform cell cycle analyses, but nowadays the technique is also commonly used in microbial studies. Multi-parameter flow cytometry studies have been performed to assess and compare cell physiological states and response to nutrient limitation in microbial batch, fed-batch and continuous cultivations, at laboratory scale as well as in industrial applications (Nebe-Von-Caron and Badley 1995; Hewitt et al. 1999; Hewitt et al. 2000; Hewitt and Nebe-Von-Caron 2004;

Sundström et al. 2004; Wållberg et al. 2005). Also more complex analyses, as semi-quantitative analysis of inclusion bodies, have been published (Wållberg et al. 2005).

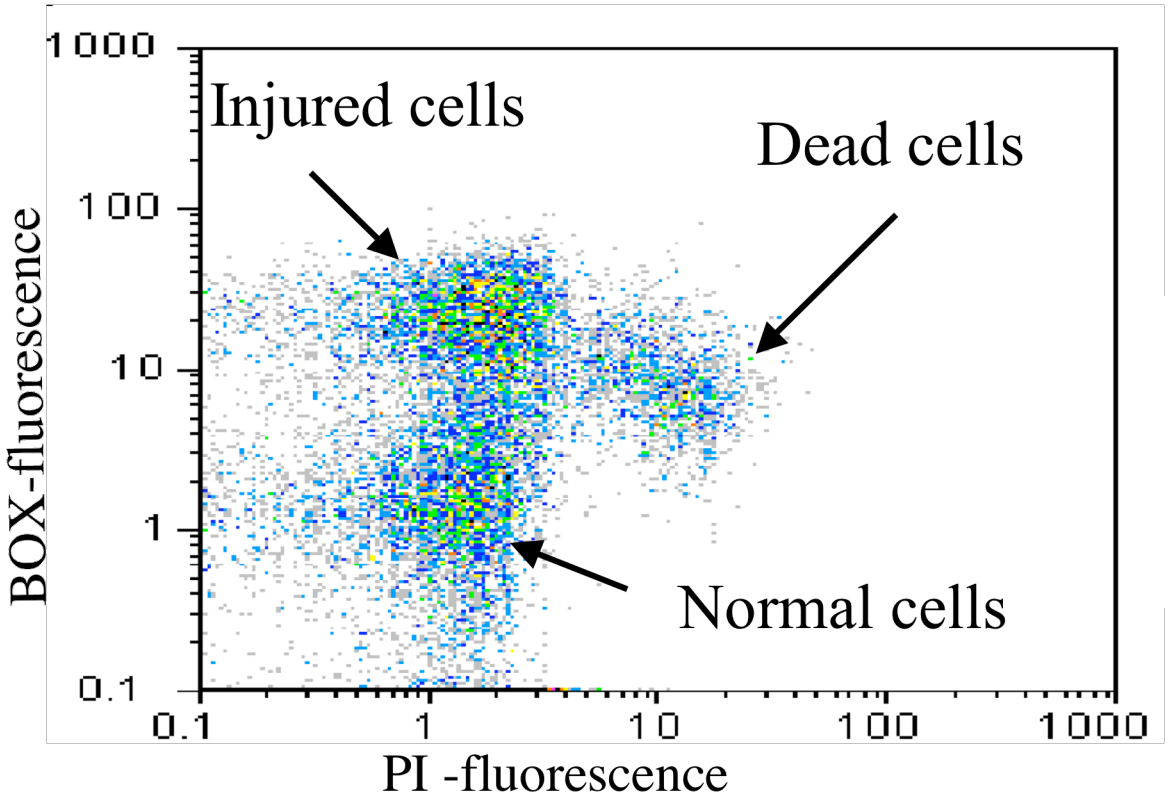


Fig. 4. PI- and BOX-staining of *E. coli* cells

Flow cytometry can offer a tool for routine at-line analysis of population heterogeneity. In heterogeneity studies fluorescent markers are used that normally do not have the ability to enter a healthy cell. See for instance Veal et al or Rieseberg et al for information on fluorescent markers used in flow cytometry, and Hewitt and Nebe von Caron or Marten-Habbena and Sass for applications with different stains (Veal et al. 2000; Rieseberg et al. 2001; Hewitt and Nebe-Von-Caron 2004; Martens-Habbena and Sass 2006). Propidium iodide, PI, which has been used in the present study, is one of the more common dyes. This molecule, which binds to nucleic acid, cannot enter a cell with intact cell membrane. A cell that rejects PI is classified as viable. Bis-(1,3-dibutylbarbituric acid) trimethine oxonol, BOX, which also has been used in this study, is lipophilic and anionic. It binds to lipid-rich intracellular compounds and can enter a cell with depolarised membrane. Figure 4 shows *E. coli* cells stained with both PI and BOX. The PI-stained cells, which are considered dead, also stain by BOX, while injured cells only stain by BOX. Examples of states or properties which can be analysed are: population heterogeneity with respect to viability (e.g. by PI staining), cell membrane depolarisation as a possible pre-stage to death (e.g. by BOX staining), and product distribution measured as inclusion body distribution

(fluorescent antibody probes) or in the cases when the products are fused to green fluorescent protein (GFP).

Cells in cultivation may segregate into subpopulations with different properties. Examples of subpopulations that have been experimentally described are the loss of plasmids (Hägg et al. 2004), loss of cell membrane polarisation and cell death (Nebe-Von-Caron et al. 2000), and loss of cell dividing ability (Sundström et al. 2004), for a review see (Hewitt and Nebe-Von-Caron 2004). However, the product quality is measured as an average value obtained from all cells. It is reasonable to assume that when cells gradually loose their cell membrane polarisation, and later on die, they have other properties than the "healthy" population. Such a gradual shift towards a depolarisation of the cell membrane and subsequent cell death probably means that the cell responds with some of the physiological stress response mechanisms. This may include effects on folding and proteolytic stability of proteins. Furthermore, segregation to a non-dividing cell state, and also cell death has been shown to be influenced by the bioreactor scale in *E. coli* processes (Hewitt et al. 2000) and are therefore dependent on the environmental operating conditions. Thus, monitoring of population segregation should be a useful tool in biopharmaceutical processes.

Flow cytometry is primarily an at-line technique, but there are some reports on more automated and on-line applications. A flow injection system interfaced with a flow cytometer and a bioreactor to perform on-line assessment of single-cell property distributions was designed by Zhao et al (Zhao et al. 1999). The performance of the system was demonstrated in three applications: monitoring of green fluorescent protein (GFP) fluorophore formation kinetics in *E. coli*, evaluation of batch growth dynamics of *E. coli* expressing GFP and determination of the distribution of DNA content in a *Saccharomyces cerevisiae (S. cerevisiae)* population by automatically staining cells. A research group at the University of Minnesota (US) developed an automated flow cytometry system with automated sampling, dilution and staining. They showed the applicability of the method for instance for measuring PHB (polyR-3-hydroxybutyric acid) in *S. cerevisiae* and *Cupriavidus necator* (Kacmar et al. 2005b), for measuring viability and cell concentration in CHO-cells (Kacmar and Srienc 2005), and cell size, viability and green fluorescence protein content in *S. cerevisiae* cultures (Kacmar et al. 2004). Such a system, which can automatically take and analyse samples continuously every 30 minutes throughout a cultivation, would be very valuable in control and monitoring of any fermentation process.

1.5 Software sensors

A software sensor is a virtual sensor, that utilises signals already available on-line and combines these signals with mathematical models with the purpose to predict parameters that are not available on-line. Obviously, this requires a model that relates the measured response to the desired parameter. A great advantage with software sensors, and also the attraction from a PAT point of view, is that they are cheap to introduce. Once the model is available they just require programme code, no expensive investment in hardware is needed. There are several process-state variables that can be made available for on-line measurement based on traditional bioprocess on-line instruments. The design of software sensors are discussed by Chéruy (Chéruy 1997). However, so far there is little information about their application for process monitoring. Thus, there is a need for research on the applicability of software sensors for quality control.

The respiratory quotient (*RQ*) is one of the most well-known and used software sensors. It reflects the distribution between respiration and other reactions that produce O_2/consume CO_2. *RQ* can describe for instance the metabolic state in a yeast-culture exhibiting over-flow metabolism, where an $RQ \approx 1$ indicates oxidative (aerobic) growth on glucose, *RQ* larger than 1 describes overflow metabolism (also aerobic), whereas an *RQ* less than 1 would indicate ethanol consumption.

More examples of software sensors suggested for monitoring and control of bioprocesses are taken up in chapter 4.

2 Analysis of inclusion body formation in Escherichia coli

2.1 Inclusion bodies

When recombinant proteins are produced with cytoplasmic expression vectors in *E. coli*, high product yields can be obtained, sometimes up to 50% of the total cellular protein (Rudolph and Lilie 1996). The product is often accumulated in inclusion bodies, especially when the target protein is eukaryotic. Typically aggregation occurs in strong expression systems, but the generation of inclusion bodies also increases with factors that result in high specific growth rate. Examples of factors that favour inclusion body formation are a high concentration of the inducing agent, the use of complex growth media and cultivation at higher temperatures.

2.1.1 Structure of inclusion bodies

Inclusion bodies are intracellular protein aggregates, which can be seen as dense particles in an electron microscope (fig. 6), however they can also be seen in an ordinary light microscope (fig.

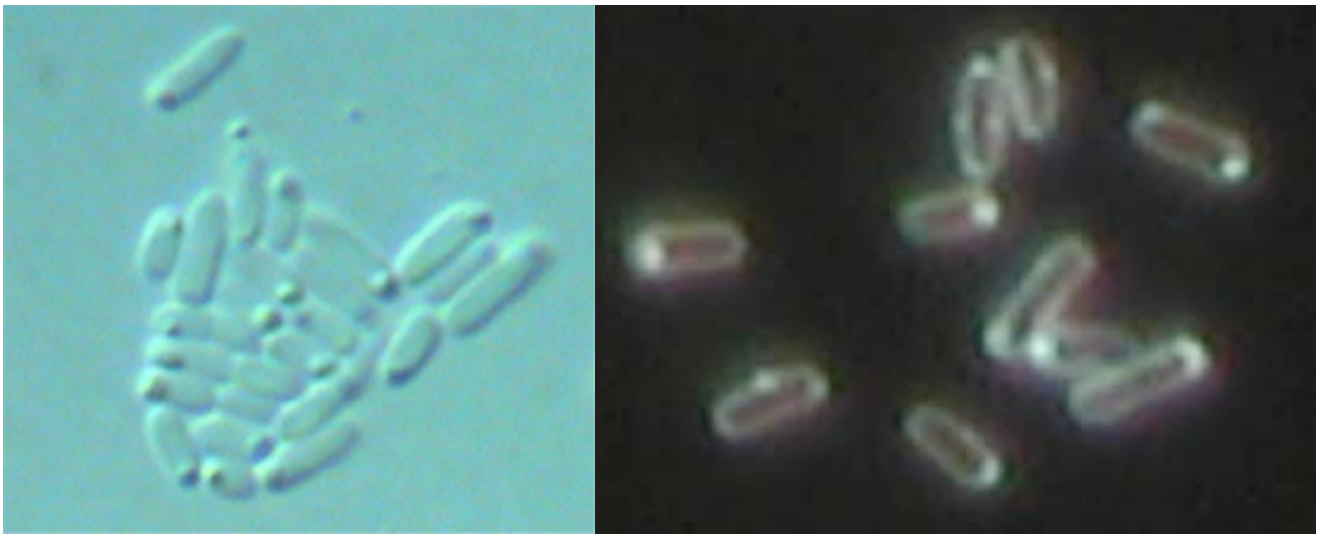


Fig. 5. Inclusion bodies of promegapoeitin in *E. coli* visualised in light microscope with differential interference contrast (left) and darkfield illumination (right) Photos by Kaj Kauko, Applied Environmental Microbiology, KTH.

5.). Inclusion bodies accumulate mainly at the ends of a bacterium and it has been suggested that minor aggregates are formed first, which then unite into a larger body at increasing concentration of unfolded protein (Carrió et al. 2000). Inclusion body production can affect the optical density, which in these cases can be seen if optical density is plotted against dry cell weight. The number

of inclusion bodies and their size is changing with the genetic background of the cell according to a review by Fahnert et al (Fahnert et al. 2004), which refer to studies that support the hypothesis that the formation of inclusion bodies is not stochastic, but involves cellular components.

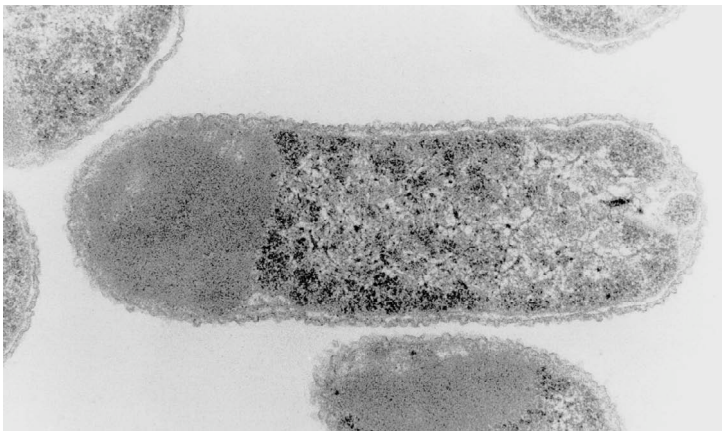


Fig.6. Inclusion body in *E. coli* as observed in an electron microscope. The inclusion body is the dense part to the left in the cell. Picture from Fahnert et al (2004) with kind permission of Springer Science and Business Media.

In most cases cytoplasmic inclusion bodies consist mainly of the incorrectly folded recombinant protein (Carrió and Villaverde 2002). Other cellular proteins (membrane proteins, RNA polymerase, ribosomal subunit proteins) and ribosomal RNA and DNA, which often are present in inclusion body preparations, are mainly integrated due to co-precipitation of cell debris while processing the inclusion bodies (Rinas and Bailey 1992), but cellular proteins may also be directly incorporated in inclusion bodies (Rinas and Bailey 1993). Large amounts of impurities in periplasmic inclusion bodies from cells grown at basic pH have been observed, while cytoplasmic inclusion bodies contained low amounts of impurities regardless of growth conditions (Valax and Georgiou 1993). Inclusion bodies are very stable and often resistant to proteases. *In vivo* solubilisation of inclusion bodies has been observed (Fahnert et al. 2004).

2.1.2 Formation of inclusion bodies

The native non-folded polypeptide undergoes a series of intra-molecular structural rearrangements leading to the mature protein (fig. 7). However, in the protein processing of many recombinant proteins the polypeptides interact with themselves and this might result in the formation of inclusion bodies. One role of chaperones is to prevent this interaction between partly or unfolded polypeptides. A high synthesis rate of a protein with slow folding characteristics may exceed the available resources of foldases and chaperones. Carrio and Villaverde (Carrió and Villaverde 2003) showed that a *dnaK* mutant had inclusion bodies twice the size of the wildtype and that IB formation was strongly suppressed in a *groEL* mutant. It has been suggested that this

indicate that the chaperones DnaK and GroE are controlling the inclusion body formation in competition, where DnaK prevents formation by reducing aggregation and GroE may take part in the inclusion body formation while transferring the protein between soluble and insoluble fractions (Fahnert et al. 2004).

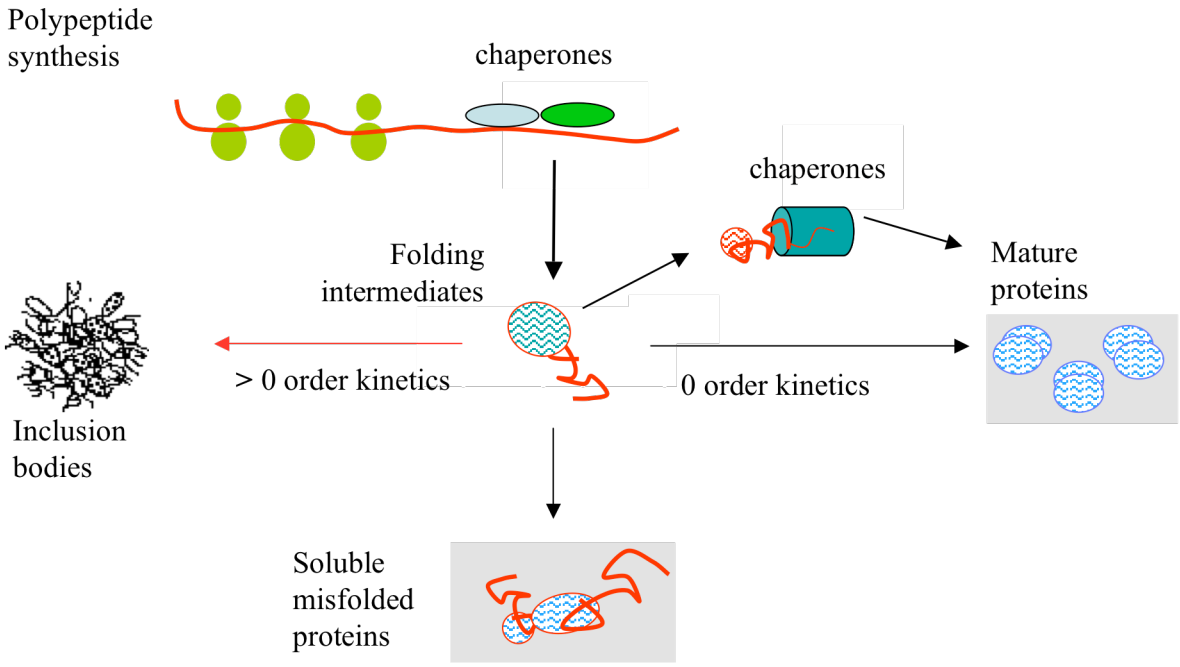


Fig. 7. Intracellular protein processing in prokaryotes.

It is mainly kinetic competition between folding and aggregation rates that govern the formation of inclusion bodies (Lilie et al. 1998). The intramolecular rearrangements leading to the mature protein follows zero order kinetics, while intermolecular reactions leading to inclusion bodies have a higher order of kinetics. A high concentration of folding intermediates is therefore a factor, which promotes inclusion body formation. There are pathways guiding the folding to the native state towards a thermodynamically stable and low energy level. Non-covalent interactions (van der Waals, ion bonds, polar interactions, electrostatic interactions, hydrogen bonds) decrease the number of possible conformations of a polypeptide. But even small changes in the primary structure of a protein may affect its solubility (Dobson and Karplus 1999), for instance if hydrophobic regions are exposed during folding this can cause aggregation of intermediates (Ellis 1997). According to (Rudolph and Lilie 1996) inclusion body formation depends on the specific folding behaviour of the protein, rather than on its general characteristics, but in their extensive

review on inclusion bodies Fahnert et al point out some structural characteristics that influences the folding rate and thereby the formation of aggregates (Fahnert et al. 2004).

For proteins containing disulfide bonds the formation of those is normally the rate-limiting step. Due to the reducing conditions in the cytosol of *E. coli*, proteins with disulfide bonds are often accumulated as inclusion bodies if produced there. Mutations can promote the formation of a correctly folded product, for instance by inactivating certain genes and thereby creating a more oxidising intracellular environment (Derman et al. 1993). Intracellular proteins usually lack disulfide bonds, whereas extracellular proteins often contain several (Stryer 1995).

Membrane proteins aggregate, even when expressed in small amounts, due to their surface-exposed hydrophobic regions. They have been considered to be hard to express and to affect the host cells (*E.coli*) negatively. Membrane proteins with correct conformation can be produced at high amounts in specific mutants of *E. coli* BL21 (Miroux and Walker 1996).

Proteins, which in homologous form are glycosylated, may often aggregate if produced in *E. coli*, since glycosylation affects the folding behaviour and solubility.

Eukaryotic proteins are prone to aggregation when expressed in bacterial systems due to differences in the protein synthesis. In prokaryotes the translation process is fast, up to 20 amino acids per second (Sorensen and Pedersen 1991), and protein folding mostly occurs posttranslationally. In eukaryotic cells the folding starts already during translation. The translation rate is much slower in eukaryotes than in *E. coli* and that is important for proper folding and glycosylation (Ujvari et al. 2001).

High synthesis rate of the target protein gives more aggregation and examples of ways to decrease inclusion body formation during cultivation are to lower the cultivation temperature or the inducer concentration. On the other hand, if the target is to produce inclusion bodies, high expression rate of the recombinant protein is optimal. The protein production rate is highly dependent on the copy number of the target protein gene, because it is the basis of the signal amplification from the gene to the protein. The transcription rate can be controlled by choice of inducible promotor and variation of inducer concentration.

2.1.3 Stress response to inclusion body formation

Overproduction of a recombinant protein in *E.coli* interferes with the cell's own metabolic processes by draining it from precursors and energy. This drainage can cause changes in metabolic fluxes and in the composition of enzymes, which in turn may induce stress responses. The induced stress response will direct cellular activities towards reorganisation of the biomass instead of growth, which may result in growth inhibition or low product yield. The extent of the stress response is governed partly by properties of the produced protein, but also by the rates of transcription and translation. (Hoffman and Rinas 2004) The response of the cell to the production of a recombinant protein depends on the competition with production of host cell proteins. If the cell is fully occupied with production of the recombinant protein it can be that no cellular response occurs, but the cell may loose its ability to divide and finally die. (Lin et al. 2001) If, on the other hand, the cell is capable of simultaneously producing its own proteins, a typical stress response (e.g. the SOS response) may be observed (Aris et al. 1998).

However, the presence of misfolded proteins induces a heat-shock like stress response, which will be followed by chaperone action (Bahl et al. 1987; Dong et al. 1995).

2.1.4 Analysis of inclusion bodies

A common method to measure the relative amount of inclusion bodies in a cell is to apply Western blot on the soluble and the insoluble protein fractions of the cell disintegrate. The inclusion body isolation from harvested and disintegrated cells is generally efficiently performed by centrifugation or filtration. If the isolated inclusion bodies harbour large amount of impurities, they can be purified by a series of washing steps, or the protein can be purified after solubilisation. However, isolation and solubilisation is often sufficient for analysis, for instance by chromatography.

In a high cell density culture of *E. coli* expressing human growth hormone as inclusion bodies, the protein formation was followed by measuring impedance (Upadhyay et al. 2001). The use of for instance green fluorescent protein (GFP) fused to the protein of interest, a so called reporter protein, has been extensively evaluated (Randers-Eichhorn et al. 1997; Albano et al. 1998; Dabrowski et al. 1999; DeLisa et al. 1999; Hisiger and Jolicoeur 2005; Nemecek et al. 2007).

In this work an approach to analyse the inclusion bodies by flow cytometry was developed.

2.2 Flow cytometry of intracellular proteins

One of the most important variables to analyse in a commercial bioprocess is product formation. It is also often one of the more time consuming and laborious analyses and the results may not be available until after the cultivation is finished. With flow cytometry it can sometimes be possible to do at-line analyses and follow product formation during the process.

Changes in the refractive index of cells have been used to follow inclusion body formation throughout processes with both forward angle light scatter (FALS) and right angle light scatter (RALS) analysis (Fouchet et al. 1994; Fouchet et al. 1995; Lavergne-Mazeua et al. 1996; Patkar et al. 2002; Lewis et al. 2004). Theoretically changes in size would mostly affect the FALS signal, but experimentally a more constant relationship was found between size and the RALS signal (Nebe-von Caron and Badley 1996). The principle that the formation of inclusion bodies should be measurable by light scatter originates in that inclusion bodies can become almost the size of the cell and, in these cases, the light scattering properties of the cell will change (Mukhopadhyay 1997).

When the product is fluorescent, or a fluorescent protein is co-expressed with the target protein, it can be possible to follow production with flow cytometry by measuring the increase in fluorescence. Patkar et al used RALS and fusion with GFP to evaluate expression systems in process development (Patkar et al. 2002). Park et al made a construction containing enhanced gfp gene, which enabled selection of highly expressing cells with flow cytometry, and concluded that the system will facilitate bioprocess development of efficient production of gutless adenoviruses (Park et al. 2004).

2.3 Present Investigation (II)

The possibility to follow the promegapoeitin (PMP) inclusion body formation during cultivation was investigated by measuring the change in light scattering properties. Figure 8 shows the protein (PMP) production in a glucose limited fed batch process, together with dry cell weight (dcw) and forward angle light scatter measurements. The reference analysis method of protein production was reversed phase HPLC. Figure 9 shows both the FALS and RALS signal during the cultivation in figure 8. Both FALS and RALS signals are increasing from the point of

induction (12,1 h process time, 4,5 h from feed-start), where PMP starts to accumulate (fig. 8), but they are also declining in the batch phase of the culture, from which the conclusion was made that the FALS and RALS signals cannot be used to monitor the formation of PMP inclusion bodies. However, as discussed by Lewis *et al*, light scattering properties of cells may be complex (Lewis et al. 2004). A possible explanation to why the FALS and RALS signals could not be used in this case, was that it could be due to instrument properties. The alignment of the light in an a modern simple instrument, like the Partec PAS (Partec GmbH, Münster, Germany) used in this study, is not quite as tight as in continuously manually aligned instruments, and it might influence the sensitivity with respect to scattered data from bacterial cells.

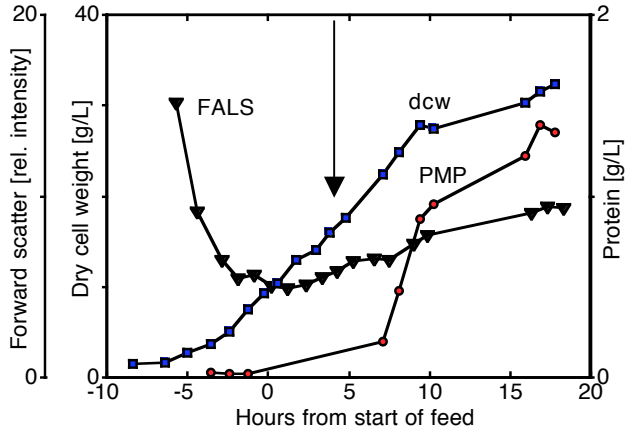


Fig. 8. Promegapoietin, PMP, produced in *E. coli* fed-batch process (circles) together with dcw (squares) and FALS (triangles) measurements. Induction at arrow.

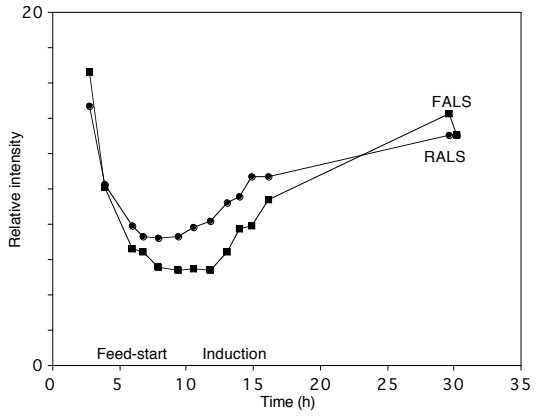


Fig. 9. Light scatter properties of PMP producing *E. coli*.

Instead, the possibility to follow the production of promegapoietin (PMP) inclusion bodies in both whole (paper I & II) and disintegrated (paper II) *E. coli* cells, by labelling with antibodies was investigated in these studies.

Inclusion bodies in a suspension of disintegrated cells were labelled with antibodies. The PMP inclusion bodies were labelled with a primary antibody and a second fluorescent antibody. The whole procedure with incubation times, washing steps and measurement with flow cytometry required 1.5 hours. Figure 10

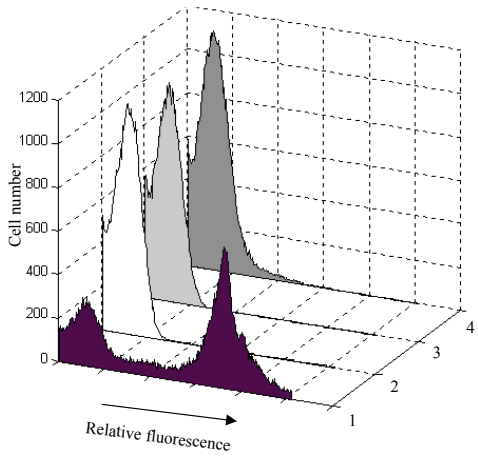


Fig. 10. Specificity test of the labelling of PMP inclusion bodies with a primary antibody and a secondary fluorescent anti-antibody. 1: both anti-bodies added. 2: control no antibodies added. 3: control only secondary antibody added. 4: cells without inclusion bodies.

shows that the labelling with antibodies of the PMP inclusion bodies was specific. This was tested with an inclusion body slurry, where the disintegrated cells first were removed by centrifugation. The first histogram (1) shows two peaks, where the one with highest fluorescence (right) represents the labelled inclusion bodies. The lower frequency peak probably corresponds to unlabeled cell debris (compare with sample 4).

The PMP formation was followed throughout a process by labelling of inclusion bodies from disintegrated cells. In this case the whole disintegrate with cells and inclusion bodies was diluted and labelled with first the primary and then with the fluorescent anti-antibody. Figure 11 shows that the PMP fluorescence peak gradually increase in fluorescence with process time. Two hours before induction the cells contained about 6.0 mg PMP per gram cell dry weight, as analysed by HPLC, probably due to a leaking promoter. The final sample contained 48 mg PMP per gram cell dry weight.

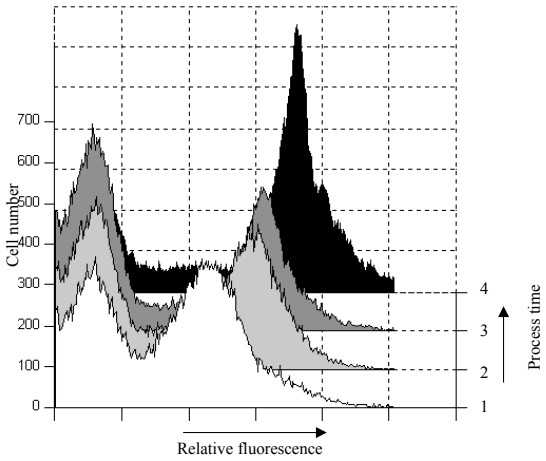


Fig. 11. Monitoring of inclusion body formation in suspensions of disintegrated cells during a fermentation process. Histograms show: 1: 2 h before induction, 2: 1.5 h after induction, 3: 3 h after induction and 4: 18 h after induction.

With reference to the discussions about viability and segregation of cells, the next approach was to examine the possibility to label the protein inside the cells and thereafter measure the distribution of protein in the population.

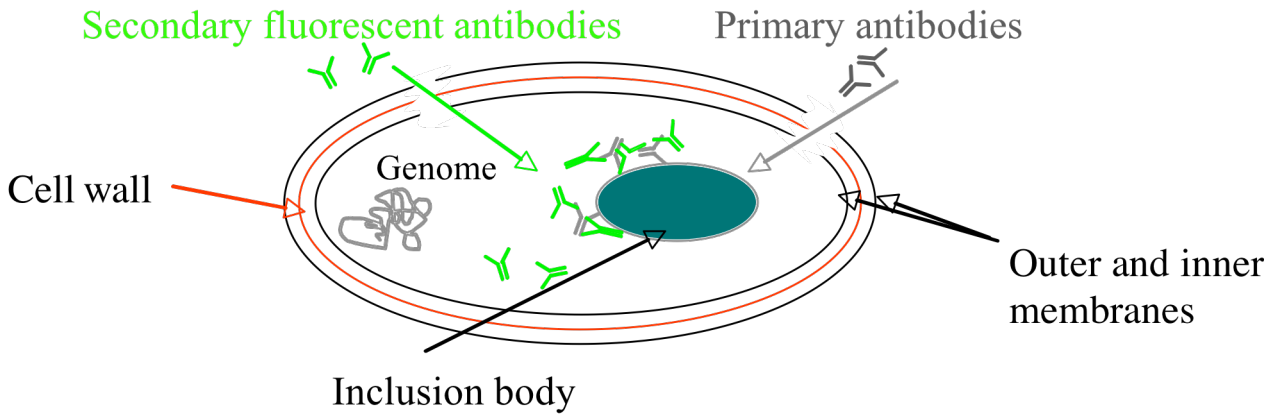
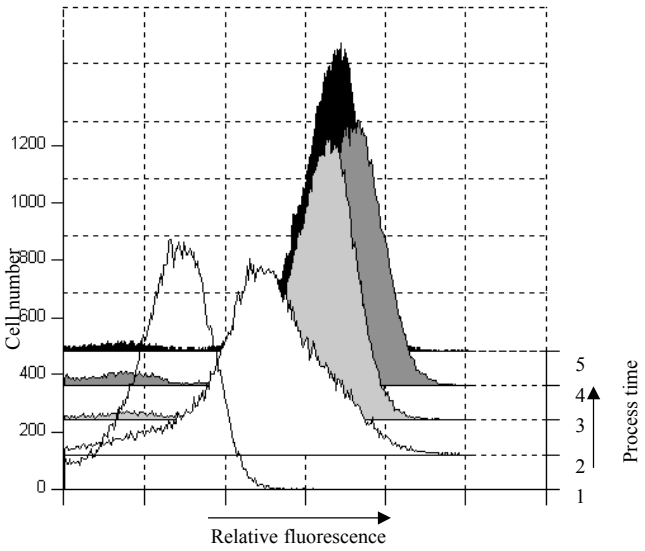


Fig. 12. Treatment with DMSO and lysosyme permeates the membranes and cell wall in *E. coli* and enables labelling of inclusion bodies with antibodies.

Promegapoietin (PMP) inclusion bodies were first labelled with a primary antibody and then secondly with a fluorescent anti-antibody (fig. 12). To do this the cells were permeabilised with dimethylsulfoxide (DMSO) and lysosyme. Treatment with DMSO for about 3 minutes (including 2 minutes centrifugation), followed by a 15 minutes incubation with lysosyme permeabilised the membranes and cell wall sufficiently to make it possible for the antibodies to enter the cytosol, while keeping the cell conformation intact. DMSO is known to easily permeabilise membranes (Anchordoguy et al. 1992), a characteristic that may be favoured by its hydrophobic groups (two



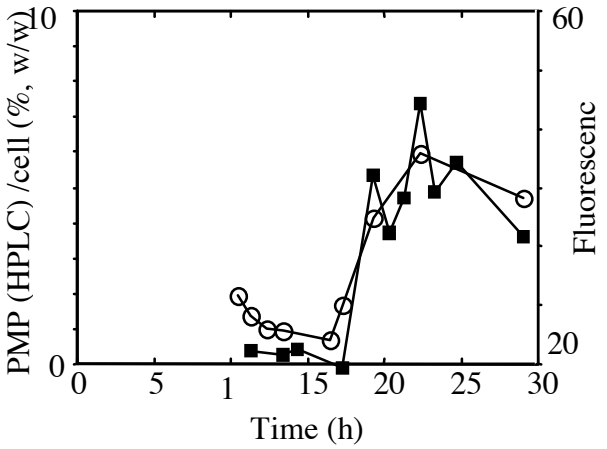


Fig. 14. Correlation between the relative fluorescence intensity measured after intracellular labelling of PMP inclusion bodies in permeabilised cells (squares) and the specific promegapoeitin concentration (% w/w, circles)

Fig. 13. Inclusion body formation in *E. coli* producing PMP followed by intracellular staining of cells permeabilised with DMSO and lysosyme and incubation with a primary and a secondary fluorescent antibody. 1: control showing the signal from cells without the PMP-plasmid. 2: Sample taken 4.6 h before induction. 3: sample taken 3.7 h after induction. 4: sample taken 7.2 h after induction. 5: sample taken 10.7 after induction.

methyl groups). Lysosyme disintegrates the bacterial cell wall by attacking peptidoglycans and hydrolyzing the glycosidic bond that connects N-acetyl muramic acid with the fourth carbon atom of N-acetylglucosamine. After permeabilisation, the cells were treated with primary antibodies and secondary fluorescent antibodies. The accumulation of PMP inclusion bodies during a fed-batch fermentation process can be seen in figure 13. The first peak (histogram 1) is a control with antibody treatment of cells lacking the PMP-producing plasmid. The second histogram shows a sample taken 4.6 hours before induction and already at this point the fluorescence had increased. This shows that the recombinant protein synthesis was not tightly regulated in this strain. Samples taken at 3.7 and 7.2 hours after induction (histograms 3 and 4) show peaks with increasing level of fluorescence that correlated well to reference HPLC data (fig. 14). The last peak (histogram 5) shows a sample taken 10.7 hours after induction. Here fluorescence intensity decreased a little and also this correlated with reference data (fig. 14). The reason for the decrease fluorescence is

not known, but one obvious guess might be proteolysis. However, since inclusion bodies are regarded very stable and quite resistant to proteolysis this is not a probable explanation. Another cause for decreasing fluorescence, could be that if structural reconformations of the inclusion bodies occur, for instance if two smaller bodies merge into one larger inclusion body, then the surface to mass proportion would change and the fluorescence would decrease. However, this scenario does not explain the decrease in the reference HPLC measurement of PMP. Yet another possible explanation could be that the cell wall, in the cells from samples taken late in the process, is more resistant to lysosyme and not as easy to permeabilise as at earlier time-points in the process. The optimization of incubation times with DMSO and lysosyme was performed with samples taken earlier in the process. This discussion could maybe be transferred also to the reference HPLC measurement of PMP, if the cells later in process would become more resistant to disintegration. Figure 15 shows a permeabilised and antibody-labeled cell, with intact conformation, visualized in fluorescence microscope.

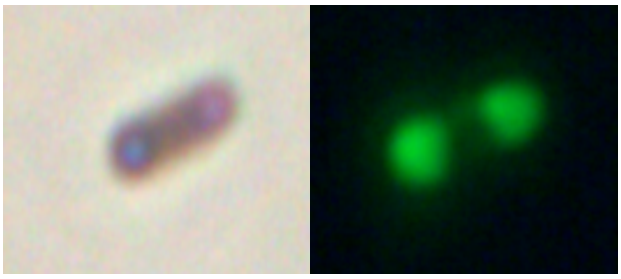


Fig. 15. Fluorescence microscopy of *E. coli* cells containing PMP inclusion bodies labeled with primary and secondary fluorescent antibody. To the left a picture showing a cell illuminated with visible light. Right picture shows the same cell illuminated with blue light. Photos by Kaj Kauko, Applied Environmental Microbiology, KTH.

This study showed that it is possible to follow inclusion body formation during a process with flow cytometric detection of the fluorescent anti-antibody, if primary antibodies against the produced protein are available. The approach to label inclusion bodies directly in disintegrated and diluted cell slurry could be adopted as a method to follow protein production qualitatively during the process, although the labelling procedure with incubation times and washings was somewhat time-consuming (1.5 h). The labelling of inclusion bodies inside the cells to follow protein production was feasible to perform, although an unexplained decrease in the relative fluorescence intensity occurred late in the process. However, it is difficult to translate this qualitative measurement into a quantitative one, since a quantitative protein analysis should give data proportional to the volume of the inclusion body, while the labelling of the spheric inclusion bodies gives a signal corresponding to the area of the body.

3 Analysis of viability of Escherichia coli cells

3.1 Earlier investigations

While working with microorganisms one will sooner or later encounter the large questions of life and death. To what extent does the population die? Why and when in the process? Do the cells lyse, and if not, are they dead or are they just resting? Of course this phenomenon has been discussed before, and will be again, for the obvious reason that this is vital for the process and the production.

In her work with high cell density fed batch cultures of *E. coli* Lena Andersson investigated growth patterns and noticed a sudden and massive drop in colony forming units (cfu). This phenomenon was shown to occur in cultivations with plasmid-free cells as well as both induced and non-induced plasmid-bearing cells, but at different time-points (Andersson et al. 1996). This characteristic drop is often found in fed-batch processes for production of recombinant proteins and sometimes interpreted as cell death (Andersson et al. 1994).

Other investigations showed that this decline in viable count was suppressed by fluctuations in glucose concentration in scale-down and large-scale reactors (Enfors et al. 2001; Rozkov et al. 2001). When using flow cytometry to analyse viability by staining with propidium iodide a corresponding drop in viability was observed in high cell density fed-batch cultures, and also this reaction was suppressed by gradients in large scale and scale-down reactors (Hewitt et al. 2000; Hewitt and Nebe-Von-Caron 2001).

3.2 Viable but not culturable cells

Many gram-negative bacteria has been shown to adapt a viable but not culturable (VBNC) state when exposed to non-favourable, stress-evoking environments (Colwell 2000). This is often referred to as a strategy of survival of the cell. When in the VBNC state, bacteria are no longer culturable on conventional growth media, but the cells display active metabolism and respiration (Rahman et al. 1994), membrane integrity (Lloyd and Hayes 1995) and gene transcription with specific mRNA production (Lleo et al. 2000), and they present cell wall modifications that may

be interpreted as a cell protection mechanism in unfavourable environmental conditions (Signoretto et al. 2002).

Chemical induction of a segregation to non-dividing state was demonstrated by Lengeler (Lengeler 1980). The author showed that the antibiotic streptozotocin rapidly and irreversibly inactivated the dividing capacity in bacteria containing the phosphenolpyruvate dependent carbohydrate phosphotransferase systems (PTS), while respiration was maintained and the cytoplasmic membrane remained impermeable. Also synthesis of RNA and proteins continued together with the uptake of carbohydrates and amino acids by diffusion or active transport. But the cells rapidly lost their ability to take up carbohydrates with the PTS systems and to synthesize inducible enzymes, e.g. β-galactosidase.

3.3 Adaptive response to changes in growth rate (The general and the stringent stress responses)

In gram-negative bacteria like *E. coli* the general stress response is regulated by the σ^S (σ^{38}, RpoS) subunit of RNA polymerase (Schweder and Hecker 2004). The *rpoS* gene and guanosine 5'-diphosphate 3'-diphosphate (ppGpp) have been implicated in the activation of the VBNC state (Boaretti et al. 2003). The shift from logarithmic to stationary growth phase causes the activation of the expression of *rpoS* (Schweder and Hecker 2004) and a large number of genes involved in the response to severe stress are regulated by the RpoS protein (Booth 1999). When *E. coli* cells in a minimal medium are starved of carbon, over 50 proteins are synthesized (during several hours), of these are 32 proteins regulated by RpoS (Booth 1999). The level of the RpoS protein is low in exponentially growing cells, but increases by stressing conditions in the environment such as nutrient starvation, osmotic stress, and high or low temperature (Schweder et Hecker). The expression of RpoS is necessary for the cells to be able to tolerate many different environmental stresses. In *E. coli* the synthesis of RpoS has been shown to be positively controlled by ppGpp. (Booth 1999) The ppGpp nucleotide functions as an alarmone in the (amino acid) starvation activated stringent response, which is related to a reduction in growth rate, ribosomal synthesis and cell size (Lengeler and Postma 1999). In growing cells the main object for the stringent response is to balance the synthesis of rRNA and tRNA with the growth rate (Booth 1999).

3.4 Flow cytometry analysis of cell concentration and viability

In microbial processes dry cell weight (dcw) of cell mass is the most often used measure of cell concentration. The laboratory work of dcw is time-consuming and, the result will not be available until the next day. The benefit of at-line analysis is obvious, but furthermore cell concentration measurements with flow cytometry gives a good statistical basis since a large number of cells are counted. The Partec volume based particle counter method, "true volumetric absolute counting", which was used in these studies, also has the advantage that it requires no calibration.

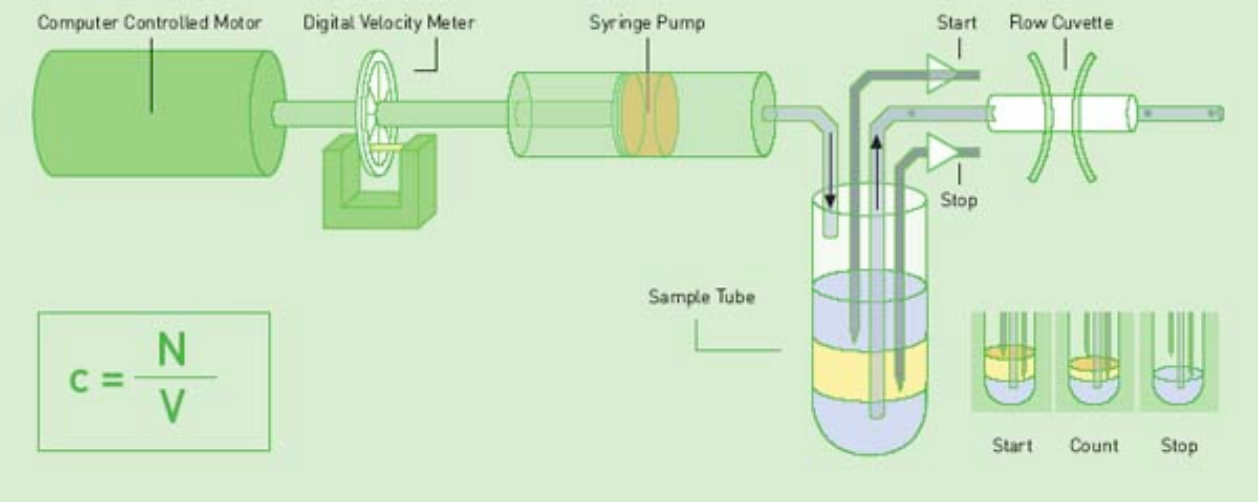


Fig. 16. The Partec volume based particle counter method is based on measurement of particles in a fixed sample volume determined by two electrodes. Figure reprinted with kind permission of Partec GmbH.

The classical method for analysing microbial viability is plating and counting of colony forming units (cfu). Apart from the fact that viability and the ability to divide are different properties, the analysis is very time- and staff consuming, and it can also be uncertain, meaning that cells that divide in one environment not always do so in another. When using flow cytometry other properties than reproduction capacity can be chosen as a measure of viability.

3.5 Present Investigation (I)

In a fermentation process for production of the recombinant protein promegapoeitin, PMP, viable count was compared by the classical method of count of colony forming units, cfu, and count of propidium iodide, PI, stained cells by flow cytometry. In the flow cytometry method cells were

assumed to be viable when not stained with PI. The basis for this assumption is that cells, which allow PI to enter the cytoplasm and attach to DNA, have permeable membranes and must be dead, while PI can not enter through an intact membrane (Hewitt and Nebe-Von-Caron 2004). Bis-(1,3-dibutylbarbituric acid) trimethine oxonol, BOX, accumulates in cells with depolarised membranes by binding to lipid-rich intracellular compounds. It is assumed to indicate a pre-stage to cell-death since it enters cells with deteriorated membrane potential (Hewitt and Nebe-Von-Caron 2004). In this study, PI and BOX-staining during *E. coli* fed-batch culture showed low amount of dead cells (PI-stained) and also very low, but slightly higher, amount of BOX-stained cells (fig.17).

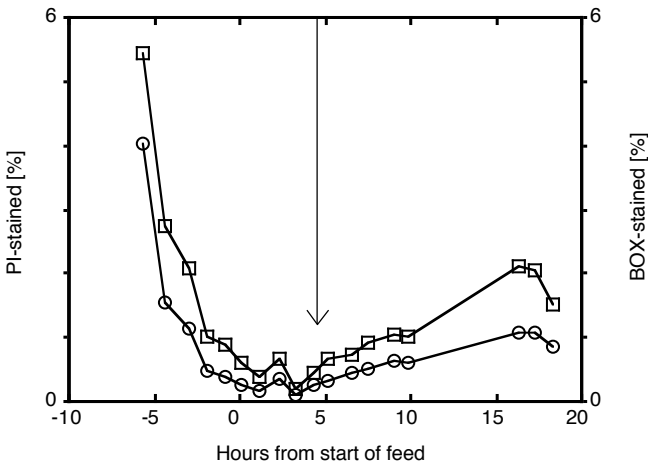


Fig. 17. PI- (squares) and BOX- (circles) stained cells in promegapoeitin producing fed-batch culture of *E. coli*. Shows that about 2% of the cells had depolarised membranes and and 1% were dead in the end of the culture.

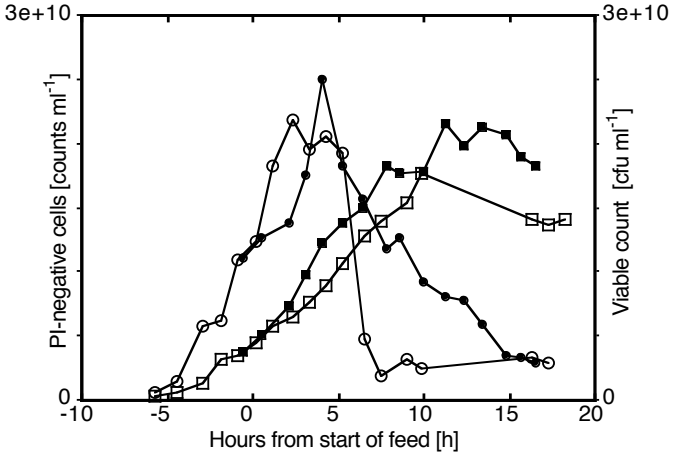


Fig. 18. Viable count by cfu (circles) and PI-negative cells (squares) in two promegapoeitin producing fed-batch cultures of *E. coli*. Filled symbols refer to one of the cultures and unfilled symbols to the other.

To investigate the sudden drop in viability reported in earlier work, the development of cfu and cells not stained by PI, PI-negative cells, were compared. While viable cells according to cfu decreased to about 10-15% of the concentration at induction, the amount of viable cells continued to increase for several hours according to the flow cytometry measurements (fig. 18). The drop in cfu correlated in time with the induction of protein production (fig. 19). Induction of the PMP production was obtained by adding nalidixic acid to the culture. Nalidixic acid is known to inhibit DNA synthesis, but in this case the number of cells according to flow cytometry count, and also dcw continued to increase.

It was also shown that the production of PMP continued at least ten hours after induction. The amount of PMP produced per viable cell mass according to the two viability analysis methods was calculated and compared with the amount of PMP produced per dcw (fig. 20). This revealed that the PMP concentration was 3-4% of the total viable mass if related to the number of PI-negative cells. However, if the total viable mass was calculated from the number of cfus the PMP concentration became as high as 40-55%, which is unrealistically high.

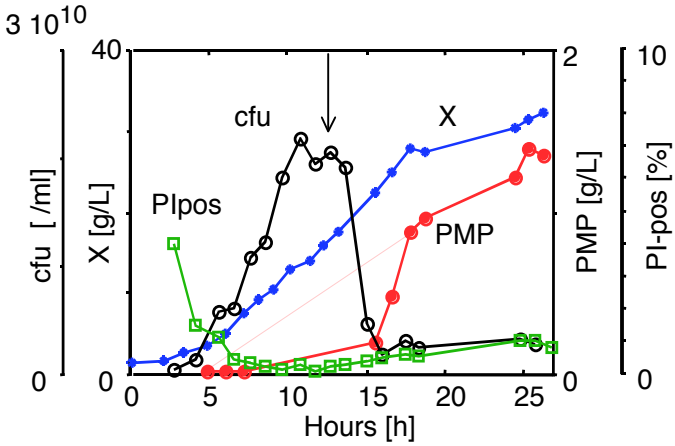


Fig. 19. Colony forming units (cfu), percentage of cells stained by PI (PIpos), cellmass (X) and recombinant protein produced (PMP) in fed-batch culture of *E. coli*. Induction at arrow.

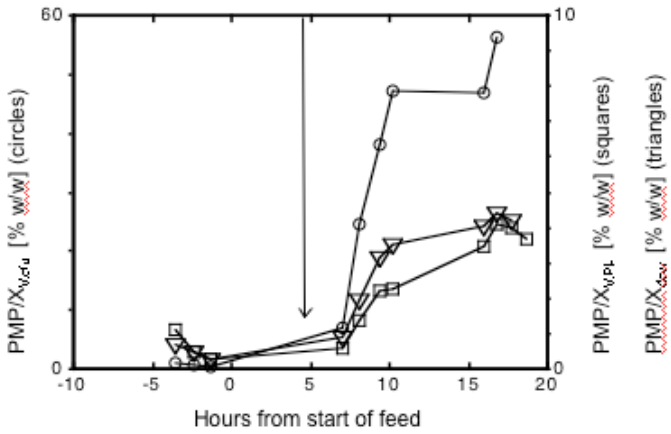


Fig. 20. Recombinant protein concentration per cell mass. Cell mass calculated according to cfu analysis ($PMP/X_{V,cfu}$ circles) and PI-staining ($PMP/X_{V,PI-}$ squares), compared to dry cell weight (PMP/X_{dcw} triangles) in promegapoeitin producing fed-batch culture of *E. coli*. Induction at arrow.

Eventually, the distribution of PMP in the cells was investigated by flow cytometry analysis of the intracellular inclusion bodies. The hypothesis was that if the total amount of PMP would have been produced by the low amount of cells surviving the induction, as indicated by the count of cfu, there would have been a large proportion of cells not producing PMP.

Figure 21 shows a test where cells with (test5) and without (test1) PMP was labelled with primary and secondary fluorescent antibody and then analysed by flow cytometry. The middle sample is a mixture of the cells with and without PMP. The monitoring of inclusion body formation during a cultivation can be seen in figure 22. The small population to the left in the histograms 3 to 5 consists of the non-producing cells. This population amounted to about 3 % of the total cell number. Hence, it was shown that almost all cells in the population (≈97%) contained PMP, and furthermore total protein analysis of the medium indicated that only about 1% of the population had lysed. This confirms that the "non-viable" cells according to viable count by cfu analysis

produced product. Therefore, they were viable, and producing, but could not divide on the agar plates.

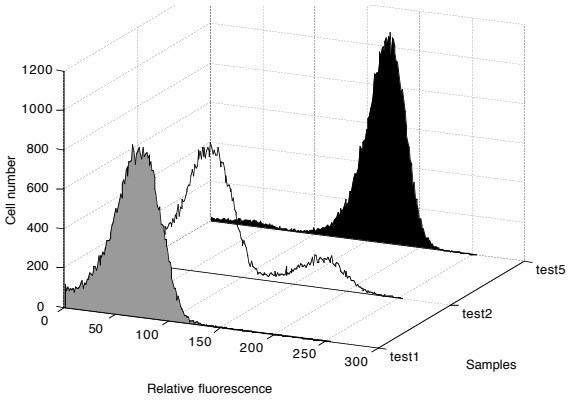


Fig. 21. Flow cytometry analysis of intracellular PMP inclusion bodies stained with fluorescent antibodies.
Sample 1: negative control of cells lacking PMP;
Sample 2: Mixture of cells containing PMP and negative control cells;
Sample 3: Cells from the end of the process.

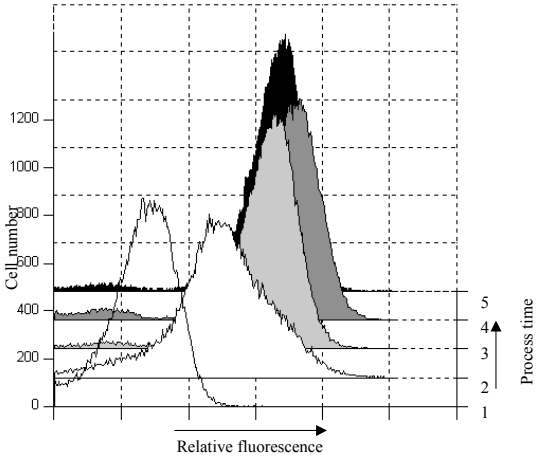


Fig. 22. Flow cytometry analysis of intracellular PMP inclusion bodies stained with fluorescent antibodies.
Sample 1: negative control of cells lacking PMP;
Sample 2: 4.6 h before induction;
Sample 3: 3,7 h after induction.
Sample 4: 7.2 h after induction.
Sample 5: 10.7 h after induction.

By using flow cytometry for population heterogeneity analysis it was possible to show that cells incapable of dividing on agar plates after induction continued to grow and to produce the induced recombinant protein.

4 SOFTWARE SENSORS FOR FERMENTATION PROCESSES

4.1 INTRODUCTION

4.1.1 Monitoring of biomass and growth rate

A software sensor, based on the on-line estimation of the ATP production rate from the oxygen uptake and the lactic acid production rate, was developed to determine the total energy requirement of animal cells in cultivation (Dorresteijn et al. 1996). Graindorge *et al*. built a software sensor for on-line estimation of substrate concentration and biomass based on a redox probe and used it to control substrate level in *Thiobacillus ferrooxidans* cultures (Graindorge et al. 1994).

Estimation of the specific growth rate (μ) based on pH titration has been presented in several cases (San and Stephanopoulos 1984; Iversen et al. 1994; Castrillo et al. 1995; Vicente et al. 1998). Such sensors are quite generally applicable for *E. coli* and *S. cerevisiae* cultures on defined media. The sensor is based on the assumption that the ammonia used for pH control is converted to biomass with a (relatively) constant yield of protons per consumed ammonium ion. Eriksen *et al*. used on-line estimation of biomass concentration and specific growth rate based on addition of NaOH to automate inducer addition and harvest of recombinant *E. coli* cultures (Eriksen et al. 2001). Liu *et al.* could estimate the biomass concentration on-line in *Zymomonas mobilis* fermentation by using the pH control and an implemented software sensor (Liu et al. 2001).

4.1.2 Monitoring of stress responses in recombinant fermentation processes

The use of software sensors to monitor stress responses is not commonly reported, but increased respiratory activity has been used as a measurement of metabolic burden from recombinant protein production (Schmidt et al. 1999; Hoffmann and Rinas 2001).

Fusions with a reporter protein such as green fluorescent protein, GFP, are applied in studies to enable monitoring of promoter regulation associated with stress on-line (Reischer et al. 2004; Nemecek et al. 2007). An array of chemical gas sensors, also know as an electronic nose, to monitor the off-gases from a bioreactor, in combination with pattern recognition methods has been used to predict metabolite and biomass concentrations (Mandenius 1999). This approach

was also shown to be useful for detection of the metabolic burden during fermentation processes (Bachinger et al. 2001).

4.2 Present investigation (III)

In this work the following software sensors were studied:

Online data processing of the ammonia reservoir weight may make biomass (X_{NH3}) and specific growth rate (μ_{NH3}) calculations applicable on-line. They can for example be useful in processes where induction at a certain biomass concentration or growth rate is wanted.

A software sensor for oxygen transfer coefficient K_LaC^* has been presented earlier (Castan and Enfors 2000). It was then shown that the sensor well described the increased viscosity caused by cell lysis. Since foaming may change K_LaC^* the software sensor was expected to also respond to foaming.

The stoichiometric coefficient $R_{O/S}$ describes the oxygen per glucose consumption ratio and it may provide data that are related to the degree of stress, for instance after induction, or at the end of the process before stress responses like the stringent response sets in.

4.2.1 Software sensor models

The model equations used in this work are summarised in Table 1, the models are then explained and the work is discussed further under each headline below.

Table 1. Models for the software sensors

Biomass and growth rate:

$$X_{NH3} = X_i + \frac{Y_{X/NH_3} C_{NH_4OH} \int F_{NH4OH} dt}{V} \left[g\, L^{-1} \right]$$

$$\mu_{NH3} = \frac{F_{NH4OH}}{\int F_{NH4OH} dt} \left[h^{-1} \right]$$

X_i: inoculum; $Y_{X/NH3}$: yield biomass per proton; C_{NH4OH}: concentration of ammonia feed; F_{NH4OH}: Feed rate of ammonia solution;

Oxygen transfer capacity:

$$K_L aC^* = \frac{Q_{in}C_{O2,in} - Q_{out}C_{O2,out}}{V100}\frac{1000}{V_m}\frac{DOT^*}{DOT^* - DOT}\left[mmol\ L^{-1}h^{-1}\right]$$

Q: air flow rate; C_{O2}: oxygen concentration; C_{CO2}: carbon dioxide concentration

C_w: water vapour concentration; V: medium volume; V_m: molar volume of oxygen; DOT: dissolved oxygen tension; DOT*: DOT in equilibrium with the air bubbles = 100 $C_{O2,in}/C_{O2,o}$

Coefficient of oxygen per substrate:

$$R_{O/S} = \frac{Q_{in}C_{O2,in} - Q_{out}C_{O2,out}}{F_S C_{S,in}}\frac{1}{100}\frac{M_S}{V_m}\left[mol\ mol^{-1}\right]$$

Q, C_{O2}, and V_m as above; F_S: Energy substrate feed rate; C_S: Energy substrate concentration in feed; M_S: Energy substrate molecular weight.

Values of constant parameters in the models are given in paper III.

4.2.1.1 Biomass concentration (X_{NH3}) and specific growth rate (μ_{NH3})

When ammonium ions are used by the cells as nitrogen source, one proton is excreted for each ammonium ion that is taken up. The stoichiometry of aerobic growth on a carbohydrate and ammonia based medium can be written as:

$$a\ (CH_2O) + b\ O_2 + \mathbf{c}\ \mathbf{NH_4^+} \longrightarrow d\ C_\alpha H_\beta O_\gamma \mathbf{N_\delta} + e\ CO_2 + f\ H_2O + \mathbf{c}\ \mathbf{H^+} \qquad (1)$$

Each mole of protons released corresponds to one mole of hydroxide (NH_4OH) consumption through titration. The concentration of produced cells is thereby related to the ammonium hydroxide consumption, which is obtained from the titration data. Hence, biomass can be calculated as:

$$X_{NH_3} = X_i + \frac{Y_{X/NH_3}C_{NH_4OH}\int F_{NH4OH}\,dt}{V} \qquad (2)$$

where X_i is the inoculum and $Y_{X/NH3}$ is the ratio d/c in equation 1.

The specific growth rate based on ammonia titration, μ_{NH3}, was in this work calculated as:

$$\mu_{NH3} = \frac{F_{NH4OH} C_{NH4OH} Y_{X/NH_3}}{(XV)_i + C_{NH4OH} Y_{X/NH_3} \int F_{NH4OH} dt} \quad (3)$$

This equation was derived in paper III. Included in equation 3 is a term $(XV)_i$ that represents the inoculum, which can be expressed in ammonia consumption units.

$$k_a = \frac{(XV)_i}{C_{NH4OH} Y_{X/H^+}} \quad (4)$$

Sensivity analyses were performed (not shown), which showed that the term k_a did not influence the calculations during the conditions prevailing in this investigation. Consequently, the calculation of μ_{NH3} could be simplified to:

$$\mu_{NH3} = \frac{F_{NH4OH}}{\int F_{NH4OH} dt} \quad (5)$$

Under other circumstances, when larger inoculums are used, the term k_a should be included in the calculation.

4.2.1.2 Oxygen transfer capacity ($K_L aC^*$)

The oxygen transfer rate (*OTR*) is usually described with the model $OTR = K_L a(C^* - C)$ where K_L is the mass transfer coefficient and *a* is the total bubble area per medium volume, both derived from the oxygen flux over the gas-liquid interface. K_L describes the diffusivity of the gas over the liquid boundary layer (D/δ). The difference in concentration between the gas-liquid interface (C^*) and the bulk liquid outside the film (*C*) is the driving force of the diffusion. The model shows that the maximum *OTR* is achieved when $C=0$, i.e. it is $K_L aC^*$. Assuming there is no significant rate of accumulation of oxygen in the gas phase above the liquid surface in the reactor, i.e normal process conditions are prevailing, the gas-liquid oxygen transfer rate equals the oxygen balance over the reactor:

$$K_L a(C^* - C) = \frac{Q_{in} C_{O2,in} - Q_{out} C_{O2,out}}{V} \quad (6)$$

This equation was further developed in paper III to show that the *OTR* capacity can be obtained from the measurements of outlet O_2 and *DOT*:

$$K_L aC^* = \frac{Q_{in}C_{O2,in} - Q_{out}C_{O2,out}}{V}\frac{DOT^*}{DOT^* - DOT} \quad (7)$$

The inlet airflow rate (Q_{in}) is usually measured with a massflow meter, but the outlet airflow rate (Q_{out}) is usually not analysed. In many cases it is the same as the inlet flow, but if the volumetric CO_2 production differs from the volumetric O_2 consumption, the flow rates are different. Also the water in the outlet airflow may contribute more than the water in the inlet. To account for these circumstances the outlet gas flow rate can instead be obtained from a mass balance of nitrogen gas:

$$Q_{out} = Q_{in}\frac{100 - C_{O2,in} - C_{CO2,in} - C_{W,in}}{100 - C_{O2,out} - C_{CO2,out} - C_{W,out}} \quad (8)$$

The *DOT** in equation 7 is obtained from

$$DOT^* = 100\frac{C_{O2,out}}{C_{O2,in}} \quad (9)$$

Application of equation 9 assumes ideally mixed air bubbles in the liquid phase, which is a reasonable approximation for small well-mixed reactors.

4.2.1.3 The $R_{O/S}$ coefficient

The software sensor describing the oxygen consumption per energy substrate consumed, the $R_{O/S}$ sensor, is calculated as oxygen consumption rate (OCR) through substrate (in this case glucose) consumption rate (GCR).

$$R_{O/S} = \frac{OCR}{GCR} = \frac{Q_{in}C_{O2,in} - Q_{out}C_{O2,out}}{F_S C_{S,in}}\frac{1}{100}\frac{M_S}{V_{O2,m}} \quad (10)$$

This calculation was further described in paper III. On the condition that no significant change in the medium glucose concentration in the reactor occurs, the feed rate may represent the substrate consumption rate. This is usually true during the quasi-steady state conditions of a fed-batch culture.

4.2.2 Biomass concentration (X_{NH_3}) and specific growth rate (μ_{NH_3})

The software sensors X_{NH3} and μ_{NH3} explore the fact that when cells utilise ammonium salts as source of nitrogen it is possible to calculate the growth from the titration of ammonia. The stoichiometry for cell growth on ammonia shows that each ammonium ion used for producing biomass results in one proton (eq.1.). These sensors are restricted by the limitation that there cannot be any other acids or bases involved in metabolism, that is, the sensors are correct when cultivating on minimal media using ammonia for titration, but not during the batch phase when by-products as acetic acid might be produced. Still, the sensors might be useful also in cultivations with enriched media, displaying fingerprints of the process if not the true value of specific growth rate or biomass. Biomass was calculated according to equation 2 during cultivation of *E. coli* and the resulting variable X_{NH3}, shown in figure 23, corresponded well to dry cell weight data.

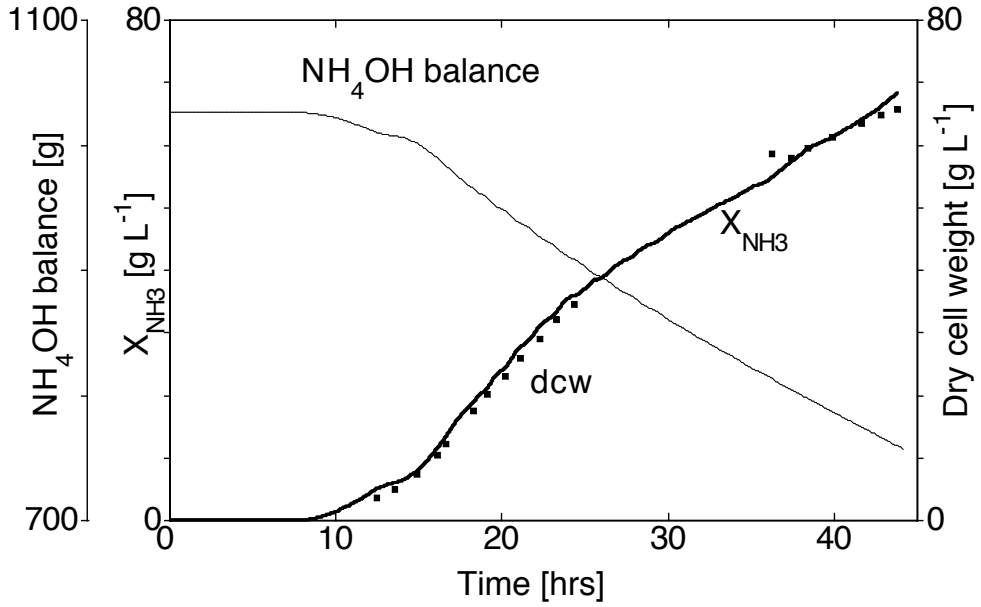


Fig. 23. The biomass sensor (X_{NH3}, continuous line) applied in an *E. coli* fed-batch cultivation on minimal medium and the biomass concentration analysed as dry cell weight (dcw, squares). The original data signal for the calculation, the NH_4OH vessel balance signal is also shown. Feeding started with an exponential feed at 12.6 h and continued with a constant feed from 16.5 h.

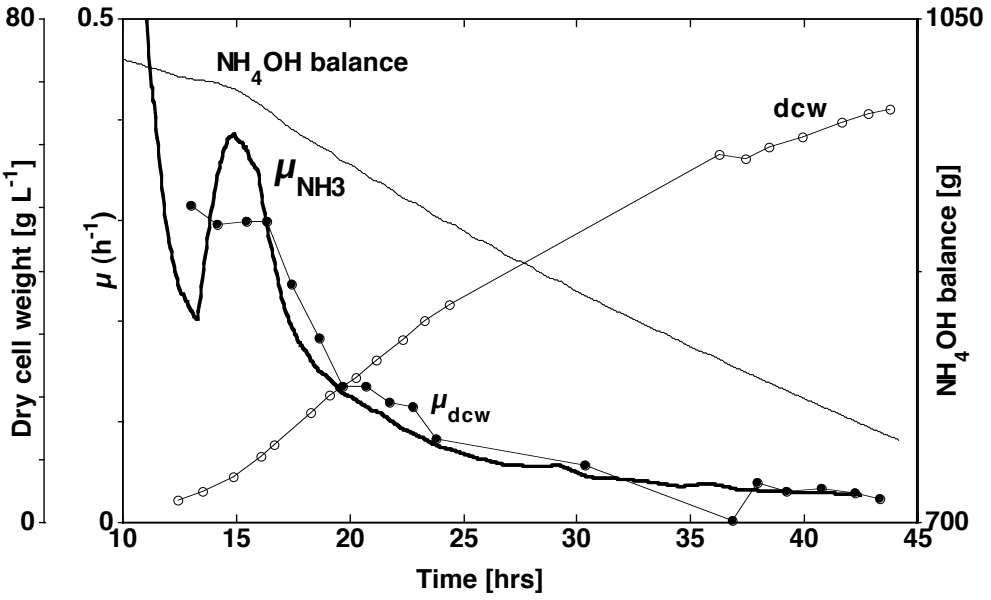


Fig. 24. Comparison between the μ_{NH3} sensor signal (thick continuous line) and μ calculated on dry cell weight data (-●-) from an *E. coli* fed-batch cultivation on minimal medium. Also shown are the measured data for the μ_{NH3} calculation, i.e. the NH_4OH vessel balance signal (thin continuous line) and the cell dry weights (-○-). An exponential glucose feed was started at 12.6h and from 16.5 h this feed was constant.

The calculation of μ_{NH3} is based solely on the signal from the balance under the ammonia vessel. In figure 24 the software sensor μ_{NH3} from an *E. coli* cultivation is compared with μ calculated from dry cell weight. The μ_{NH3} signal fits the μ-calculation from off-line values well and gives a smoother curve. This is probably due to small errors in dcw raw data, which are enlarged at calculation.

A great advantage with these software sensors for calculation of biomass concentration and specific growth rate is that they provide continuous data without any manual analyses during the process. A limitation is the calculations at low biomass, which becomes uncertain since the NH_4OH-solution will be titrated with relatively large time intervals at very low growth rate. The already mentioned restriction that other acids or bases can interfere, means that true values of specific growth rate and biomass can be obtained only in the substrate limited feed phase.

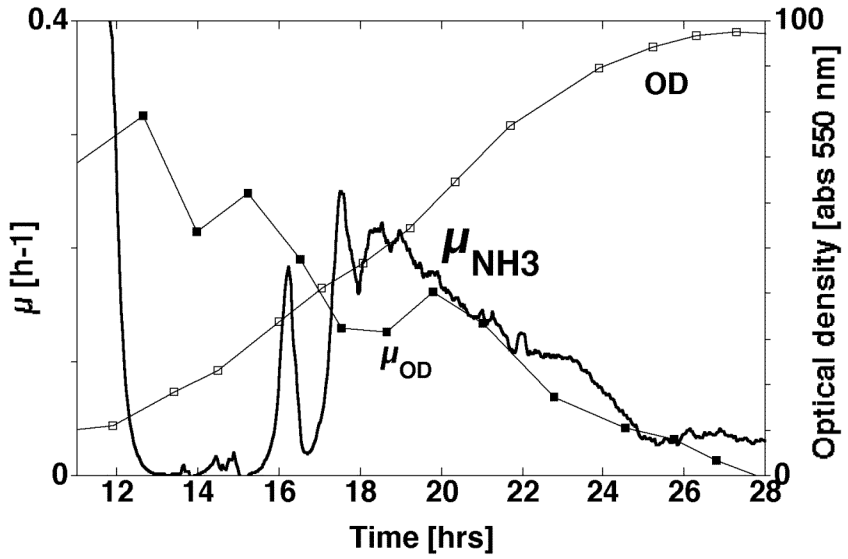


Fig. 25. The μ_{NH3} sensor signal from a cultivation of *E. coli* using a complex medium with yeast extract, compared to μ calculated from OD_{550} measurements. Glucose feeding was started at 13.5 hrs. Point of induction was at 18 hrs.

Figure 25 shows the μ_{NH3}-sensor applied in a cultivation of *E. coli* in a medium supplemented with yeast extract. In this case the μ_{NH3} tuned in with μ calculated from OD (μ_{OD}) after the induction at 18 hrs, but the signal did not agree with the calculation of μ_{OD} during the batch phase, or in the beginning of the feed phase. A probable explanation to this can be an initial production and consumption of acetic acid, and metabolism of other acids and bases originating from the rich medium. If this is the case the μ_{NH3}-sensor may still be used as a fingerprint of the process when using complex media, even if it does not show a true value of specific growth rate.

4.2.3 Oxygen transfer capacity (K_LaC^*)

The calculation of K_LaC^* relies on the analysis of oxygen and carbon dioxide content in the outgoing gas, the gas flow rates and the DOT-signal (eq. 7). K_LaC^* was calculated during cultivations of *E. coli* on minimal medium. Figure 26 shows the signal during the constant feed phase. The arrows marked **a** indicate expected responses in K_LaC^* due to increases in stirring (from 500 to 700 rpm at the first occasion and from 700 to 800 rpm the second time). At the time-point marked with arrow **c**, a gradual, but substantial increase of the signal started, which was not provoked by any changes in the process control. At the same time a distinct volume-increasing foaming was observed. Froth, which in contrary to surface foam, is an effect of increased dispersion of the bubbles. More bubbles give an increased gas/liquid interface area for diffusion, and it is the *a* in K_LaC^* that increases. After providing a stable signal for about ten hours, K_LaC^* reacts explicitly to the additions of an anti-foam agent (arrows **d**). Anti-foaming agents act by inducing bubble coalescence, thereby reducing the specific interface area *a*.

In his thesis from 2001 Andreas Castan showed that a sudden, and apparently un-provoked, decrease in *KLaC** was due to cell lysis. At higher viscosities both K_L and a in K_LaC^* decreases. This is due to that the boundary layer which oxygen has to diffuse through increases, and also that the bubbles become larger which decreases the total gas/liquid interface for diffusion. Another possible use of the K_LaC^* sensor might be to monitor the increased viscosity caused by filamentous microorganisms or polysaccharide production.

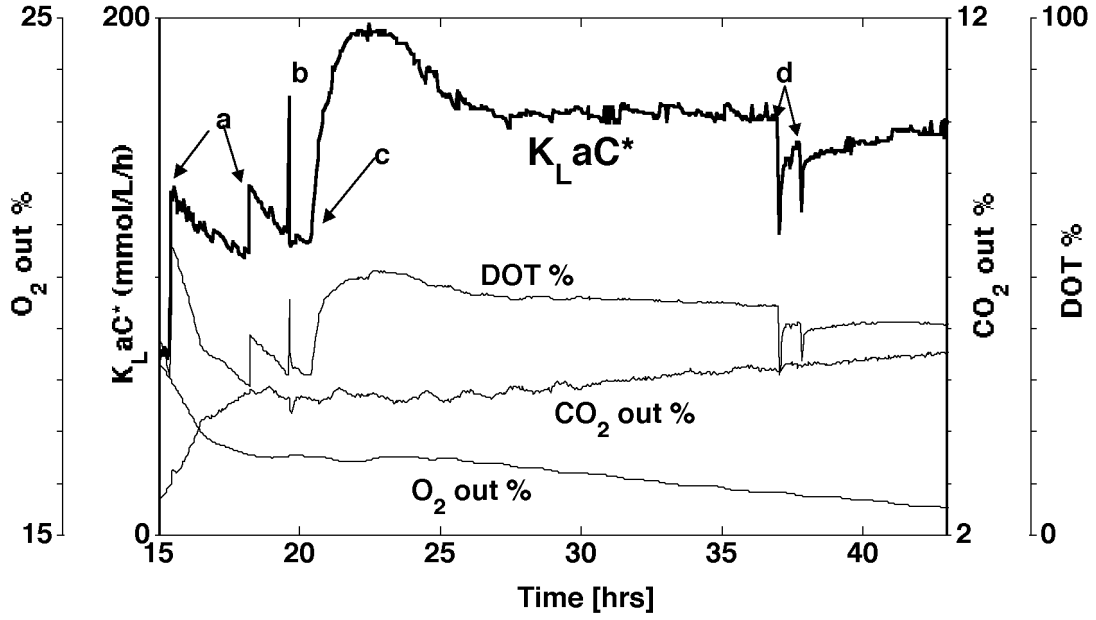


Fig. 26. The signal of the K_LaC^* sensor during an *E. coli* cultivation on minimal medium. Changes in K_LaC^* represents: *a* – increase of stirrer speed, *b* – adjustment of feed hose, *c* – development of froth in the medium, *d* – antifoam additions. Also shown are the input data for K_LaC^* calculation: DOT, $CO_{2,out}$ and $O_{2,out}$. Constant feed started at 16.5 hrs.

During certain conditions the difference between DOT* and DOT is small, implying that measurement errors will have a great impact in those cases. The same applies for the oxygen content in the outgoing gas which, for instance in the beginning of a culture, can have a value close to the inlet value resulting in large errors. This occurs at very low oxygen consumption. Also, DOT* can be difficult to estimate. DOT* is the partial pressure of dissolved oxygen in the liquid, which is in equilibrium with the oxygen concentration in the air bubble, meaning it is not identical to the value calibrated as 100% when the medium is saturated with air. In this work an ideal mixing of the bubbles in the reactor was assumed and therefore DOT* was obtained from the outlet oxygen analysis (eq. 9).

4.2.4 The $R_{O/S}$ coefficient

In fed-batch fermentations with constant glucose feeding the $R_{O/S}$ steadily increases, as can be seen in figure 27.

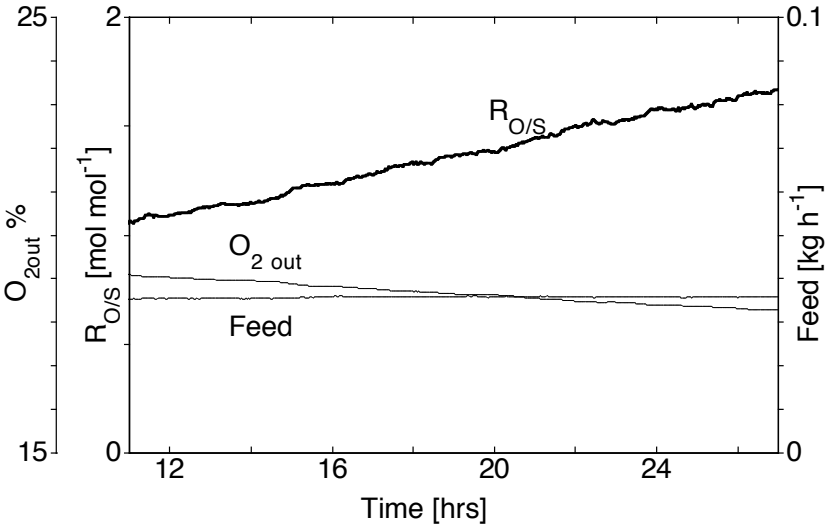


Fig. 27. Signal of the $R_{O/S}$ software sensor from an un-stressed fed-batch cultivation of *E. coli* with constant feed. Also shown are the substrate flow rate and the outlet oxygen concentration. The gas-flow rate was constant.

This can be explained by looking at the specific glucose consumption rate (fig. 29) and the part of energy metabolism constituting of maintenance in a simple illustration of the substrate flux (fig. 28). The glucose taken up by the cells is used both for cell growth (anabolism) and for energy metabolism. The maintenance part of the substrate uptake, is the part that does not result in new biomass. Maintenance is often explained as the energy required by the cell to keep concentration gradients over the cell membrane and for replenishing of proteins and other cell components that are degraded in the cell, but if extra-cellular products are secreted, this also becomes part of the maintenance. The maintenance coefficient, q_m, is assumed to be constant as long as no stress influences the cell. In a fed-batch cultivation with constant feed rate, as biomass increases, the degree of substrate limitation is constantly increasing, which results in a decreasing specific glucose uptake rate (q_S) (fig. 29). With declining q_S follows declining specific growth rate, μ. During non-limiting conditions only a small part of the energy metabolism constitutes of maintenance. For *E. coli* W3110 q_{Smax} is about 1.4 g g^{-1} h^{-1}, while q_m is about 0.04 g g^{-1} h^{-1} (Xu et al. 1999). But when q_S declines, the portion of the total energy metabolism allocated to maintenance increases if the maintenance coefficient is assumed to remain constant. This is illustrated in figure 28.

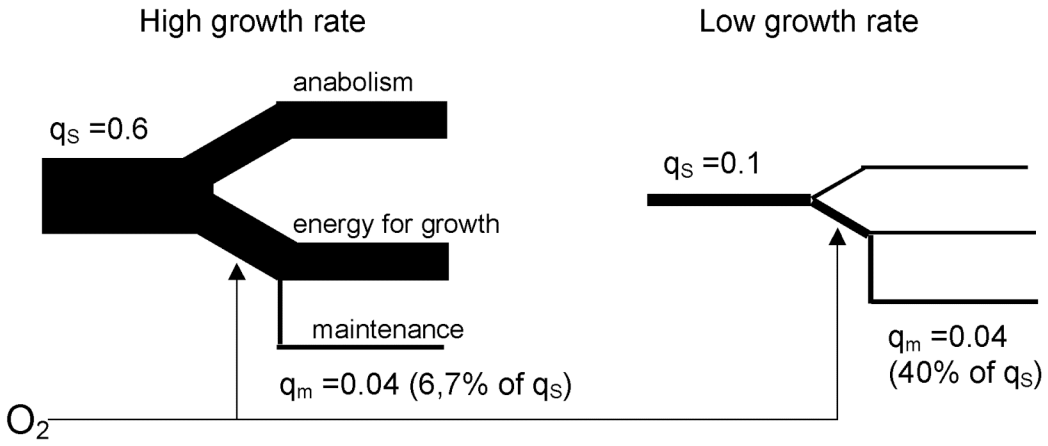


Fig. 28. Schematic illustration of the increasing oxygen per glucose consumption at declining growth rate in a fed-batch culture with energy-substrate limitation. Oxygen consumption is mainly connected to energy metabolism (respiration). At high specific glucose uptake rate, the flux to maintenance constitutes only about 6.7% in *E. coli* (left panel). If this flux to maintenance is prioritized at declining specific substrate consumption rate the demand for oxygen per consumed glucose ($R_{O/S}$) will be higher (right panel).

When the organism grows on a carbohydrate the molecular oxygen consumed from air is mainly used for respiration, while the cellular oxygen is derived from the carbon source. The oxygen consumption rate therefore becomes proportional to the part of the substrate flux that is used for energy metabolism (fig. 28). Since this flux takes a larger part of the total substrate uptake when q_S declines, the oxygen consumption per substrate consumption, i.e. $R_{O/S}$, increases in fed-batch

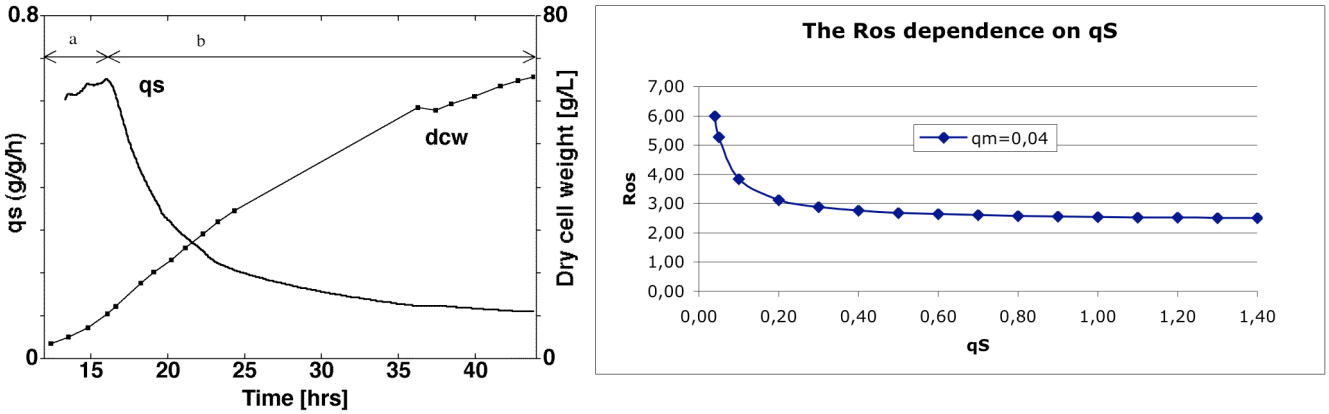


Fig. 29. Biomass concentration and specific glucose consumption rate in an *E. coli* fed-batch process with initial exponential feed with exponent 0.3 h^{-1} (a) followed by a constant feed rate (b). (left panel)
Calculation of the $R_{O/S}$ software sensor dependence on qS (right panel). Calculation assumes: 0.48 g carbon per g cells, qm=0.04, yield of cells per substrate excluding maintenance 0.5, 0.40 g carbon per g substrate.

processes with constant feed rate. This is also shown in a theoretical calculation in figure 29. If stress occurs forcing the cells to work harder to keep gradients and replenish necessary cellular components for survival, the maintenance demand increases, and $R_{O/S}$ should subsequently also increase.

So, the calculation of the stoichiometric coefficient of oxygen per substrate, $R_{O/S}$, depends on the substrate consumption rate, and since it responds to changes in the maintenance requirements, our hypothesis was that this software sensor could be used as an indicator of stress in the culture.

When stress was imposed on cultures similar to the unstressed culture in figure 27, by a sudden decrease in pH, the $R_{O/S}$ sensor responded with an increase in the signal as can be seen from figure 30. A raise of pH to 6 did not seem to lower the experienced stress much, but the signal did gradually return to the baseline after a restoration of pH to 7, see also figure 31.

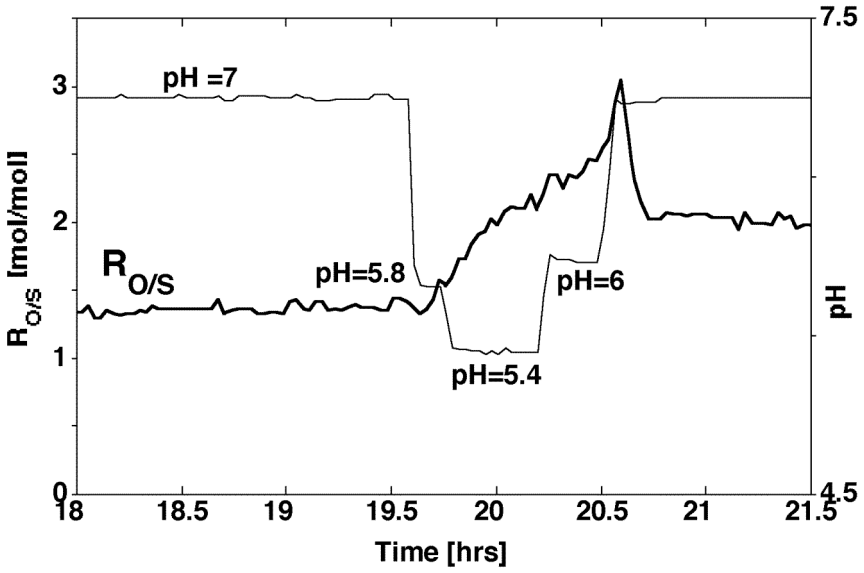


Fig. 30. $R_{O/S}$ software sensor from a cultivation with *E. coli* where stress was induced by decreased pH.

In figure 31 a stressed and an unstressed culture is compared. The signal response to pH-decrease is very clear and distinct. About three hours later the cells were exposed to another stress, the

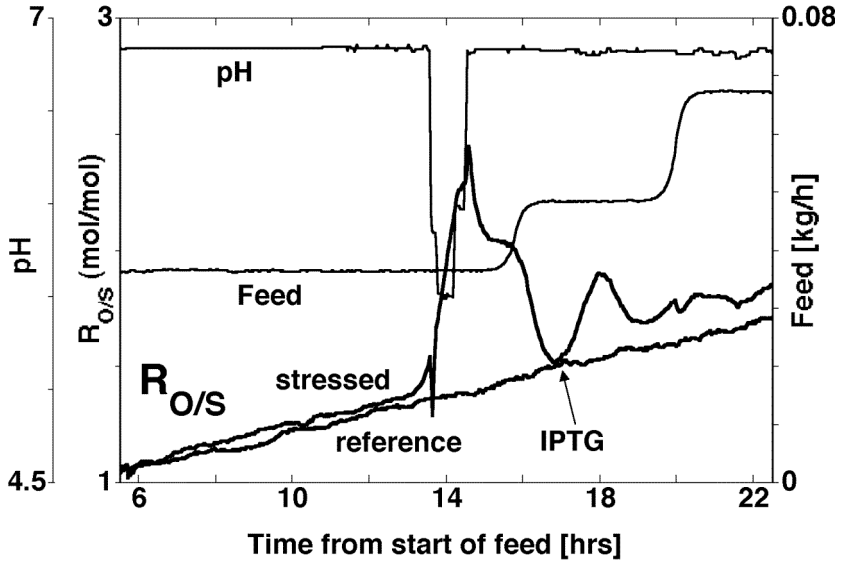


Fig. 31. The response of the software sensor $R_{O/S}$ in stressed and reference *E. coli* cultivations respectively. pH was shifted by addition of phosphoric acid. The arrow indicates addition of IPTG corresponding to 450 mM.

addition of isopropyl-β-D-thiogalactopyranoside (IPTG). The common lac-operon inducer IPTG also gave a transient response of the $R_{O/S}$ sensor, but the signal was not completely restored, maybe indicating that this stress did impose some sustained effect on the maintenance demand.

For the software sensor $R_{O/S}$ to be valid it is presumed that the substrate is consumed at the same rate as it is added, that is substrate limiting conditions prevail.

The software sensors X_{NH3} and μ_{NH3}, which utilises base titration data to estimate biomass and specific growth rate were shown to correlate well with the off-line analyses during cultivation of *E. coli* W3110 using minimal medium. In rich medium the μ_{NH3} sensor was shown to give a signal, which probably is influenced by metabolic shifts. Thus it may be useful as a fingerprint of the process, at least from the time of induction. The software sensor $K_La C^*$ was shown to respond to foaming in culture, which probably caused increased air bubble dispersion. The $R_{O/S}$ coefficient, which describes the oxygen to substrate consumption, was shown to give a distinct response to stress caused by lowered pH and addition of the inducing agent IPTG.

5 PARALLEL CULTIVATIONS

5.1 Multi-bioreactor systems

A bottleneck in the research on the correlation between analysis and process quality is the demand for large quantities of data and the relatively slow rate of fermentation processes. Several bioreactor-producing companies offer bench-scale multi-bioreactor systems for parallel fermentations. Such systems are suitable for rational experimental design through which statistically significant correlations between the measured variable and process quality parameters can be obtained. The combination of multi-bioreactor systems and on-line analyses is also suitable for process optimisation. In this way different batches of complex raw materials, e.g. yeast extract and hydrolysates, can be compared and evaluated in one experiment. Other parameters of interest for process optimisation with a multi-bioreactor system are feed-profiles, induction strategies and strain constructs.

Multi-bioreactor cultivations demand a high degree of automation since the work otherwise becomes very labour-intensive. Automation of fermentation processes puts a demand on individual monitoring and control of the reactors to enable for instance automated start of feed or induction. Software sensors can contribute to the automatic monitoring of such cultivations. The demand on individual control and monitoring of the reactors results in the production of very large data-sets that need to be processed for evaluation of the process.

In this study a commercially available six-unit bioreactor system with 400 ml initial volume per reactor was used. The system possesses a high degree of individual reactor control. Software sensors for determination of biomass (X_{NH3}) and growth rate (μ_{NH3}), both based on base titration data, and the stress sensor ($R_{O/S}$) was applied. Furthermore, based on the logged variables and the biomass software sensor several total and specific rates and the biomass yield coefficient were calculated. The system generates a primary log-file with 84 measured variables logged against process time, which after the additional calculations increases to 150-200 variables. A specific software was therefore developed to enable flexible data presentation.

5.2 Greta description

The parallel six-reactor system Greta is built up by two main units: one unit for preparation of medium and liquids for CIP (cleaning-in-place), and the reactor unit (fig. 32). Each bioreactor can be individually controlled with respect to the standard parameters depicted in figure 2.

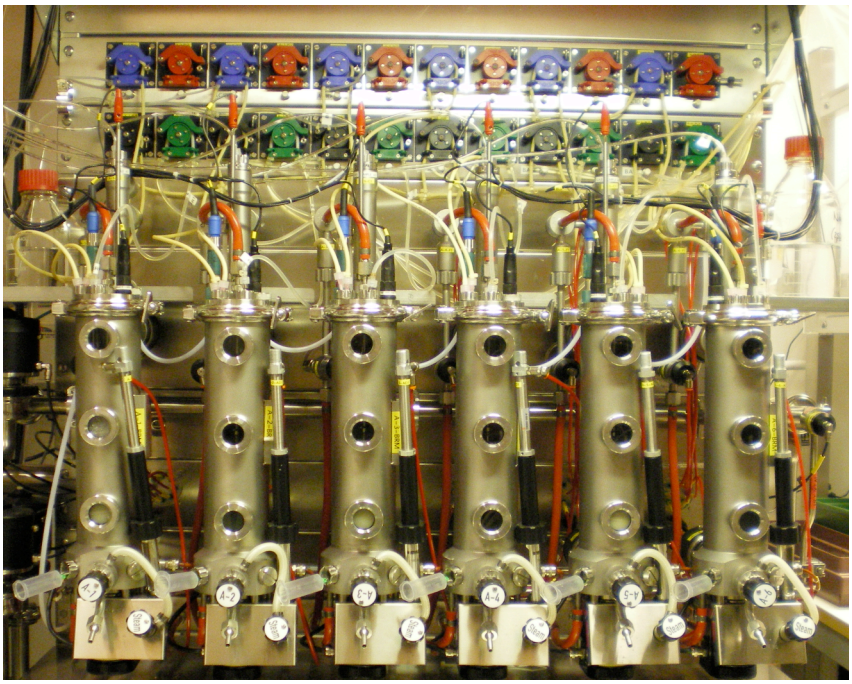


Fig. 32. The six parallel set-up of reactors

5.2 Evaluation and presentation of data (IV)

For evaluation and display of the large amount of logged and calculated variables from the system, two MATLAB (The MathWorks, Inc.) toolboxes have been developed at the department, SimPlot and Autoreport. The primary log-file from the multi-reactor system produces a data-matrix of 84 columns with a density of 1 row per second. This matrix was then extended with calculated variables with the SimPlot application. The matrix can be complemented with off-line data like product and cell concentration. Filtering of the balance data for calculation of flow rates was performed off-line with MATLABs forward and reverse digital filter *filtfilt.m*. For graphical presentation of the variables in different combinations the Autoreport toolbox was developed. Figure 33 shows an example of how a graphical report can be specified.

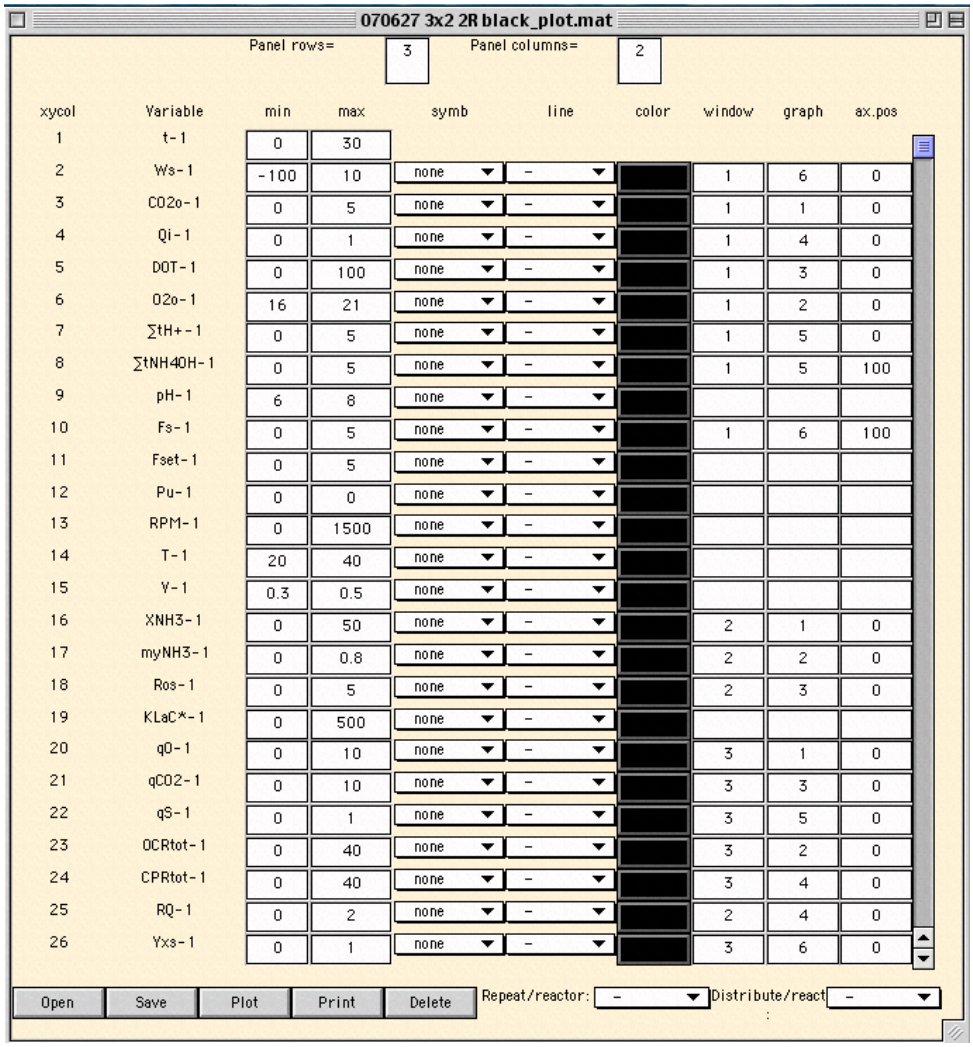


Fig. 33. The plot specification format of the MATLAB application Autoreport adapted for the cultivations performed in this study. The window displays the settings for one reactor at a time, here number 1. The example specification shown here results in plots in three different windows. Window 1 will display a panel of six graphs with logged data (fig. 34), window 2 will display four graphs plotting the software sensors (not shown) and window 3 will show plots of the total and specific rates, and yield (fig. 40), which were calculated for this *E. coli* fed-batch cultivation.

5.3 Present Investigation (IV)

The software sensors used in this study was presented in chapter 4. However, one important difference between the cultivations performed in papers I-III and in the manuscript IV, is the lack of a balance signal for the ammonia solution used for titration in the parallel cultivations. So in this case a logged variable representing the accumulated run-time of the titration pump was used instead of the signal from the balance in the calculations of biomass and specific growth rate. One expected use of this system is to examine the possibility to evaluate different media with parallel cultivations, so the first cultivations were made with both minimal and yeast extract enriched media.

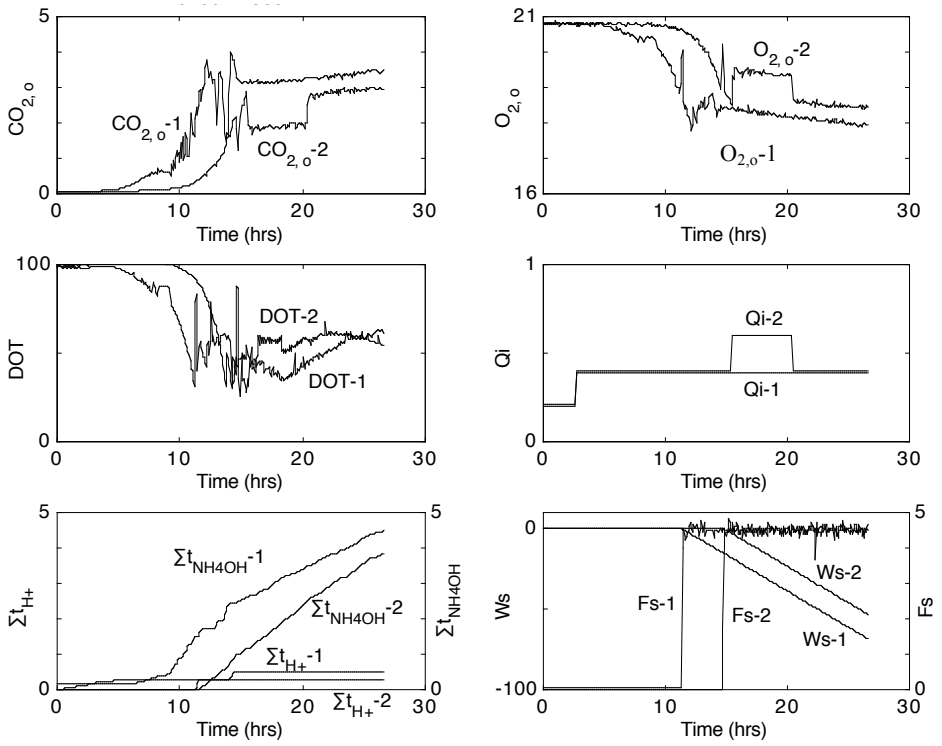


Fig. 34. A selection of the logged data from two of the six reactors of the multi-parallel system. Variables labelled with -2 represent minimal medium cultures of *E. coli* and variables labelled -1 represent cultures supplemented with 5g/L yeast extract.

In figure 34 a selection of the logged data from two of the six reactors is displayed. Bioreactor no. 1 was supplemented with yeast extract and bioreactor 2 was a cultivation on minimal medium. In the reactor supplemented with yeast extract the cells grew faster and the batched glucose was depleted earlier. The figure clearly demonstrate the different performance of the two cultivations.

Another way to present the data is to compare one variable for all reactors. Figure 35 shows an example of CO_2 in the outlet air. Although started with 20 times larger inoculum, the minimal medium cultures (reactors 2, 6) display a longer lag phase than the cultures with yeast extract (reactors 1, 3, 5). Reactor 5 was inoculated with 20 times larger inoculum then reactors 1 and 3, which results in shorter lag phase. Reactor 4 was not inoculated at all. In reactor 5 the feed was started at 9.95 hrs, at which time the initial amount of glucose was already depleted. In reactors 1 and 3 the feed was started at 11.35 hrs. In minimal medium cultivations in reactors 2 and 6, the feed was started at 14,7 and 15,3 hrs, respectively. Between 16 and 21 hrs the aeration in reactor 2 was elevated and this is reflected in the signal of carbon dioxide content in the outgoing gas flow.

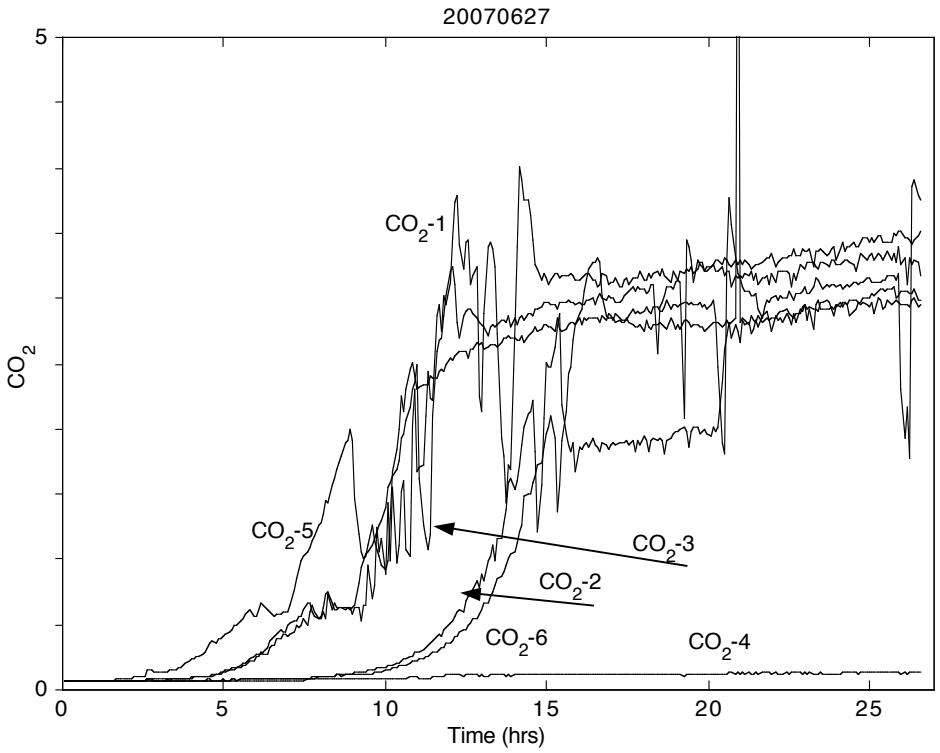


Fig. 35. Comparison of the CO_2 concentration in 6 cultivations with different media and inoculum size. Reactor 2, 4 and 6: minimal medium. Reactor 1, 3 and 5: yeast extract enriched medium. Reactor 2 and 6 (minimal media) and 5 (rich media) were inoculated with 20x larger inoculum than reactor 1 and 3 (rich media). Reactor 4 was not inoculated at all.

Since the parallel cultivations were performed with both minimal medium and yeast extract enriched medium, it was possible to compare the signals from the cultures with and without yeast extract.

When comparing the respiratory pattern in two cultures, one with and one without yeast extract (fig 36) the CO_2 production and O_2 consumption in the minimal medium produced smooth curves that were proportional (fig 36 left), meanwhile the corresponding curves for the yeast extract enriched medium included two short and transient intervals with an altered respiration pattern (fig 36, right), which probably reflects metabolic shifts.

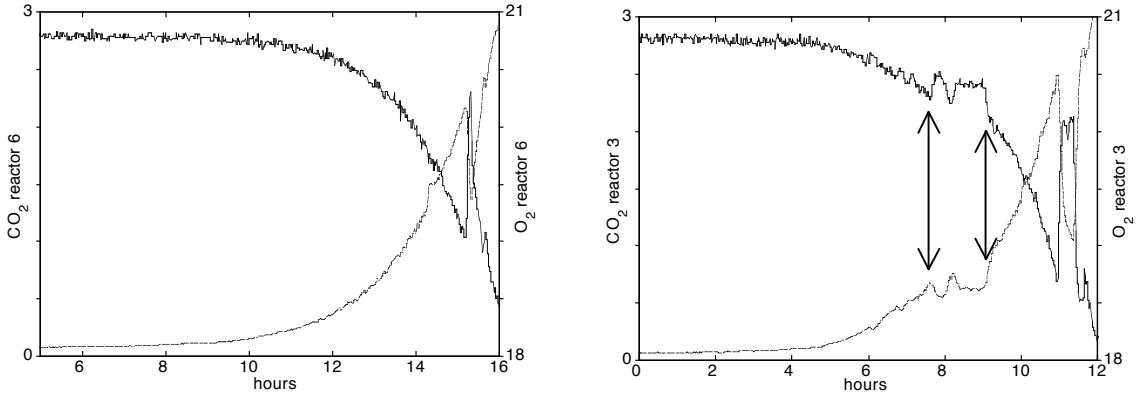


Fig 36. The respiration pattern in two parallel cultures with (right) and without (left) yeast extract is compared. Changed respiration rate in the yeast extract enriched medium was located at two periods of time (indicated by arrows).

The model for calculation of biomass was adjusted to use accumulated titration time rather than reservoir weight. The integral in equation 2 was obtained from the summed time of ammonia titration as

$$\int F_{NH4OH}\,dt = \Sigma t_{NH_4OH} \mathrm{P}_{NH_4OH/t} \qquad (11)$$

where $\sum t_{NH4OH}$ represents the accumulated run-time of the titration pump and $P_{NH4OH/t}$ describes the titration pump flow rate (grams of solution titrated per time).

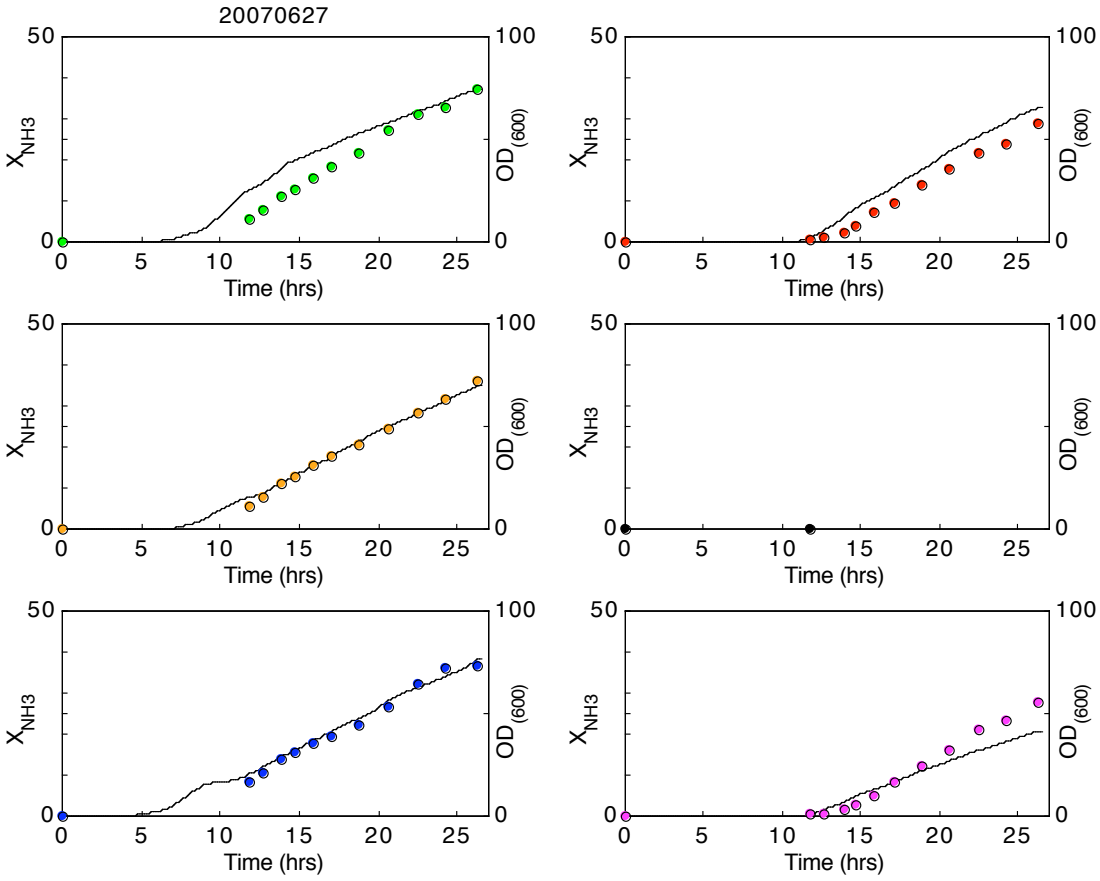


Fig.37. The software sensor calculating biomass, X_{NH3}, compared with OD_{600} measurements in six reactors. Reactors supplemented with yeast extract (1, 3, 5) in left column and reactors with minimal medium (2, 4, 6) in right column.

As can be seen from figure 37, where X_{NH3} and OD are plotted, the agreement between the software sensor and the OD analyses varies between the reactors. When the OD measurements were plotted together, the enriched medium cultivations and the minimal medium cultivations, very nicely grouped together respectively, (not shown). One possible explanation could be found in the calculation based on titration data, where a common factor for the base pump flow rate was used in the calculations. It is possible that an individual calibration factor for each pump would improve correlation with OD. Also, the calculation of X_{NH3}, unlike the calculation of μ_{NH3}, depends also on the volume, which in this case was estimated as the initial volume plus the amount of substrate fed to the reactor. Further investigations are needed to evaluate the reason for these differences in correlation.

Also the formula for the μ-softsensor was modified to use the accumulated run-time of the titration pump. In analogy with equation 5 the specific growth rate was obtained from:

$$\mu_{NH3} = \frac{d(\Sigma t_{NH4OH})}{dt} \frac{1}{\Sigma t_{NH4OH})} \tag{12}$$

An example of μ_{NH3} from two identical cultures with minimal and rich medium, respectively, is shown I figure 38. The software sensor μ_{NH3} is more robust than the software sensor X_{NH3}, since it requires only the signal from base titration for calculation. The limitation of these sensors is the condition that no other acids or bases can be metabolised for the calculations to be correct (discussed in paper III). This is not a valid assumption in the case where enriched medium is used. Still, the parameter can be used for estimating growth rate in cultures with rich medium.

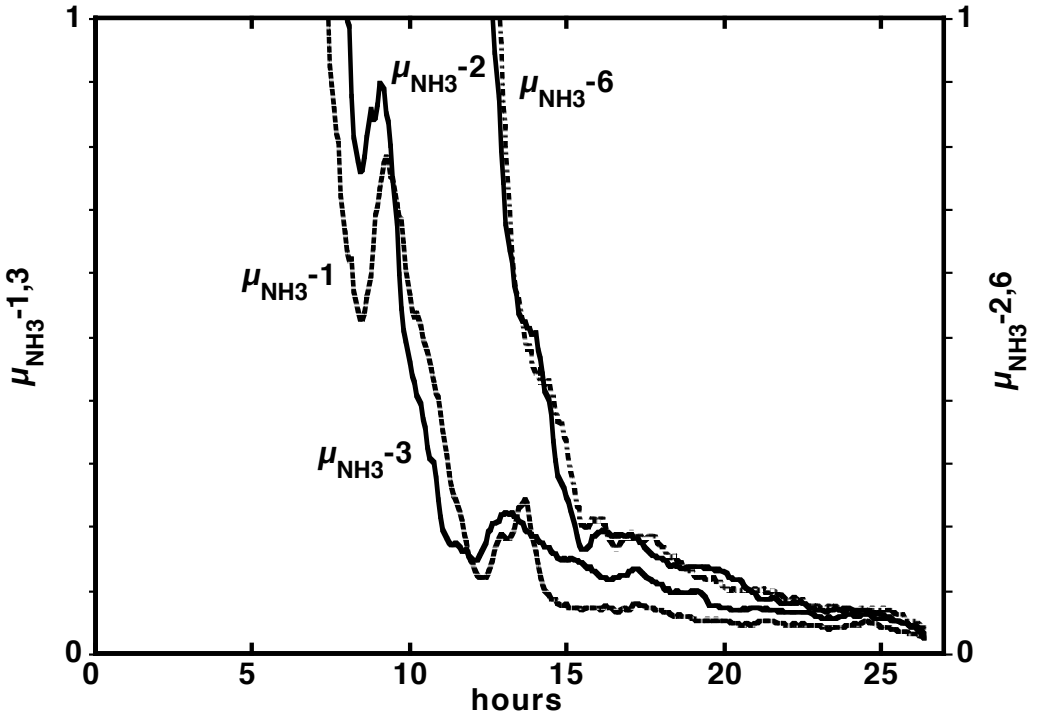


Fig. 38. The software sensor μ_{NH3} signals from four of the six bioreactors plotted together. The signals group the cultures with supplementation of yeast extract together (reactors 1 and 3) and the cultures with minimal medium (reactors 2 and 6) with each other.

In figure 39 the software sensors $R_{O/S}$ is displayed for two of the cultures with enriched medium and RQ is shown for reactors 1 and 2. The difference in medium is clearly reflected by the RQ sensor. As described earlier the $R_{O/S}$ variable, in non-stressed constant-fed cultures like these, will continue to increase from start of feed throughout the culture as a reflection of the increasing

maintenance demand coupled to the increasing degree of substrate limitation. Here, this was shown true also for the enriched medium cultures.

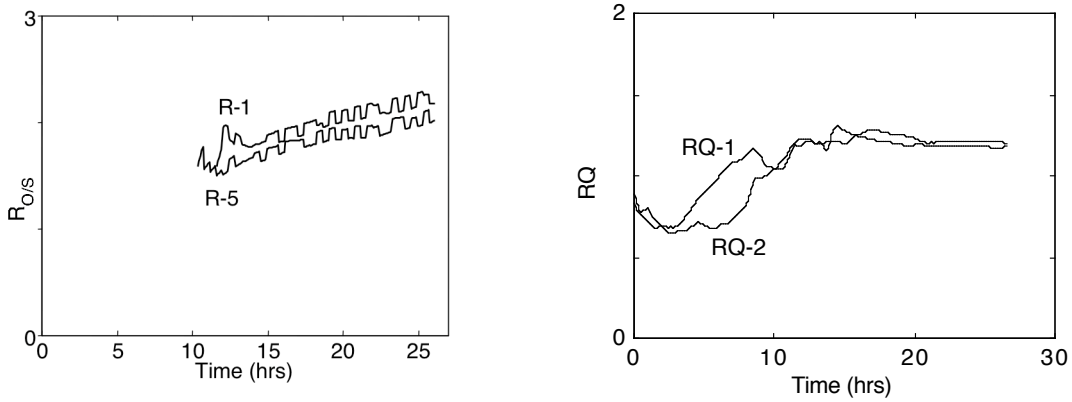


Fig. 39. 1x2 plot panel displaying the software sensors $R_{O/S}$ and RQ for two of the six bioreactors. Variables labelled -1 and 5 refer to reactors with rich medium and variable labelled -2 refer to reactor with minimal medium.

When it becomes possible to calculate biomass on-line with software sensor X_{NH3}, this also enables the calculation of specific reaction rates. Figure 40 shows the specific rates for oxygen consumption, q_O, carbon dioxide production, q_{CO2} and substrate consumption, q_S, and the yield of biomass per consumed substrate $Y_{X/S}$.

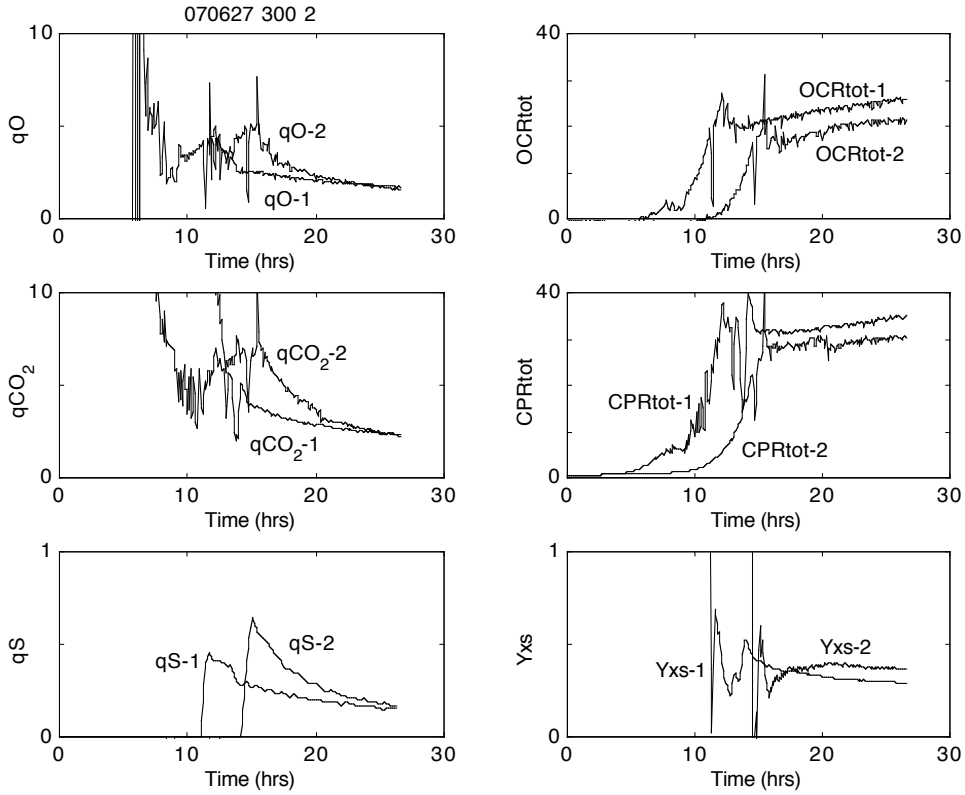


Fig. 40. 3x2 plot panel displaying the specific rates *qO*, *qCO2*, and *qS* together with total oxygen consumption rate (*OCR-tot*), total carbon dioxide production rate (*CPR-tot*) and the yield of cells per consumed substrate ($Y_{X/S}$). Variables labelled -1 refer to reactor with rich medium and variables labelled -2 refer to reactor with minimal medium.

The introduction of the multi-bioreactor system combined with the use of software sensors and the toolbox for handling and evaluation of the data showed the applicability of such systems for high throughput process development. Additional experiments and further development of the software sensors for this system will surely reveal the reason for the discrepancies in the biomass estimation. With a future introduction of statistical design of experiments and multivariate data analysis the system would be a even more valuable tool for process development.

6 CONCLUDING REMARKS

Flow cytometry has been shown to be a potent technique for population heterogeneity analysis. By labelling inclusion bodies with fluorescent antibodies it was possible to monitor the product accumulation in each cell of a sample population. In an *E. coli* culture with a severe drop in viability, according to classical colony forming units analysis, it was shown that almost all cells in the population continued to produce a recombinant protein, which aggregated as inclusion bodies. This can be utilised to study activities in so called viable-but-not-culturable cells.

The use of software sensors, which combine signals already available on-line with mathematical models, should be a valuable tool for process development. It was shown that a software sensor for estimation of biomass concentration from titration data correlates well with off-line analyses, at least in minimal medium. This opens the possibility to calculate and monitor a number of specific rates of substrate consumption and product formation. Such variables describe the activity per cell unit, which together with other software sensor signals, like the $R_{O/S}$ signal may become tools for a more sophisticated development of PAT for the fermentation industry.

The possibility to obtain more information throughout the process using software sensors, without sampling and off-line analysis, generates a demand for efficient tools for data evaluation by multivariate analysis and data presentation.

7 NOMENCLATURE AND ABBREVIATIONS

Nomenclature

Symbol	Unit	Description
μ	$[h^{-1}]$	specific growth rate
μ_{NH3}	$[h^{-1}]$	specific growth rate calculated from base titration data
a	$[m^{-1}]$	specific area of air bubbles $[m^2\ m^{-3}]$
C	$[mol\ m^{-3}]$	O_2 conc. in bulk liquid
C*	$[mol\ m^{-3}]$	O_2 conc. at gas/liquid interface
C_{CO2}	% (v/v)	carbon dioxide concentration in gas phase
C_{NH4OH}	% (w/w)	concentration of ammonia feed
C_{O2}	% (v/v)	oxygen concentration in gas phase
$C_{S,in}$	$[g\ kg^{-1}]$	concentration of substrate (glucose) in feed
C_W	% (v/v)	water vapour concentration in gas phase
DOT	%	dissolved oxygen tension
DOT*	%	DOT in equilibrium with the air bubbles in the medium
F_{NH4OH}	$[g\ h^{-1}]$	feed rate of ammonia solution
F_S	$[kg\ h^{-1}]$	feed rate of substrate solution
GCR	$[mol\ h^{-1}]$	substrate (glucose) consumption rate
k_a	[g]	initial biomass in NH_4OH units
K_L	$[m\ h^{-1}]$	oxygen transfer coefficient
K_La	$[h^{-1}]$	volumetric oxygen transfer coefficient
K_LaC^*	$[mmol\ L^{-1}\ h^{-1}]$	oxygen transfer capacity
M_S	$[g\ mol^{-1}]$	molecular weight of substrate
OCR	$[mol\ h^{-1}]$	oxygen consumption rate
OTR	$[mmol\ L^{-1}\ h^{-1}]$	oxygen transfer rate
Q	$[L\ min^{-1}]$	air-flow rate
q_{CO2}	$[mmol\ g^{-1}\ h^{-1}]$	Specific carbon dioxide production rate
q_{O2}	$[mmol\ g^{-1}\ h^{-1}]$	Specific oxygen consumption rate
q_m	$[g\ g^{-1}\ h^{-1}]$	maintenance coefficient
q_S	$[g\ g^{-1}\ h^{-1}]$	specific substrate consumption rate
q_{San}	$[g\ g^{-1}\ h^{-1}]$	part of q_S used for anabolism
q_{Sen}	$[g\ g^{-1}\ h^{-1}]$	part of q_S used for energy metabolism
$q_{Sen,g}$	$[g\ g^{-1}\ h^{-1}]$	part of q_{Sen} used for biomass synthesis
$R_{O/S}$	$[mol\ mol^{-1}]$	the ratio of oxygen consumed per C/energy-substrate
RQ	$[mol\ mol^{-1}]$	the respiratory quotient
V	[L]	medium volume
$V_{O2,m}$	$[L\ mol^{-1}]$	molar volume of oxygen
$V_{CO2,m}$	$[L\ mol^{-1}]$	molar volume of carbon dioxide
W_{NH4OH}	[g]	weight of ammonia solution reservoir
X	$[g\ L^{-1}]$	biomass concentration
X_{NH3}	$[g\ L^{-1}]$	biomass concentration calculated from base titration data
$Y_{X/NH3}$	$[g\ g^{-1}]$	yield of biomass per consumed NH_4OH
$Y_{X/S}$	$[g\ g^{-1}]$	yield of biomass per consumed substrate

Indent *i* is used for initial values. Indents *in* and *out* refer to in- and outgoing flows, resp.

Abbreviations

BOX	bis-(1,3-dibutylbarbituric acid) trimethine oxonol
cfu	colony forming units
dcw	dry cell weight
FALS	forward angle light scatter
GFP	green fluorescent protein
IPTG	isopropyl-β-D-thiogalactopyranoside
OD	optical density
PAT	Process Analytical Technology
PI	propidium iodide
PMP	promegapoietin (a recombinant protein)
RALS	right angle light scatter
VBNC	viable but not culturable (cells)

8 ACKNOWLEDGEMENTS

VINNOVA and the consortium of industrial and academic partners in the Bioman project, and the Swedish Centre for Bioprocess Technology (CBioPT) for financing and participating in these projects.

My supervisor Professor Sven-Olof Enfors, for accepting me as a PhD-student, for guiding me through the work and for sharing your profound knowledge with me.

For last minute, and nevertheless enthusiastic, reading of this thesis I would like to express my gratitude to Professor Lena Häggström.

Co-workers Erika Ledung and Fredrik Wållberg, for your contribution to this work and for great teamwork, sometimes with long work-nights, but always enthusiastic. Special thanks to Erika for your generous friendship. All participants in the Bioman project for nice cooperation, and special thanks to diploma worker Petteri Pulkkinen. Bo Norrman for inspiring discussions, mind-maps, beer and tango ☺. Chris Hewitt for your excellence in flow cytometry.

To all the people at the Department of Bioprocess Technology, present and oldies: thank you for Friday-fika, lunch discussions, beer nights (and for brewing beer with me!), Krabba, Christmas parties, bowling, curling, canoeing and much more. Especially, I would like to thank Andres for passionate discussions on subjects both off and on research, Gen for your scientific enthusiasm, Gunilla for coffee-breaks and nice chats, Tommy for technical and problem-solving support, Ela for help with analyses and for being so kind-hearted, Marita for administrative support and more nice chats, and Eric for readily solving any computer problems, Bato for always keeping the labs tidy ☺, Magda and Yanling for nice conversations at lunch and fika. Katrin and Emma for being there, especially the last year.

Marie and Erika, my very missed roommates! Marie, thank you for sleepovers and for being such an inspiring person. Erika, without our refreshing talks and your warm friendship this would have been so much harder. Thank you both for dragging me out to jog and gympa.

My family, for always supporting me and believing in me, even when I didn't.

Urban for everything! Anna and Adam because you are the best children ever! ♥

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