Real-time monitoring and control of the specific growth rate in yeast fed-batch cultures based on Process Analytical Technology monitoring tools such as biocalorimetry and spectroscopy

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Abstract

Key features of bioprocesses, such as product quantity and quality, but also cell physiology can be related to the growth characteristics of the organism under study. The specific growth rate, a key variable, cannot be measured directly, but might be estimated and inferred from other measurable variables such as biomass, substrate or product concentrations. The present thesis reviews techniques for real-time estimation and control of the specific growth rate in microbial fed-batch cultures by focusing on its importance in the development of processes for the production of high-value products such as recombinant proteins. Existing models and monitoring techniques are discussed before comparing two particular approaches, developed within the scope of this thesis, to estimate the biomass concentration and the specific growth rate of yeast cells in real-time, based on spectroscopic methods on the one hand and on heat flow measurements on the other. Particular emphasis is given to changes that need to be undertaken when adapting the initial strategy, developed for a process with *Kluyveromyces marxianus*, to different type of yeast cells such as *Candida utilis* or *Pichia pastoris* or *Saccharomyces cerevisiae*. For both control strategies, controller errors of less than 20 % were achieved, allowing ton control the specific growth rate of the four different yeast strains at a constant setpoint.

Acknowledgments

First of all, I would like to thank Prof. Ian W. Marison for offering me the incredible opportunity to carry out the work that resulted in present PhD thesis. His ideas and suggestions as well as the infrastructural framework he provided, ensured perfect conditions for me to grow - not only from a scientific point of view, but also from a personal one. I would like to thank furthermore Dr. Michal Dabros and Dr. Senthilkumar Sivaprakasam for their guidance and help that made this work possible. As we all know, the first months of a PhD are the most important ones. My thanks are also extended to Prof. Urs von Stockar who allowed me to assist his lectures here at DCU and to learn even more from him through our lunch time conversations. Special thanks go to all my fellow "LiBers" that came, went and stayed over the past years: Dr. Micheal "Mick" Whelehan, Brian Freeland, Amanda "Mandy" Murphy, Lise Castella, Dr. Milena Carlot, Dr. Pirkko "Pimu" Muhonen, Nora Engels, Bianca "Blanchette" Tassinari, Karen Byrne, Harriet "Harri" Cole, Roisin Foley, Dr. Siobhan Hennessey, Sharon Conaghan, Michael O'Donohoe, Dr. Amir Maghsoudi, Yannick Dufour, Sinead Doherty, Aurelie Demont, Niamh Sheridan and Timothey O'Regan. I am specially thankful to Adel Hama and Katie-Marie Hughes who contributed to the present work and to Tanja "Tabu" Buch for proof-reading the present thesis. I also would like to thank Mary Rafter, as well as the staff team of the School of Biotechnology at DCU, in particular David Cunnigham and Marta Perez. Further, I would like to thank the staff of HES-SO Valais Sion, in particular Dr. Kurt Eyer, Dr. Simon Crelier, Dr. Sergio Schmid and Dr. Alain Grogg, as well as Julien Pott and Gordana Pistoletti for providing me with the necessary background for starting this work. While I would like to thank my fellow Phd students from DCU for making our daily student live enjoyable, I am particularly thankful to Ana Carolina Maganha de Almeida for our discussions about the world in general and about science in particular. I am also extremely thankful to my family, Eva Maria Kissling, Josef, Andreas, Anja and Timo Schuler, "vorallem fuers geduldigi Zuelosae bim Chloenae". And last, but not least, moechte ich Tobias Mueller fuer seine sowohl technische als auch emotionale Unterstuezung danken.

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Chapter 1

Introduction

People grow through experience if they meet life honestly and courageously. This is how character is built. [1]

[1] Eleanor Roosevelt, Diplomat (1884-1962)

1.1 Thesis aim, importance, strategy and scope

The work undertaken throughout this PhD project is related to a research project (Project Number 08/IN.1/B1949) funded by the Science Foundation Ireland (SFI) and entitled "On-line Monitoring & Control of High Density Cultures" (Principle Investigator: Prof. Ian W. Marison).

1.1.1 General objectives of Project 08/IN.1/B1949

In the following lines, the general objectives of the project, as described in the SFI proposal, are itemized before the actual objectives of the thesis work are explained in a more detailed manner.

- The project is based on the enhanced accuracy and reliability of the real-time measurements of process variables for the purpose of designing and implementing a versatile feedback control strategy for critical process parameters in fed-batch cultures.
- The potential of several process monitoring devices as well as associated data enhancement techniques should be assessed in order to provide a firm basis for the careful choice of the appropriated monitoring system.
- The feedback platform should be employed to develop an integrated bioprocess where for instance recombinant protein production should be optimized or where relevant, critical process parameters are controlled in order to ensure the quality of the end-product of the process.

1.1.2 Particular focus of the present thesis

Content of the previous Section (1.1.1) suggests that the overall objective of the project funded by SFI is to develop an integrated bioprocess for the optimal production of recombinant proteins in line with recommendations of the Food and Drug Administration (FDA) about Process Analytical Technology (PAT). In order to achieve the development of such

a process, a certain number of process variables need to be investigated and correlated to "critical quality and performance attributes of raw and in-process materials and processes with the goal of ensuring final product quality" [3]. Two main aspects should be kept in mind. Firstly, not all process variables, despite initial assumptions, are critical and secondly, not all critical variables can be monitored and controlled with currently available techniques. Moreover, certain process variables might be process-specific.

A top-down approach would suggest to focus on a particular quality attribute, for instance the glycosylation pattern of a recombinant protein, to investigate all relevant process variables and to finally develop an appropriate strategy to consistently produce a product of reliable quality. In the present thesis, an other approach has been chosen, by first determining a particular variable to be controlled, looking then for a particular set of techniques to measure the actual value of the controlled quantity and by finally associating a manipulated variable to complete the control strategy [4, 5]. The variable of choice in this project is the specific growth rate. Indeed, being closely related to four main aspects of bioprocessing (Section 2.2) and relevant to bioprocesses independently of the nature of biological system under consideration, the specific growth rate can be considered a key variable for achieving overall process control for enhanced product quality.

A common feature of several important expression systems such as *Escherichia coli* or Chinese Hamster Ovarian (CHO) cells (Figure 1.1) is the production of overflow metabolites in the presence of high carbon-source concentrations. While different strategies are available to avoid the on-set of overflow metabolism (Section 5.1), controlling the specific growth rate below a critical value, specific to each system, is a valid approach [6, 7] strengthening the cogency of the control platform suggested throughout the present thesis.

The aim of the present thesis is to critically analyze existing strategies to monitor and control the specific growth rate in microbial fed-batch cultures and to explore ways of achieving such control by regulating an exponential substrate feed in response to real-time measurements of process variables gathered either by spectroscopic or biocalorimetric techniques. It should be kept in mind that the control strategy should be as simple as possible and should rely on the fewest number of measurements possible (*lex parsimoniae* [8]).

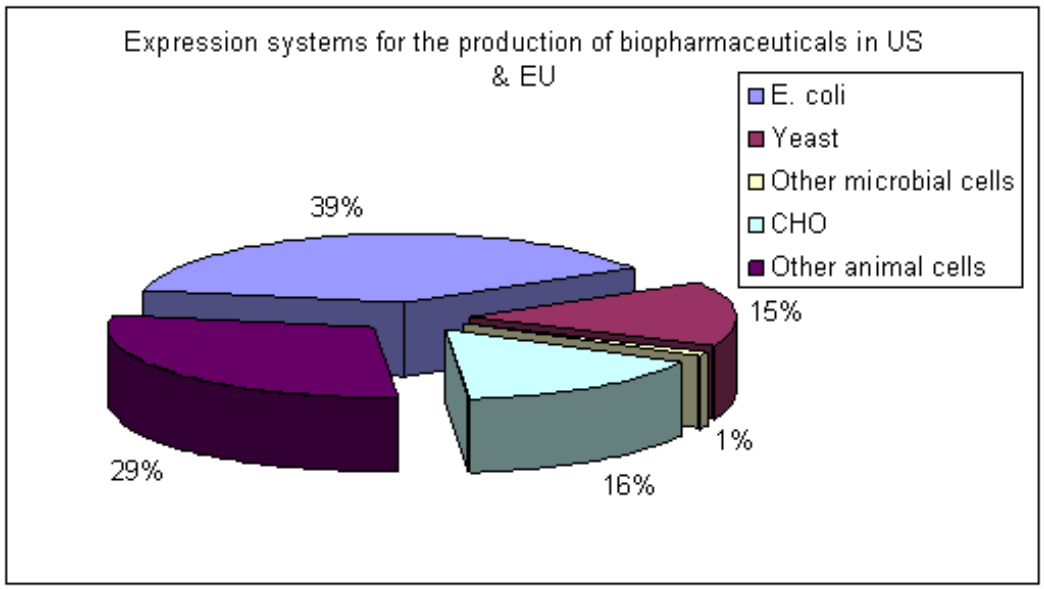

Figure 1.1: Important expression systems for the production of biopharmaceuticals, such as recombinant proteins; figures adapted from [9].

1.2 Thesis outline

The present thesis includes eight chapters and is structured in the following manner:

Chapter 2 is complementary to Section 1.1.2 and provides a comprehensive literature review of currently available techniques for real-time monitoring and control of the specific growth rate in microbial fed-batch cultures, thereby developing the rationale of the present thesis and highlighting its novelty.

Chapter 3 deals with the different Process Analytical Technology (PAT) monitoring tools used throughout the present study.

Chapter 4 contains the sequence of steps in the development of a platform for simple control of the specific growth rate in fed-batch processes based on enhanced real-time measurements of biomass by dielectric spectroscopy. The study should serve as a basis to all subsequent chapters.

Chapter 5 expands the application of the developed feedback control platform to fed-batch cultures of the Crabtree-negative yeast cells.

Chapter 6 presents the development of a biomass and specific growth rate estimator based on calorimetric measurements that should be incorporated into an alternative feedback platform for the control of the specific growth rate.

Chapter 7 investigates the potential of biocalorimetry as general applicable Process Analytical Technology (PAT) tool for monitoring and control of yeast fed-batch cultures.

Chapter 8 summarizes the conclusions from chapters 4 - 7 and gives an outline of further applications of the present findings. Furthermore, challenges are highlighted and potential solutions discussed before drawing an overall conclusion of the work carried out within the scope of this thesis.

Chapter 2

Real-time monitoring and control of the specific growth rate in microbial fed-batch cultures: current state and perspectives [1]

One looks back with appreciation to the brilliant teachers, but with gratitude to those who touched our human feelings. The curriculum is so much necessary raw material, but warmth is the vital element for the growing plant and for the soul of the child. [2]

[1] The present chapter is adapted from work published in *Applied Microbiology and Biotechnology*; Schuler MM, Marison IW - Real-time monitoring and control of the specific growth rate in microbial fed-batch cultures: current state and perspectives [10]

[2] Carl Jung, Psychiatrist (1875-1961)

Understanding the growth characteristics of micro-organisms is an essential step in bioprocessing, not only because product formation may be growth associated, but also because they might influence cell physiology and thereby product quality. The specific growth rate, a key variable of many bioprocesses, cannot be measured directly and relies on the estimation through other measurable variables such as biomass, substrate or product concentrations. Techniques for real-time estimation of the specific growth rate in microbial fed-batch cultures are discussed in the present chapter. The advantages and limitations of different models and various monitoring techniques are discussed, highlighting the importance of the specific growth rate in the development of fast, reliable and robust processes for the production of high-value products such as recombinant proteins.

2.1 Introduction

Since Jacques Monod's observations of microbial growth and the mathematical description he derived from his experimental data, the time-dependent variation of the number of cells ($\frac{dN}{dt}$) as well as the variation of the cell concentration over time in relation to the actual cell concentration (μ), has fascinated, sometimes even obsessed, many researchers [11]. The early stages of research of microbial growth focused on the reproduction, the expansion and generalization of Monod's equation and later on the correlation between the specific growth rate and a particular cellular state or set of culture conditions. Interest in the determination and the control of the specific growth rate is constantly growing, predominantly on account of the importance of this particular process variable in a variety of bioprocesses, in particular since the introduction of the Process Analytical Technology (PAT) initiative [3] by the Food and Drug Administration (FDA) in 2004. So far, PAT applications have been more related to small molecule synthesis, formulation and fill-finish processes, however, the four main principles of the initiative can as well be applied to monitor and control produc-

tion processes of biologicals such as recombinant proteins. As pointed out by Gnoth and co-workers [12]: “A desirable application of PAT is to understand the impact of measurable and controllable process variables on product characteristics that are not directly measurable in real time”’. The specific growth rate is without a doubt, as shown in Section 2.2 of the present chapter, related to important product characteristics, rendering its control in bioprocesses crucial.

The present review focuses on the specific growth rate in relation to microbial fed-batch cultivation processes for both biomass production and value-added product production such as recombinant proteins or secondary metabolites. In a first part, the importance of the specific growth rate (μ) in bioprocesses will be highlighted, then ways of modeling and estimating μ will be presented before control strategies involving the specific growth rate will be outlined and finally the limitations of the current techniques, as well as ways of achieving improvements in the near future will be presented. In the last step, the relevance of the analysis of the current situation in relation to the objectives of the present thesis is highlighted, specifying the scope of the work undertaken.

2.2 Importance of the specific growth rate in bioprocesses

The specific growth rate (μ) is an essential process variable, above all because it is characteristic of the physiological state of micro-organisms and related to the biosynthesis of many products of interest [13]. With regard to bioprocesses, μ has an influence on four main aspects: biomass production, product quantity, cellular state and product quality.

2.2.1 Maximal biomass production

In the production of single-cell proteins for instance, where biomass is the final product, one would expect that the most efficient operation is to run the process at the maximal specific growth rate, to ensure maximal productivity. Even where the product is not directly related to growth, such as in the case of recombinant protein production, the final cell concentration is still paramount. Together with the specific cellular yield, it will determine the volumetric

yield of the substance of interest, for example recombinant ovine growth hormone (r-oGH) in *Escherichia coli* [14].

2.2.2 Product formation rate and product quantity

In terms of product formation, three particular cases might be considered as stated by Luedeking and Piret; growth-associated, non-growth-related and partially growth-associated products. Extracellular heteropolysaccharides (HePS) for instance are growth-associated substances produced by many species of lactic acid bacteria (LAB) [15] as are Xanthan gum, produced by *Xanthomonas campestris* [16] or poly-hydroxysbutyrate (PHB) by *Alcaligenes eutrophus* [17]. The ability to maintain cells in a particular metabolic or physiological state related to μ, promoting a particular metabolism for the production of secondary metabolites, such as prodigiosins [18], is of particular relevance to biotechnology and pharmaceutical industries. Generally speaking the specific productivity, the recombinant protein production rate and the secretory expression in carbon-limited fed-batch cultures is often inversely correlated to the specific growth rate of the micro-organism [19], hence the importance of maintaining low specific growth rates, at least during protein expression phase, to maximize product yield [14, 20, 21, 22, 19, 23, 24, 25].

2.2.3 Physiological state of cells

Several physiological attributes of cells vary as a function of the specific growth rate. Thus, Paredes-Lopez and co-workers [26] established a hyperbolic dependence of the cellular lipid content on the specific growth rate in *Candida utilis*, while Lange *et al.* [27] showed that the carbohydrate fraction stored within *Saccharomyces cerevisiae* cells decreases with decreasing values of μ. They also demonstrated that the protein content in these cells decreased with higher values of μ, except in ethanol-limited cultures, where an increase in protein content was observed [26]. Not only the physiological state of the cells is determined by the growth rate that a particular medium composition can support, also the protein synthesizing machinery is influenced by it [11, 12]. RNA synthesis and content within cells is reported to increase with the specific growth rate [27, 28]. Plasmid stability, of particular in-

terest in the recombinant protein production using *E. coli* or *Bacillus subtilis*, has also been associated with the specific growth rate [23, 29, 25]. The protein synthesizing ability of cells, also depending on μ, is of particular importance for recombinant protein production. At the same time, the metabolic burden associated with recombinant protein production can decrease the growth rate significantly [14, 30]. High specific growth rates and the presence of high C-source concentrations can lead to overflow metabolite production in some of the most important microbial expression systems, so-called Crabtree-positive organisms such as *S. cerevisiae* or *E. coli*. The presence of overflow metabolites such as acetate or ethanol frequently leads to the inhibition of both growth and protein formation [12, 29, 31]. To a certain extent, the specific growth rate also determines the overall physiological state and fitness of an organism, affecting for instance the sensitivity of pathogens to solar disinfection when cultured at higher growth rates [32].

2.2.4 Product quality

The relation between μ and product quality is probably most relevant in industrial bioprocesses within the scope of PAT. As highlighted in Section 2.2.3, the specific growth rate affects the rate of protein expression and plasmid stability, usually yielding higher specific protein productivity at lower specific growth rate. But productivity is not the only metric to characterize a bioprocess. Product quality is, at least for the production of therapeutics, even more important than the titer. A number of studies have highlighted the effect of changes in the specific growth rate on the glycosylation pattern [2] or on the secretory expression [25] of certain recombinant proteins. Characterization of product quality is predominately carried out off-line, frequently once the process is finished. The information obtained by end-product characterization methods such as mass-spectroscopy, HPLC/UPLC, affinity or bio-activity assays is of utmost relevance to product quality, however, measurements are only available off-line and with delay [33]. By developing monitoring and control strategies to determine the specific growth rate in real-time in microbial cultivation processes and by relating this particular process variable to end-product quality attributes, bioprocessing would go a long way to improving bioprocess understanding and control.

2.3 Estimating the specific growth rate in bioprocesses

Before addressing the topic of monitoring and controlling the specific growth rate, it is important to highlight that this particular process variable cannot be measured directly, but solely estimated from other variables. Hence the estimation of μ is dependent on reliable process measurements on the one hand and on accurate, yet simple and robust models on the other. Therefore, this section will focus on available models, their advantages and limitations. Then the discussion will be centered on the measurements that are required to provide reliable inputs for the models. In this context, emphasis will be given to real-time measurements in bioprocesses.

2.3.1 Models

The complexity of living cellular systems renders obtaining meaningful information about all biological rates an impossible task. Segregated, structured cell models have been developed [34, 35, 36], but are seldom applied for estimating or controlling the specific growth rate in real-time [37], The main difficulty lies in accurately measuring or estimating all the different metabolites, rates or yields involved. Unstructured, unsegregated kinetic models are the most simple models encountered. They are applicable to many situations of practical interest. As highlighted by Faria *et al.* [16], these simple models are based on balances over substrate(s), biomass and product(s) concentrations in the form of a system of differential equation to describe, among others, μ. Indeed, μ is dependent on a myriad of factors, such as pressure, temperature, pH, inhibitors, but also substrate, product and biomass concentration [38]. However, most of these factors (T, pH, pressure) are kept constant during fed-batch cultures, therefore, only biomass, substrate and product concentrations really matter. Developed in 1949, Monod's growth equation is the most common way to express the dependency of growth on the concentration of the growth-limiting substrate [39]:

$$\mu = \frac{\mu_{max} S}{K_s + S} \tag{2.1}$$

This empirical Equation (2.1) is very simple, but it is, to a certain extent, sufficient to

Table 2.1: Three generalized equations to describe the variations of biomass (x), substrate (s) and product (p) as a function of culture time in fed-batch cultures. D is the dilution rate, F is the feed rate, $Y_{x/s}$ is the biomass yield, q_p is the specific product formation rate and V is the reaction volume.

$$\frac{dx}{dt} = (\mu - D)x$$

$$\frac{ds}{dt} = Ds_i - x\frac{\mu}{Y_{x/s}}$$

$$\frac{dp}{dt} = q_p X(V_0 + Ft)$$

describe most experimental data for growth [37]. With a slight modification, the model can even describe substrate or product inhibition or diauxic growth [37]. Several similar models have emerged over time. Some forms of unstructured models are only valid in steady-state cultures or during the exponential phase of batch cultures and might fail during any transient condition. Since mainly fed-batch cultures are considered within the scope of this thesis, the most important equations are summarized in Table 2.1. Papers mentioned in Table 2.2 and Table 2.3 make all use of a form or the other of these equations.

As early as 1976, Cooney [40], in his work involving *S. cerevisiae*, pointed out that the bottleneck for the development of cost- and time-efficient cultivation strategies lies in the lack of reliable sensors to monitor biomass, substrate and product concentration. He also has foreseen the potential of soft-sensors to estimate those quantities, along with non-measurable variables such as the specific growth rate. Along with his co-workers, he suggested the use of off-gas analysis (paramagnetic gaseous oxygen analyzer, IR carbon dioxide analyzer and air flow meter) for the estimation of the oxygen uptake rate (OUR), the carbon dioxide evolution rate (CER) and a simple weighing scale to determine the nitrogen consumption rate (NCR), combining the three to estimate biomass concentration variation and the specific growth rate over the time of a culture. Oner and co-workers [41], investigating the growth patterns of *Lactobacillus bulgaricus* and *Streptococcus thermophilus* showed that the utilization of spline functions to mathematically smooth the data before estimating the specific growth rate, along with product formation and multiple-substrate con-

sumption rates, gives reliable results for fed-batch culture characterization. Furthermore, the technique proposed allows checking the consistency of the data and detecting gross errors. Neeleman and co-workers [42] explored different recursive and filtering methods to improve the traditional Monod model-based approach for estimating the specific growth rate. Lubenova [43] suggested the use of the oxygen uptake rate as a sole function to estimate the biomass concentration and the specific growth rate as a function of culture time. The particularity of this research consists in taking the time derivative of the oxygen uptake rate to create the model. The simulation was verified off-line with experimental data from a *Streptomyces clavuligerus* culture.

Unstructured models, such as the four studies presented above, are useful in many situations, but they do have limitations [37]. In the case of changes in morphology and physiological stages [44], structured models should be applied to take into account variations in biomass activity and composition [45]. Esener *et al.* [46] predicted that the next generation growth models will mainly be structured models. The state-of-the art literature in bioprocess monitoring and control tends to disagree. It is true that moving towards metabolic flux analysis can bring improvements to the estimation of the specific growth rate [47]. A basic, unstructured model can be improved by adding, for instance, an ATP balance to its description [48]. When expanding the model by including different metabolic fluxes and their distribution [49, 50, 51, 52], the choice of the right level of detail is crucial, but might in reality depend on the availability of measurements. Examples of applications of both structured and unstructured models in industrial bioprocesses are rather sparse. The required measurements, the pre-conditioning of the data or the need of appropriate computing tools for data acquisition, computation and data distribution might be lacking or implementation is hindered by the difficulties in choosing a suitable model and adequate simplifications for the system [53].

Kinetic and stoichiometric models allow to infer unknown components [54] and to understand biological phenomena. Some of the models even consider time-varying parameters since cell metabolism changes during the course of a culture (ex. shift to product synthesis) [30], hence the importance of the appropriate combination of sensors, data analysis and

consistency check to gather solid process parameters in real-time [55]. Accurate measurements, gathered in real-time, are the basis for reliable models and robust control strategies. Section 2.3.2 is therefore dedicated to techniques that allow inferring valuable information about the different process variables.

Data-driven models, such as artificial neural networks (ANN) are not widely employed for the estimation of the specific growth rate. ANN have a tremendous application potential, especially in industrial processes where an enormous amount of data is available for model training and validation. ANN have been extensively applied to estimate biomass [56, 57, 58] and substrate concentrations [59] or oxygen up-take rate [19], however, probably due to the lack of off-line values in sufficient number for training and validation, examples of real-time estimations of μ by ANN or similar methods are rare.

2.3.2 Measurements

Off-line measurements are inevitable for model validation and observation purposes. The different reference methods used in microbiology are: dry cell weight (DCW), optical density (OD), packed cell or packed mycelium volume, colony forming unit counting and cell number evaluation (manual counting, Coulter, Casy etc.). Section 2.3.2 is dedicated to the different possibilities to gather real-time information about the different input variables for the models presented in Section 2.3.1. For unstructured models, μ can be expressed as a function of X, S or P or a combination of them [60]. Potential process analyzers to quantify these variables and to infer the specific growth rate in real-time in bioprocesses are outlined in Table 2.2 and discussed throughout Section 2.3.2. Other sensors exist to quantify either substrate, biomass or product concentration and could potentially be applied to gather data for the estimation of μ.

Substrates Using information about the oxygen consumption rate (OUR) of microbial cultures to infer the specific growth rate is an interesting and widely used approach. Both off-gas analyzers and dissolved oxygen probes are commonly used in industrial processes and can be exploited to gather enhanced process knowledge. While off-gas analysis for

OUR determination is a method of choice, studies have been carried out where traditional dissolved oxygen measurements served as a basis for the computation of the oxygen uptake rate via a stationary liquid phase balance [61]. Gnoth and co-workers [12] pointed out that for OUR and carbon dioxide evolution rate (CER) from off-gas analysis, cumulative values with reduced noise influence are best for further estimation computations. Carbon source concentration measurements are more difficult to obtain. Fourier transform mid-infrared (FTIR) and near-infrared (NIR) spectroscopy is gaining importance in the real-time monitoring of metabolites [62, 63, 64]. In comparison to at-line or off-line High Performance Liquid Chromatography (HPLC), spectroscopy is faster in terms of measurement time, however, the limit of quantification is higher. Base consumption rate (BCR) or more generally nitrogen-source consumption rate, determined by the simple use of a weighing scale is a widely underestimated tool that should receive more attention in a close future and can be an interesting, robust input variable into for instance ANN models for both biomass and specific growth rate estimation.

Products Four main categories can be considered when considering product formation rate: carbon dioxide evolution, metabolic heat production, overflow metabolites production and recombinant protein or other high-value products formation. CER is probably the most frequently characterized product formation rate and has also been used for μ estimations (Table 2.2). FTIR and NIR spectroscopy could, as mentioned in Section 2.3.2, be an alternative to at-line HPLC or Gas Chromatography (GC) for the quantification of overflow metabolites such as acetate, ethanol or citric acid, but might also be considered for determining the amount of recombinant protein produced in real-time [65]. Reaction heat evolution rates can be determined by micro-calorimetric [66, 67, 68] and bench-scale calorimetric devices [69, 70, 71, 72], but also using simple instrumentation such as temperature probes and mass flow meters [6, 7], particularly at large-scale [73, 74].

Biomass Having real-time information about the biomass concentration of a microbial cultures is probably the most attractive way to estimate the specific growth rate. Indeed, if growth is characterized by the increase of biomass, having an accurate and reliable

measurement of the latter allows direct inference of the first without relying on any additional process information. However, it is difficult to measure biomass concentration accurately and reliably in real-time. Many processes depend on the measurements of other process variables to infer the biomass concentration, rendering the calculation of the specific growth rate an estimation based on estimated values and increasing the probability of fault occurrence through error propagation. Dielectric and NIR spectroscopy are the two main real-time monitoring technique for biomass concentration. The first has received a lot of attention over the past two decades [75, 76, 77, 78], while the latter is still underexploited [62, 63]. Three other direct biomass quantification techniques that have only received marginal attention so far are real-time optical density and turbidity measurements and focused beam reflectance measurements [79]. Viscosity measurements, RNA content and real-time measurements of NADH by fluorescence spectroscopy could be alternative techniques to indirectly correlate process information with biomass concentration.

The more accurate and reliable process data is available, the better the estimation of the specific growth rate will be, especially if the system is (over-) determined and redundant, allowing for enhanced measurement reliability for instance through data reconciliation [98]. Extended and Unscented Kalman Filters (EKF and UKF respectively), standard in the theory of state estimation in nonlinear systems, can alternatively be used to improve the reliability of measured or estimated quantities. The stability and convergence however is, especially in the case of EKF involving the linearization of a non-linear system, difficult to estimate [43, 99].

The development of real-time monitoring techniques for the specific growth rate allows the observation of deviation from expected growth patterns [32]. Of particular interest are the important oscillations in the values of μ that are observed by several authors [80, 100, 81, 88, 84, 85], even during the exponential phase in batch cultures. Claes and co-workers tried to deal with the oscillations by applying Butterworth filters [81]. It remains however so far unclear if oscillations, only observed when monitoring μ continuously, are an artifact of measurements or if they have a biological cause. Considerable filtering might lead to a smoother signal, but maybe also to a consequent loss of important biologically meaningful

Table 2.2: Real-time monitoring tools for quantifying unknown process variables used to estimate the specific growth rate in microbial cultures.

Unknown	Sensor
Biomass evolution rate	
	Dielectric spectroscopy [80, 81, 82, 83, 17, 84, 85]
	NIR [33, 86]
Substrate consumption rate	
Oxygen uptake rate	
	Acoustic gas analyzer [87]
	Bluesens technology [13]
	(quadrupole) Mass spectroscopy [30]
	Paramagnetic gaseous oxygen analyzer [40, 88, 19, 89, 33, 85, 90, 55]
	Polarographic electrode [91, 92, 89]
Glucose consumption rate	
	Flow injection analysis (FIA) [93]
	Multi-wavelength fluorescence spectroscopy [87]
	Weighing scale & carbon source analyzer [40]
Methanol consumption rate	
	Frings Alkosens / FC-2002 [20, 94]
Nitrogen uptake rate	
	Weighting scale [40, 95, 19, 87, 86, 55]
Product formation rate	
Carbon dioxide production rate	
	Acoustic gas analyzer [87]
	Bluesens technology [13]
	IR carbon dioxide analyzer & air flow meter [40, 19, 85, 90, 55]
	(quadrupole) Mass spectroscopy [30]
Acetate production rate	
	at-line GC [96]
	at-line HPLC [86]
Ethanol production rate	
	at-line GC [96]
	FTIR spectroscopy [95]
	IR Exhaust gaz [91]
	Multi-wavelength fluorescence spectroscopy [87]
	NDIR (BINOS 4B2 Leybold-Heraeus) [97]
Heat production rate	
	Temperature probes & mass flow meters [6, 7]
Recombinant protein production rate	
	GFP by 2D fluorescence spectroscopy [85]

information.

Throughout Section 2.2, the importance of the specific growth rate has been outlined and related to different important process and product attributes. In Section 2.3, different potential and applied monitoring strategies for the specific growth rate have been discussed. While industrial examples of real-time μ estimations are close to inexistent, research groups investigate more and more means of exploring and exploiting information related to the specific growth rate. An effort needs still to be made to introduce advanced monitoring techniques such as IR, dielectric spectroscopy or on-line HPLC in development and production environment to track process variables. Once these sensors have been established, the estimation of more complex process variables will naturally take place through the appropriate combination and implementation of advanced hard- and soft-sensors. Reactor parameter control such as temperature, pH or agitation speed control are nowadays considered as standard but still received particular attention in research in the early 1990es. Given enough time, advanced PAT process analyzers as well as estimations of critical process variables such as the specific growth rate will find their place in industrial applications.

2.4 Controlling the specific growth rate in bioprocesses

In Section 2.2, the importance of the control of the specific growth rate was highlighted, the current section explores how control can be achieved. Robust control strategies rely on reliable models and generally simplifications in the mathematical description of the cellular system are made when developing models for control [37, 101]. In order to keep the organism in a particular physiological state and ensure the production of certain species of compounds, there is usually one particular object of control, the regulation of the biomass growth rate [101]. Furthermore, the control of μ can be considered as an effective strategy for process optimization [102] since most of the biochemical reactions for product formation are either directly or indirectly associated with cell growth or cell state and that their kinetics strongly influence process performance [103]. The most common way of controlling the specific growth rate is to take advantage of continuous and perfusion cultures [104]

Table 2.3: Feedback control strategies with focus on the monitoring technique employed.

Control	Monitoring
Fuzzy logic control with two sets of rules for 3 states	Exhaust gas (including ethanol), dissolved oxygen [91]
	Turbidity probe, Off-gas, Ethanol [97]
Corrected feed-forward	At-line lactose concentration by colorimetry [105]
Step-wise changes to a predefined, exponential feed rate	Ethanol & acetate by at-line GC [96]
Control ethanol concentration at constant low value	FTIR, BCR [95]
	Off-gas, BCR [19]
	Off-gas, dissolved oxygen [106]
	Off-gas (MS) [30]
Adaptive proportional feedback action on feed rate	Off-gas [13]
Adaptive PI control	Heat flow [6]
Stage changes of exponential feed rate	Absorption spectrum for surfactant [107]
	FIA [93]
Control of ethanol feed	Off-gas, heat [108]
Switching feed rates	[109]
PID on predetermined feed rate	Heat flow, off-gas [110]
PI control action on exponential feed rate	Heat flow [7]

where the biological variable, μ, is controlled by regulating a physical variable, namely the dilution rate. This chapter, as well as the thesis as a whole, focuses on the control of μ in fed-batch cultures.

In control theory, the system under consideration can be broken down into three main parts: a. the controlled variable b. the associated manipulated variable and c. the sensed value of the controlled variable. The sensed value of the controlled variable is of particular interest within the scope of this project since control relies on different monitoring techniques or even on the appropriate combination of techniques to sense or estimate the specific growth rate. Available techniques to measure associated process variables and to estimate the specific growth rate have been extensively discussed in Section 2.3. As for the associated manipulated variable, the most common one is the carbon and energy substrate feed rate, even though feed rate of gaseous substances such as oxygen and methane

can also be considered [111]. Feeding strategies in fed-batch cultures can be divided into: a. fixed feed trajectories b. *a priori* calculation of the feed trajectories (based on kinetic models) c. predictive control with feedback at intervals (dependent on sampling) d. feedback control in real-time [112, 92] . Existing strategies that have been successfully applied to regulate feed rates in fed-batch cultures have been discussed by Lee [113]. As an example of fixed carbon source feed rates, Cheng and co-workers [114] have explored the potential of pseudo-exponential feed regulation. Feed-forward control by *a priori* calculation of an exponential feed trajectory, based on a simple combination of mass balances and Equation (2.1) has been widely exploited to maintain μ constant throughout the culture [101, 22, 23, 2, 86, 107, 115, 93]. The mathematical description of the exponential feed trajectory is given in Equation (2.2)[3] .

$$F = F_0 e^{(\mu t)}; F_0 = x_0 V_0 \frac{\mu}{Y_{x/s} s_F} \tag{2.2}$$

A predetermined feed rate, obtained off-line by the use of genetic algorithms, has been successfully applied at industrial scale to maximize biomass production while keeping to a minimum the formation of overflow metabolites [116]. As for feedback control, Smet and co-workers stated that "the primary goal of a substrate feedback controller for a fed-batch fermentation process is not to stabilize the process globally, but rather to optimize it while keeping an inherently unstable type of behavior under control" [17]. Feedback or closed-loop control strategies can be achieved by: a. on/off control b. combinations of proportional, integral and derivative feedback control or c. adaptive feedback control. From Table 2.3, it can be concluded that on/off control is not widely applied to maintain the specific growth rate constant, although this type of control can be useful to regulate other types of process variable within certain limits [117]. Adaptive control is more commonly found. Soons and co-worker [92] pointed out that there are two categories of adaptive control. Either the error term is the driver for the adaptation or the adaptive expression is linked to the measured or estimated process state. The latter approach has been explored by Dochain and

[3]For details and definitions, refer to Chapter 4

co-workers [38]. Jobe *et al.* [96] compared feed forward control as given by Equation (2.2) with closed-loop control. While the latter controllers yield better results, they also imply a more complex approach, mainly in terms of *a priori* knowledge of the process, control parameter tuning [89] and monitoring instrumentation. Indeed, as highlighted by Gnoth and co-workers [12]: "feedback control only makes sense where the current value of the controlled variable can be determined accurately and fast enough to determine the deviation from the target set-point". The adaptability of the organisms makes the control task even more difficult as pointed out by Arndt *et al.* [118]. However, Table 2.3 as well as Figure 2.1 prove that close control via feedback strategies is possible even though appropriate controller tuning is of high importance, for instance to avoid overshooting and consequent carbon-source accumulation.

Theoretical consideration of μ control and associated data enhancement techniques have been explored by several authors [38, 119, 42, 120, 101, 121, 122]. Indeed, application of some mathematical techniques such as parameterization methods could improve product titer by 28.32 % compared with an existing empirical approach [123]. Mathematical techniques are of utmost importance to provide measurements with enhanced reliability and reduced noise. Ways to achieve noise reduction and increase reliability include data reconciliation [64], filters [81] or ANN [124]. In summary, over the past two decades, important realizations have been achieved in terms of controlling the specific growth rate. Limitations to existing strategies are the facts that process kinetics are most often poorly understood, that the processes are time-varying and that real-time sensors (hard- and soft-sensors) are still not systematically implemented in industrial bioprocesses.

2.5 Conclusion

Throughout this chapter, the importance of the specific growth rate in bioprocesses has been described. Techniques for modeling and estimating the specific growth rate have been presented. It has been demonstrated that improvements in measurement techniques of associated process variables such as biomass, metabolites and substrate concentrations, but also

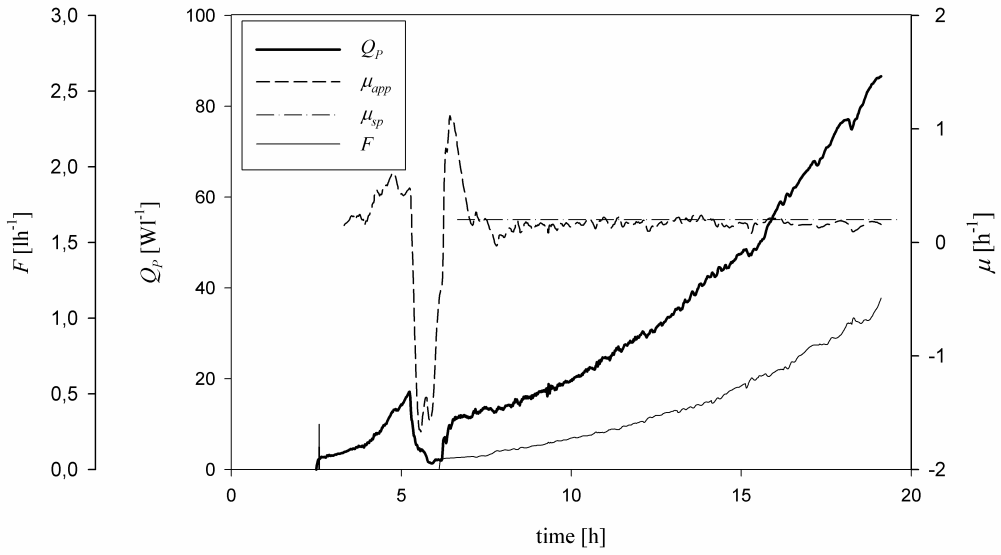

Figure 2.1: Excerpt of a fed-batch culture of *Escherichia coli* where the specific growth rate was maintained at a sufficiently low level to avoid overflow metabolite production - Reprinted from [6], with permission from Elsevier.

metabolic heat, allow to establish a firm ground for control strategies. The fields of industrial microbiology and bioprocessing are at an early stage of control of the specific growth rate, though the number of successful applications of strategies to control μ by regulating a given substrate feed rate are increasing. A tendency to move toward dual-stage fed-batch processes can be observed [17, 23, 125, 24]. Indeed, fed-batch cultures where the specific growth rate is first maintained at a value that allows maximizing the specific biomass productivity without leading to overflow metabolite formation, then maintained at low values after induction to ensure highest specific product formation rate and product quality might be economically interesting, leading to an increased overall productivity of the process.

The analysis of the current situation in terms of available real-time monitoring and

control strategies to regulate the specific growth rate in microbial fed-batch cultures leads to the conclusion that:

- a growing interest in the topic can be observed at sight of the increasing number of publications in the field 2.3 in the last years
- a quest for simple control strategies can be perceived [126]
- reliable real-time measurements are key for the development of robust control strategies

Keeping these three main points in mind, a set of suitable real-time monitoring techniques and an adequate control strategy will be discussed in Chapter 3 and applied throughout Chapters 4, 5, 6, 7.

Chapter 3

Theoretical considerations about the analytical tools chosen for the study [1]

Life is growth. If we stop growing, technically and spiritually, we are as good as dead. [2]

[1] The present chapter is adapted from work submitted for publication in *Springer Book Series - M3C*; Marison IW, Hennessey S, Foley R, Schuler MM, Sivaprakasam S, Freeland B - The choice of suitable on-line analytical techniques and data processing for monitoring and control of bioprocesses and from work intended for submission in *New Biotechnology*; Schuler MM, Byrne K, Foley R, Freeland B, Marison IW - Investigation of the potential of bench-scale biocalorimetry as a monitoring tool for CHO DP12 cell cultures

[2] Morihei Ueshiba, Martial Artist (1883-1969)

The development of a robust control strategy relies on reliable real-time measurements. Generally required characteristics of real-time measurement devices for bioprocess are discussed within this chapter. Furthermore, reasons for the choice of devices and techniques in the scope of this thesis are presented. Additionally, application examples, advantages and drawbacks of the employed methods are presented and emphasis is given each time to the potential industrial application of the presented technique.

3.1 Introduction

In order to be able to construct a reliable platform for the control of the specific growth rate in microbial fed-batch cultures as suggested in Chapter 2, appropriate process monitoring tools need to be chosen. Monitoring devices available for the three main, global variables (biomass, substrates and products) have been discussed in Section 2.3.2 and are schematically summarized in Figure 3.1.

In addition to those more advanced monitoring features, the ”holy, ancestral trinity” of bioprocess monitoring, namely pH, pO_2 and temperature should not been forgotten. Indeed, heat transfer challenges, heavily investigated and successfully addressed in the early stages of bioprocessing are a core part in creating an appropriate bioprocess environment. While nowadays the reaction temperature is routinely monitored with high accuracy and controlled with precision at the optimal growth conditions for the organism under consideration, changes in temperature are taking more and more importance. Temperature shifts or cold-shock strategies are employed for recombinant protein production in mammalian expression systems [127, 128] and to a certain extent also with microbial systems [129]. Lowering the culture temperature to $32\,^{\circ}\mathrm{C}$ instead of $37\,^{\circ}\mathrm{C}$ in some CHO lines for instance, will result in a decreased specific growth rate, however yield an increased specific recombinant protein productivity. Furthermore, an effective strategy to avoid protein aggregation during recombinant protein production by micro-organisms, is to decrease the temperature

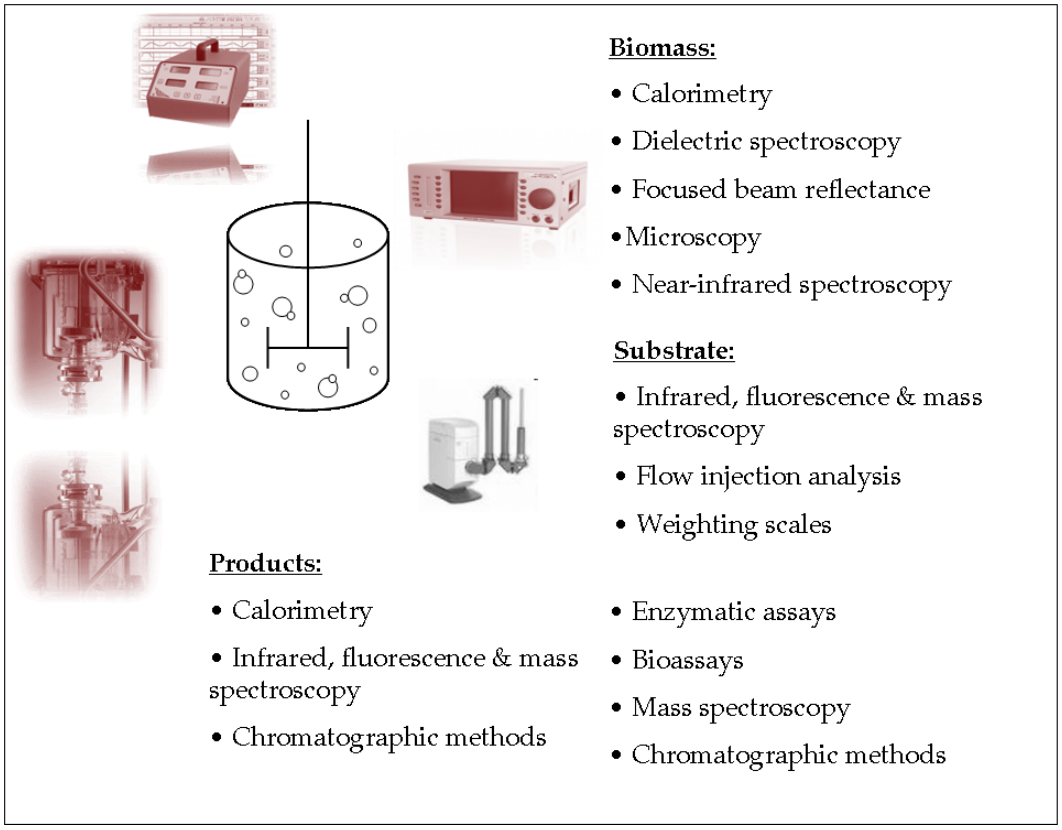

Figure 3.1: Non-exhaustive list of monitoring devices for bioprocesses.

down to 25 °C [130]. Providing efficient temperature monitoring and control is therefore paramount for successful industrial bioprocessing. Maintaining the pH at the optimal value for growth is also considered as a standard in both research and industry. However, in some particular cases, an uncontrolled, however monitored situation in terms of pH can be profitable. For instance, in acetone-butanol-ethanol fermentations [131, 132], a change in pH is the inductive driving force leading to the formation of the product of interest. It has already been mentioned in Section 2.3.2 that the level of dissolved oxygen (DO) can actually be part of effective strategies for monitoring the specific growth rate [61]. DO-stat cultures have also been identified as interesting approaches to enhanced recombinant protein production [133]. Maintaining a sufficient level of dissolved oxygen within the reactor environment is essential in order to avoid oxygen-limited conditions.

While temperature, pH, pO_2, stirring and aeration rate monitoring and control obey to well-established standards, for all additional variable monitoring techniques, some particu-

Table 3.1: Expected features and characteristics of monitoring techniques for bioprocesses

- Reliable
- Sensitive
- Non-invasive
- Non-destructive
- Stable, rapid and simple in use
- Not compromising sterility
- Biologically inactive

lar characteristics are required. These requirements are summarized in Table 3.1. Process analyzers, as described by the PAT initiative [3], should provide process related information, if possible of multivariate nature, through non-destructive, non-invasive real-time measurements.

Within the scope of this thesis, a choice needed to be made in terms of monitoring tools, searching for a technique that satisfies as many as possible of the characteristics mentioned above (Table 3.1). Biocalorimetery and a combined use of dielectric and mid-infrared spectroscopy were chosen, mainly due also to availability of devices and lab-inherent expertise and logistics. The present chapter will mainly describe biocalorimetry and dielectric spectroscopy starting with their evolution in bioprocessing monitoring and highlighting their importance in relation to the monitoring of the specific growth rate. Additionally, data reconciliation, a powerful engineering tool, yet underestimated in bioprocessing will be discussed since it is of relevance to Chapters 4 and 5. Finally, a short and very shallow introduction to control theory will be given to place the control strategy developed within the scope of this thesis in perspective with existing standards. Emphasis will also be given to the discussion about the boundaries of control.

3.2 Process Analytical Technology (PAT) process monitoring tools

3.2.1 Biocalorimetry - heat flow calorimetry

Over the past three decades, biocalorimetry has made a significant impact as a tool for bioprocess monitoring and control applications. Bench-scale calorimeters as well as micro-calorimetry devices have been extensively employed to fingerprint metabolic singularities of microbial cultivations, such as cell growth, product formation, nutrient limitations and diauxic growth. Improving the resolution of the measured heat flow signal in bench-scale calorimeters is however required to monitor weakly exothermic bioprocess systems such as animal cell cultivations.

Applications of heat flow calorimetry for monitoring and control

"Heat generation and absorption is a truly universal feature of chemical, biochemical and also biological processes" [134]. Heat exchange rates can provide quantitative information about the on-going process and have been used in many instances to characterize cellular processes. Process monitoring by calorimetry can be split into two main categories: the use of micro-calorimetric devices for either off- or at-line characterization of heat of reaction rates and in-line monitoring of cultures by the use of bench-scale calorimeters. The latter have been extensively applied to microbial processes to measure the metabolic activity [135, 69, 70, 71], to investigate thermodynamic efficiency during transitions [136, 137] or to assess microbial activity under different culture conditions [138, 139]. Several authors exploited the heat signal to control microbial cultures [140, 69, 141, 142, 67, 143, 144] or to study a particular type of enzymatic activity within it [145]. On the other hand, application of bench-scale calorimetry to monitor mammalian cell cultures or anaerobic microbial fermentations are rather scarce. Micro-calorimetry seemed so far more appropriate to measure the rather low heat generation rate of mammalian cells or anaerobically growing micro-organisms. Indeed, researchers at the University of Wales intensively studied heat flow signals generated by CHO 320 cells producing interferon-γ [146, 147, 148, 149] using heat conduction (micro-) calorimeters. Such devices have a lower detection limits (less than 2

mW/L [66]) than bench-scale calorimeters and can even be used to follow encapsulated cells [68]. Given the lower detection limit, micro-calorimetry seems more appropriate to monitor the weakly exothermic nature of cell cultures. However, the use of bench-scale calorimeters such as the BioRC1 or the eRC1 from Mettler-Toledo, offering a sufficient sensitivity (Figure 3.2) for aerobic cultures carried out throughout this study, can be beneficial in particular with regards to temperature regulation for recombinant protein production. Optimized recombinant protein production through temperature shifts is a commonly used strategy in *E. coli* and CHO cell cultures [129, 127, 150, 128]. The bench-scale calorimetry environment enables tight control over temperature modulation while simultaneously providing a tool to monitor the metabolic activity of the cells. In this sense, bench-scale calorimetry can be considered as a key part of a control platform for integrated recombinant protein production optimization and potentially be transferred to large-scale, since the sensitivity in the heat signal improves with any scaling up due to increasing ratio of heat producing volume to heat exchanging surface [151].

Challenges associated with heat flow calorimetry

Two main challenges are associated with calorimetry and discussed in the following two paragraphs.

Non-specificity of the calorimetric signal Calorimetric measurements are relatively simple to obtain, depending on the devices used, and can even allow for the identification of micro-organisms or the distinction between different strains [152]. Data obtained can also be used to detect particular events such as a shift from oxidative to fermentative metabolism or nitrogen-limited growth [153]. However, the heat evolution rate is representative of the global metabolism of the organisms of the culture under study. In this sense, heat flow measurements do not allow, compared to dielectric spectroscopy or chromatographic methods, the detection of a particular compound, but rather the detection of the sum of all metabolic reactions leading to a change in the heat evolution. Care must therefore be taken in the interpretation of the calorimetric data. It might be helpful to check obtained values with the-

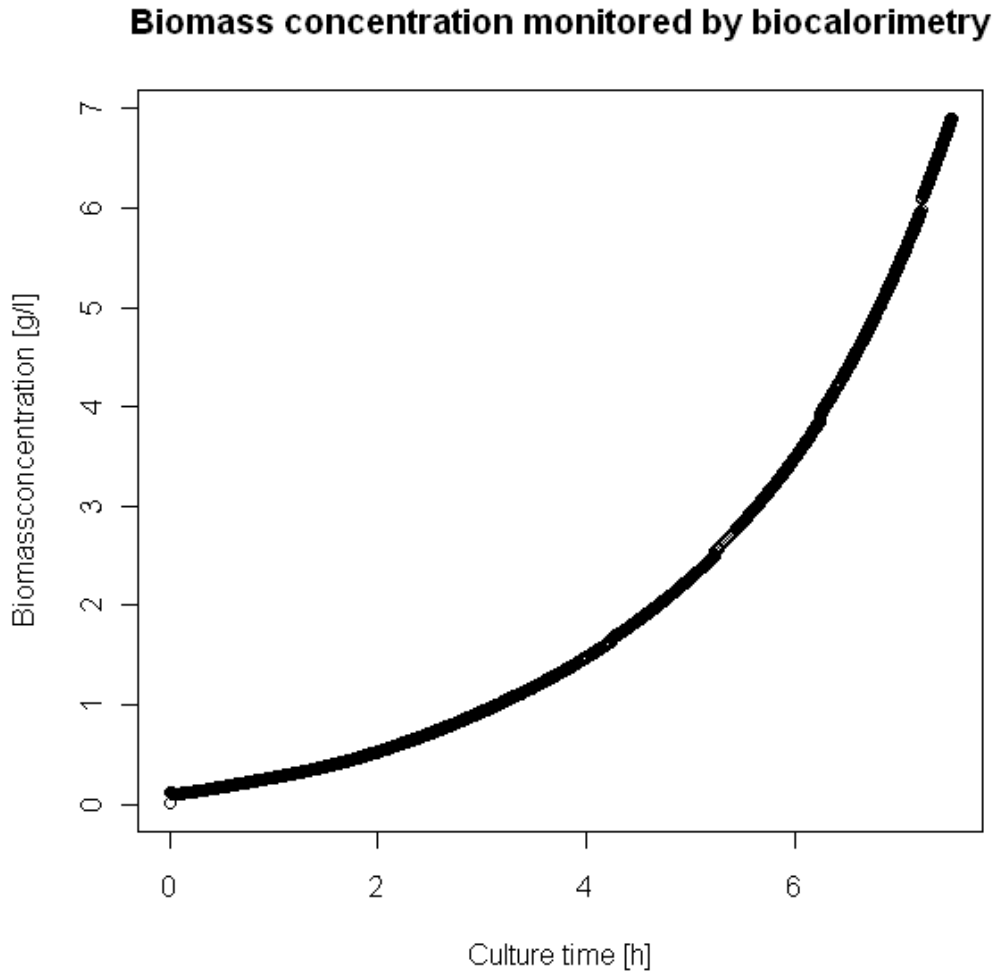

Figure 3.2: Example of application of bench-scale biocalorimetry (BioRC1) to monitor the biomass evolution, estimated based on heat flow measurements as described in Chapter 6, in a batch culture of *Kluyveromyces marxianus* (Adapted from [72]).

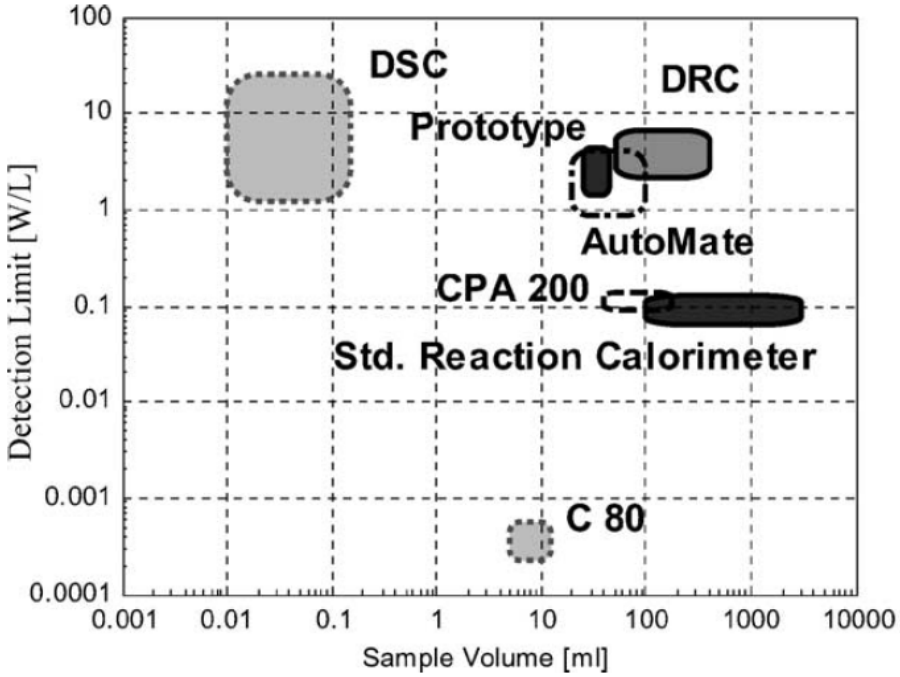

Figure 3.3: Comparison of the detection limit of different commercially available reaction calorimeters [157].

oretical predictions [154, 155] and to have additional measurements to confirm metabolic particularities such as the on-set of fermentative stage by determination of the respiratory quotient (RQ) by off-gas analysis [97, 156].

Sensitivity of the technique As previously mentioned, the sensitivity of bench-scale calorimetry, as employed throughout this study, is within the require range for monitoring and controlling high cell density cultures of aerobically growing micro-organisms [158, 70, 140]. However, the detection limit is around $100mWl^{-1}$ for most commercially available devices (Figure 3.3) and around $50mWl^{-1}$ for aerated conditions in specially modified bench-scale calorimeters [158]. Such values are at the limit of the requirements for monitoring weakly exothermic reactions, such as anaerobic cultures or mammalian cell cultures (Figure 3.4). Much higher sensitivity can be achieved in micro-calorimeters [153, 159], but they do not allow the same type of operation than traditional reactors, rendering simultaneous monitoring of heat flow and other important process variables nearly impracticable.

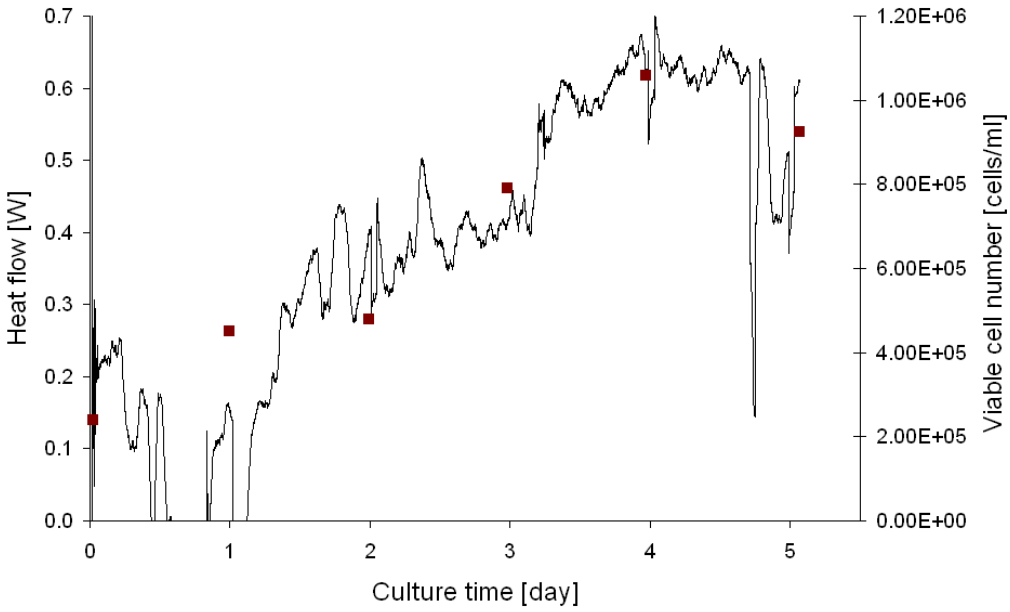

Figure 3.4: Example of application of bench-scale calorimetry for monitoring purposes of mammalian cell cultures (CHO DP12 cells - batch culture carried out in April 2012 (DP12eRC1B04)). The continuous black signal represents the heat flow in watts, while the red squares represent the number of viable cells per milliliters determined off-line by manual cell counting.

Potential of industrial application and use within the scope of the thesis

Industrial applications of calorimetry to monitor or control microbial bioprocesses are close to non-existent. However, the way leading to an industrial application is paved. As already mentioned, increased sensitivity can be achieved through scaling up of the process [151] and several groups have shown how a set of temperature probes and mass flow controllers can actually transform any large-scale bioreactor into a biocalorimeter [73, 74, 160, 161].

The simplicity of the sample preparation, measurement and interpretation, mentioned throughout several presentations at the last two International Society for Biological Calorimetry Conferences (ISBC) [162, 163], is an attractive feature of this particular process analyzer. For aerobic yeast cultures, all main characteristics of an ideal process analyzer (Table 3.1) are actually satisfied, rendering biocalorimetry the method of choice for the creation of a versatile and powerful platform for controlling the specific growth rate of micro-organisms in fed-batch cultures.

3.2.2 Dielectric Spectroscopy

Dielectric spectroscopy exploits the complex electrical characteristic of viable cells. Any such complex, passive, electrical system can be defined by two characteristics: capacitance measured in Farads (F) and conductance measured in Siemens (S). Dielectric spectroscopy can provide information on the total viable cell volume; only cells with intact membranes act like capacitors and contribute thereby to the signal when placed in an electrical field. Obtaining information about the viable cell volume is important, since monitoring the growth of the organism of interest can be crucial to the process, for instance to determine the appropriate time for recombinant protein production induction. In addition, some interesting bioprocess products are growth-related. The measurement of the evolution of the viable cell volume can allow identifying the specific product formation rate. In order to design control strategies to maintain a particular specific growth rate or act on the product formation rate, it is crucial to make *in situ* measurement of biomass or bioactivity.

Challenges associated with dielectric spectroscopy

Dielectric spectroscopy originated from impedance measurement techniques and is used extensively in different fields, such as electronic engineering and chemistry. The first papers related to the biological application mentioned the use of radio-frequency impedance to measure viable biomass [164]. Since its origin in the biotechnology area, the technique is gaining importance, particularly since the edition of the PAT initiative, as shown in a recent review [165].

Commercially available dielectric spectrometers are built to fit within other clean in place (CIP) and sterilize in place (SIP) equipment and allow *in situ* monitoring providing real-time information through high-frequency measurements. Most available devices are highly customized and include a wide range of approved filtering and data pre-processing techniques. The instruments are versatile and applicable to all type of cells. On the other hand, capacitance measurements show dependence on temperature, pressure, mixing rate, aeration rate, reactor volume, probe position and proximity to metal components. Obtained data is however reasonably stable if all above mentioned parameters are kept as constant as possible [166]. The validity and reliability of the gathered process information is highly dependent on the correlation to off-line or alternative measurements as discussed later. Despite the advantages of dielectric spectroscopy as a process analyzer, its application is found in the academic field rather than in industry. Dielectric spectroscopy finds its application in fields such as molecular biology (monitoring of the transfection efficiency [167, 168]), protein folding [169] or food technology [170]. Alternative but related methods include dielectrophoresis [171, 172], electrochemical impedance spectroscopy [173].

Initial work in the area was mostly concerned with improving the instrumentation and the mathematical translation of the signal [75, 80, 174] as well as the exploration of the dielectric proprieties of cells [175, 176]. A series of papers was dedicated to exploring the frequency-dependence of the capacitance measurements and the appropriate techniques to extract meaningful information out of the obtained data [80, 177, 76, 178]. Several types of corrections for changes in conductivity of the medium were proposed and the most appropriate ones are now built into the most recently developed devices. Once the technique had

reached a sufficient level of reliability and maturity, a vast amount of processes involving commercially relevant cell types (animal cells [179, 180], plant cells [181] and industrially exploited microbes) were monitored with the aim of drawing correlations [90] and developing prediction models [64]. The method became more and more popular for monitoring process singularities or particular events such as storage of intracellular compounds [182], virus production within infected animal cells [183] or bacteriophage production [184]. Different authors reviewed the application of dielectric spectroscopy to bioprocess monitoring [181, 178, 77, 185, 165].

The challenges associated with dielectric spectroscopy have received a lot of attention over the years and can be divided into two main topics that are discussed in details in the next paragraphs: a. reference techniques for use in correlations to transform the physical measurement (in Farad or Farad/m) into process related information and b. data processing.

Reference methods for use in dielectric spectroscopic correlations The quest for a golden reference standard for the correlation of the capacitance reading to relevant biological information is still on-going. Regardless of the nature of cells considered, the capacitance of a lipid membrane is on average $0.51\mu F\ cm^{-2}$ of membrane area [80]. The capacitance measurement still needs to be transformed into relevant information depending on the aim of the study. Research groups have described several correlation methods over the past two decades (Table 3.2). Xiong *et al.* [90] compared the most common techniques, namely optical density at 600 nm, dry cell weight (DCW), packed mycelial volume and colony forming units (CFU), and obtained linear correlations in the range $3.1 \cdot 10^6 cells\ ml^{-1}$ to $9.2 \cdot 10^9 cells\ ml^{-1}$ for *Saccharomyces cerevisiae* with all techniques. Cell counting methods, especially when associated to Trypan blue staining to differentiate between viable and non-viable cells, are commonly used to correlate capacitance measurements to cell number or cell viability for animal cells. However, if Crystal Violet for nuclei counting is used, deviation from linearity can be observed if cells tend to be multi-nucleated under a specific culture conditions [186]. Neves *et al.* [187] observed linear correlations of capacitance for DNA, packed mycelial volume and CO_2 production during exponential phase, while

Table 3.2: References techniques used for correlating dielectric spectroscopic signals to biological meaningful variables.

Technique	Reference
Cell counts & staining methods	[90, 180, 186]
Cell diameter & cell size	[189, 168, 182]
Colony Forming Unit (CFU)	[90]
Cross sectional area	[182]
Dry Cell Weight (DCW)	[181, 190, 187, 166, 85, 90]
DNA content	[187]
Nucleotide (NTP) content	[191]
Packed (mycelium) volume	[181, 90]
OD/turbidity	[166, 85]
Viscosity measurements	[187, 84]

correlations between DCW and capacitance measurements tended to be linear during exponential, transition and stationary phase. Whatever method is used, it should be noted that the correlations tend to be linear as long as no major metabolic, physiological or morphological changes occur within the culture [90]. Indeed, as mentioned previously, dielectric spectroscopy is not only a tool to measure viable cell volume, but also to detect changes during biotechnological processes [168, 183]. Maskow an co-workers [188] discussed the reasons and implication of non-linear correlations between biomass and capacitance correlations in the scope of bioprocess monitoring and control.

Data processing Finding and exploiting the appropriate correlation method is not the only challenge to be tackled when implementing dielectric spectroscopy for monitoring purposes. Signal noise and lack of robustness are the principal hurdles that need to be overcome. The first issue has been extensively addressed through implementing the appropriate filtering techniques [80, 177, 76, 178]. Stirring noise can be filtered out using a low pass filter with a time constant of $1s$ [75]. Most manufacturers of dielectric spectrometers have integrated the appropriate filters in the provided software. However, there is still a need to correct for changes in conditions over time. Predictive modeling [81] as well as data reconciliation [64] can improve the reliability of the measurements.

Range of measurement and limits of application

Dielectric spectroscopy gives real-time information about cell volume and viable cell number and depending on the application even insight into changes in physiology or morphology of cells. Commercially available devices have different ranges of validity and linearity of measurements and also find different applications. xCELLigence for instance allows for real-time monitoring of cultures in 96-well plates and is mainly used for mammalian cell culture [192] at micro-scale. Aber Instruments as well as the more recent but very similar Fogal range of devices find their application in the monitoring of cultures of all type of organisms, but mostly at lab-scale. Aber devices are very commonly used in brewing industry where they are even well-established control tools. Devices from Hewlett-Packard [190, 193, 166, 194, 195] and EG-G (Edgerton, Germeshausen & Grier) [177], as well as some custom-made micro-devices [196, 176, 197] only play a marginal role in the field of bioprocess monitoring by dielectric spectroscopy. The most commonly devices encountered have validity ranges from $2 \cdot 10^5 cells\ ml^{-1}$ to a maximum of $1 \cdot 10^9 cells\ ml^{-1}$ corresponding to approximately $1 - 200 gl^{-1}$ of dry cell weight for yeast cells. When working with Gram-positive organisms it should be kept in mind, that the α-dispersion, not relevant when working with animal cells or Gram-negative bacteria, plays an important role [177, 80]. Cultures involving highly conductive medium cannot be monitored by traditional dielectric spectrometer that have an upper conductivity limit of $100 mS cm^{-1}$ [90]. The sensitivity of the method, especially at low cell concentration, is limited [198].

Potential of industrial application and use within the scope of the thesis

Monitoring and process understanding is one part of PAT, process control is another. Dielectric spectroscopy is definitively an interesting tool to gain better process understanding and to monitor one of the most vital process parameters in biotechnology. Applications in the area of bioprocess control are less common. A combination of capacitance and heat flow measurements has been suggested and simulated by Guan et al. [146] and later applied to control the bacterial conversion of toxic substrates into polyhydroxyalkanoates [66]. Important correlations between capacitance reading and a particular consumption or production

rate can be drawn. For instance, Noll *et al.* [191] correlated capacitance to glutamine consumption rate and based a successful feeding strategy on this particular correlation. Dielectric spectroscopy measurements were used to trigger automated cell harvest [85]. Justice et al. [165] reviewed the different control application of dielectric spectroscopy at lab-scale over the past few years. Dielectric spectroscopy is not yet widely applied in industrial production processes for biologicals, probably due to requirements in terms of data pre-processing, filtering or predictive modeling, On the other hand, in the brewing industry, another important part of the biotechnology sector, the technique is used both for monitoring and control. Interesting fields of application are automatic pitching rate control or automatic yeast recovery control to minimize biowaste [199]. Applications in pharmaceutical processes are also reported by Eli Lilly (*Pichia pastoris* cultures for recombinant protein production) [78] and by Novo Nordisk (processes involving the cultivation of BHK cells) [200]. The need to find an appropriate correlation technique and to develop a suitable technique to reduce noise may outweigh the advantages that dielectric spectroscopy has in some cases, making its implementation in the production plant rare at the present time.

The advantage of having a direct real-time measurement of the biomass concentration is an appealing feature of dielectric spectroscopy and has contributed to apply this technique throughout this work. Furthermore, the technique satisfies several points highlighted in Table 3.1. Indeed, the device is rapid and simple to use, biologically inactive, non-destructive and sterilizable. It is considered as "invasive" according to the classification of Schuegerl [201] as it is in direct contact with the culture broth, hence with the product of interest (biomass or value-added product). The sensitivity and reliability of the device is debatable at low cell concentration levels, but for the scope of this work (high density yeast cultures where the reliability of the dielectric signal is enhanced by data reconciliation (Section 3.3)), it is sufficient. For studies described in Chapters 4 and 5, the dielectric signal was correlated with DCW values or OD measurements, once a reliable correlation factor between DCW and OD values was obtained.

3.2.3 Fourier transformed (mid-) infrared (FTIR) spectroscopy

Fourier transformed (mid-) infrared spectroscopy is a powerful tool for real-time monitoring of substrates, metabolites and products concentrations in bioprocesses and has been the subject of several theses under the supervision of Prof. Ian Marison in the past as well as at the present time. Details about applications of monitoring and control based on FTIR spectroscopy as well as the limits of this methods have been widely discussed in those theses [202, 203, 204] and in a more recent paper [205] by a member of the group and will not be re-iterated here.

3.3 Data reconciliation

Raw process measurements are not flawless. In order to design suitable and robust control strategies, reliable and consistent data is required. The fermentation step for the production of a therapeutic glycoprotein for instance, can generate several thousand data points, emanating from different process analyzers. Some process variables are unmeasured, others measurements are redundant. Process data might also contain gross errors, which are non-random noise that might be attributed to calibration errors, malfunction in devices or post-calibration drifts. These errors might lead to discrepancies in the energy and mass balances of the system under study. Data reconciliation, also called data rectification or data confrontation, is one way of dealing with non-random noise and unmeasured process variables and consists in adjusting raw process measurements in order to eliminate known errors and measurement noise [206]. The method might also include coaptation or the practice of estimating the values of unmeasured variables from measured process data [207].

Data reconciliation is a well-documented approach, widely applied in industrial facilities such as refineries or bulk chemical production plants. Kuehn *et al.* [208] described data reconciliation in his paper about Mathematics of control as early as 1961. Since then, alternative mathematical approaches for the optimization problem have been explored, different statistical tests for the gross error detection have been discussed and a multitude of applications of data reconciliation in the field of engineering have been suggested.

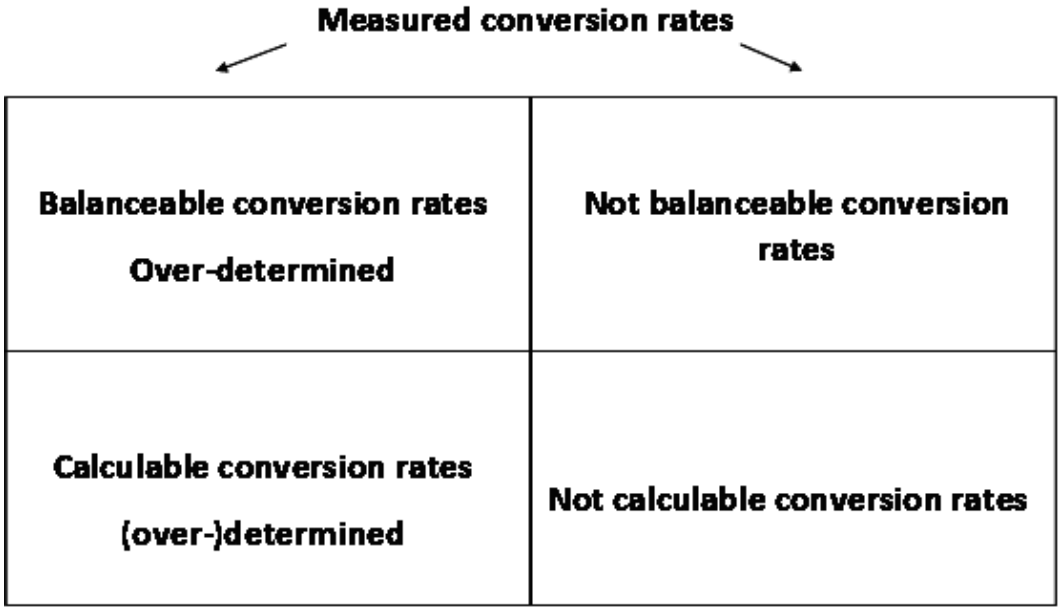

Figure 3.5: Classification of rates according to van der Heijden and co-workers [211].

Through the PAT initiative, the FDA advises the pharmaceutical and biotechnology industries to embrace the "design of manufacturing processes using principles of engineering, material science and quality assurance to ensure acceptable and reproducible product quality and performance" [3]. The implementation of data reconciliation in industrial bioprocesses, over the whole life-cycle of the products in question, would perfectly satisfy this particular point of the PAT framework. The method gains even more importance when considered within the scope of the Guidance for Industry Process Validation; General Principles and Practices, published by the FDA in January 2011 [209]. A main point of the new guidance is to deal with inherent process variations and variability. The source of variability can be either error in measurements or external disturbances such as shift in process parameters, ambient temperature fluctuations or drifts in instruments [210]. If all measurement errors are identified and eradicated by data reconciliation, anomalies in the process can be detected and readily managed. Most importantly however, having access to a set of consistent and reliable data is paramount for effective control of bioprocesses.

3.3.1 Principles

The methodology for data reconciliation is the same whether it is applied to a refinery or to the metabolism of a cell. In all cases, a clear formulation of the problem and a description of the system or a model of the process is required. Modeling energy and mass flows for a cellular system can be a challenge.

According to Noorman et al. [98], the overall aim of data reconciliation can be divided into three main points:

- Calculation of unknown state variables from measured quantities
- Detection and removal of inconsistencies from the data set
- Improvement of the accuracy of the measured data

In every application field, whether it is in industrial chemistry or mechanical engineering, data reconciliation is based on mass and energy conservation principles. However, it is particular to the biotechnology field to conventionally use conversion rates of metabolic pathways to describe the on-going process. When working with cellular systems, the conservation equations required for data reconciliation can be derived from mass and energy balances using metabolic stoichiometry.

According to Heijden et al. [211, 212], four major steps should be considered in any data reconciliation routine:

- Selection of measured and non-measured conversion rates
- Classification of conversion rates into four categories (Figure 3.5)
- Calculation of the optimal estimations for the measured and non-measured rates
- Gross error detection, diagnosis and estimation

Table 3.3: Definitions associated with data reconciliation.

Redundant:	Two or more (but not necessarily all) of the measured conversion rates are balanceable
Determined:	All non-measured rates can be calculated from the measured ones
Under-determined:	At least one non-measured rate cannot be calculated from the measured ones

Classification of conversion rates

Data reconciliation, and subsequent gross error detection, is possible if a system is over-determined and in order to avoid confusion, some conventions, according to Heijden et al. [211, 212] are stated in Figure 3.5. These conventions should allow the development of a harmonized and simplified methodology. Some associated definitions are listed in Table 3.3.

Once the cell or reaction system is defined and its rates are classified, the laws of conservation inherent to the model used will lead to formulation of a so-called conservation matrix [98] with a certain number of constraints and a certain number of net conversion rates. From this initial conservation matrix, the redundancy of the system can be calculated, a redundancy matrix and a constraining vector can be inferred and used for the next steps of error detection and data reconciliation. A detailed mathematical explanation can be found in the work of Noorman *et al.* [98] or Heijden *et al.* [211, 212]. The computational approach corresponding to data reconciliation can be viewed as an optimization problem or a weighted least-square minimization of the measurement's adjustments [213, 214, 215]. The applied algorithm allows the calculation of the optimal estimate for each measured and non-measured component and finally statistical inference, such as maximum likelihood techniques or χ^2 tests [216, 217, 50, 27], allows detection, and even eradication of gross errors. The application of a simple, yet powerful, multidimensional χ^2 test renders the set of estimates more reliable and internally consistent, allowing the detection of instrumental malfunctions or process disorders [218].

An important aspect of data reconciliation is the choice of the appropriate system de-

scription and the palette to choose from is rather broad. Models describing the process of suspension cultivation of organisms producing commercially relevant products are common in literature and range from black box over metabolic to synthetic mechanistic models [219, 220, 59]. Detailed metabolic systems have an advantage over black box models as they provide extra balances through knowledge of the biochemistry of the system, leading to a higher redundancy and more balancing possibilities [98]. However, unstructured models based on macroscopic information (elemental and energy balances and entropy inequalities) provide a good starting point for industrial applications due to the simplicity of the models [46]. A simple Monod-model combined with a linear law for substrate removal can provide an adequate description for microbial growth, particularly in pseudo-steady state situations where kinetic expressions are not relevant and where the behavior of the system is mainly fixed by energetic parameters.

The choice of the right level of detail is crucial, but might in reality also be influenced by the availability of measurements. Indeed, for long time the difficulties of quantifying all relevant components (especially biomass or bioactivity) in real-time hindered industrial application of real-time data reconciliation and gross error detection. Recent advances in the development of sensitive real-time process analyzers should now be taken advantage of, as they will render the available data set even more reliable through the use of appropriate data reconciliation. Commonly used bench-scale process analyzers, or “lab analyzers”, such as FTIR-, mass- or dielectric spectroscopy based techniques can provide sufficient information to perform carbon, nitrogen, degree of reduction or charge balances [221, 96] while less common analyzers such as biocalorimetry [136, 137] or fluorescence spectroscopy [222] can supply additional measurements for performing heat [141] or adenosine triphosphate (ATP) balances [48]. When information about different additional metabolites is present, the system description can be extended by including different metabolic fluxes and their distribution [51, 50, 223, 52].

Challenges inherent to data reconciliation

Applications of data reconciliation to steady state processes have been widely reported [40, 27]. A dynamic approach for transient experiments bears an additional set of challenges. For instance, accumulation needs to be accounted for and in certain cases non linear systems need to be dealt with. In his work, Herwig [54] discussed both the advantages of dynamic process over steady state conditions and the inherent challenges before stating the requirements for efficient real-time balancing that are reported below:

- "Multiple process analyzers that provide high frequency measurements of the key variables to be included in the model of the process to be monitored
- A robust way of calculating balances without neglecting accumulation terms
- A simple method to check data consistency in order to:
  - Obtain a reliable description of the state of the process and of the different rates
  - Check for gross errors
  - Reconcile the measurements"

Nowadays, potentially all requirements are fulfilled, rendering data reconciliation an excellent basis for control and in-process decision making.

3.3.2 Potential of industrial application and use within the scope of the thesis

Examples of applications of data reconciliation in industrial bioprocesses are rather sparse. Perhaps the required pre-conditioning of the data or the need of appropriate computing tools for data acquisition, computation and data distribution are lacking or implementation is hindered by the difficulties in choosing a suitable model and adequate simplifications for the system [53]. However, when used appropriately, data reconciliation can turn out to be an excellent basis for enhanced process understanding and for the development of control strategies and a powerful tool for decision-based bioprocessing through the creation of a consistent and reliable data set [54, 96, 224, 64, 225, 55]. Implementation of

data reconciliation can, in theory, be done for existing processes without any changes to current installations and would not burden the companies with regulatory uncertainty. Emphasis should be put on transferring existing knowledge from academia to industry, after all "it should be common practice to adjust raw measurements taken from a process so that known errors and measurements noise are eliminated" [206]. While this particular way of processing data has been successfully applied for real-time data evaluation in projects presented in Chapters 4 and 5, it would required a few additional months of work to transfer and deploy such a real-time data reconciliation routine in the experimental setup presented in Chapters 6 and 7.

3.4 Bioprocess control

Control is essential and inherent to the bioprocessing industry. Every single step of a process needs to be controlled, since in the end, for instance for the case of recombinant proteins production, patients' lives are at stake. The final conversion step in the production of glucose syrup needs to be controlled in order to optimize the yield and to ensure the viability of the process. The feeding of methanol and oxygen in aerobic cultures process of methanotrophic yeast needs to be controlled in order to ensure protein production induction while avoiding explosions. In each of these very different processes, control is required, but is it the same type of control? Obviously an appropriate control strategy can only be developed if the specifications are well-known and well defined. It seems therefore important to start with a series of questions that are essential to establish a reliable control strategy:

- Why is control required?
- What exactly needs to be controlled?
- What are the tools required to identify or quantify the subject of control?
- What timeframe is acceptable for the control response?

Control theory defines three essential parts common to any control strategy [4]; the controlled variable, the associated manipulated variable and the sensed value of the controlled variable. The latter part, concerning the "sensed value of the controlled variable", is particularly interesting within the scope of this chapter, since control relies on different monitoring techniques or even on the appropriate combination of techniques [69] to sense or estimate biological attributes that can sometimes not be measured directly, such as the specific growth rate.

3.4.1 Available control strategies

A traditional process, involving suspension cultures of an organism of interest producing a commercially relevant product, is normally run under specific culture conditions where a few parameters are kept constant by well-established control. These traditional constant culture parameters are usually process temperature, pH, agitation, aeration rate or initial concentrations of medium components. The control of these variables, common to other, more mature industrial areas such as chemical processing, has been improved and optimized over years. Sometimes strategies including shifts of these variables over the time of a culture are also applied. Temperature is decreased to induce protein production in *Escherichia coli* cultures [129] or in *Pichia pastoris* cultures [226], pH is changed [227] to induce a physiological shift within the cells or the agitation, the aeration rate or even the gas in-let composition is changed in order to maintain a constant dissolved oxygen level within a reaction vessel [228].

Control strategies for bioprocesses can be classified depending on the control strategy applied:

- Open loop control [229, 230, 69, 105, 96, 55]
- Closed-loop control
  - On/off control [117]
  - Combination of proportional, integral, derivate feedback [231, 141, 108]
  - Adaptive feedback control [88, 232, 233, 101, 123]

Any of the above applied strategies can either be applied to continuous process control such as maintaining a particular specific rate throughout the cultivation process [101] or to specific, crucial time-point changes, such as recombinant protein production induction [234, 128] or cell harvest triggering [235, 236]. When looking at control from this point of view, the classification leads to two main categories: a. continuous control of a given variables and b. decision making.

Control of physiological key variables such as the specific growth rate, the specific carbon source up-take rate or a given cellular and metabolic state can be considered as "high" level control in comparison to the control of culture conditions as mentioned at the beginning of the section. However, most control strategies are based on similar types of manipulated variables. The feed rate or the concentration of a given compound in one form or another is probably the most widely used manipulated variable [113]. The control strategies applied however, differ from project to project and are dependent on the aim of the control but also on the acceptable timeframe of the system response. Indeed, the same feeding strategy might not be used when using methanol, a potentially cytotoxic compound [237] or a milder form of inductor such as IPTG (isopropyl -D-1-thiogalactopyranoside) [232, 85]. Slow growing mammalian cells do not need the same type of control as cultures of cells with a much shorter cell cycle, such as *Bacillus* genus [238].

A sound process understanding, a simple, robust, and hence a comprehensive model of the process and the appropriate type of process analyzers combined to the right multivariate data analysis technique are paramount for designing the appropriate control strategy. The number of reviews providing an in depth analysis of the broad and challenging topic of control in bioprocesses have increased considerably since the launch of the FDA's PAT initiative [165].

3.4.2 Process analyzer combination and bioprocess control in the scope of the PAT initiative

"Effective control of all critical quality attributes" [3] is one of the points addressed by the PAT initiative. Several attributes also require several process analyzers. When dealing with complex systems, such as living organisms producing highly complex molecules, it is essential to use several tools to capture as much of this complexity as possible. Furthermore, multivariate data analysis, as well as data-driven modeling techniques should lead to better process understanding hence to better process control.

3.5 Conclusion

Monitoring and control of biotechnological processes can be broken down into four prime components; the measurements of critical process parameters, estimators and models to describe non-measurable process parameters, control strategies based on measurements and estimators to control critical process variables as well as to operate the process at an optimal level and finally the response system, such as the process or the organism under study. Each of these four parts needs to be well defined and well understood in order to ensure an integrated approach to bioprocessing control. In Chapter 3, features and limitations of the process analyzers applied to bioprocesses within the scope of this thesis have been highlighted and application examples have been discussed, thereby opening up space for conceiving further applications of the presented concepts and methods proposed throughout Chapter 4 to Chapter 7.

Chapter 4

Simple control of specific growth rate in biotechnological fed-batch processes based on enhanced on-line measurements of biomass [1]

You have to do your own growing no matter how tall your grandfather was. [2]

[1] This chapter is published in *Bioprocess and Biosystems Engineering*: Dabros M, Schuler MM, Marison IW - Simple control of specific growth rate in biotechnological fed-batch processes based on enhanced on-line measurements of biomass (With kind permission from Springer Science & Business Media: [239])

[2] Abraham Lincoln, 16th President of the United States (1809-1865)

Reliable control of the specific growth rate (μ) in fed-batch fermentations depends on the availability of accurate on-line estimations of the controlled variable. Due to difficulties in measuring biomass, μ is typically estimated using reference models relating measurements of substrate consumption or oxygen uptake rate (OUR) to biomass growth. However, as culture conditions vary, these models are adapted dynamically, resulting in complex algorithms that lack the necessary robustness for industrial applicability. A simpler approach is presented where biomass is monitored using dielectric spectroscopy. The measurements are subjected to on-line balances and reconciled in real-time against metabolite concentrations, base consumption and off-gas composition. The reconciled biomass values serve to estimate the growth rate and a simple control scheme is implemented to maintain the desired value of μ. The methodology is developed with the yeast *Kluyveromyces marxianus*, tested for disturbance rejection and validated with two other strains. It is applicable to other cellular systems with minor modifications.

4.1 Introduction

On-line monitoring and control of biotechnological processes is becoming standard procedure in the light of increasing demand and governmental regulations regarding product quality and consistency. Fed-batch processes, which are used extensively in industrial pharmaceutical applications, can be greatly improved through optimization [101]. From the control point of view, the most important parameter to manage is usually the specific growth rate (μ). The productivity of a process and product quality are frequently strongly growth-related and suffer from even relatively small deviations from the optimal growth rate [89, 231]. In addition, the biosynthesis of many products is closely dependent on reaching and maintaining the appropriate growth profiles [92, 12, 88]. In processes involving strains prone to metabolic bottlenecks, such as *Escherichia coli* or *Saccharomyces cerevisiae*, con-

trolling the growth rate below a critical value is also necessary to prevent the formation of overflow metabolites causing decreased biomass productivity [95, 240, 96, 232]. Hence, the ability to closely monitor and maintain the desired growth rate at various stages of a fed-batch process is essential in order to maximize productivity and ensure consistent product quality. Achieving dependable process monitoring and control fully contributes to the development of the field of Process Analytical Technology (PAT) [3].

The main obstacle in controlling the specific growth rate comes from the difficulties in measuring accurately the concentration of biomass on-line. Biomass is one of the most important parameters to follow during a culture yet at the same time, it is one of the most challenging analytes to measure on-line [241, 242]. As a result, feeding strategies for fed-batch processes are often based on open-loop dynamics derived from experimental optimization [101]. Closed-loop growth rate control requires some form of on-line mechanism to estimate the controlled variable. Since direct on-line analyzers are frequently unavailable or not sufficiently reliable, biomass concentration is typically estimated by monitoring an auxiliary parameter, such as substrate consumption, the oxygen uptake rate (OUR) or dissolved oxygen (DO) levels [101, 231, 96]. Reference models, containing various relevant parameters that need to be identified for the specific process, are then used to estimate the biomass concentration and the specific growth rate. A frequent problem with such models is that they inherently suffer from uncertainties caused by the continually changing state of the culture [89, 231, 42]. In order to minimize the resulting model-process mismatches, many authors turn to various types of model adaptation algorithms [101, 89, 92] but as a result, the controllers and adaptation mechanisms often end up being highly complex and lack the necessary robustness for industrial applicability.

A much more straightforward and systematic way of estimating and controlling the specific growth rate in real-time is possible provided that reliable instrumentation and methodology are available for direct measurements of biomass. Dielectric spectroscopy has become one of the most promising techniques for on-line biomass monitoring, exhibiting selective detection of viable biomass, short measurement intervals and non-invasive, *in-situ* operation [64]. Claes and Van Impe [81] and November and Van Impe [82] used a dielectric

spectrometer to measure biomass concentration and implemented an observer-based estimator for the specific growth rate. Following careful parameter tuning, the resulting algorithm provided relatively reliable estimates of μ, though some difficulties were encountered with oscillations in the results as well as with estimator stability at low biomass concentrations.

Besides the challenges of measuring biomass and estimating μ, precise control of an exponential feed rate is often hindered by issues related to the controller stability. Classical proportional or proportional-integral controllers require dynamic parameter adaptation (gain scheduling), which can lead to a loss of robustness resulting from the exponential nature of process disturbances. Additionally, gain scheduling itself is inherently process-specific and may show weak adaptability to other strains or process conditions.

In this work, an alternative approach is presented to address both of the above-mentioned issues. During fed-batch cultures of three different strains of yeast, biomass concentration measurements provided by dielectric spectroscopy are enhanced in accuracy by reconciling them in real-time against on-line measurements of the concentrations of medium metabolites and off-gas composition [64]. The reconciliation algorithm is based on the closure of mass and elemental balances, while a statistical test is used to confirm the integrity of the balances throughout the process. The reconciled measurements of biomass are used to estimate the specific growth rate and a simple proportional-integral feed-back control strategy is implemented to maintain μ at a desired setpoint value. Because the controller gains are included in an exponential form, they require no dynamic adaptation, providing a simpler and more robust control scheme. The principal aim of this study is to highlight the value of reliable on-line measurements of biomass concentration in regulating the specific growth rate using a simple control approach. In short, rather than focusing on the development of complex reference models, parameter estimators or control strategies, this article proposes to concentrate on improving the estimation accuracy of the controlled variable. Furthermore, the simple but highly efficient process control strategy proposed in this study may be considered as a Process Control Tool in the realm of the PAT initiative since it intends “to monitor the state of a process and actively manipulate it to maintain a desired state” [3].

4.2 Materials and methods

4.2.1 Chemicals

All chemicals used in this study were purchased from Sigma Aldrich (Sigma Aldrich, St. Louis, USA).

4.2.2 Cell strain and culture conditions

Cell strain

The study was centered on fed-batch cultivations of the wild-type strain of the yeast *Kluyveromyces marxianus* (DSM 5422, DSM, Braunschweig, Germany). This organism was chosen mainly due to its Crabtree-negative metabolism and simple growth requirements. Source cells were stored at $-80\,^{\circ}\mathrm{C}$ in $1.8ml$ aliquots.

Inoculum preparation

The reaction inoculum was obtained by suspending cells from one aliquot into a 1-liter Erlenmeyer flask containing $100ml$ of a sterile complex pre-culture medium ($20gl^{-1}$ glucose, $10gl^{-1}$ yeast extract, $10gl^{-1}$ peptone), incubating the flask for 24 hours at $30\,^{\circ}\mathrm{C}$ and 150 rpm, centrifuging to remove the complex medium and re-suspending the cells in 10 ml of a sterile solution of $9gl^{-1}$ NaCl.

Reactor description, media composition and culture conditions

The cultures were grown at $30\,^{\circ}\mathrm{C}$ in a 3.6-liter laboratory bioreactor (Bioengineering AG, Wald, Switzerland) with a working volume of $2.6l$, equipped with a double 6-blade Rushton-type agitator, baffles, temperature and pH probes and control mechanisms, air inlet and outlet ports, a base inlet port, a feed inlet port and a sampling port. The outlet air passed through a condenser to minimize liquid loss by evaporation. A solution of 4M NaOH was used to automatically maintain pH 5; no acid control was necessary. The reactor was sterilized *in-situ* with deionized water at $121\,^{\circ}\mathrm{C}$ for 20 minutes, drained when cool and filled with $2.0l$ of sterile medium. The cells were first grown in the batch mode until depletion of

Table 4.1: Composition of the batch and feed media for *C. utilis* and *K. marxianus* .

Component	Batch medium [gl^{-1}]	Feed medium [gl^{-1}]
Glucose	10	300
$(NH_4)_2SO_4$	5	50
KH_2PO_4	3	35
$MgSO_4 \cdot 7H_2O$	0.5	3
Component	Batch medium [mll^{-1}]	Feed medium [mll^{-1}]
Trace elements & vitamins solutions	5	15
Polypropylene glycol 2000 (Antifoam)	0.5	2

the carbon and energy source, at which point medium feed was started. Table 4.1 outlines the composition of the defined batch and feed media. The media were sterilized by filtration and supplemented with vitamins and trace elements as prescribed by Verduyn *et al.* [243] and Cannizzaro *et al.* [95].

The batch phase of the culture was carried out with an agitation speed of 800 rpm and an inlet air flow rate of $2.5lmin^{-1}$ ($1.25vvm$). The maximum specific growth rate observed during the batch phase was in the order of $0.5h^{-1}$. The agitation speed was raised to 1200 rpm during the fed-batch phase in order to enhance oxygen transfer. A detailed account of liquid volumes entering the reactor (base and medium feed) and leaving it (samples) was made in order to have a continuous inventory of the reactor volume throughout the experiment. Samples of $12ml$ were taken at regular intervals and biomass dry cell weight (DCW) determined by passing a known volume of the sample through a pre-weighed 0.22 μm pore filter, washing twice with demineralized water and drying the filter to constant weight at $100\,^{\circ}\mathrm{C}$. Optical density measurements were performed spectrometrically at 600 nm (Spectronic Helios-Epsilon, Waltham, MA, USA).

4.2.3 Process monitoring and data reconciliation

The dielectric instrument used in this work to follow the evolution of the biomass concentration on-line was the Biomass Monitor 210 from Aber Instruments (Aberystwyth, UK). Details pertaining to the measurement principle are described elsewhere [241]. The spectrometer was equipped with a 12 mm probe installed inside the bioreactor and sterilized

in-situ. A built-in crosstalk correction algorithm for small fermenters, provided by Aber Instruments was applied to minimize the influence of conductivity variations on the capacitance measurements. The Biomass Monitor was switched on two hours before starting culture experiments to allow stabilization of the signal. The instrument was calibrated prior to the experiment using data from preceding fed-batch cultures. A linear correlation model was established between biomass dry cell weight and dual-frequency capacitance values obtained at $500kHz$ and $10MHz$. During the culture, measurements were taken every 20 seconds and averaged over the routine interval of 2 minutes. A program developed in-house using LabVIEW 7.1 (National Instruments, Austin, TX) was used to collect and store the acquired data.

In parallel to the biomass measurements, the CO_2 and O_2 composition of the culture off-gas was measured using a laboratory-scale gas analyzer (Duet, Advanced BioSystems Ltd, UK), while the major medium components (glucose, glycerol, ethanol, ammonium and phosphates) were monitored using a Fourier-transform mid-Infrared (FTIR) spectrometer (ReactIR 4000, Mettler Toledo, Greifensee, Switzerland). In addition, the amounts of base consumed for pH control and of medium fed into the bioreactor were registered on-line by placing the reservoirs containing base and medium on analytical balances (PG5001-S, Mettler-Toledo, Greifensee, Switzerland) interfaced to LabVIEW. From the on-line measurements, a set of on-line mass and elemental balances (carbon, nitrogen, degree of reduction and charge) were set-up [118, 243, 64]. The balances served as constraints for a data reconciliation algorithm which adjusted all measurements to their best estimates. The balances and data reconciliation routines were carried out in real-time at an interval of 2 minutes in MATLAB 7.5 (The MathWorks, Inc., Natick, MA, USA) producing, at each interval, a reconciled value of the biomass concentration. Exact details of the method can be found elsewhere [118]. The implementation of the complete setup is described in Section 4.2.6.

4.2.4 Specific growth rate estimator

The reconciled biomass concentration values were used to estimate the controlled variable, μ_{est}, on-line. Typical ways of calculating the instantaneous specific growth rate using measurements or estimates of the biomass concentration, c_x, usually involve the use of some form of the following equation [42, 96]:

$$\mu_{est}(t) = \frac{r_{x,t}}{c_{x,t}} = \frac{\ln \frac{c_{x,t}}{c_{x,t-1}}}{t_t - t_{t-1}} \tag{4.1}$$

In Equation 4.1, r_x is the reaction rate. The drawback of using these estimators is that they are extremely vulnerable to noise in the values of c_x, rendering the controlled variable μ_{est} highly unstable [12]. Therefore, the measurements of biomass need to be filtered using one of many available signal smoothing methods. In this work, satisfactory results and stability were achieved by filtering c_x over a moving window, t_{int}, of 20 minutes and calculating the specific growth rate over this interval:

$$\mu_{est}(t) = \frac{\ln \frac{c_{x,t}}{c_{x,t-20min}}}{t_{int}} \tag{4.2}$$

The 20-minute interval was chosen based on a compromise between allowing enough time for the filter to be effective and ensuring that the interval was not significant compared to the growth dynamics of the organism (doubling time at μ_{max} for *Kluyveromyces marxianus* is approximately 2 hours).

4.2.5 Controller design

The majority of authors investigating the control of fed-batch fermentations agree that the implementation of classical controllers is challenging because: a) continuously changing process conditions make it difficult to model the process and to identify controller parameters; b) the presence of living cells implicates additional factors related to cell metabolism and morphology, which may influence the system response [12]; and c) controllers working with processes that change exponentially often lack stability because possible process disturbances are exponentially increasing [92, 244]. In this work, we propose to use a slight

variation on the classical PI controller which, to our knowledge, has not been reported previously in literature. The control law is based on a simple feed-forward / feedback principle, where the proportional and integral terms are included directly in the exponential term of the growth equation, as explained below. The main motivation of this approach is that the inherent simplicity of the formulation should contribute to greater controller robustness.

The feed-forward part of the controller is designed by combining substrate and biomass balances to yield the following expression:

$$F_{FF}(t) = F_0 e^{(\mu_{est} t)} \tag{4.3}$$

In Equation 4.3, the initial feed rate F_0 is expressed as:

$$F_0 = x_0 V_0 (\frac{\mu_{sp}}{Y_{x/s} s_F}) \tag{4.4}$$

In Equation 4.4, x_0 and V_0 are, respectively, the initial biomass concentration and reactor volume, $Y_{x/s}$ is the biomass yield coefficient and s_F is the substrate concentration in the feed solution [96]. The values of x_0, V_0 and $Y_{x/s}$ are obtained at the beginning of the feeding stage from reconciled process measurements.

The feedback component is based on the tracking error (ϵ):

$$\epsilon(t) = \mu_{sp} - \mu_{est}(t) \tag{4.5}$$

The tracking error is calculated at each interval. Including the proportional feedback term yields the following relation:

$$F_{PI}(t) = F_0 e^{(\mu_{sp} + K_P \epsilon(t)) t} \tag{4.6}$$

$$F_{PI}(t) = F_0 e^{((\mu_{sp} + K_P \epsilon(t) + K_I \int_0^t \epsilon(t) dt) t)} \tag{4.7}$$

K_P is the proportional gain. The proportional feedback term should correct for any

potential inaccuracies contained in F_0. On the other hand, the classical shortcoming of proportional-only control is that, except in presence of a pure capacity process, its use invariably leads to a non-zero steady-state offset [245]. A simple way of compensating for this effect is to add an integral term which enables the removal of the residual steady-state error. The proportional-integral controller takes the following form:

$$F_{PI}(t) = F_0 e^{((\mu_{sp}+K_P\epsilon(t)+K_I\int_0^t \epsilon(t)dt)t)} \tag{4.8}$$

K_I is the integral gain. Though effective at removing offsets, the most common weakness of PI controllers is the occurrence of oscillatory behavior and instabilities related to an excessive increase in the integral gain (windup). Therefore, the value of K_I must be chosen carefully. The difficulty of choosing and tuning both K_P and K_I stems from the fact that possible disturbances to μ are exponentially increasing. In this study, the controller parameters were chosen experimentally following simple manual tuning rules [246] where four different controller constant combinations were tested. Finally, a value of 1.5 was chosen for K_P while K_I was set to 0.5.

4.2.6 Implementation

Figure 4.1 shows the control block diagram proposed in this study. A remote-controlled peristaltic pump was used to implement the controller action, $F(t)$, and supply feed medium. The pump was calibrated before the experimental work and a simple corrective mechanism was included in the code to ensure that the amount actually fed (monitored on-line) corresponded to the theoretical feed integral, calculated at each interval.

The overall implementation flowchart is shown in Figure 4.2. All programs in MATLAB and LabVIEW, as well as the dynamic interfaces between the different programs, were coded in-house and tested rigorously before the study.

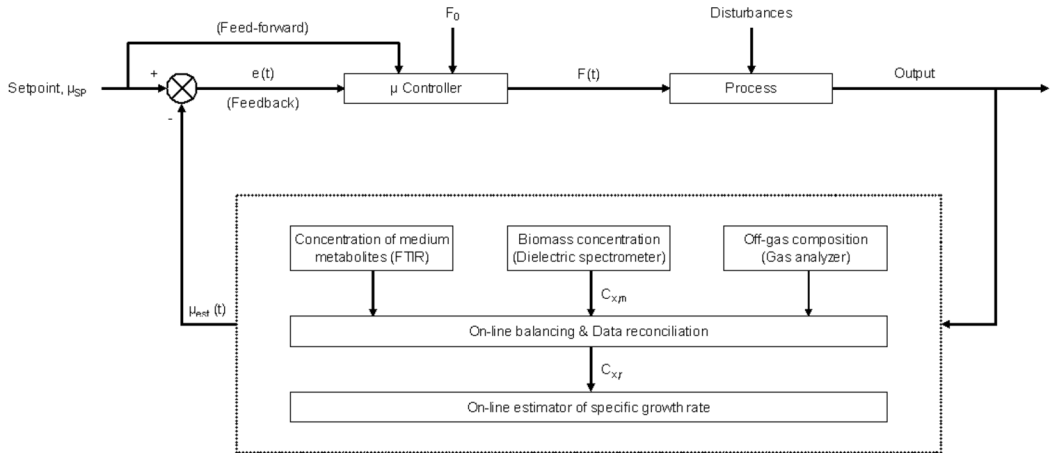

Figure 4.1: Proposed control block diagram. The measured and reconciled biomass concentration is represented by $c_{x,m}$ and $c_{x,r}$, respectively.

4.2.7 Disturbance rejection experiment

In addition to the experiments described above, the control system was subjected to an induced process disturbance. A fed-batch culture was carried out at a constant specific growth rate setpoint of $0.15h^{-1}$, maintained using the PI controller. In order to simulate a potentially realistic process disturbance, reactor cooling was switched off for a period of about two hours, causing the culture temperature to rise as a result of cellular catabolism. The purpose of this experiment was to assess the ability of the system to handle the alterations in the physiology and metabolism of the cells and to maintain tight control despite the disturbance.

4.2.8 Assessment of general applicability

In the final stage of this study, the proposed control approach was tested on different yeast strains, namely *Candida utilis* and *Pichia pastoris*. The aim of this set of experiments was to assess whether the methodology developed for the model organism (*Kluyveromyces marxianus*) could be applied without major modifications to other strains growing on different media or with different process parameters.

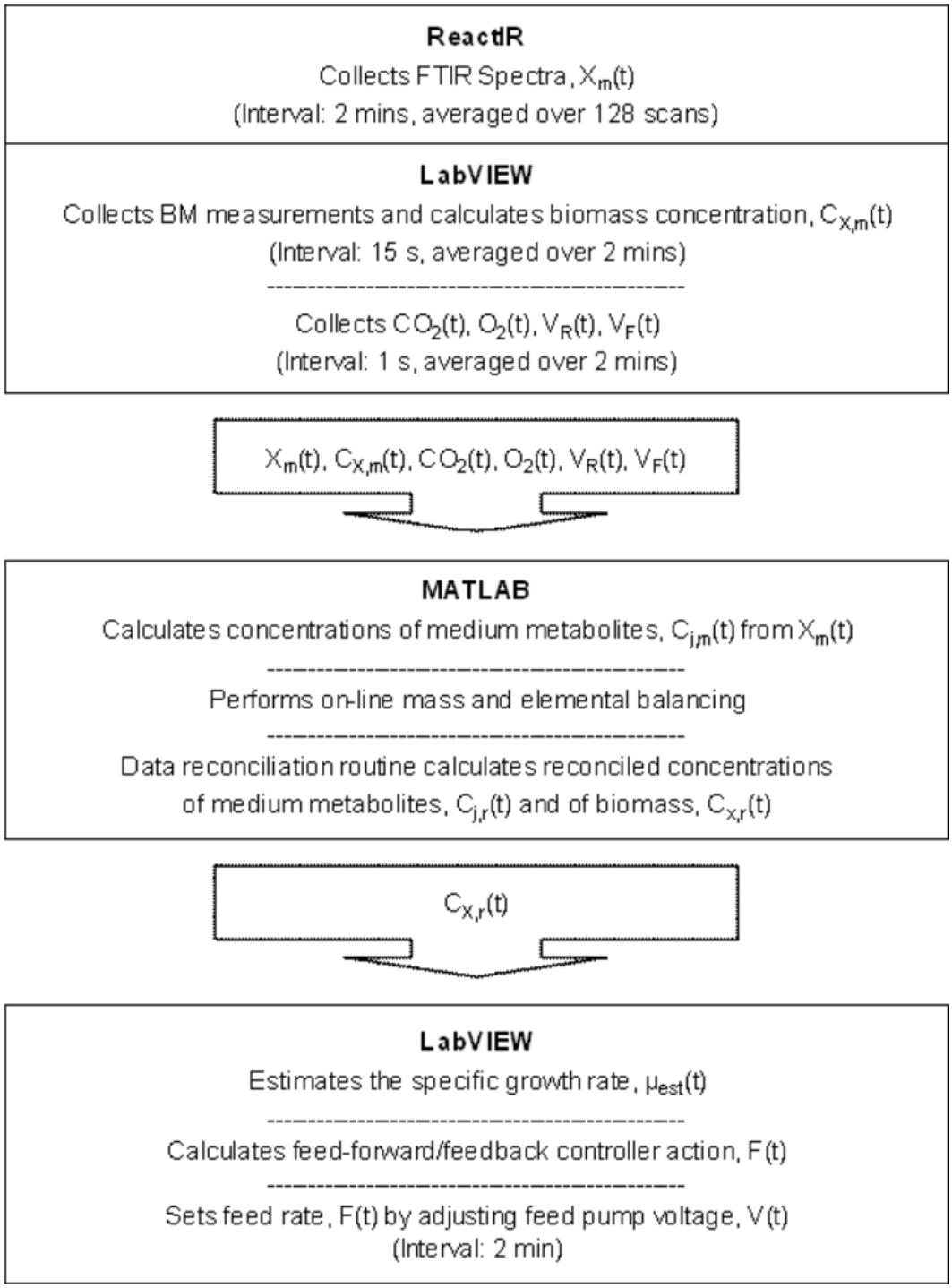

Figure 4.2: Implementation flowchart. The variables included in the arrow boxes represent those that are passed from one program to another via a dynamic interface.

4.3 Results and Discussion

In total, three types of cultures were run to evaluate the three controller types mentioned above (feed-forward, proportional feedback and proportional-integral feedback). In each case, the specific growth rate setpoint was changed three times during the fed-batch stage of the culture in order to test the ability of the controller to track the changing setpoint. Due to reactor volume and aeration considerations, the fed-batch phase was limited in duration to 12 hours at the investigated growth rates. A 20-minute adaptation phase was allowed following each setpoint change to allow the μ estimator to stabilize. The controllers were evaluated by measuring the average absolute tracking error values (ϵ_{av}) for each setpoint period.

4.3.1 Feed-forward controller

Figure 4.3 shows the reconciled profiles of glucose, ethanol, ammonium and biomass obtained during a culture which was run to test the feed-forward controller. Exponential growth of biomass and consumption of glucose can be observed during the batch phase of the culture (until a culture time of 7.5 h). The fed-batch phase, initiated manually upon the depletion of glucose, is characterized by further biomass growth with no residual glucose but sufficient ammonium concentration to prevent nitrogen starvation. The amount of ethanol produced during the culture was insignificant, as expected with the Crabtree-negative strain used.

The reconciled profile of the biomass concentration (Figure 4.3, thick continuous line) appears to match quite well with the reference values obtained off-line (Figure 4.3, circles). In fact, the reconciliation routine led to a decrease of 64% in the standard error of prediction (SEP) of biomass compared to the SEP obtained using raw biomass measurements. This agreement was comparable for the other experiments undertaken and further results are not shown here.

Precise determination of the error in the estimated value of the specific growth rate is impossible since its true value is not known on-line [81]. The accuracy of the μ estimator

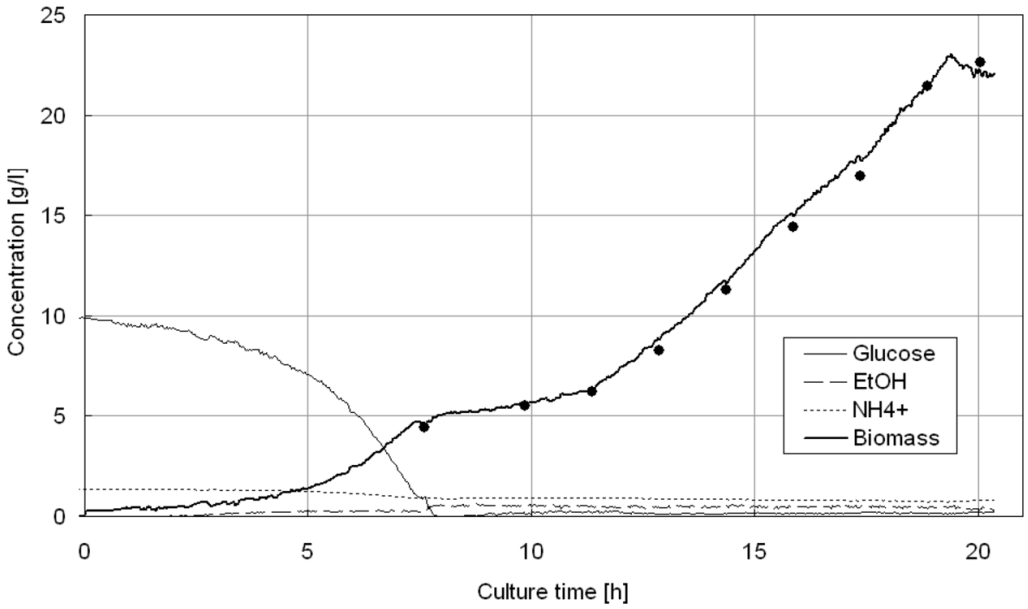

Figure 4.3: Reconciled profiles of the main metabolites and biomass obtained in the first culture. The circular points represent the off-line (dry cell weight) concentration of biomass.

can be confirmed post-culture by calculating the reference specific growth rate based on off-line measurements of the biomass concentration. In this experiment, the average error between the estimated (μ_{est}) and the reference values (μ_{ref}) of the specific growth rate was 7.7%, which confirms the validity of the on-line biomass measurements. Similar results were obtained for the remaining experiments and are not shown here.

Figure 4.4 shows the evolution of the estimated growth rate obtained using the feed-forward controller compared to the setpoint values of $0.1h^{-1}$, $0.2h^{-1}$ and $0.15h^{-1}$.

The average absolute values of the tracking error, ϵ_{av}, were equal to $0.040h^{-1}$, $0.018h^{-1}$ and $0.032h^{-1}$, respectively for the three setpoints, yielding a mean overall controller error of 23.4%. Attaining stability after implementing the first setpoint of $0.1h^{-1}$ took longer than for the latter two setpoints, most likely due to the significant drop in the specific growth rate from μ_{max}. Examining the response after the implementation of the second and third setpoints, it was observed that the controller adaptation ability did not depend on whether μ_{sp} was increased or decreased.

The oscillations in the specific growth rate that occur around the setpoints in Figure 4.4

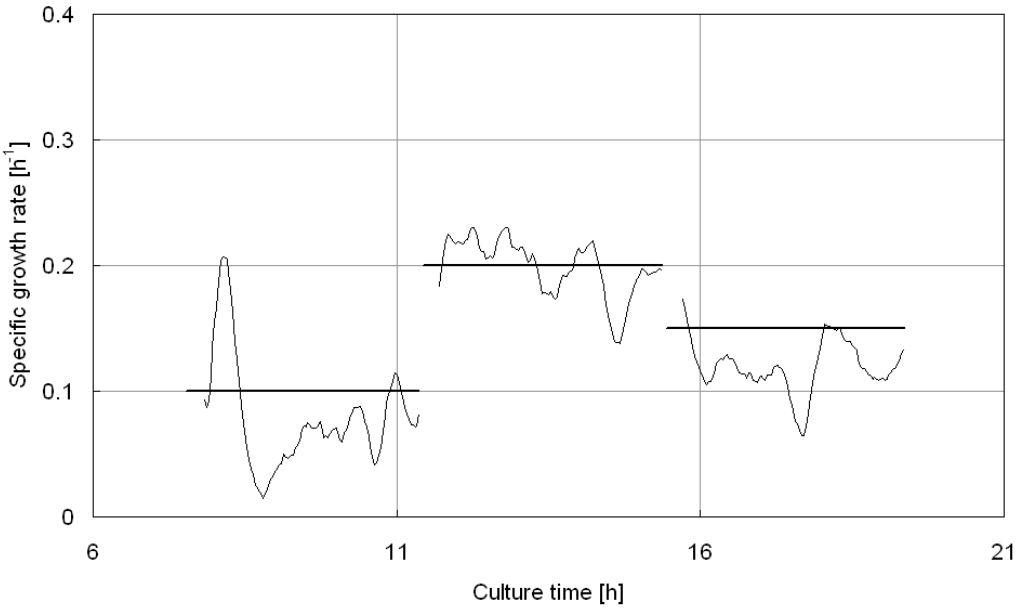

Figure 4.4: Setpoint (thick line) and estimated (thin line) specific growth rate obtained during a fed-batch culture controlled using the feed-forward controller alone.

are not caused by the action of the controller but are related to the natural biosynthetic patterns of the cells. In Figure 4.5, the evolution of the biomass concentration and the estimated specific growth rate are plotted for the batch phase of the culture. It can be observed that even towards the end of the batch phase, when the cells would be expected to be growing steadily at μ_{max}, oscillations in the specific growth rate similar to those seen in Figure 4.4, can be observed. Thus, it can be concluded that these inherent oscillations are independent of the actual value of the specific growth rate.

A natural drawback of a simple feed-forward controller is that it lacks the necessary feedback mechanism to correct setpoint tracking errors and steady-state offsets that may be due to, for example, inaccuracies in the value of F_0. For this reason, the effect of implementing a feedback control action was determined.

4.3.2 Proportional feedback controller

Figure 4.6 shows the evolution of the estimated growth rate obtained using the proportional feedback controller. The experimental setup and the values of the tested setpoints were the

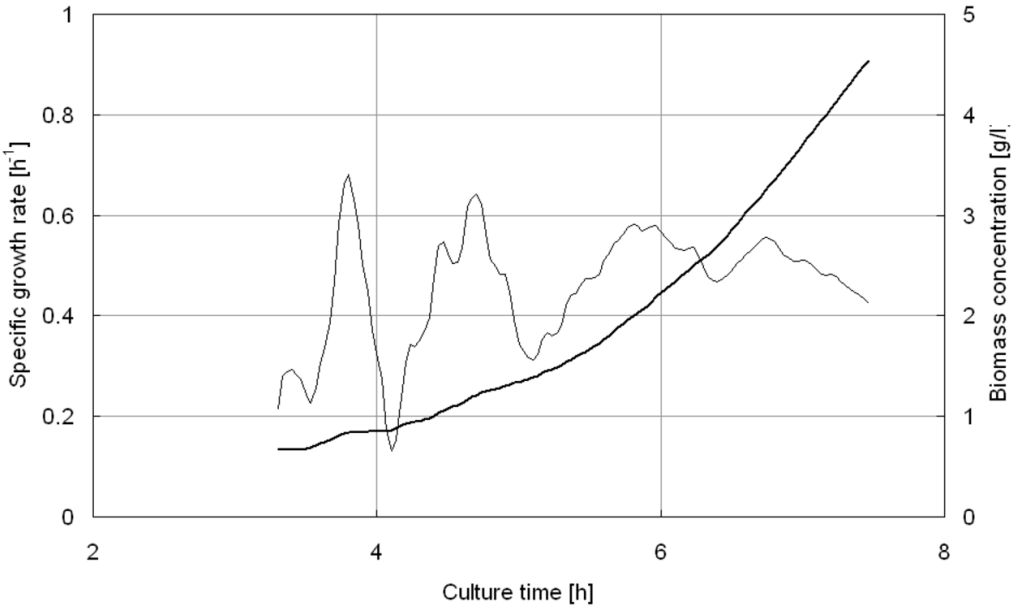

Figure 4.5: Biomass concentration (thick line) and specific growth rate (thin line) recorded during batch growth.

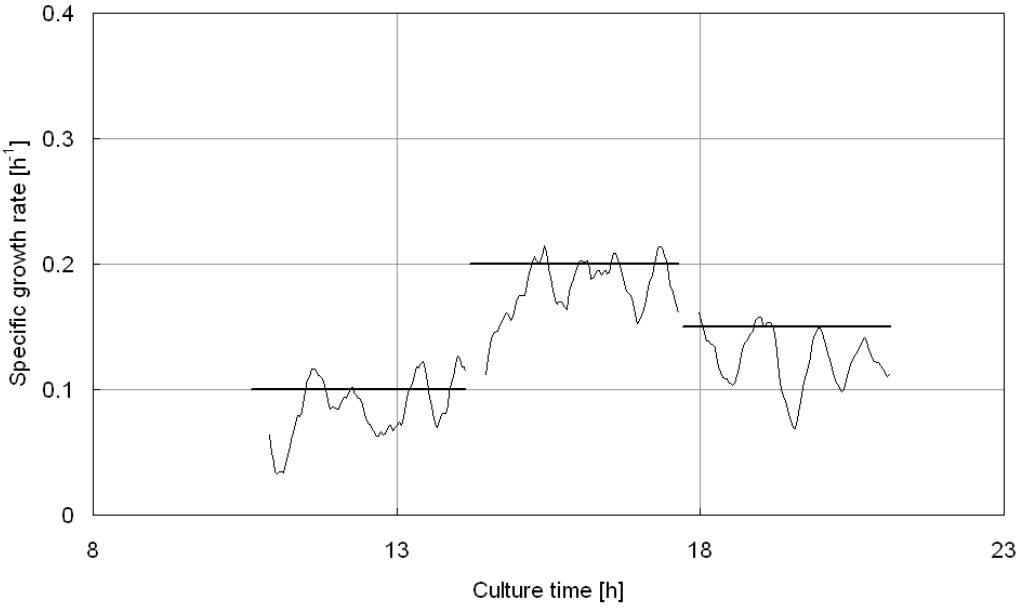

Figure 4.6: Setpoint (thick line) and estimated (thin line) specific growth rate obtained during a fed-batch culture controlled by the P feedback controller.

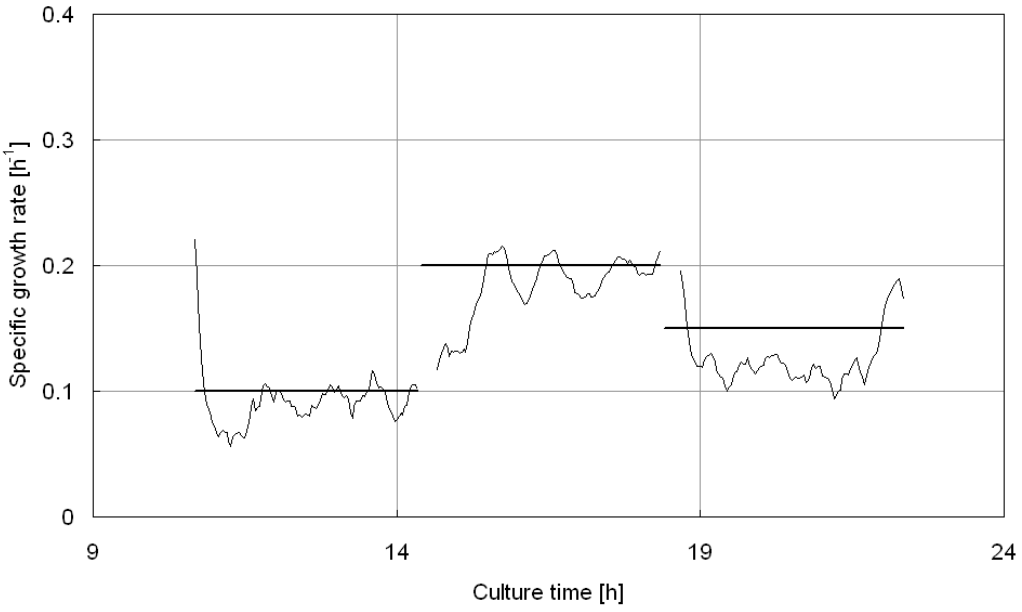

Figure 4.7: Setpoint (thick line) and estimated (thin line) specific growth rate obtained during a fed-batch culture controlled by the PI feedback controller.

same as in the case of the feed-forward controller.

Including the proportional feedback term allowed for closer setpoint tracking and reduced the mean overall controller error of 17.1%. The values of ϵ_{av} obtained for the three setpoints were $0.022h^{-1}$, $0.021h^{-1}$ and $0.028h^{-1}$, respectively. Nevertheless, persisting offsets between μ_{sp} and μ_{est} were observed during most of the culture duration. This outcome was expected with a proportional feedback controller, as mentioned in the Section 4.2.5. In addition, however, another phenomenon was observed: towards the end of the culture, the estimated specific growth rate remained consistently below the setpoint. The most likely reason for this trend is that with the cumulative addition of the feed solution and the base, the reactor volume increased enough to cause the value of F_0 to be appreciably underestimated. As a result, the feed-forward action of the controller was not adequate for the actual reactor volume, causing the negative offset. In fact, the same phenomenon could already be seen during the culture run with the feed-forward controller alone (Figure 4.4). Such errors should be corrected effectively by adding the integral term to the controller, as is shown in the following section.

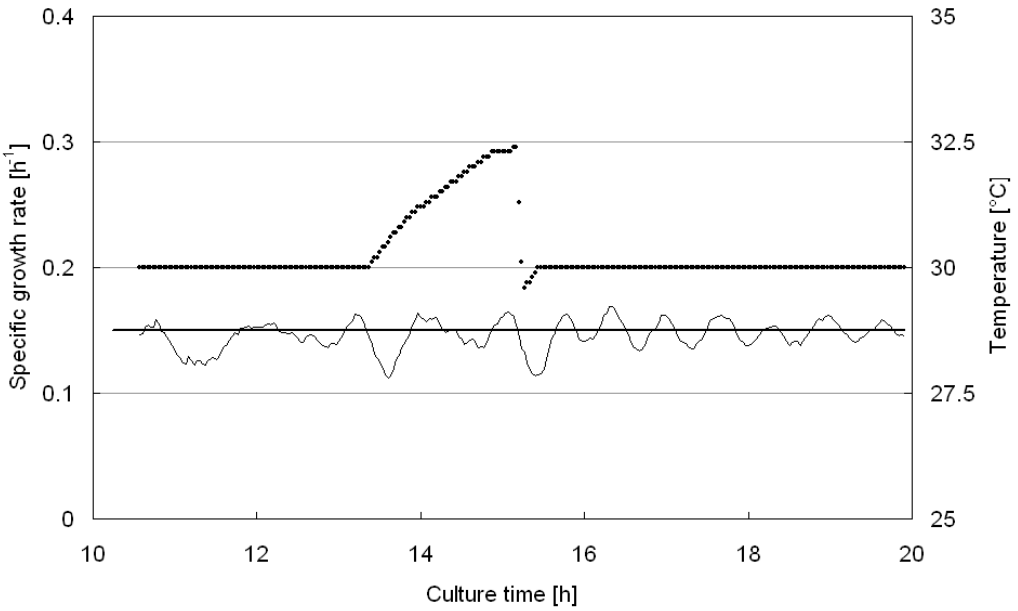

Figure 4.8: Controller response to a temperature disturbance (circular points) in the process caused by a simulated malfunction of the reactor cooling mechanism between culture time of 13.4h and 15.4h.

4.3.3 Proportional-integral feedback controller

Figure 4.7 shows the evolution of the estimated growth rate obtained using the proportional-integral feedback controller under identical experimental conditions to those used with the other controller types.

The introduction of the integral term appears to have helped in reducing the offset in the response and in reaching the setpoint more effectively. Noticeable improvement in setpoint tracking was noted for the first two setpoint values of $0.1h^{-1}$ and $0.2h^{-1}$, with the values of ϵ_{av} equal to $0.016h^{-1}$, $0.021h^{-1}$, respectively. Again, presumably due to the rapidly rising reactor volume, as well as an unintended oxygen limitation, the controller experienced some difficulties in keeping up with the setpoint towards the end of the culture, producing a mean error of $0.032h^{-1}$. Following an increase in the agitation rate, the integral action of the controller did allow correction of the error and the setpoint was eventually reached towards the end of the experiment, albeit at the cost of producing a slight windup of the integral term. Overall, the mean error obtained with the PI controller was lower than that noted for

Table 4.2: Summary of the full results of the average tracking error ϵ for the three controllers studied.

Controller type	$\epsilon\ [h^{-1}]$		
	$\mu_{sp} = 0.1h^{-1}$	$\mu_{sp} = 0.2h^{-1}$	$\mu_{sp} = 0.15h^{-1}$
Feed-forward	0.040	0.018	0.032
P feedback	0.022	0.021	0.028
PI feedback	0.016	0.021	0.032

Table 4.3: Summary of the full results of the mean controller error for the three controllers studied.

Controller type	Mean controller error [%]
Feed-forward	23.4
P feedback	17.1
PI feedback	15.8

the P controller, as summarized in Table 4.2 and Table 4.3, containing the full results for the three controllers studied.

From the results obtained, it can be concluded that applying the feedback mechanism appreciably enhances the controller performance. The corrective action of the feedback terms compensates for potential errors in the variables defining the feed-forward component of the controller: X_0, V_0, $Y_{X/S}$. The integral term of the PI controller effectively removes impending offsets inherent to proportional feedback controllers and contributes to more effective setpoint tracking. On the other hand, it can also lead to controller destabilization, for example, in cases of a persisting controller error like the one observed towards the end of the culture shown in Figure 4.7. It is, therefore, recommended that an integral reset option be considered in order to override the effects of a potential controller windup. Finally, a dynamic adaptation of the term F_0 should decrease the risk of controller error caused by variations in reactor volume.

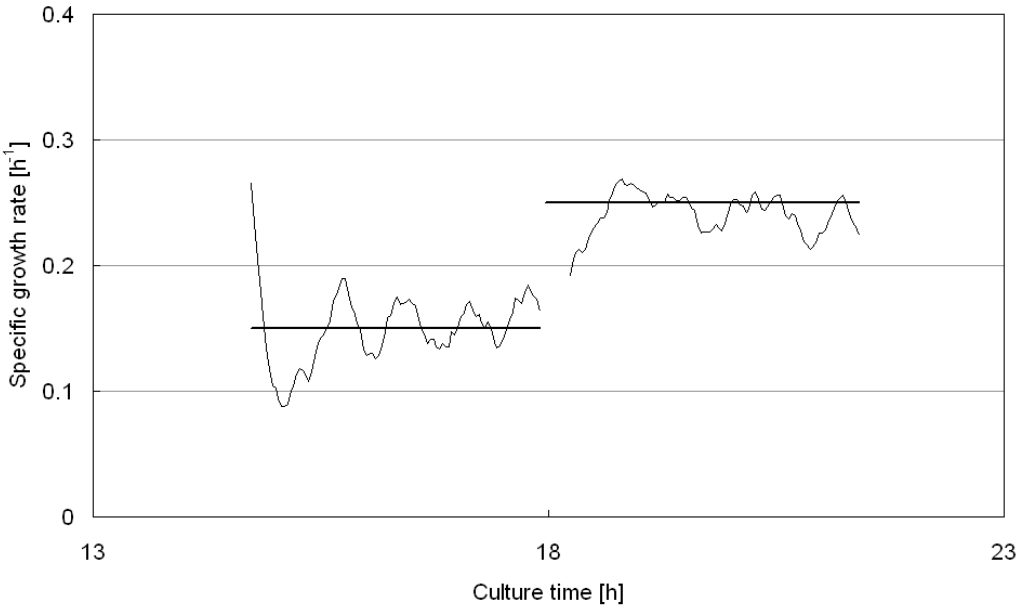

Figure 4.9: Setpoint (thick line) and estimated (thin line) specific growth rate obtained applying the feed-back PI controller during a fed-batch culture of *Candida utilis*.

4.3.4 Disturbance rejection

The disturbance rejection experiment was carried out by switching off the process chilling system at culture time, $t = 13.4$h, as shown in Figure 4.8. The disturbance was maintained for a period of about two hours, during which the reaction temperature rose by $2.5\,^{\circ}\mathrm{C}$, before the cooling mechanism was switched back on. Figure 4.8 shows the evolution of μ_{est} before and after the induced disturbance.

Analyzing Figure 4.8, the PI controller ensured effective setpoint tracking with no offsets and showed rather good disturbance rejection abilities in the face of the sudden change in reaction temperature. The natural oscillations in the estimated specific growth rate were subtly more pronounced at the beginning of the disturbance (t = 13.4 h), as well as after the reactor cooling mechanism was turned back on (t = 15.4 h). Remarkably though, following the slightly erratic response at t = 13.4 h, the controller was able to regain stability for the remainder of the disturbance duration. Finally, a complete return to stability can be noted following the removal of the disturbance.

4.3.5 General applicability

The general applicability of the proposed control strategy was tested by running a fed-batch culture of the yeast *Candida utilis* (strain DSM 2361 from DSMZ, Braunschweig, Germany) using the same batch and feed media as those outlined in Table 4.1. The controller was tested at two different specific growth rate setpoints of 0.15 h^{-1} and 0.25 h^{-1}. The cultivation was carried out at a pH value of 4, reported to be the optimal pH for this yeast [26]. The methodology and algorithm proposed in Section 4.2.2 were not modified in any way in this experiment. Figure 4.9 shows the evolution of the estimated growth rate obtained using the proportional-integral feedback controller to maintain two setpoints of $0.15h^{-1}$ and $0.25h^{-1}$.

The proposed control strategy was quite efficient in controlling the specific growth rate for this organism, despite the differences in culture parameters. The mean controller error was 10.0%, with average absolute values of the tracking error, ϵ_{av}, equal to $0.022h^{-1}$ and $0.014h^{-1}$, respectively for the two setpoints. The success of this experiment was expected considering that the two yeast strains are very similar, grow on the same culture media and exhibit similar types of metabolism. Therefore, another experiment was carried out using the yeast *Pichia pastoris* growing on a different medium (composition outlined in Table 4.4) for both the batch and the fed-batch phases.

The strain PPC43AZ of *P. pastoris* [247, 2] was grown at a pH value of 5, using glycerol as the main carbon and energy source and NH_4Cl as the nitrogen source. The media were sterilized by filtration and supplemented with vitamins and trace elements as prescribed by Jungo [2].

Because of the different medium constituents, the mass and elemental balances had to be modified in the data reconciliation routine. The control approach proposed in Section 4.2.2 remained unchanged. Figure 4.10 shows the evolution of the estimated growth rate obtained using the proportional-integral feedback controller to maintain two setpoints of $0.1h^{-1}$ and $0.15h^{-1}$ (lower values of μ_{sp} were chosen for this experiment since the μ_{max} for this strain grown on glycerol is in the order of $0.24h^{-1}$ [**?**]).

The PI controller was successful at maintaining the specific growth rate at the two ex-

Table 4.4: Composition of the batch and feed media for *P. pastoris* .

Component	Batch medium $[gl^{-1}]$	Feed medium $[gl^{-1}]$
Glycerol	20	300
NH_4Cl	15.26	91.56
KH_2PO_4	5.62	33.72
$MgSO_4 \cdot 7H_2O$	1.18	7.08
Component	Batch medium $[mll^{-1}]$	Feed medium $[mll^{-1}]$
Trace elements & vitamins solutions	5	15
Polypropylene glycol 2000 (Antifoam)	2	4

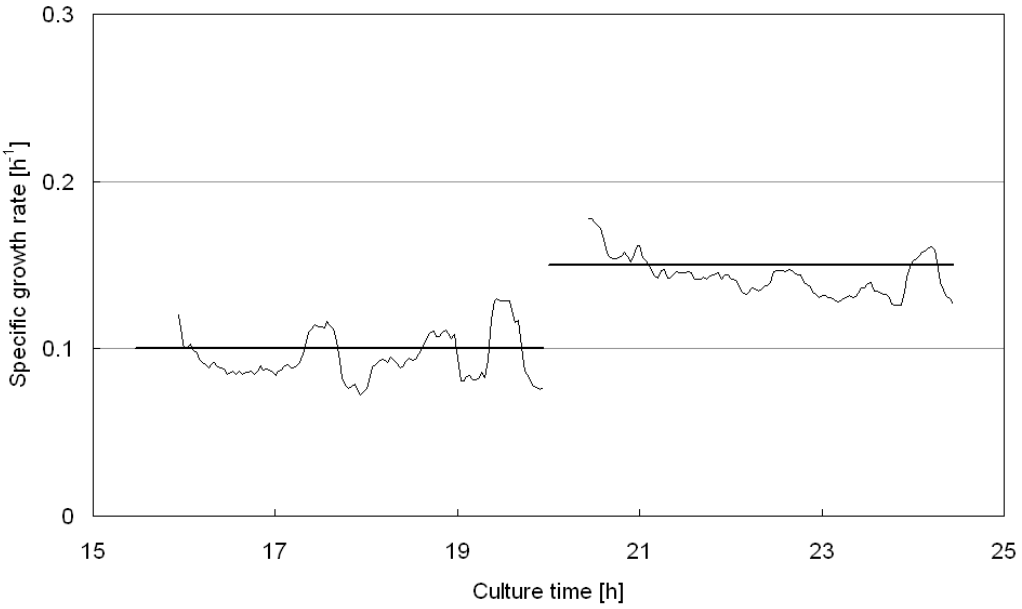

Figure 4.10: Setpoint (thick line) and estimated (thin line) specific growth rate obtained applying the feed-back PI controller during a fed-batch culture of *Pichia pastoris*.

perimental setpoints. The mean controller error was 10.2%, with average absolute values of the tracking error, ϵ_{av}, equal to $0.013h^{-1}$ at $\mu_{sp} = 0.10h^{-1}$ and $0.011h^{-1}$ at $\mu_{sp} = 0.15h^{-1}$.

4.3.6 Discussion

Any system, as a whole, is only as strong as its weakest element. Breaking down the structure of the proposed methodology into its prime components, and subsequently analyzing each element in terms of performance and robustness, gives an insight into the system's limitations. In this study, four prime components were identified: the on-line biomass measurements, the specific growth estimator, the controller and the response system.

The accuracy of the on-line measurements of biomass concentration, further enhanced by on-line balancing and data reconciliation, is indisputable. Low SEP values, as mentioned before, suggest that the measurements obtained with this method are satisfactory. Accurate on-line biomass concentration measurements provide, in turn, a reliable basis for the estimation of the specific growth rate. Nonetheless, instantaneous on-line μ estimators can become problematic and unstable in the presence of even minimal measurement noise. Consequently, a trade-off between noise and estimator lag must be made. In this work, a 20-minute moving window was allowed to filter the biomass measurements and minimize noise in the estimator outputs. With the yeast strain used in this study, this interval was a good compromise (doubling time at μ_{max} is approximately two hours) but it would need to be revised if other organisms were to be used. In the case of slower-growing organisms, such as mammalian cells, the moving window could even be increased without compromising the controller action. However, with fast growing bacteria, moving average filtering would have to be done over a much narrower interval. On the other hand, if the cells grow faster, the significance of noise relative to the actual changes in biomass concentration would also drop. Alternatively, different filtering techniques, such as the extended Kalman filter [106, 121] could prove useful in this application.

Next to the estimator issues discussed above, the weakest link in the proposed methodology is without a doubt the response system itself. Cellular growth is very difficult to control due to the wide array of complicated physiological and metabolic factors involved.

Indeed, biological systems are highly unpredictable and the supply of the limiting substrate is by no means the only factor influencing the specific growth rate. In this context, the proposed feedback controller design is certainly adequate and increasing its complexity would probably only result in decreased robustness and a higher vulnerability to destabilization. For successful technology transfer and industrial applicability, suitable equilibrium needs to be found between the simplicity of the control system and the resulting flexibility.

4.4 Conclusion

The implementation of a simple variant of the proportional-integral feedback controller to maintain a constant specific growth rate was successfully demonstrated. By including the proportional and integral feedback gains directly in the exponential term governing the feeding regime, sufficiently stable control could be achieved without adaptation or scheduling mechanisms. The absence of complex adaptive properties makes the proposed control strategy more generally applicable and a suitable candidate for industrial implementation. Indeed, even the feed-forward controller alone is considered by the FDA a flexible tool designed to manage inherent process variability, despite the fact that the same guidance urges the use of feedback control as an ultimate aim during process development [3].

In this work, the growth rate was controlled by regulating the glucose supply. The proposed methodology should be tested with a different limiting substrate, for example the nitrogen source. Future studies should also investigate the general applicability of the approach presented in this work by testing the proposed algorithm on different organisms, particularly mammalian cells. Finally, the methodology presented here could be used to control or prevent the formation of overflow metabolites in Crabtree-positive strains like *S. cerevisiae* or *E. coli*.

Despite significant advances in research on fermentation control, only very simple control strategies are currently applied in industry [12]. Often, it is the lack of or the low quality of measured data that is at fault, making it necessary to implement complicated process models and control strategies at the cost of robustness. Indeed, successful application

of closed-loop control depends a great deal on the availability of reliable measurements of the controlled variable [12]. The aim of this work was to tackle this issue from an angle that is different from the usual one: by focusing on improving the quality of the measured variables rather than building up the complexity of the modeling and control systems. It was shown that dependable on-line measurements of the biomass concentration, verified by on-line balancing and data reconciliation, allowed for an accurate estimation of the specific growth rate and the implementation of a simple, robust control strategy that satisfies the criteria set by the PAT initiative for industrial settings.

Chapter 5

Preventing overflow metabolism in Crabtree-positive yeast cultures through feedback control of the specific growth rate [1]

It is only because of problems that we grow mentally and spiritually. [2]

[1] This chapter is intended for submission to *Biochemical Engineering Journal* or *New Biotechnology* (Schuler MM, Dabros M, Marison IW).

[2] Morgan Scott Peck, Psychiatrist (1936-2005)

At growth rates above a particular critical value, Crabtree-positive organisms exceed their respiratory capacity and enter diauxic growth metabolism. Excess substrate is converted reductively to an overflow metabolite, resulting in decreased biomass yield. To prevent this scenario, Crabtree-positive organisms are typically grown in a fed-batch mode at a growth rate below the critical value, μ_{crit}. This approach is hindered by two major challenges: accurately estimating the current specific growth rate and controlling it below μ_{crit}. Continuous and non-invasive monitoring and control of a small number of essential critical process parameters is crucial in order to overcome these difficulties. In this chapter, the specific growth rate of *Saccharomyces cerevisiae* is estimated from enhanced on-line biomass concentration measurements obtained with dielectric spectroscopy and reconciled in real-time to satisfy a series of mass and elemental balances. A simple feed-back control scheme is implemented to maintain the specific growth rate at a setpoint below μ_{crit}, while on-line FTIR spectroscopy measurements provide early detection of overflow metabolites and might be considered for regulating the controller action. The proposed approach is not only significant in terms of enhanced productivity of either biomass or a target product, but also a step towards the creation of a process control tool as defined by the FDAs initiative for industrial Process Analytical Technology (PAT).

5.1 Introduction

The shift from a purely respiratory to a respiro-fermentative metabolism in Crabtree-positive, facultative anaerobe micro-organisms occurs in oxygen limited conditions or in response to the presence of excess carbon substrates saturating the respiratory bottleneck. The limited respiratory capacity of Crabtree-positive strains can have significant repercussions in industrial applications in terms of decreased productivity [248, 249] and poor product quality [249, 250]. In the case of biomass production, for instance for Single Cell Proteins (SCP)

or mass production of baker's yeast in food industry, fermentative metabolism results in a lower biomass yield, leading to reduced productivity. With regard to recombinant protein production, an additional problem arises. The presence of overflow metabolites, such as ethanol, or acetate in the case of *Escherichia coli* , can inhibit both growth of the organism and recombinant protein quality [31].

Overcoming the drawbacks related to the Crabtree effects in industrial processes has been a concern since the early 1920s. Carbon-source-limited fed-batch cultures are a common mean of avoiding the negative effects of the change in metabolism from respiration to fermentation. The choice of type and timing of feed design, as well as the overall control behind it varies widely. Maintaining a particular critical value of glucose in the medium while monitoring the process through off-gas analysis was an early attempt by Cooney *et al.* [40] to increase productivity. A similar approach was taken by Axelsson and coworkers ([248] and later by Akesson *et al.* [251] using a tubing technique for sensing the ethanol concentration and a simple dissolved oxygen probe for indirect detection of acetate in the culture broth respectively. With enhanced sensitivity and accuracy in measuring techniques such as infrared or mass spectroscopy, the detection of the overflow metabolite, such as ethanol is simplified. The improvements in the devices lead to the development of a different approach to control the metabolism of microbial cells in fed-batch cultures. Regulating the extracellular ethanol concentration was the main idea in the work proposed by Valentinotti *et al.* [240] and Cannizzaro *et al.* [95]. The idea of maintaining a certain amount of overflow metabolite as basis of control was even taken a step further towards industrial application by a group proposing a control strategy at technical scale based on a similar approach [108]. Approaches considered to date were rather focused on measuring and controlling either the carbon-source or the overflow metabolism product concentration [40, 248, 251, 240, 95, 117, 108] in yeast-based processes. Additionally, a couple of groups worked on processes involving *Escherichia coli* cultures [228, 252, 253, 254]. Papers reporting findings in relation to the causes of the Crabtree effect, to overflow metabolism occurrence and to its regulation generally concern the traditional yeast *Saccharomyces cerevisiae* [1, 255, 249, 256].They systemically link the shift from purely oxidative to respiro-

fermentative metabolism in the presence of high sugar concentrations to a critical value of specific growth rate. Nevertheless, only few groups tried to work on controlling the specific growth rate as close as possible to this critical value, maximizing thereby the biomass yield [6, 7]. The most common approach is to work at a certain distance using linear feed strategies or exponential feed forward with a lower μ setpoint, compromising however, in both cases, maximal biomass productivity.

Whatever system and control strategy is chosen, the challenge is to select a suitable monitoring technique for the appropriate process variable and to develop a control strategy with sufficient flexibility and simplicity to allow industrial implementation. The availability of reliable measurements for the sensed value of the controlled quantity is the decisive criterion in the choice of the approach. In the present work, focus is given to the combined use of reconciled on-line biomass concentration values from dielectric spectroscopy and continuous Fourier transformed infrared (FTIR) measurements of substrate concentrations to develop a simple feedback control scheme to maintain the specific growth rate at a setpoint below its critical value (μ_{crit}) and the carbon substrate concentrations within the limits of the respiratory capacity of the organism under study. *Saccharomyces cerevisiae*, one of the most common, Carbtree-positive yeast expression systems, was chosen to assess the effects of the proposed control strategy on the minimization of overflow metabolite formation and on the biomass yield. The principal aim of this study is to highlight that the developed platform for the control of μ can be used to first confirm in a single culture the critical value (μ_{crit}) for the specific growth rate. Following this initial step, the process can be controlled at a maximal value $\mu_{sp,crit}$. The advantage of the proposed strategy is twofold. On the one hand, the specific growth rate can be be maintained at a maximal value just below (μ_{crit}) with the purpose of ensuring maximal biomass yield. On the other hand, the proposed control strategy allows maintaining constant growth rate for optimal recombinant protein production for instance while ensuring that no overflow metabolite production might hinder maximal growth or influence the expression rate of the target molecule. The proposed approach is therefore not only significant in terms of enhanced productivity of either biomass or a target product, but also a step towards the creation of a reliable process

control tool for Quality by Design (QbD) as defined by the FDAs initiative for industrial PAT.

5.2 Material and methods

5.2.1 Chemicals

All chemicals used in this study were purchased from Sigma Aldrich (Sigma Aldrich Ireland Ltd. Arklow, Ireland).

5.2.2 Cell strains and culture conditions

Cell strain

A Crabtree-positive strain *Saccharomyces cerevisiae* (CBS 8066, Centraalbureau voor Schimmelcultures (CBS), Utrecht, The Netherlands) was chosen for this study, mainly due to the fact that it is one of the preferred, Crabtree-positive yeast production and expression systems in academia and in industry. Cells were preserved as $1.8ml$ aliquots in a $20gl^{-1}$ glycerol solution at $-80\,^{\circ}\mathrm{C}$.

Inoculum preparation

The preculture was obtained by suspending thawed cells from a $1.8ml$ aliquot in a 1-liter, baffled shakeflask containing $100ml$ of preheated, sterile, complex preculture medium (pH 5) containing $20gl^{-1}$ of glucose, $10gl^{-1}$ of yeast extract and $10gl^{-1}$ of peptone and incubating the flask in an orbital shaker incubator (SHEL LAB S19, Sheldon Manufacturing, Cornelius, USA) for 24 hours at $30\,^{\circ}\mathrm{C}$ and at 150 rpm. $90ml$ of the culture broth were then centrifuged at 3000 rpm for 10 minutes, the supernatant discarded and the cell pellet suspended in $10ml$ of a sterile saline solution ($9gl^{-1}$ of NaCl).

Reactor description, media composition and culture conditions

Cultures were grown at $30\,^{\circ}\mathrm{C}$ for *S. cerevisiae* in a 3.6-liter laboratory bioreactor (Bioengineering AG, Wald, Switzerland), with a working volume of 2.6 liters, equipped with

Table 5.1: Composition of the batch and feed media for culturing *S. cerevisiae* .

Component	Batch medium [gl^{-1}]	Feed medium [gl^{-1}]
Glucose	10	300
$(NH_4)_2SO_4$	5	50
KH_2PO_4	3	35
$MgSO_4 \cdot 7H_2O$	0.5	3
Component	Batch medium [mll^{-1}]	Feed medium [mll^{-1}]
Trace elements & vitamins solutions	5	15
Polypropylene glycol 2000 (Antifoam)	0.5	2

a double, 6-blade Rushton-type agitator, baffles, temperature and pH probes and control mechanisms, air inlet and outlet ports, base and feed inlet ports, a pO_2 probe, a recycling loop to the FTIR spectrometer detector cell and a sampling valve at the bottom of the reactor. The outlet air was passed through a condenser to minimize liquid loss by evaporation. A $4M$ NaOH solution was used to maintain the pH at 5 via a pH control unit (Bioengineering AG, Wald, Switzerland). Prior to inoculation, the bioreactor, as well as the recycling loop, the sampling valve and the air outlet, were sterilized *in-situ* at $120\,^{\circ}\mathrm{C}$ for 20 minutes with deionized water, cooled down to room temperature, drained and filled with sterile batch medium (Table 5.1). Once all process parameters reached the target value, data acquisition was started and the reactor inoculated with the cells obtained from the preculture (Section 5.2.2). Cells were grown in batch mode until depletion of the carbon and energy source when an exponential C-source-limited feed (composition outlined in Table 5.1) was started. Both media were prepared with deionized water, pH adjusted as required and sterilized by filtration (Steritop, $0.22\mu m$ pore size, Millipore, Billerica, USA) and supplemented with sterile antifoam solution, trace elements and vitamins as described by Verdyun *et al.* [243] and Cannizzarro *et. al* [95].

5.2.3 Monitoring, data acquisition and control strategy

Off-line analysis

Samples ($10ml$) were taken at regular intervals (60 minutes) during the feed phase of the cultures in order to determine off-line the biomass concentration. Biomass dry cell weight

Table 5.2: Process parameters kept constant during the cultures of *S. cerevisiae* in this project.

pH	5
Temperature	$30\,^{\circ}\mathrm{C}$
Aeration rate (air)	$2.5\ lmin^{-1}$
Agitation (batch phase)	800 rpm
Agitation (fed-batch phase)	1200 rpm

(DCW) was determined by filtering a known volume of the culture broth through a pre-weighed $0.22\mu m$ pore size filter (GSWP $0.22\mu m$ Nitrocellulose membrane filters, Millipore, Billerica, USA), drying the filter to constant weight and subsequently reweighing it [63]. Optical density measurements were performed spectrometrically at 600 nm (Spectronic Helios-Epsilon, Waltham, MA, USA). Glucose and ethanol concentrations of the samples were quantified by HPLC (Agilent Instruments 1200, Agilent Technologies Ltd. Cork, Ireland) equipped with a refractive index detector, thermostatted at $30\,^{\circ}\mathrm{C}$ with a Supelcogel C-610H column (Sigma Aldrich Ireland Ltd. Arklow, Ireland) equipped with a guard column and using a $0.027\%\ v/v\ H_2SO_4$ mobile phase for isocratic elution at $0.5mlmin^{-1}$ for 32 minutes. Calibration curves for each component were prepared for each run using five synthetic standards. An internal standard ($30gl^{-1}$ of isopropanol) was run with each sample and each standard.

Data acquisition and process control

The dielectric instrument used in this work to follow the evolution of the biomass concentration on-line was the Biomass Monitor 210 from Aber Instruments (Aberystwyth, UK). Details of the measurement principle are described elsewhere [241]. The spectrometer was equipped with a 12 mm probe installed inside the reactor. A built-in crosstalk correction algorithm for small reactors was applied to minimize the influence of conductivity variations on the capacitance measurements. The Biomass Monitor was switched on two hours prior the experiments to allow stabilization of the signal. The dielectric signal was correlated to biomass concentration values after the first experiment. A linear correlation model was es-

Table 5.3: Summary of the assumptive specific growth rates for *S. cerevisiae* [1], as well as of the setpoints for the different experiments for this study.

	$[h^{-1}]$
Assumptive μ_{crit}	0.28
First setpoint	0.26
Second setpoint	0.28
Third setpoint	0.30

tablished between DCW and dual-frequency capacitance values obtained at 500 kHz and 10 MHz. During the culture, measurements were taken every 20 seconds and averaged over the routine interval of 2 minutes. A program developed in-house using LabVIEW 7.1 (National Instruments, Austin, TX) was used to collect and store the acquired data. In parallel to the biomass measurements, the CO_2 and O_2 composition of the culture off-gas was measured using a laboratory-scale gas analyzer (Duet, Advanced BioSystems Ltd, UK), while the major medium components (glucose, ethanol, ammonium and phosphate) were monitored using a Fourier-transform mid-Infrared (FTIR) spectrometer (ReactIR 4000, Mettler Toledo, Greifensee, Switzerland). In addition, the amounts of base consumed for pH control and of medium fed into the bioreactor were registered on-line by placing the reservoirs containing base and medium on analytical balances (PG5001-S, Mettler-Toledo, Greifensee, Switzerland) interfaced to LabVIEW. From the different real-time measurements, a set of on-line mass and elemental balances (carbon, nitrogen, degree of reduction and charge) were set-up [118, 64]. The balances served as constraints for a data reconciliation algorithm which adjusted all measurements to their best estimates. The balances and data reconciliation routines were carried out in real-time at an interval of 2 minutes in MATLAB 7.5 (The MathWorks, Inc., Natick, MA, USA) producing, at each interval, a reconciled value of the biomass concentration. Exact details of the method can be found elsewhere [64, 239]. The reconciled biomass values were then used to estimated in real-time the specific growth rate, allowing the determination of the error term between the actual value of μ and its setpoint, principle driver of the control strategy as explained in Chapter 4. Process parameters such as temperature, pH, agitation and aeration were kept constant (summarized in Table 5.2).

5.2.4 Methodology

For the present project, the following approach was chosen. Literature was first consulted to determine the assumptive critical specific growth rate for the strain under study. With this particular value in hand, the different setpoints for the initial testing were chosen. The assumptive critical specific growth rate, as well as the different setpoints are summarized in Table 5.3.

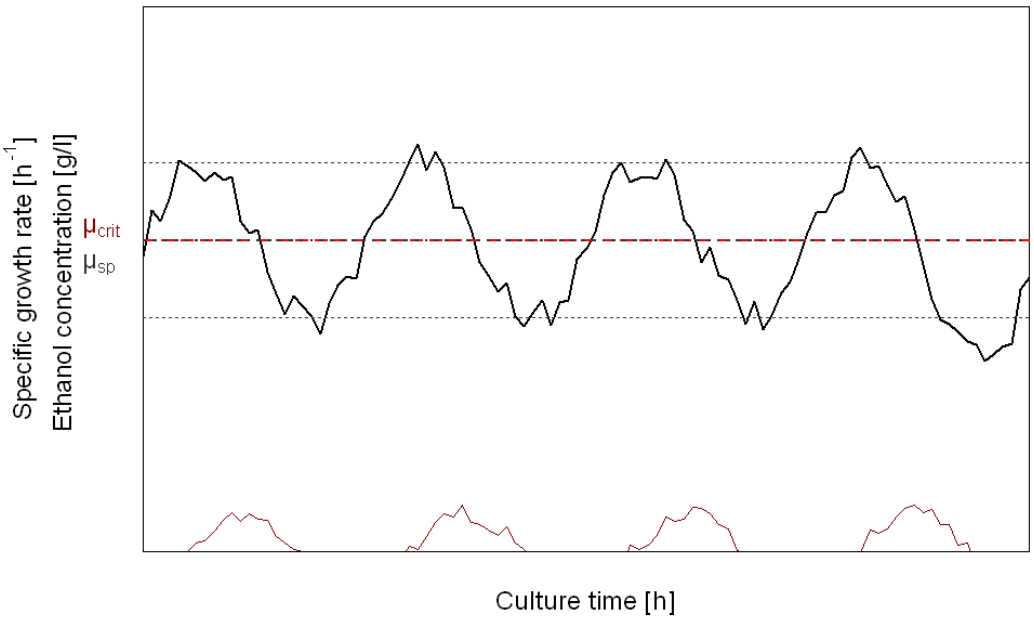

Figure 5.1: Illustration of the specific growth rate in a hypothetical culture of *S. cerevisiae* , taking into account oscillations that were observed during the development of the control strategy (Chapter 4). If μ is set to the critical value where the shift from pure oxidative to fermentative metabolism occurs, oscillations in the growth rate around the control setpoint would lead to substantial production of overflow metabolites such as ethanol (thin red line).

Results from Chapter 4 showed that the developed control strategy invariably leads to oscillations around the setpoint. These oscillations need to be taken into account. Indeed, if the μ is maintained at its critical value where the shift from purely oxidative to respiro-fermentative metabolism occurs, oscillations will lead to substantial production of undesired overflow metabolites and eventually a decrease in the specific growth rate below the setpoint (Figure 5.1).

However, if the setpoint for μ is chosen slightly below, but as close as possible to its

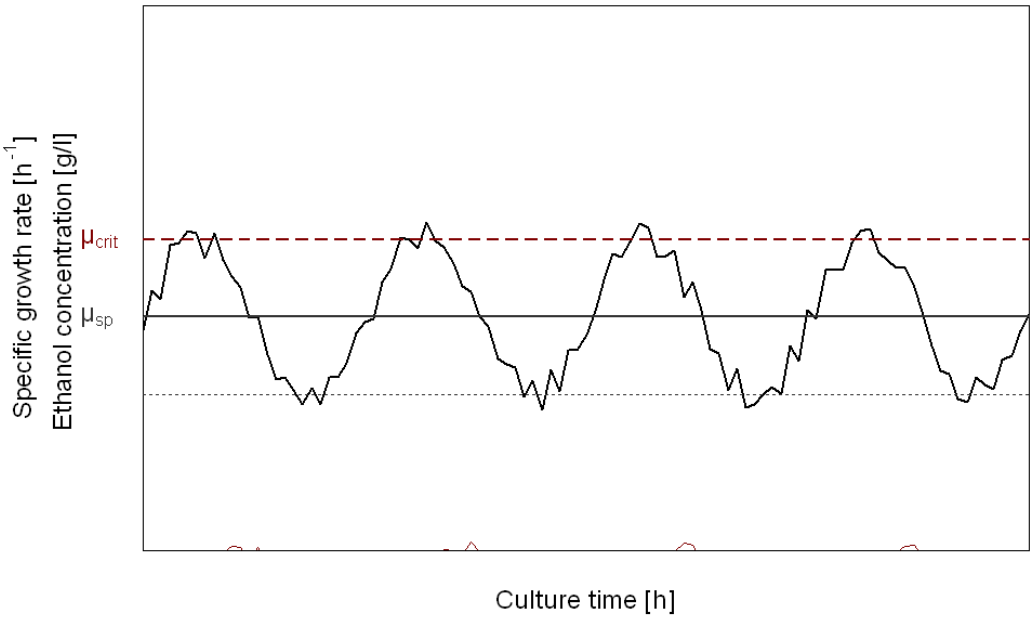

Figure 5.2: Illustration of the specific growth rate in a hypothetical culture of *S. cerevisiae* where the specific growth rate was set slightly below μ_{crit}. The oscillations, inherent to the developed control strategy would not exceed the assumptive critical value and the amount of overflow metabolites produced (thin red line) would be kept to a minimum.

critical value, the production of ethanol in response to overflow metabolism can be kept as low as possible (Figure 5.2). Therefore, the methodology described in Figure 5.3 will be applied. The specific growth rate will be first set slightly below the critical value, then to the assumptive critical value as found in literature and mentioned in Table 5.3 and finally to a level just above the critical specific growth rate. It is expected that ethanol, overflow metabolite produced by *S. cerevisiae* via fermentative metabolism, will form and accumulate within the culture broth, leading to a drastic decrease in the specific growth rate. The controller, as developed within the scope of the present work is not able to deal with such a situation and the process will get out of control, demonstrating the limits of the present control strategy. After the initial confirmation of the critical threshold for occurrence of fermentative metabolism, the specific growth rate will be maintained at a setpoint slightly below the critical value (first setpoint of the initial experiment) to prove that only very small amounts of ethanol will be formed and that the controller is able to maintain the specific growth rate within its limits over a certain amount of time.

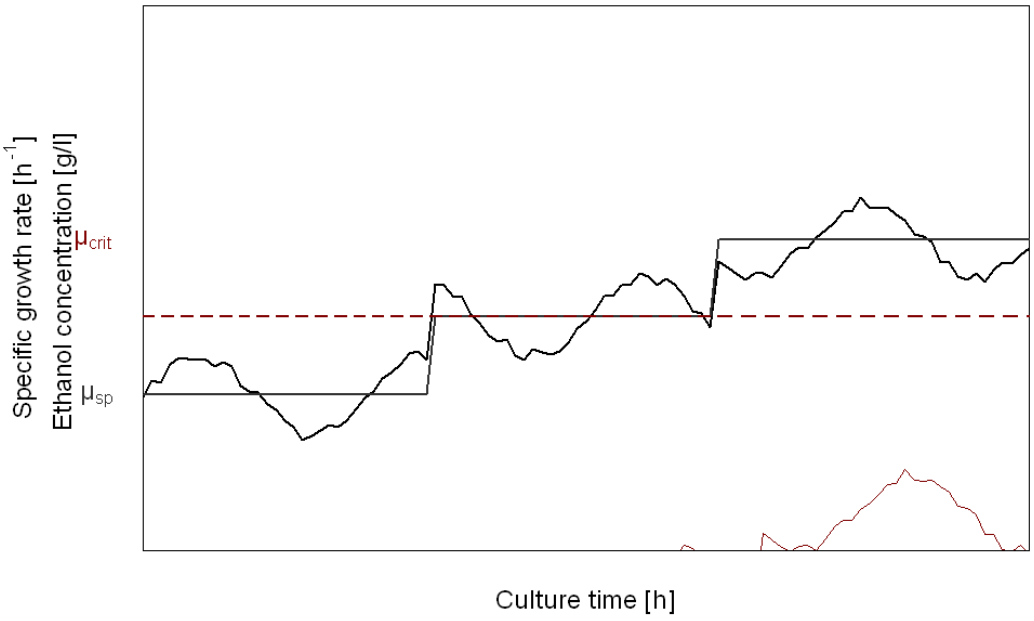

Figure 5.3: Illustration of the specific growth rate in a hypothetical culture of *S. cerevisiae* to underpin the methodological approach chosen for this project. The setpoint for μ would first be set slightly below its critical value, then to its critical value and finally slightly above its critical value. At the setpoint above the critical value, production of ethanol, as a consequence of the fermentative metabolism, is expected.

5.3 Results and discussion

Two types of cultures were run during this study. The initial fed-batch culture aimed to determine the critical specific growth rate μ_{crit} at which the organism changes its metabolism from pure oxidative respiration to fermentation. The specific growth rate was therefore set to a given value close to the assumptive μ_{crit} obtained from literature and then increased above this critical value for confirmation purposes. The on-line measurements from the FTIR spectrometer allowed to follow in real-time the appearance of the overflow metabolites which were confirmed off-line by HPLC analysis at the end of the experiment. Furthermore, for each setpoint, the biomass yield was calculated. Once data mining allowed to determine μ_{crit}, a fed-batch was then carried out at a certain, maximal tolerable value $\mu_{sp,crit}$, $0.02h^{-1}$ below μ_{crit} to assess that no substantial amount of overflow metabolites were produced when applying the proposed feedback control strategy.

5.3.1 Determination of the critical specific growth rate

The estimated specific growth rate, as well as the on-line determined ethanol concentration together with the off-line values from HPLC experiments are displayed in Figure 5.4. While the overflow metabolite concentration stays minimal throughout the two first setpoints, it reaches maximal values of $2.3gl^{-1}$ when the setpoint is above the critical assumptive value of the specific growth rate ($0.28h^{-1}$). Furthermore, the controller is not able to maintain the imposed setpoint when the ethanol concentration increases. The biomass yield decreases by 7% when cells display respiro-fermentative metabolism during the last setpoint (Table 5.4). The on-line values from the FTIR spectroscopic measurements differ from the values determined off-line determined via HPLC. This implies that the created FTIR calibration model is not optimal. The off-line measurements were used to optimize the calibration model in order to be more accurate and reliable for the following experiments. The standard error of prediction (SEP) for ethanol after optimization was $0.128gl^{-1}$.

Table 5.4: Summary of the biomass yields for each of the setpoints in the first experiment (Figure 5.4).

Setpoint number	Biomass yield $Y_{x/s}$ $[-]$
1	0.52
2	0.51
3	0.48

5.3.2 Fed-batch cultures at constant specific growth rate minimizing overflow metabolite production.

A fed-batch culture was carried out with the validated and optimized calibration model for ethanol detection by FTIR spectroscopy at a $\mu_{sp,crit}$ value $0.02h^{-1}$ below μ_{crit}. The response of the test system to the developed controller action is illustrated in 5.5. The concentration of the overflow metabolite, ethanol, is maintained below $0.2gl^{-1}$ and the specific growth rate oscillated constantly around the imposed setpoint for the first 16 hours of the culture. However, around 18 hours of culture, a decrease in the specific growth rate and a rise in the ethanol concentration occurs.

5.3.3 Discussion

The proportional-integral controller, developed in Chapter 4, to maintain a constant specific growth rate in Crabtree-negative yeast cultures, was successful at maintaining constant rates for μ, allowing to determine first the critical threshold value for switch to fermentative metabolism and then to maintain the specific growth rate below this value, ensuring purely oxidative growth conditions.

Nevertheless, some particular points should be considered. Firstly, the concentration of the carbon and energy source in the batch medium is higher than the limit identified as leading to fermentative metabolism. Therefore, during the batch phase, diauxic growth of *S. cerevisiae* can be observed (data not displayed). The concentration of glucose has been chosen such that at the end of the batch phase a substantial amount of biomass is already formed, leading to more reliable biomass measurements. However, it could be of interest to

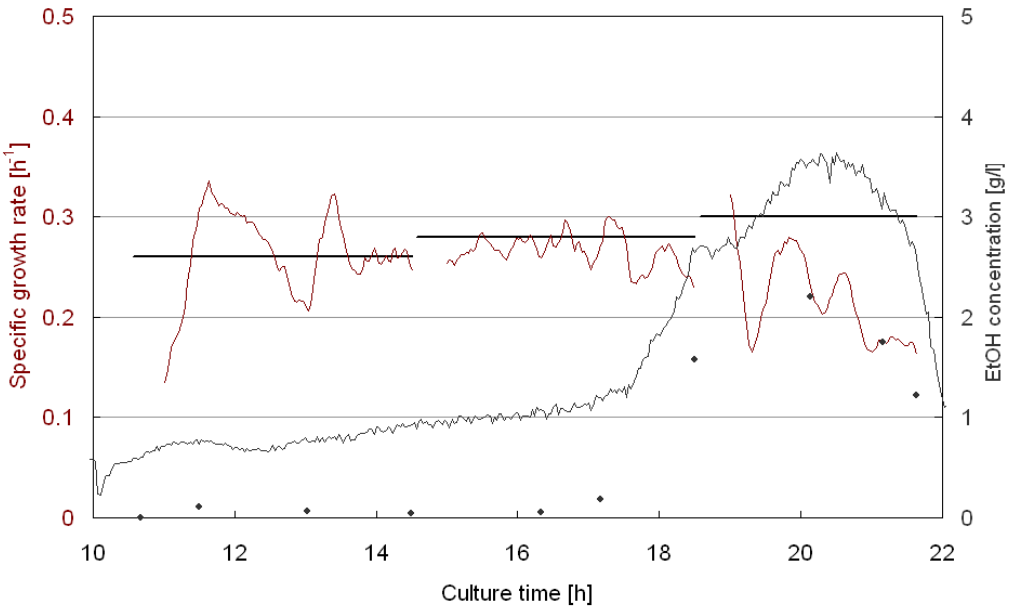

Figure 5.4: Determination of the critical specific growth rate for *S. cerevisiae*. Above the value of $0.28h^{-1}$, ethanol accumulation starts to be important. The FTIR measurements (continuous gray line) show and offset compared to the off-line determination of the ethanol concentration by HPLC (gray dots).

start with a glucose concentration of less than $2gl^{-1}$, leading to purely oxidative metabolism in the batch phase and a smoother transition to the fed-batch phase. The high initial oscillations of the specific growth rate and the resulting controller errors observed during the first setpoint (Figure 5.4 and Table 5.5) could thereby be reduced. A critical point concerns also the level of dissolved oxygen. This particular process parameter is, within the scope of this study, only monitored, but not controlled. However, *S. cerevisiae* will, like most yeast cells, start to ferment in microaerobic and anaerobic conditions. No particular studies were carried out to determine the critical oxygen concentration required to ensure fully aerobic conditions for the growth of *S. cerevisiae*, therefore it is not totally clear whether microaerobic conditions above a certain culture time would not play a role in destabilizing the controller. A last particular point is related to the model development for the interpretation of the spectral data acquired by FTIR spectroscopy. It is commonly accepted that the more samples are used to prepare a model, the more reliable and accurate the model will be [62, 63, 257, 33]. Chemometric model development, optimization and comparison

for spectroscopic methods is a field on its own [205]. Within the present project, a reliable chemometric model is paramount for a satisfying estimation of the specific growth rate on the one hand and for required performance of the controller on the other. However, it is important that efforts for model development are kept to a minimum. Therefore a simple 64 synthetic sample approach stipulated by Brereton [258] and standard in chemometrics [205] was chosen to develop a model that was then optimized with concentration values obtained from off-line samples from the first experiment by HPLC quantification (Figure 5.5), leading to satisfying results for the second experiment. Adapting the present prediction model for the detection of substrates and metabolites by FTIR spectroscopy to other microbial processes might represent an other challenge all together. For instance *Escherichia coli* cells, an important expression system for recombinant protein production (Figure 1.1), are cultured at 37 °C and mainly produce acetate as an overflow metabolite. Therefore, even though the same defined medium might potentially be used for culturing *E. coli* cells, a new chemometric model for the real-time quantification of substrates and metabolites by FTIR spectroscopy would need to be created taking into account temperature [259, 260, 205] and additional metabolites.

The controller errors in this study, summarized in Table 5.5, are comparable to those obtained for Crabtree-negative yeast cultures (Chapter 4) and below 20%, the arbitrarily chosen maximal tolerable controller error for the present work. The oscillations around the setpoint seem more pronounced than those observed by Biener and co-worker [7] during their study of the control of the specific growth rate of *S. cerevisiae* . The presented approach certainly requires *a priori* knowledge of the growth characteristics of the strain under study, which might not be necessary when focusing on the control of the overflow metabolite itself [240, 95]. Nevertheless, the proposed control strategy is a valid approach to tackle simultaneously the challenge of avoiding the formation of overflow metabolites and of controlling the specific growth rate at a given setpoint.

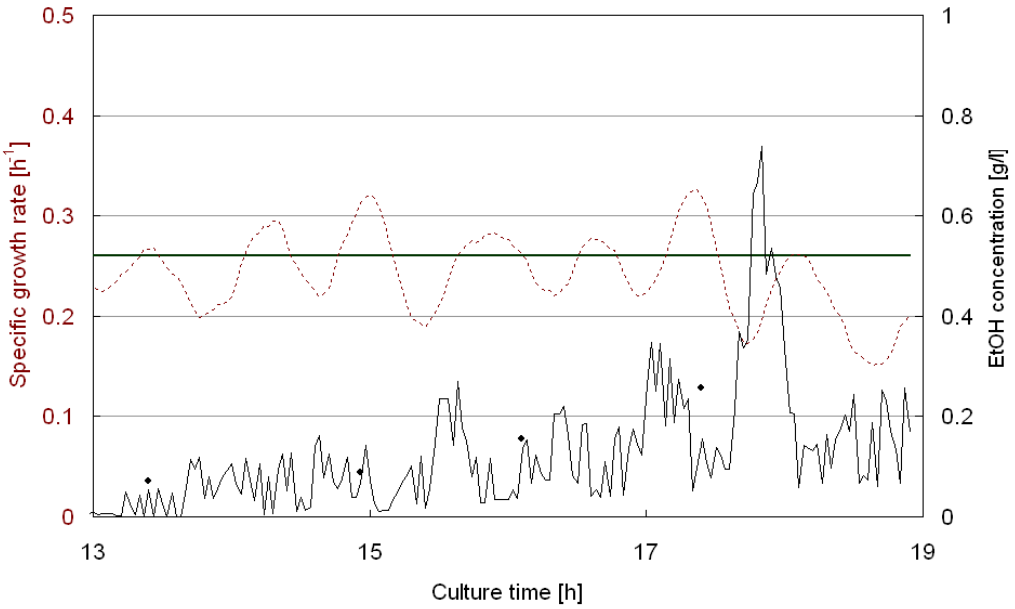

Figure 5.5: Culture of *S. cerevisiae* where the specific growth rate was set to $0.26h^{-1}$, which is $0.02h^{-1}$ below the critical specific growth rate, in order to prevent the formation of overflow metabolites.

Table 5.5: Summary of the controller error for the specific growth rate from experiments displayed in Figure 5.4 and Figure 5.5.

Experiment	Controller error [%]	Overall controller error [%]
I Figure 5.4		15.5
Setpoint I	12.5	
Setpoint II	6.1	
Setpoint III	28.0	
II Figure 5.5		14.2

5.4 Conclusion

By culturing *S. cerevisiae* at three different constant specific growth rates in fed-batch cultures, the present work successfully confirmed the assumptive critical specific growth rate where the strain under study switches from a purely oxidative to a fermentative metabolism and starts producing overflow metabolites (ethanol in this particular case) in response to excess carbon-source present in the culture medium. Furthermore, maintaining the specific growth rate constant at a $\mu_{sp,crit}$ value $0.02h^{-1}$ below μ_{crit} allowed to successfully culture *S. cerevisiae* in purely oxidative conditions, allowing for high biomass yield and low production of overflow metabolites such as ethanol. The present study shows the potential of transferability and general applicability of the control strategy developed in Chapter 4, since no particular changes or adaptations to the controller are necessary for its application to Crabtree-positive yeast cell cultures. The present results are positive indications that the simple feedback control strategy developed within the scope of this thesis could potentially be taken a step further and applied to other important, Crabtree-positive expression systems for recombinant protein production such as *Escherichia coli*.

Chapter 6

Biocalorimetry as a Process Analytical Technology process analyzer; robust in-line monitoring and control of aerobic fed-batch cultures of Crabtree-negative yeast cells [1]

Take chances, make mistakes. That's how you grow. Pain nourishes your courage. You have to fail in order to practice being brave. [2]

[1] This chapter is published in *Journal of Thermal Analysis and Calorimetry*: Sivarprakasam S, Schuler MM, Hughes KM, Hama A, Marison IW - Biocalorimetry as a process analytical technology process analyzer; robust in-line monitoring and control of aerobic fed-batch cultures of Crabtree-negative yeast cells, 2010 [143]

[2] Mary Tyler Moore, Actress (1936 -)

Control of bioprocesses requires reliable and robust on- or in-line monitoring tools providing real-time information on process dynamics. Heat generation related to metabolic activity of living systems is currently gaining importance in bioprocess industry due to its non-invasive and essentially instantaneous characteristics. The present study deals with monitoring and control of pure aerobic fed-batch cultures of three Crabtree-negative yeast strains, *Kluyveromyces marxianus*, *Candida utilis* and *Pichia pastoris*, based on in-line measured, metabolic heat flow signals. A high resolution biocalorimeter (BioRC1) was developed from a standard bench-scale heat flow calorimeter (RC1) [158]. The BioRC1 is equipped with in-line (dielectric spectroscopy, pH probe and dissolved oxygen probe) and at-line (exit gas analyzer) sensors to characterize the growth behavior of the yeast cells. Both metabolic heat flow and biomass profiles exhibited similar behavior proving the significance of employing heat flow signal as a key-parameter for the system under investigation. A simple estimator for biomass concentration and specific growth rate was formulated based on heat flow values. In order to evaluate the potential of calorimetry as a reliable and powerful process monitoring tool, the robustness, reliability as well as the broad applicability of the developed estimators was assessed through comparison with off-line measurement techniques and showed promising results for general applicability with a wide range of bioprocesses.

6.1 Introduction

Biotechnological processes are rather complex systems especially regarding effective monitoring and control. Control of biotechnological processes requires reliable real-time measurements of critical process parameters. During the last years several sensors and controllers have been developed and implemented for in-line monitoring and control of bioprocess systems [201]. Invasive mode of operation, time delay in measured signals and difficulties in integrating lab-scale tested systems into existing industrial bioprocesses are

the major drawbacks of custom available sensors [87]. Recently, biocalorimetry has been found to have potential for real-time bioprocess monitoring due to its non-invasive and instantaneous mode of operation [160]. Indeed, heat signals during a bioprocess provide a global insight into metabolic activity of living cells [158], [261]. In the case of a pure aerobic respiratory metabolism where the substrate is entirely converted into biomass, water, carbon dioxide and heat, the signal measured by a biocalorimeter (BioRC1) can be related to the actual biomass concentration as well as to the specific growth rate. Product quality, as well as the productivity of a process is influenced by the specific growth rate and its fluctuation. Controlling this critical process parameter is an important step towards Quality by Design (QbD) as encouraged by the FDA initiative [3]. Several authors so far attempted to employ calorimetry as an analytical tool to monitor and investigate various bioprocesses [262, 70, 263]. However, literature related to applications of heat signal as a basis for control of the specific growth rate fed-batch cultures of yeast cells are scarce. While most reported research applied heat flow signals as an indicative measurement to start the nutrient feeding at the end of batch phase [264], the actual measurements used to control the substrate feed rate of the process were then heat-derived parameters such as the oxycalorific equivalent or combined parameters such as heat flow signals combined with carbon dioxide evolution rate [134, 140]. In this work, a reliable biomass and specific growth rate estimator is developed based on heat flow measurements. The principle aim of the study is to highlight the importance of a creating biomass and specific growth rate estimator based on simple process parameter measurements. An additional step includes the improvement of the reliability and the robustness of the developed estimators in order to ensure, at a later stage, an established basis for bioprocess control. In accordance with classical biothermodynamics it is possible to use the calorimetric heat flow signal as a sole variable to control microbial fed-batch culture since, for pure aerobic bioprocesses involving Crabtree-negative organisms, the cell growth is entirely based on its respiratory capacity and further characterized by the absence of by-product formation. Moreover, the heat generation rate due to the metabolic activity of living cells is directly proportional to the growth rate at any instant of time unless there is a substrate limitation [153]. This study is therefore focusing on the aerobic batch and

fed-batch cultures of Crabtree-negative yeasts, namely *Kluyveromyces marxianus*, *Candida utilis* and *Pichia pastoris*. These microbes were chosen as model organisms due to their simple growth requirements as well as to the fact that they do not suffer from any metabolic bottlenecks enabling the direct correlation between heat generation rate and specific growth rate in its simplest way. Model development and initial test were carried out with *K. marxianus*, while *C. utilis* and *P. pastoris* were used to test-proof the general applicability of the designed estimators to different Crabtree-negative yeast strains. The development of a robust biomass and specific growth rate estimator based on heat-measurements has its importance in the framework of the FDA's PAT initiative, since it gives a real-time insight into the on-going industrial process. In fact, it has been proven that small modifications to a standard large-scale bioreactor allow the acquisition of in-line calorimetric measurements [73]. The proposed biomass and specific growth rate estimator might therefore present a simple, but robust and non-invasive way of monitoring an industrial microbial bioprocess.

6.2 Material and methods

6.2.1 Chemicals

All chemicals used in this study were purchased from Sigma Aldrich (Sigma Aldrich, St. Louis, USA).

6.2.2 Cell strains and culture conditions

Cell strains

A wild-type yeast strain, *Kluyveromyces marxianus* (DSM 5422, DSMZ, Braunschweig, Germany) was chosen for this study, mainly due to its Crabtree-negative metabolism. For the assessment of the general applicability of the developed estimators, two other yeast strains were cultured, namely *Candida utilis* (DSM 2361, DSMZ, Braunschweig, Germany) and *Pichia pastoris* (hSA producing strain, Invitrogen, Carlsbad, USA). All strains were preserved as $1.8ml$ aliquots in a $20gl^{-1}$ glycerol solution at $-80\,^{\circ}\mathrm{C}$.

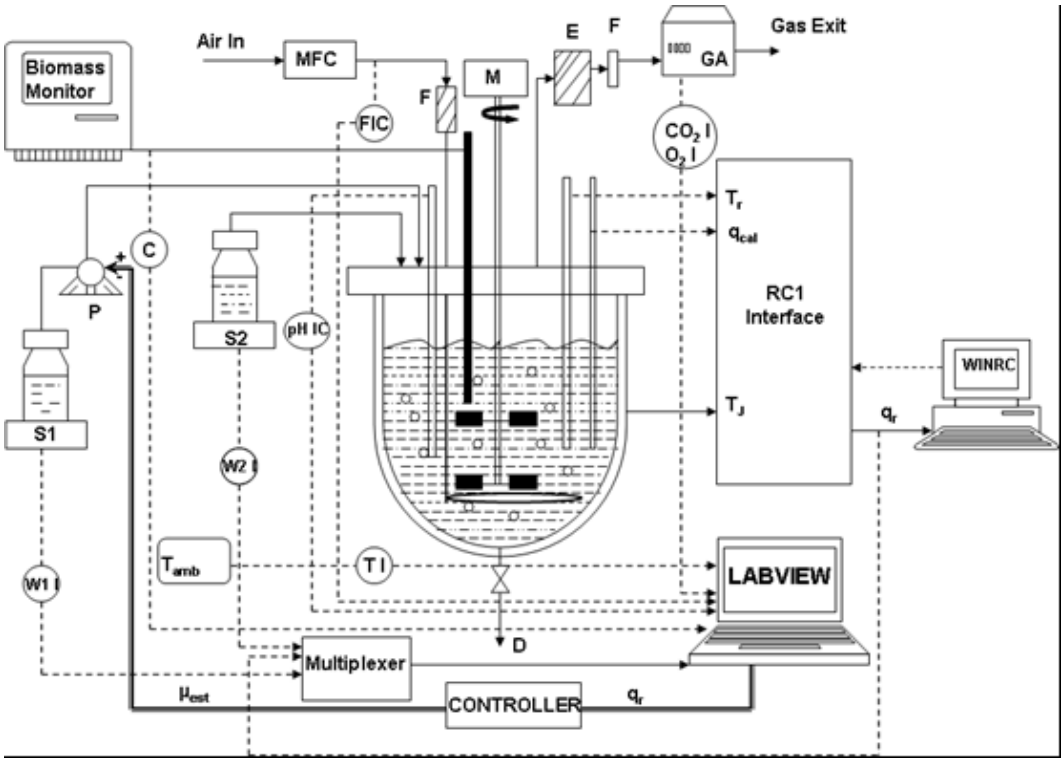

Figure 6.1: Schematic representation of the experimental setup around the BioRC1system: C; Capacitance, D; Drain valve, E; Entrainer, F; Membrane Filter, GA; Gas analyzer, M; Motor, MFC; Mass flow controller, P; Peristaltic pump, S1, S2; Balances, T_{amb}; Ambient temperature probe, T_r; Reaction temperature, T_j; Jacket temperature, q_{cal}; Calibration heat flow, q_r; Net metabolic heat flow, W1, W2; Mass, μ_{est}; Estimated growth rate,. Dotted lines represent the in-line measured values for LABVIEW. Double line represents the estimated control parameter from LABVIEW.

Inoculum preparation

The preculture was obtained by suspending cells from a $1.8ml$ aliquot in a 1-liter, baffled Erlenmeyer flask containing $100ml$ of preheated, sterile, complex preculture medium (pH 4 for *C. utilis* and pH 5 for *K. marxianus* and *P. pastoris*) containing $20gl^{-1}$ of glucose for *K. marxianus* and *C. utilis* and $20gl^{-1}$ of glycerol for *P. pastoris*, $10gl^{-1}$ of yeast extract and $10gl^{-1}$ of peptone and incubating the flask in an orbital shaker incubator (SHEL LAB S19, Sheldon Manufacturing, Cornelius, USA) for 24 hours at $30\,^{\circ}\mathrm{C}$ and at 150 rpm. Ninety milliliters of the culture broth were then centrifuged at 3000 rpm for 10 minutes, the supernatant discarded and the cell pellet suspended in $10ml$ of a sterile saline solution ($9gl^{-1}$ of NaCl).

Reactor description, media composition and culture conditions

The preculture was used to inoculate a 2-liter BioRC1 (modified RC1, Mettler Toledo, Greifensee, Switzerland) with a working volume of $1.3l$, equipped with a Rushton-type agitator, baffles, pH probe and controller, gas inlet and outlet ports, a base inlet port, a port for a capacitance probe (Biomass Monitor, ABER Instruments, Aberysthwyth, UK) and a sampling valve. The aeration rate was set to $2.5lmin^{-1}$ using a thermal massflow controller (5850E, Brooks, The Netherlands) and the air was sterilized by passage through a $0.22\mu m$ filter before entering the biocalorimeter. Outlet gas was passed through a Wolff bottle followed by a $0.22\mu m$ filter before entering a gas analyzer (Duet, Applied BioSystems Ltd, UK). The measured values of O_2 and CO_2 were corrected for water vapor according Duboc and von Stockar [141] and used to evaluate in real-time the oxygen up-take rate (OUR), the carbon dioxide evolution rate (CER) as well as the respiratory quotient (RQ). A solution of $4M$ NaOH was used to maintain the pH at 5. Acid control was not necessary due to the composition of the culture medium and the cell metabolism. The operating principle of the BioRC1 has been reported previously in literature [136]. All experiments were performed with an initial working volume of $1.3l$ and the reaction temperature was maintained constant at $30\,^{\circ}\mathrm{C}$. Agitation rate was maintained at 800 rpm throughout all cultures. A detailed account of liquid volumes entering the reactor (base and medium feed) and leaving it (samples) was made gravimetrically (Analytical Balances, Mettler Toldedo, Greifensee, Switzerland) and filed through a LabVIEW (LabVIEW 8.2, National Instruments, Austin, USA) program in order to have a continuous inventory of the reactor volume throughout the experiment. Figure 6.1 is a schematic representation of the experimental set-up including all the instruments, their accessories and their connections. Prior to inoculation, the bioreactor was sterilized in-situ at $121\,^{\circ}\mathrm{C}$ for 20 minutes with de-ionized water using an automated WINRC sterilization program (WINRC Software, Mettler Toledo, Greifensee, Switzerland), cooled down to room temperature, drained and filled with sterile batch medium. Cells were grown in batch mode until depletion of the carbon and energy source. For the assessment of the growth rate estimator, a fed-batch culture of *K. marxianus* was carried out, where an exponential feed mode, described elsewhere [239] was started after the end

Table 6.1: Composition of the batch and feed media for culturing *C. utilis* and *K. marxianus* .

Component	Batch medium $[gl^{-1}]$	Feed medium $[gl^{-1}]$
Glucose	10	300
$(NH_4)_2SO_4$	5	50
KH_2PO_4	3	35
$MgSO_4 \cdot 7H_2O$	0.5	3
Component	Batch medium $[mll^{-1}]$	Feed medium $[mll^{-1}]$
Trace elements & vitamins solutions	5	15
Polypropylene glycol 2000 (Antifoam)	0.5	2

Table 6.2: Composition of the batch and feed media for culturing *P. pastoris* .

Component	Batch medium $[gl^{-1}]$	Feed medium $[gl^{-1}]$
Glycerol	10	500
$CaSO_4 \cdot 2H_2O$	0.59	-
K_2SO_4	9.1	-
$MgSO_4 \cdot 7H_2O$	7.45	-
KOH	2.06	-
NH_4Cl	9	40
Component	Batch medium $[mll^{-1}]$	Feed medium $[mll^{-1}]$
H_3PO_4 85%	13.35	-
Trace elements & vitamins solutions	4.35	-
Polypropylene glycol 2000 (Antifoam)	1	-

of the batch phase. Table 6.1 and Table 6.2 outline the composition of the defined batch and feed media. The media were prepared with de-ionized water, sterilized by filtration (Steritop, $0.22\mu m$ pore size, Millipore, Billerica, USA) and supplemented with sterile antifoam solution, trace elements and vitamins as prescribed by Verdyun *et al.* [243] and Cannizzarro *et al.* [95] for *K. marxianus* and *C. utilis* and suggested by Invitrogen for *P. pastoris*.

6.2.3 Monitoring, data acquisition and control strategy

Off-line analysis

Samples ($10ml$) were taken at regular intervals during the cultures in order to determine off-line the biomass concentration. Biomass dry cell weight (DCW) was determined by filtering a known volume of the culture broth through a pre-weighed $0.22\mu m$ pore size

filter (GSWP $0.22\mu m$ Nitrocellulose membrane filters, Millipore, Billerica, USA), drying the filter to constant weight and subsequently reweighing.

Data Acquisition and Process Control

Data acquisition of the process parameters from the different probes and sensors was carried out through a FieldPoint (Model - FP 2000, National Instruments, USA) and an interfacing hardware (NB-MID-32X, National Instruments) with a PC. A LabVIEW program (LabVIEW 8.2, National Instruments, Austin, USA) was developed in-house for data acquisition, storage and display as well as for calibration and process control. The acquired raw values, saved in a separate file, were continuously averaged over 50 points and used for the real-time calculations of the baseline heat flow, the metabolic heat flow, the biomass concentration, the specific growth rate, the nutrient feed rate and the error term for the feedback control loop for the control strategy under development.

6.2.4 Biomass and specific growth rate estimator

Specific growth rate estimators are commonly based on Monod-derived models, requiring reliable direct biomass measurements such as capacitance measurements [82] or indirect estimations based on oxygen up-take rate [43]. However, a heat-yield based approach may be employed as stated by von Stockar *et al.* [265]. The detailed development for the heat-based biomass estimator and the specific growth rate estimator for fed-batch cultures, described in Equation (6.1) and Equation (6.2) may be found in the appendix.

$$x_t V_t = x_0 V_0 + \frac{Q_t - Q_0}{Y_{Q/x}} \tag{6.1}$$

Equation (6.1) describes the estimation of biomass at a given time t as a function of the cumulative heat release and the heat yield coefficient.

$$\mu_{est,t} = \frac{q_r}{x_0 V_0 Y_{Q/x} + (Q_t - Q_0)} \tag{6.2}$$

Equation (6.2) describes the estimation of the specific growth rate as a function of the

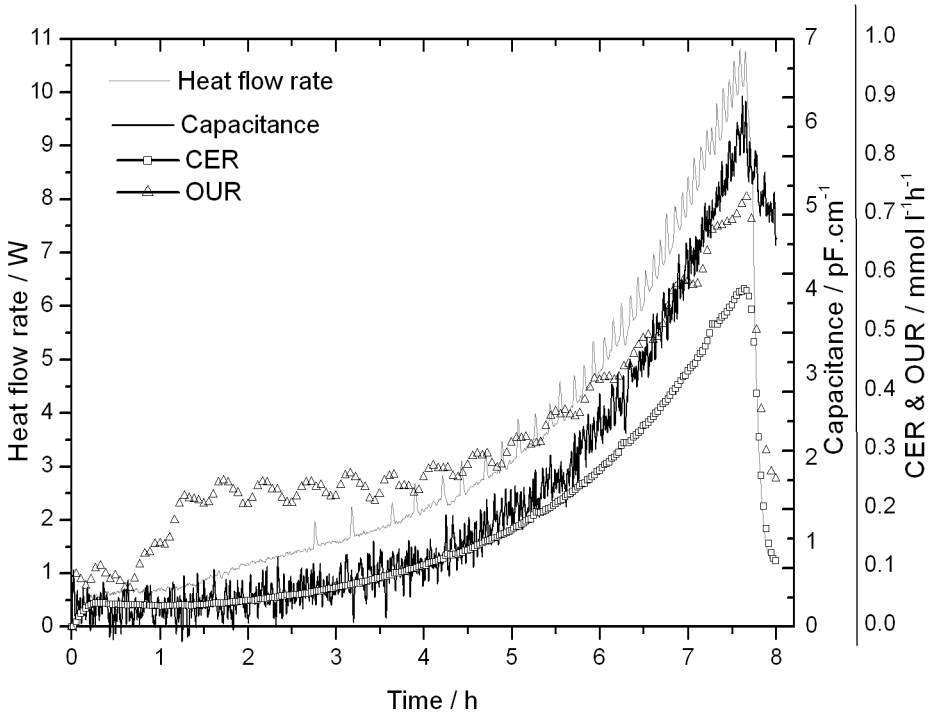

Figure 6.2: Comparative profiles of heat flow rate, capacitance, oxygen up-take rate (OUR) and carbon dioxide evolution rate (CER) for a batch cultivation of *K. marxianus* in BioRC1.

initial biomass concentration, the heat yield coefficient, the cumulative heat production and the heat production rate.

The drawback of instantaneous estimators is their extreme vulnerability to noise in the values serving as a basis for the estimation. Therefore the measurements need to be smoothed by applying for instance moving-point averaging or mathematical filtering techniques. Throughout this study, a moving-point average of 50 points was applied systematically to all raw data. Several different moving-point averages were applied and the influence on the stability of the measurements studied. The results indicated that 50 points seemed to be a good compromise between stability and robustness of the measurements without losing vital information through over-smoothing of the signal.

6.3 Results and Discussion

The present study can be divided into four different parts. In a first step, the different on-, in- and at-line monitoring techniques included in the reactor setup, as described in Section 6.2 were qualitatively evaluated in terms of sensitivity to noise and information provided. Additionally, correlation studies, relating the heat flow rate to different off-line biomass measurements, were conducted to justify the use of calorimetric data as a basis of the development of a reliable biomass and specific growth rate estimator for Crabtree-negative yeast cultures and experimental heat yield coefficient were inferred from batch cultures of the different strains. In a third part, the biomass estimator based on heat measurements is assessed regarding reliability and robustness by comparing the results to off-line dry cell weight measurements and the specific growth rate estimator was also tested in a fed-batch experiments using a control strategy developed in previous work [136]. In a final step, the proposed biomass estimator was tested on two different Crabtree-negative yeast strains, *C. utilis* and *P. pastoris*, under different culture conditions in terms of pH and medium composition namely.

6.3.1 Qualitative evaluation and comparison of different monitoring techniques

Figure 6.2 depicts comparative profiles of heat flow rate, capacitance, oxygen up-take rate (OUR) and carbon dioxide evolution rate (CER) for a batch cultivation of *K. marxianus*. While the OUR, CER and heat flow measurements fingerprint the instantaneous metabolic activity of the organism under study, the dielectric spectroscopy data provides an estimation of the total viable cell volume based on capacitance measurements. While CER, OUR and heat flow measurements offer the same type of information about the metabolic state and activity of the cells, they display fundamental differences in terms of robustness, reliability and sensitivity. Furthermore, the OUR profile in this particular case differ significantly from the CER and heat profiles. The sensitivity and robustness of exhaust-gas based metabolic profiling relies, as does every monitoring technique, on the availability of reliable raw data.

Table 6.3: Summary of experimentally determined and reported [2] heat yield coefficients for three different Crabtree-negative yeast strains on two different C-sources.

Strain	C-source	Average experimental heat yield ($Y_{Q/x}$)[kJg^{-1}]	Heat yield reported in literature ($Y_{Q/x}$)[kJg^{-1}]
K. marxianus	Glucose	16.8 ± 0.5	19.96
C. utilis	Glucose	14.6 ± 0.2	11.73
P. pastoris	Glycerol	5.8 ± 0.2	11.66

However, the gas analyzer used in the scope of this study has a limited sensitivity and provides inconsistent data for oxygen concentration measurements throughout the culture. Fluctuations due to noise disturbances can be observed in capacitance and heat flow measurements, highlighting the importance of suitable data pre-treatment and processing. Furthermore, the dielectric spectroscopy data refers to a correlation between the capacitance measurements and any suitable off-line determination of biomass to provide an estimation of the total viable cell volume. The instantaneous and universal nature of the heat flow measurements suggest that biocalorimetry is the most promising and reliable of the techniques studied for the monitoring of the process under study.

6.3.2 Correlation studies

Now that the use of calorimetry as an appropriate process analyzer has been qualitatively evaluated and justified, correlations need to be drawn between the measured heat flow rate and off-line biomass concentration to check the linearity of relationship between the two variables. Biomass profiles based on DCW measurements and heat flow rate exhibit similar behavior proving the significance of employing the heat flow signal as a key-parameter for monitoring the system under investigation. These profiles enabled the calculation of the experimental heat yield for the different organisms cultured throughout this study. Table 6.3 summarizes the average heat yield coefficient value for each yeast strain and allows the comparison with experimental heat yield coefficient from literature. As expected in absence of any additional carbon sources, linear correlations (R^2 above 0.96 for all studies) are observed. The experimentally determined heat yield values were used for the estimation of the biomass concentration and the specific growth rate as described in Equation (6.2).

Table 6.4: Summary of the correlation factors between the estimated biomass concentration based on the metabolic heat flow measurements and the off-line determined biomass based on Dry Cell Weight (DCW) for the three different yeast strains under study.

Strain	Correlation factor [-]	R^2 value
K. marxianus	1.21	0.99
C. utilis	0.94	0.97
P. pastoris	0.60	0.97

6.3.3 Reliability assessment of the biomass estimator

In order to be able to assess the quality of the developed estimator, it was important to find an appropriate, well-established alternative biomass measurement technique to confirm the values obtained through the heat flow rate. The heat-based model, as described in Section 6.2, represents the biomass concentration and is therefore expressed in units of grams of biomass per liter of culture broth. In order to facilitate comparison, validation technique should represent biomass in the same units. Even though Dry Cell Weight (DCW) measurements are prone to gross errors and poor sensitivity, their validity as an off-line validation method is commonly admitted [266, 267, 90, 87]. Table 6.4 summarizes correlation plots between DCW and the heat flow based biomass estimation for each strain under study. The correlation between DCW and heat-based biomass estimation is between 0.6 and 1.2 for the three strains under study. A one-to-one correlation factor is an indication that the heat-based model is actually able to predict, estimate or measure the biomass determined by DCW in a realistic and tangible way. The accuracy of the estimation is difficult to assess since the biomass measurement based on DCW are not always very accurate themselves and inherently overestimate the actual biomass concentration through accumulation of cell debris over time. Moreover, the DCW estimates the whole amount of biomass in the bioreactor where else the heat flow based model estimates the viable, metabolically active biomass. Therefore, it could be assumed that DCW measurements systematically overestimate the real biomass concentration which would be reflected by a correlation factor smaller than one between these measurements and the heat-based values. The overestima-

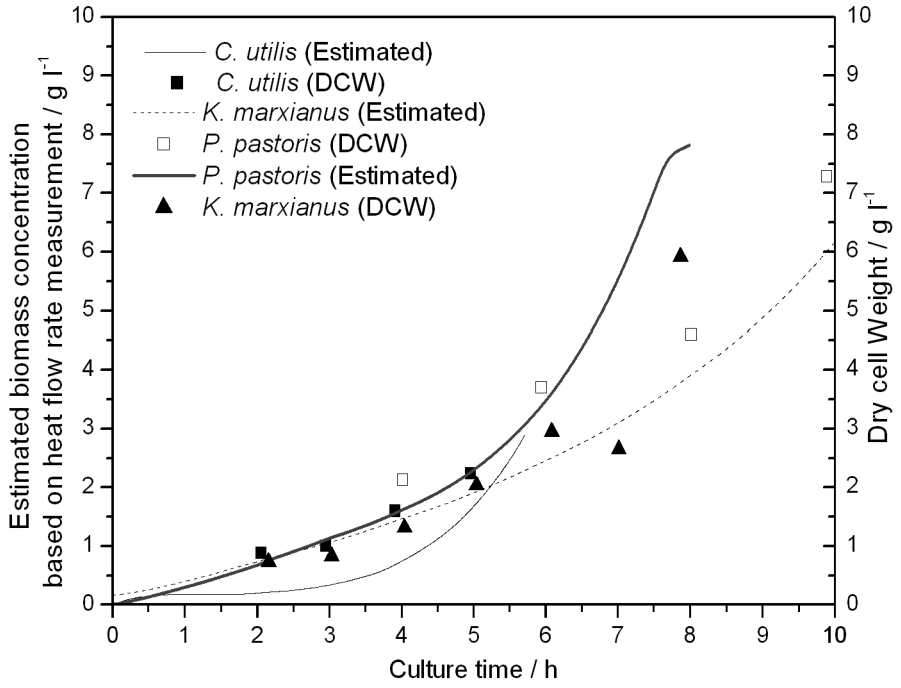

Figure 6.3: Comparative profiles of biomass concentration as a function of time for *C. utilils*, *K. marxianus* and *P. pastoris*; the lines (thick black line for *C. utilis*, thin black line for *P. pastoris*, dotted line for *K. marxianus*), represent the on-line estimated biomass concentrations based on the measurements of the metabolic heat flow while the discrete points (empty squares for *C. utilis*, filled squares for *P. pastoris* and triangles for *K. marxianus*) represent the off-line determination of the biomass concentration.

tion of the real biomass concentration through the off-line values compared to the measured metabolic activity can be observed in two out of three cases, namely for *P. pastoris* and *C. utilis*. However, a slight overestimation of the biomass based on heat flow measurements can systematically be observed for *K. marxianus*. It is unclear for the moment if the unexpected overestimation for this strain has a biological meaning. Nevertheless, microscopic observations have shown that the strain tends to form hyphae throughout the culture. The inherent dimorphism of *K. marxianus* could be part of a plausible explanation, but further studies may need to be conducted to determine the validity of this hypothesis. Alternative explanations might be based on findings regarding internal storage materials [268].

In an additional step, the heat-based biomass estimator was tested on-line in batch cul-

tures for each yeast strain. The evolution of the biomass concentration in the batch phase of cultures of *Candida utilis*, *Kluyveromyces marxianus* and *Pichia pastoris* is shown in Figure 6.3. As expected, according to correlation results shown in Table 6.4, the biomass estimator shows a certain ability to depict the actual biomass concentration evaluation at least at some stages of the process. Particular attention has to be paid to the smoothness of the heat based biomass values. In fact, during the batch phase, the only disturbance to the system is the occasional base addition and the sampling. Therefore, the heat flow signal is relatively stable, leading also to a smooth biomass concentration measurement. The developed biomass estimator based on heat flow measurements is reliable and robust as long as the disturbances applied to the system are kept to a minimum.

6.3.4 Specific growth rate estimator and controller robustness

The μ estimator is, according to Equation (6.2), derived from the biomass estimator based on calorimetric measurements. To assess the performance of the developed estimator, a fed-batch was carried out, controlling the specific growth rate to a desired set-point ($0.2h^{-1}$). The average specific growth rate based on heat was calculated ($0.21h^{-1}$) and the mean tracking error evaluated ($0.03h^{-1}$) in order to assess the robustness of the specific growth estimator. The setpoint was then maintained for over 5 hours in order to determine the long term stability of the specific growth rate estimator as well as of the controller,. The off-line estimation of μ, based on the application of the Monod model for cell growth involving the biomass concentration obtained from DCW measurements, showed concordance with the heat-based values. However, Figure 6.4 indicates that the response (black line) is noisy, giving evidence for the need for additional stabilization of the controller through mathematical techniques in order to create an estimator with a high potential as a robust control platform for microbial fed-batch cultures. Even though more appropriate techniques may be available, the simple application of a Savitzky-Golay filter over the heat flow measurements showed to improve the stability of the specific growth rate estimator, highlighting the importance of mathematical treatment for enhanced data robustness.

6.3.5 Assessment of broader applicability

The results in Section 6.3.3 highlighted that the developed biomass estimator can be applied to different Crabtree-negative yeasts. It is estimated that little modification of the method for biomass estimation and filtering is required to enable application to other organisms as long as they do not suffer any overflow metabolism leading to a fermentative metabolism. This transferability of the developed model allows a fast development of monitoring and control strategies for different types of organisms since the only important parameter to be known prior a first run is the appropriate heat yield coefficient of the system. This value can be obtained from the literature for several of the most commonly used organisms growing on different C-sources [265] or calculated theoretically based on the molecular composition of the biomass of the strain involved [269]. Furthermore, after an initial run, the heat yield coefficient can be experimentally determined for the given culture conditions. The developed biomass estimator is thereby a simple and flexible tool for in-line monitoring of Crabtree-negative suspension cultures and a firm foundation for the creation of control strategies for fed-batch cultivation.

6.4 Conclusion

The implementation of a simple, heat-based biomass and specific growth rate estimator for yeast cell cultures in a bench-scale calorimeter has been successfully demonstrated. The continuous in-line estimation of the biomass gives a real-time insight into the on-going process and serves as a starting point for the development of simple, but flexible control strategies for setting the specific growth rate at a desired level in fed-batch cultures of Crabtree-negative yeast cells. The potential of the developed biomass estimator as a live window into the bioprocess, has its importance in the PAT framework as it highlights the potential of biocalorimetry as a non-invasive PAT process analyzer and as a firm basis for the development of a PAT platform for monitoring and control.

A PAT platform for monitoring and control of microbial fermentations can be broken down into several components, namely the process parameters and their monitoring, the

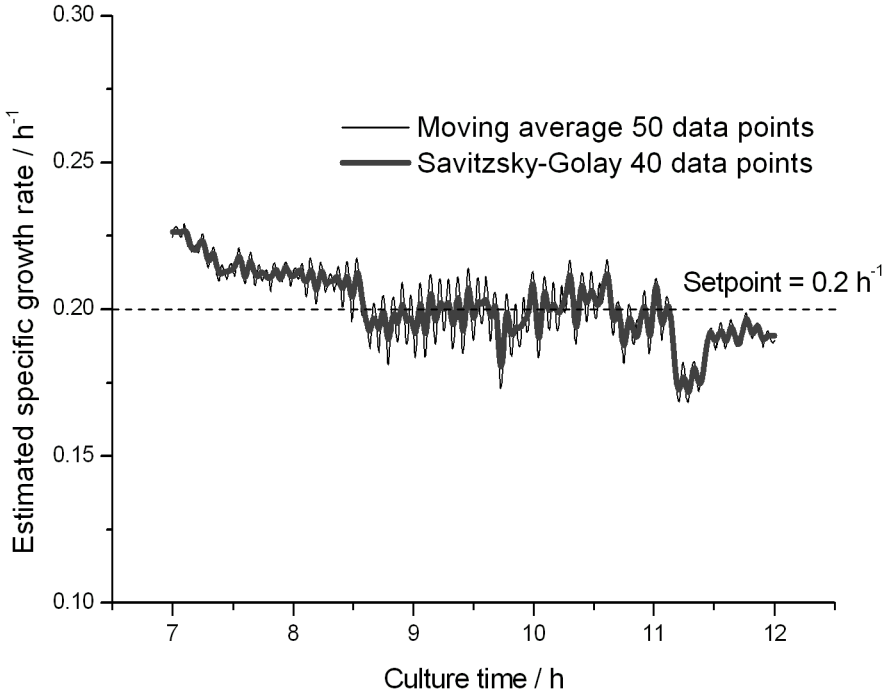

Figure 6.4: Evolution of the specific growth rate profile based on the developed estimator throughout a fed-batch culture of *K. marxianus*, where the black line represents the original data and the grey line data after application of a Savitzky-Golay filter.

monitoring tools, the control strategy and the response system.

The present study shows that a reliable and robust estimation of process parameters such as biomass and specific growth rate is possible within the limits stated by this report. As for the monitoring tool and the control strategy, the present work highlights the potential of biocalorimetry as a PAT tool to successful and controlled microbial fermentations. Indeed, the developed biomass estimator based on heat flow measurements proves to be efficient in monitoring the growth of Crabtree-negative micro-organisms such as *K. marxianus*, *C. utilis* or *P. pastoris*. However, only through the use of carefully chosen averaging and modeling techniques, it becomes feasible to apply this biomass estimator as well as the derived growth rate estimator, as a basis for a control strategy in order to maintain a given specific growth rate for a Crabtree-negative yeast. The investigations are in an early stage of development, but the high potential for industrial application of this calorimetry-based μ-estimator should be highlighted. Indeed, even though a calorimeter, as presented in this paper, could be considered as a sophisticated and costly process analyzer, gathering the necessary on-line heat flow measurements from an industrial bioreactor in order to infer the rate of heat production requires only minimal changes and the addition of some common instrumentation such as temperature probes, calibration heaters and flow meters. The transformation of an industrial bioreactor into a tool enabling the measurement of heat flow has indeed already been achieved as reported by several authors [73, 74].

The most important part in terms of components of a PAT platform, however, is the response system. Industrial bioprocesses for the development of pharmaceuticals or food products involve for instance organisms such as *Escherichia coli* or *Saccharomyces cerevisiae*, displaying a more complex metabolic behavior than the microbes under study. Issues related to metabolic activity of Crabtree-positive organisms render a direct correlation between heat production and biomass evolution, as presented in this paper, more complex. However these challenges could be addressed by the use of a second monitoring tool allowing the measurement of the biomass or the metabolic state of the cellular system.

This work underlines the importance of a thought through choice of the model organism in order to minimize difficulties inherent to the response system and their impact onto the

monitoring and control platform. Indeed, it was an interesting choice to use Crabtree-negative yeast cells in the first instances in order to avoid dealing with both respiratory and fermentative metabolism. However, it was not foreseen that other characteristics, such as the dimorphism of *K. marxianus* could also influence the outcome of the study.

The aim of subsequent work is the improvement of the reliability and the robustness of the biomass and growth rate estimation based on heat in order to achieve at a later stage close control of the latter. This objective is pursued in two distinctive ways at the moment by developing on one hand an artificial neural network model for biomass estimation through heat measurements and by creating, on the other hand, an energy-balance based on on-line data reconciliation system. Both approaches have their validity, advantages, as well as drawbacks and will be tested and compared in order to achieve the most consistent possible biomass and specific growth rate estimator which would enable the creation of a PAT biocalorimetry platform for highly controlled fed-batch cultures of Crabtree-negative yeasts.

6.5 Appendix

6.5.1 Metabolic heat flow model

Precise measurements of the heat generated by the metabolic activity of living cells are a prerequisite for reliable bioprocess control based on calorimetry. A heat balance over the BioRC1 setup paved the way for an accurate determination of the metabolic heat generation rate and can be stated as follows:

$$\text{Heat inflow} = \text{heat accumulation} + \text{heat outflow} \tag{6.3}$$

Equation (6.3) can be written as well as:

$$C_{p,r}\frac{dT_r}{dt} = q_r - q_f + q_s - q_g + q_d - q_l \tag{6.4}$$

For isothermal conditions as those encountered during fermentations, the heat accumu-

lation stated in Equation (6.4) equals zero, leading thereby to Equation (6.5).

$$q_r + q_c + q_s = q_f + q_g + q_d - q_l \tag{6.5}$$

Equation (6.5) displays the details of the heat flow balance over the reactor, where qr represents the heat generated by the metabolic activity of the cells, q_c the calibration power, q_s the energy input due to the stirring, q_f the heat flow through the reactor wall, q_g the heat flow caused by the aeration, q_d the heat input due to dosing and q_l the heat flow through the reactor head assembly by radiation and conduction. Equation (6.5) can be equaled to the variation of the total heat capacity of the reactor as a function of time.

For constant operating conditions, q_c and q_g are observed to be constant. Moreover, q_c, the calibration power used for the specific heat transfer coefficient determination is kept at constant at 9 W (standard calibration heater, Mettler Toledo, Greifensee, Switzerland). The heat flow through the reactor wall into the jacket fluid q_f is determined in-line by the WINRC software as stated in Section 6.2 during the calibration step preceding each experiment.

$$q_f = UA(T_r - T_j) \tag{6.6}$$

The three parameters, q_s, q_g and q_c are assumed to be constant [153, 136] throughout a given fed-batch experiment, hence, they may be grouped and represented by q_b, the baseline heat flow, which is measured in terms of q_f at steady state operating conditions before the inoculation at each fed-batch run.

Liquid additions in terms of base consumption, as well as substrate consumption during feed phase need to be accounted in the overall heat balance since they are contributing to disturbances in the measured heat flow signal. Heat flow contributions due to liquid additions can be taken in account as given in Equation (6.4).

$$q_d = m_{base}C_{p,base}(T_r - T_{base}) + m_{substrate}C_{p,substrate}(T_r - T_{substrate}) \tag{6.7}$$

Equation (6.7) describes the heat term due to liquid additions, where m_{base} and $m_{substrate}$ are the mass flow rate of the base and of the substrate feed solution respectively, $C_{p,base}$ and $C_{p,substrate}$ the specific heat capacity of the base and of the substrate feed solution respectively and T_{base} and $T_{substrate}$ the temperature of the base and of the substrate feed solution. Equation (6.7) was evaluated in real-time in a LabVIEW program, assuming T_{base} and $T_{substrate}$ to be equal to the ambient temperature (T_a) which is continuously monitored.

Heat loss through the non-thermostatted parts of the reaction setup is depending both on the surrounding temperature T_a and the heat transfer coefficients of the different set-up parts. A proportional model for the estimation of q_l was developed based on measurements of the ambient temperature as described in Equation (6.8).

$$q_l = \alpha(T_r - T_a) \tag{6.8}$$

The factor α in Equation (6.8) is provided by Mettler-Toledo for a given setup [270]. The previous equations are brought together and result in Equation (6.9) describing the metabolic heat flow rate. The q_r values were estimated in LabVIEW and averaged over 5 points to decrease the influence of the short-term noise on the heat flow signal. The metabolic heat flow measurements serves as a basis to the elaboration of a heat-based biomass and specific growth rate estimator.

$$q_r = q_f + q_d + q_l - q_b \tag{6.9}$$

6.5.2 Biomass and specific growth rate estimator

Specific growth rate estimators are commonly based on Monod-derived models, requiring the reliable direct biomass measurements such as capacitance measurements [82] or indirect estimations based on oxygen up-take rate [43] for example. However, a heat-yield based approach, relating the heat production rate to the rate of any component i, may be interesting as stated by von Stockar *et al.* [153]

$$Y_{Q/i} =\mid \frac{q_r}{r_i} \mid \tag{6.10}$$

In the specific case of biomass production rate, the relationship between the metabolic heat flow rate and the heat yield can be expressed as the ration of heat release and the biomass production rate:

$$Y_{Q/x} = \mid \frac{q_r}{r_x} \mid \tag{6.11}$$

Since the heat release rate is a parameter that is monitored on-line as described by Equation (6.11) the biomass production rate can be represented by the following equation:

$$r_x = \frac{dx}{dt} = x\mu \tag{6.12}$$

$$dx = \frac{q_r}{Y_{Q/x}} dt \tag{6.13}$$

Combing and rearranging Equation (6.11) and Equation (6.12) leads to Equation (6.13), describing the biomass production rate in gram of cells per unit time. This equation can be integrated (Equation (6.14)) leading to Equation (6.15)

$$\int_{x_0}^{x_t} dx = \int_0^t \frac{q_r}{Y_{Q/x}} dt \tag{6.14}$$

The solution of Equation (6.14) can substitute the term for biomass in Equation (6.11) leading to a new form of:

$$x_t = x_0 + \frac{Q_t}{Y_{Q/x}} \tag{6.15}$$

The solution of the integration displayed in Equation (6.14) is shown in Equation (6.15), leading to an estimation of the biomass at a given time t as a function of the cumulative heat release Q_t and the heat yield coefficient.

$$Y_{Q/x} = \frac{q_r}{\mu x_0 + \frac{Q_t}{Y_{Q/x}x_0 + (Q_t - Q_0)}} \tag{6.16}$$

$$\mu_{est,t} = \frac{q_r}{Y_{Q/x}x_0 + (Q_t - Q_0)} \tag{6.17}$$

Equation (6.17) permits to express the specific growth rate as a function of the heat production rate q_r, the cumulative heat release Q_t at a given time t and the heat yield coefficient.

Equation (6.17) gives an instantaneous estimation of the specific growth rate at a given time t as long as the reaction volume does not change. Since the different experiments in this study were carried out in fed-batch mode, the changes in volume during the reaction must be taken in account. When accounting for the changes in reaction volume, Equation (6.13) is modified as follows:

$$d(xV_r) = \frac{q_r}{Y_{Q/x}}dt \tag{6.18}$$

Equation (6.16), giving the relationship between the heat yield coefficient, the heat release rate and the variation of biomass and reaction volume, might be again integrated leading to Equation (6.19) which gives an instantaneous estimation of the specific growth rate at a given time t. The solution of can substitute the term for biomass. By modifying the equation, the specific growth rate might be estimated, relating the heat production rate q_r, the cumulative heat release Q_t at a given time t and the heat yield coefficient.

$$\mu_{est,t} = \frac{q_r}{x_0 V_0 Y_{Q/x} + (Q_t - Q_0)} \tag{6.19}$$

Chapter 7

Investigation of the potential of biocalorimetry as a general applicable Process Analytical Technology tool for monitoring and control of microbial cultures [1]

If we're growing, we're always going to be out of our comfort zone. [2]

[1] This chapter is published in *Applied Microbiology and Biotechnology*: Schuler MM, Sivaprakasam S, Freeland B, Hughes KM, Hama A, Marison IW; Investigation of the potential of biocalorimetry as a general applicable Process Analytical Technology tool for monitoring and control of microbial cultures[144]

[2] John Calvin Maxwell, Author (1947 -)

Biological reaction calorimetry, also known as Biocalorimetry has lead to extensive applications in monitoring and control of different bioprocesses. In this work, a simple estimator for biomass and growth rate was formulated based on in-line measured metabolic heat flow values and tested in a unique bench-scale calorimeter (BioRC1), improved to a sensitivity range of 8 mWl^{-1} in order to facilitate monitoring of even weakly exothermic biochemical reactions. A proportional-integral feedback control strategy based on these estimators was designed and implemented to control of growth rate of *Candida utilis*, *Kluyveromyces marxianus* and *Pichia pastoris* by regulating an exponential substrate feed. Maintaining a particular specific growth rate throughout a culture is essential for reproducible product quality in industrial bioprocesses and therefore a key sequence for the step from Quality by Analysis to Quality by Design as stated by the FDA's Process Analytical Technology initiative. The present study highlights the potential of biocalorimetry as a reliable biomass monitoring tool and as a key part of a robust control strategy for aerobic fed-batch cultures of Crabtree-negative yeast cells in defined growth medium. Presenting controller errors of less than 4% in the best cases, the approach presented within the scope of this work paves the way for the development of a generally applicable PAT platform for monitoring and control of microbial fed-batch cultures.

7.1 Introduction

The specific growth rate (μ), describing the variation of the cell concentration over time in relation to the actual cell concentration, is an important process parameter. Its correlation to the metabolic state of the cells and thereby to other cell-related process variables, such as product quality attributes is undeniable. Studies have highlighted the effect of changes in the specific growth rate on the glycosylation pattern [271] or over the secretory expression [25] of recombinant proteins and describe the importance of maintaining threshold values

of μ in order to avoid overflow metabolite production [272]. Moreover, arbitrary induction of a given cell state in order to produce a specific compound, such as prodigiosins [18], is of particular relevance to the biotechnology and pharmaceutical industry. A particular process parameter can be defined as "critical" as soon as it is related to a critical product quality attribute [273]. The control of critical process parameters is a prerequisite in the step from Quality by Analysis to Quality by Design as highlighted by the FDA's initiative for Industrial PAT [3].

Research is often restricted to continuous cultures, where the biological variable μ is regulated by the dilution rate (D), a physical parameter, in order to study growth rate related phenomena and effects [274, 275, 276]. However, fed-batch cultures are gaining importance since they allow maintenance of μ at a given setpoint by the choice of an appropriate control strategy. Various types of control strategies for fed-batch cultures have been proposed by different research groups for the past two decades, as shown by Lee *et al.* [113]. The application of complex soft-models, such as ANNs or fuzzy-control, give interesting results in terms of controller stability and adaptivity. Simulations with such soft-models perform well in certain cases, however there is a potential risk of loosing biologically relevant information. Stoichiometric or mechanistic models provide more information about the cellular state, especially if there is a switch from a pure black box model to a parametric based one. It should, however, be kept in mind, that simplicity is a keyword when the developed control strategy is intended for industrial application. Indeed, some studies on complex model-predictive control showed interesting results at simulation level but proved to be difficult to implement in practice [118]. In this study, there was a quest for a simple control strategy, in order to ensure a facilitated applicability to actual fed-batch cultures. A slight variation of a simple, exponential feeding strategy, described in previous work [239] and adopted here. This control strategy enables a constant specific growth rate to be maintained without extensive soft-model based adaption calculations.

However, a control strategy is only as good as the measurements used to estimate the control variable. Therefore, all strategies to control μ rely on the availability of reliable biomass measurements or estimations. Direct measurements of biomass can be achieved

through capacitance readings [81], [277] or by means of near-infrared spectroscopy [278]. Off-gas analysis or metabolite detection by FTIR measurements for instance enables indirect biomass estimation through the calculation of different rates, such as oxygen up-take rate, carbon dioxide evolution rate or C- or N-source consumption rates. Heat evolution rate, inherent to every process involving living organisms, has been investigated as a powerful monitoring tool over the past 25 years [279], [153], [184]. Biocalorimetry enables a deeper insight into the metabolic state of cells and brings a greater process understanding in general. However, attempts to use metabolic heat flow as sole measurement to estimate and control the specific growth rate throughout microbial fed-batch cultures are scarce. Indeed, heat measurements were used either in combination with other parameters, such as carbon dioxide evolution rate or in a derived form, such as the oxy-calorific equivalent [140], [134], to control the substrate feed rate into the system. The presented work is an investigation of the potential of biocalorimetry as a tool to provide reliable measurements to create a simple control strategy for μ.

The current chapter is centered around three different Crabtree-negative yeast strains, *Candida utilis*, *Kluyveromyces marxianus* and *Pichia pastoris*, grown in a modified, high sensitivity biocalorimeter. The organisms were chosen due to their simple growth requirements as well to the fact that they do not suffer from any known metabolic bottleneck, enabling the direct correlation between heat generation rate and specific growth rate in its simplest way [265]. A simple proportional-integral feed-back strategy, where the specific growth rate, estimated from metabolic heat flow measurements, as well as the error term of the controller are embedded in the exponential term of an equation described in previous work [239] is presented. The development of a simple strategy to control the specific growth rate of cells in fed-batch processes has its importance in the realm of the PAT framework in terms of process understanding and process control. Moreover, two independent research groups [74], [73] have shown the feasibility of large-scale calorimetry bringing the proposed control strategy a step closer to the development of a general applicable, heat flow measurement based platform for industrial control of the specific growth rate of microbial cells in fed-batch cultures.

7.2 Material and methods

7.2.1 Chemicals

All chemicals used in this study were purchased from Sigma-Aldrich (Sigma-Aldrich, St. Louis, USA).

7.2.2 Cell strains and culture conditions

Cell strains

Two wild-type yeast strains, *Kluyveromyces marxianus* (DSM 5422, DSMZ, Braunschweig, Germany) and *Candida utilis* (DSM 2361, DSMZ, Braunschweig, Germany) were chosen for this study, due to their Crabtree-negative metabolism. For the assessment of the general applicability of the developed control strategy, another yeast strain was cultured, namely *Pichia pastoris* (hSA producing strain, Invitrogen, Carlsbad, USA). All strains were preserved as $1.8ml$ aliquots in a $20gl^{-1}$ glycerol solution at $-80\,^{\circ}\mathrm{C}$.

Inoculum preparation

The preculture was obtained by suspending cells from a $1.8ml$ aliquot in a 1-liter, baffled Erlenmeyer flask containing $100ml$ of preheated, sterile, complex preculture medium (at pH 4 for *C. utilis* and at pH 5 for *K. marxianus* and *P. pastoris* respectively) containing $20gl^{-1}$ of glucose for *K. marxianus* and *C. utilis* and $20gl^{-1}$ of glycerol for *P. pastoris*, $10gl^{-1}$ of yeast extract and $10gl^{-1}$ of peptone and incubating the flask in an orbital shaker incubator (SHEL LAB S19, Sheldon Manufacturing, Cornelius, USA) for 24 hours at $30\,^{\circ}\mathrm{C}$ and at 150 rpm.Culture broth (90 ml) were then centrifuged at 3000 rpm for 10 minutes, the supernatant discarded and the cell pellet resuspended in 10 ml of a sterile saline solution ($9gl^{-1}$ of NaCl).

Reactor description, media composition and culture conditions

The preculture was used to inoculate a 2-liter BioRC1 (modified RC1, Mettler Toledo, Greifensee, Switzerland) with a working volume of $1.3l$, equipped with a 6-blade Rushton-

type agitator, baffles, a pH probe and controller, gas inlet and outlet ports, a base inlet port, a port for a capacitance probe (Biomass Monitor, ABER Instruments, Aberysthwyth, UK) and a sampling valve. The aeration rate was set to $2.5 l min^{-1}$ using a thermal mass-flow controller (5850E, Brooks, The Netherlands) and the air was sterilized by passage through a $0.22 \mu m$ filter before entering the biocalorimeter. Outlet gas was passed through a Wolff bottle followed by a $0.22 \mu m$ filter before entering a gas analyzer (Duet, Applied BioSystems Ltd, UK). The measured values of O_2 and CO_2 were corrected for water vapor according Duboc and von Stockar [141] and used to evaluate in real-time the oxygen uptake rate (OUR), the carbon dioxide evolution rate (CER) as well as the respiratory quotient (RQ). A solution of 4 M NaOH was used to maintain the pH at 4 and at 5 respectively. Acid control was not necessary due to the composition of the culture medium and the cell metabolism. The operating principle of the BioRC1 has been reported previously in literature [136]. All experiments were performed with a initial working volume of $1.3 l$ and the reaction temperature was maintained constant at $30\,^{\circ}\mathrm{C}$. Agitation rate was maintained at 800 rpm throughout all cultures. A detailed account of liquid volumes entering (base and medium feed) and leaving the reactor (samples) was made gravimetrically (Analytical Balances, Mettler Toldedo, Greifensee, Switzerland) and acquired through a LabVIEW (LabVIEW 8.2, National Instruments, Austin, USA) program in order to have a continuous inventory of the reactor volume throughout the experiment.

Prior to inoculation, the bioreactor was sterilized *in-situ* at $121\,^{\circ}\mathrm{C}$ for 20 minutes with deionized water using an automated WINRC sterilization program (WINRC Software, Mettler Toledo, Greifensee, Switzerland), cooled down to room temperature, drained and filled with sterile batch medium. Cells were grown in batch mode until depletion of the carbon and energy source. For the assessment of the growth rate estimator, fed-batch cultures were carried out, where an exponential feed mode was started after the end of the batch phase. Table 7.1 and Table 7.2 outline the composition of the defined batch and feed media. The media were prepared with deionized water, sterilized by filtration (Steritop, $0.22 \mu m$ pore size, Millipore, Billerica, USA) and supplemented with sterile antifoam solution, trace elements and vitamins as prescribed by Verdun *et al.* [243] and Cannizzarro *et. al* [95] for *K.*

Table 7.1: Composition of the batch and feed media for culturing *C. utilis* and *K. marxianus* .

Component	Batch medium [gl^{-1}]	Feed medium [gl^{-1}]
Glucose	10	300
$(NH_4)_2SO_4$	5	50
KH_2PO_4	3	35
$MgSO_4 \cdot 7H_2O$	0.5	3
Component	Batch medium [mll^{-1}]	Feed medium [mll^{-1}]
Trace elements & vitamins solutions	5	15
Polypropylene glycol 2000 (Antifoam)	0.5	2

Table 7.2: Composition of the batch and feed media for culturing *P. pastoris* .

Component	Batch medium [gl^{-1}]	Feed medium [gl^{-1}]
Glycerol	10	500
$CaSO_4 \cdot 2H_2O$	0.59	-
K_2SO_4	9.1	-
$MgSO_4 \cdot 7H_2O$	7.45	-
KOH	2.06	-
NH_4Cl	9	40
Component	Batch medium [mll^{-1}]	Feed medium [mll^{-1}]
H_3PO_4 85%	13.35	-
Trace elements & vitamins solutions	4.35	-
Polypropylene glycol 2000 (Antifoam)	1	-

marxianus and *C. utilis* and suggested by Invitrogen for *P. pastoris*.

7.2.3 Monitoring, data acquisition and control strategy

Off-line analysis

Samples ($10ml$) were taken at regular intervals during the cultures in order to determine off-line the biomass concentration. Biomass Dry Cell Weight (DCW) was determined by filtering a known volume of the culture broth through a pre-weighed $0.22\mu m$ pore size filter (GSWP $0.22\mu m$ Nitrocellulose membrane filters, Millipore, Billerica, USA), drying the filter to constant weight and subsequently reweighing. Glucose and glycerol concentrations of the samples were quantified by HPLC (Agilent Instruments 1200, Agilent Technologies Ltd. Cork, Ireland) with a Supelcogel C-610H column (Sigma-Aldrich, St. Louis, USA)

equipped with a guard column and using a $0.027\%v/v\ H_2SO_4$ mobile phase for isocratic elution. Calibration curves for each component were prepared for each run using five synthetic standards. An internal standard ($30gl^{-1}$ of isopropanol) was run with each sample and each standard.

Data Acquisition and Process Control

Data acquisition of the process parameters from the different probes and sensors was carried out through a FieldPoint (Model - FP 2000, National Instruments, USA) and an interfacing hardware (NB-MID-32X, National Instruments) with a PC. A LabVIEW program (LabVIEW 8.2, National Instruments, Austin, USA) was developed in-house for data acquisition, storage and display as well as for calibration and process control. The acquired raw values, saved in a separate file, were continuously averaged over 50 points and used for the real-time calculations of the baseline heat flow, the metabolic heat flow rate, the biomass concentration, the specific growth rate, the nutrient feed rate and the error term for the feedback control loop.

Biomass and specific growth rate estimator

Specific growth rate estimators are commonly based on models derived from Monod's equation for cellular growth, requiring reliable direct biomass measurements such as capacitance measurements or indirect estimations based on oxygen up-take rate for instance [43]. However, a heat yield based approach may be employed as stated by von Stockar *et al.* [134]. The detailed mathematical development for the heat-based biomass estimator and the specific growth rate estimator for fed-batch cultures, described in Equation (7.1) and Equation (7.2) has been described in previous work [143].

$$x_t V_t = x_0 V_0 + \frac{Q_t - Q_0}{Y_{Q/x}} \tag{7.1}$$

$$\mu_{est,t} = \frac{q_r}{x_0 V_0 Y_{Q/x} + (Q_t - Q_0)} \tag{7.2}$$

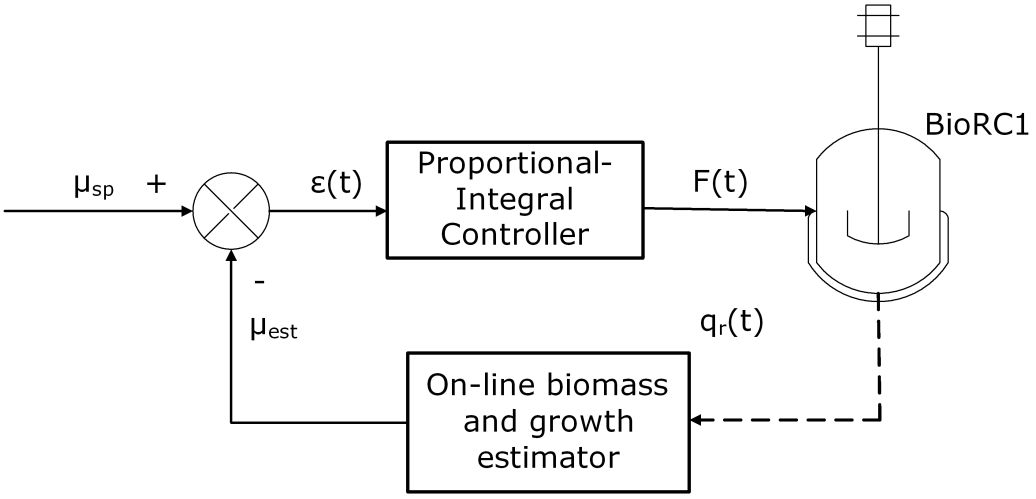

Figure 7.1: Control block diagram of the feedback control strategy based on heat flow measurements (dotted line).

Equation (7.1) relates the biomass concentration at a given time (x_t in gl^{-1}) and the actual culture broth volume (V_t in l) to the biomass concentration at the end of the batch phase (x_0 in gl^{-1}) and the culture volume at this very same moment (V_0 in l), the cumulative metabolic heat production at the end of the batch (Q_0 in kJ), the cumulative metabolic heat production at a given time (Q_t in kJ) and the heat yield coefficient ($Y_{Q/x}$ in kJg^{-1}). The specific growth rate at a given time ($\mu_{est,t}$) is a relation between the same physical quantities and the instantaneous heat flow rate (q_r) due to the metabolic cell activity as given in Equation (7.2).

Control strategy design

Independently of the control strategy, each controlled quantity or controlled variable is associated with a manipulated variable. Furthermore, a sensed variable, which is the measured value of the controlled variable, needs to be taken in account [280]. In the particular case of this study, the controlled variable is μ, the specific growth rate and the associated manipulated variable is the flow rate of a C-source-limited substrate feed. The sensed variable is the metabolic heat flow rate. Measurements of the heat flow rate, acquired through the BioRC1, are used to estimate the actual state of the controlled variable as indicated in Equation (7.2). The difference between the setpoint for the specific growth rate μ_{sp} and its actual

value μ_{est} is evaluated and used to define the tracking error ϵ as defined in Equation (7.3). The tracking error is included into the feedback loop, proper to a closed-loop system.

$$\epsilon(t) = \mu_{sp} - \mu_{est}(t) \tag{7.3}$$

In this work, a variation on the classical PI controller was designed, based on previous work [239] where proportional and integral terms were included in the exponential term of the equation for feed rate. The equation for feed rate was obtained by combining substrate and biomass balances as suggested by other authors [113], [239]. The feed-forward component of the closed-loop control strategy is described in Equation (7.4).

$$F_{FF}(t) = F_0 e^{(\mu_{est} t)} \tag{7.4}$$

The initial feed rate F_0, as shown in Equation (7.5) is a function of the initial biomass concentration (x_0) at the start of the feed for a given setpoint, the actual reactor volume (V_t), the biomass yield coefficient ($Y_{x/s}$) and the substrate concentration (s_F) in the feed solution.

$$F_0 = x_{t-1} V_{t-1} (\frac{\mu_{sp}}{Y_{x/s} s_F}) \tag{7.5}$$

A proportional-integral feedback control, described in Equation (7.6) was chosen, since a proportional controller, except in the presence of a pure capacity process, invariably leads to a non-zero steady-state offset. A simple way of removing the offset effect of the proportional controller is to add an integral term. The action of this controller is based on the summing of the instantaneous error over time and therefore responding to accumulated errors from the past, enabling to remove the residual steady-state error. However, oscillatory behavior may be expected since this type of controller is more prone to instability.

$$F_{PI}(t) = F_0 e^{((\mu_{sp} + K_P \epsilon(t) + K_I \int_0^t \epsilon(t)dt)t)} \tag{7.6}$$

The control block diagram developed during this study and implemented in a LabVIEW

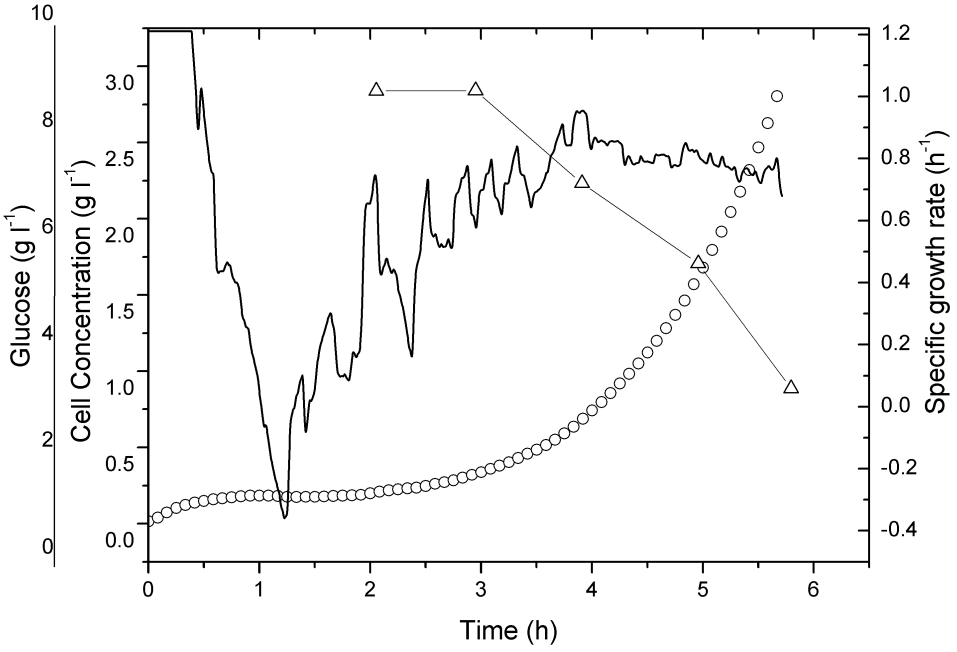

Figure 7.2: Batch culture of *C. utilis*, illustrating the glucose (triangles) and biomass (circles) concentrations as well as the specific growth rate (dotted line) over time.

program is shown in Figure 7.1. The control action, $F_{PI}(t)$ was executed by a remote-controlled peristaltic pump.

7.3 Results and discussion

The present section is divided into five different parts, highlighting first the potential of biocalorimetry as a biomass monitoring tool (Section 7.3.1) before emphasizing the importance and limits of data pretreatment (Section 7.3.2). The differences in controlled and uncontrolled situations are shown in Section 7.3.3, followed by a discussion about the performance of the controller in section 7.3.4, and finally the applicability of the developed strategy is assessed to another yeast strain in section 7.3.5.

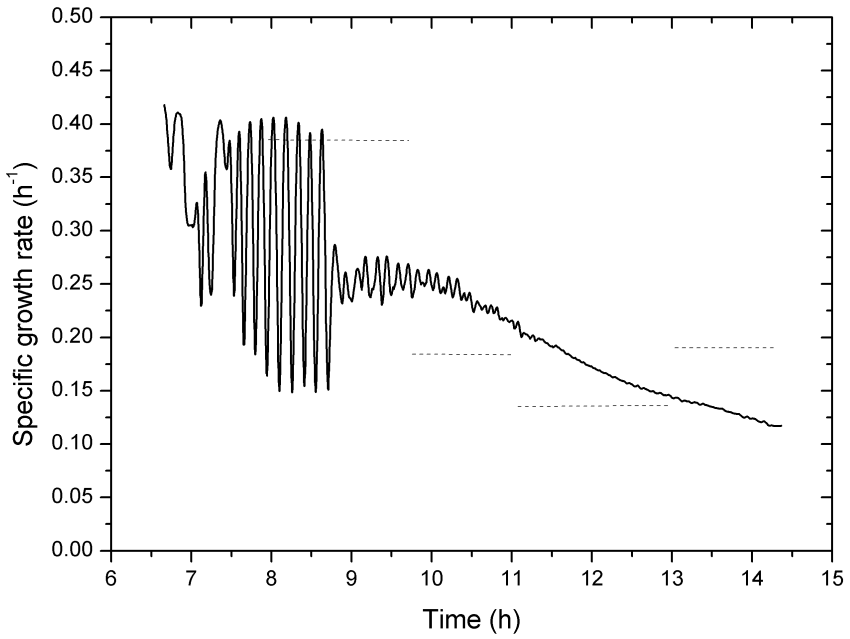

Figure 7.3: Study of the influence of different filtering techniques on the specific growth rate during a culture of *K. marxianus*. Moving-point averages with values ranging from 50, 80 and 100 were chosen.

7.3.1 Validity of the biomass and specific growth rate estimator

The applicability of biocalorimetry as a monitoring tool for biomass in microbial cultures has been assessed in previous work [143] for the three yeast strains under study, and is illustrated in Figure 7.2 for a batch culture of *Candida utilis*. Glucose concentration is decreasing as biomass, predicted by the biomass estimator based on calorimetric measurement, increases as the culture evolves. The estimated specific growth rate shows initially abnormally high values, due to heat signal disturbances inherent to the inoculation procedure. however, after the first hour of culture, the specific growth rate starts to follow an expected pattern, increasing until reaching a constant, slightly oscillating values, μ_{max} during the exponential growth phase.

7.3.2 Importance and limits of data pretreatment & controller constant tuning

Raw μ estimations might consist of noise which will have an effect on the controller action. Simple mathematical data pretreatment, such as a moving-point average or a high/low pass filter, can smoothen the signal. However, if the data pre-processing is too dominant, a potential loss of essential biological information might occur. Therefore, it is important to find an appropriate trade-off. Several different moving-point averages as well as some built-in (LabVIEW) high/low pass filters were tested during a fed-batch and the most suitable one was finally chosen and applied to all cultures that followed. The response of the system is clearly affected by the applied data processing technique (Figure 7.3). Indeed, an increase in the number of points averaged results in a decrease in the peak-to-peak amplitude. A 100-points moving average corresponds, in the case of a data point sampling interval of 10 seconds to a total average time of 16.7 minutes. Considering the doubling time of yeast cells at maximal growth rate and moving-point average values reported elsewhere [55], [239], a 16.7 minutes windows was regarded as acceptable.

Furthermore, an important point of a feedback control strategy is the choice of the appropriate values for K_P and K_I. Based on previous experiments [239], several different values for K_P (Figure 7.4) and later for K_I were tested and the response of the controller qualitatively construed. Finally, K_P was set to 0.1 and K_I to 0.15.

7.3.3 Differences in controlled and non-controlled situation

The specific growth rate (Figure 7.5) results in less oscillations during the feed phase where a given setpoint value is imposed to the system than in the batch phase, where the cells are free to grow at their maximal specific growth rate. Previous work [239], [90] has shown that oscillations in the values of the specific growth rate, even at the end of the batch phases, are present. The shift from a highly oscillating system in the uncontrolled situation to a smoother signal in the controlled one, without any change in the data processing, points out that the controller, despite oscillations in the response, actually has a stabilizing action on the system in comparison to the naturally occurring behavior during the batch phase.

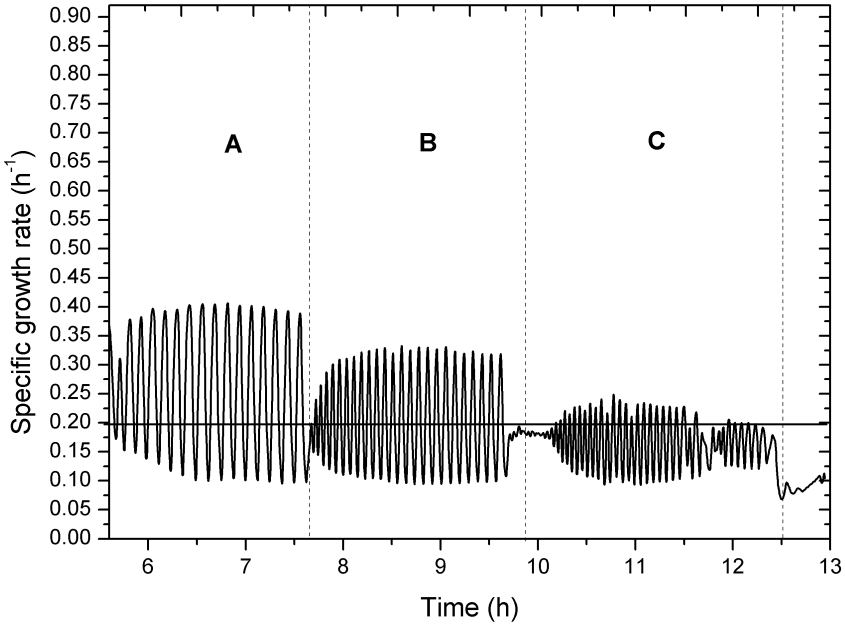

Figure 7.4: K_P tuning during a fed-batch culture of *K. marxianus*. The K_P value were set to 1 (region A), 0.5 (region B) and 0.1 (region C). A K_P value of 0.1 leads to the least marked oscillations.

7.3.4 Experimental robustness and longterm stability of the controller

To assess the performance of the developed control strategy, a series of fed-batches were carried out with *K. marxianus*, controlling the specific growth rate to a desired setpoint. In a very first attempt, only the feed-forward part of the controller was used, in a second step, the proportional feedback part was brought into play and finally, the proportional-integral feedback controller, as described in Equation (7.6) was tested. During the first cultures, two different setpoints were imposed ($\mu_{sp1} = 0.2h^{-1}$ and $\mu_{sp2} = 0.15h^{-1}$), where only one setpoint was assessed during an additional culture in order to observe the long term stability of the control strategy.

For each setpoint, the average specific growth rate was calculated, the mean tracking error evaluated and the overall controller error estimated in order to assess the robustness of the specific growth estimator under the given culture conditions. The results of these *K. marxianus* cultures are summarized in Table 7.3.

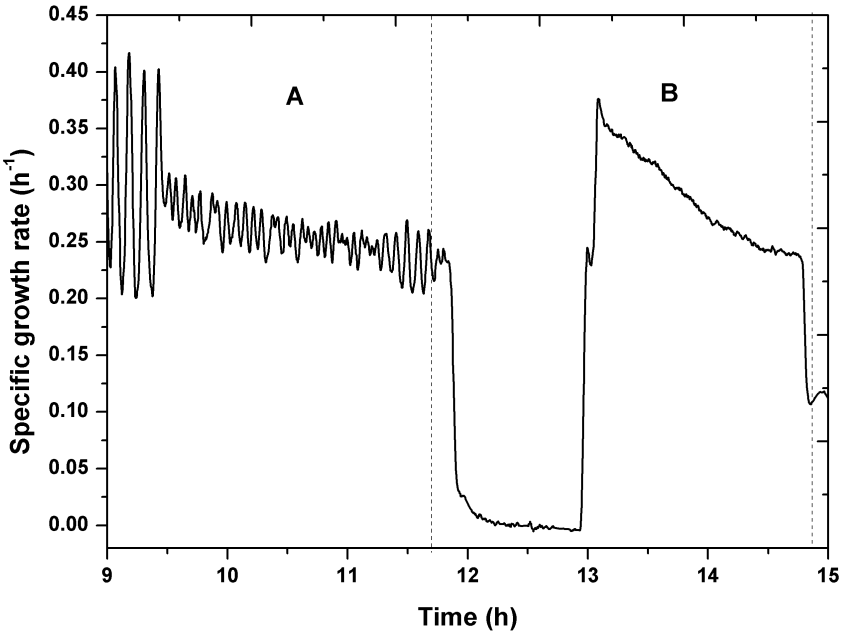

Figure 7.5: Illustration of differences in the oscillating behavior of the specific growth rate in batch (region A) and feed phases (region B) during a culture of *K. marxianus*.

Table 7.3: Summary of the different average values for the specific growth rate, the average tracking error (ϵ) as well as the overall controller error.

Controller	Setpoint $[h^{-1}]$	Average μ $[h^{-1}]$	Average ϵ $[h^{-1}]$	Controller error $[\%]$
Feed-forward	0.2	0.27	0.07	37.16
	0.15	0.23	0.08	54.17
P	0.2	0.24	0.02	18.14
	0.15	0.17	0.17	10.73
PI	0.2	0.25	0.05	24.26
	0.15	0.16	0.01	3.73
PI	0.2	0.21	0.03	3.48

The feed-forward control strategy (Equation (7.4)) that was implemented in this part of the study showed a satisfactory ability to control the specific growth rate at a constant value. However, as expected for a control strategy without any feedback term, the setpoint is not reached. Feed-forward control lacks the ability to compensate for overshooting within a reasonable frame of time, resulting in high controller errors for each setpoint (37.16% and 54.17%).

A proportional feedback controller invariably leads to an offset in the response, except in the case of a pure capacity process. Despite the risk of ending up with a unstable controller by adding an integral term, the proportional-integral feedback term was implemented. The resulting PI-controller showed a surprisingly high stability and resulted in a decreased controller error for all setpoints (Table 7.3).

The overall controller error is much higher in the first setpoint than in the second one. This more pronounced controller error could be related to the importance of the moment of the feed starting at the end of the batch phase. Indeed, if too much time elapses before the controller is switched on, cells will enter the stationary phase. As a result, a delayed response of the system which might result in an accumulation of substrate and in a later

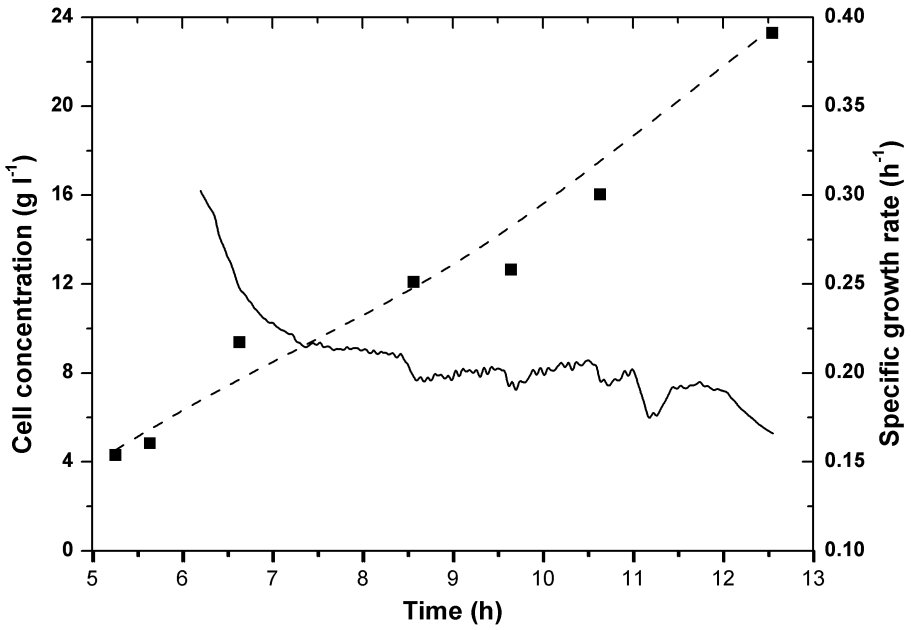

Figure 7.6: Feed phase of a fed-batch culture of *K. marxianus* where the specific growth rate (continuous line) was set to $0.2h^{-1}$ using the developed PI-feedback control strategy. Biomass concentration were measured off-line by DCW method (squares) and estimated in continuously in real-time (dotted line).

stage to an overshooting of the system, as illustrated in Figure 7.8 and Figure 7.5. However, the end of the batch phase should indeed be easy to detect since it is accompanied by a sudden drop in the heat signal as reported in the past by several authors. Therefore a robust, systematic and automated way of controlling the onset of the first setpoint should be implemented.

The robustness of the developed μ-estimator implemented in the control strategy is highlighted in Table 7.3. The average tracking error shows to be not higher than $0.08h^{-1}$. Furthermore, the response is very smooth, as in Figure 7.6 giving evidence for a high potential as a robust control platform for microbial fed-batch cultures.

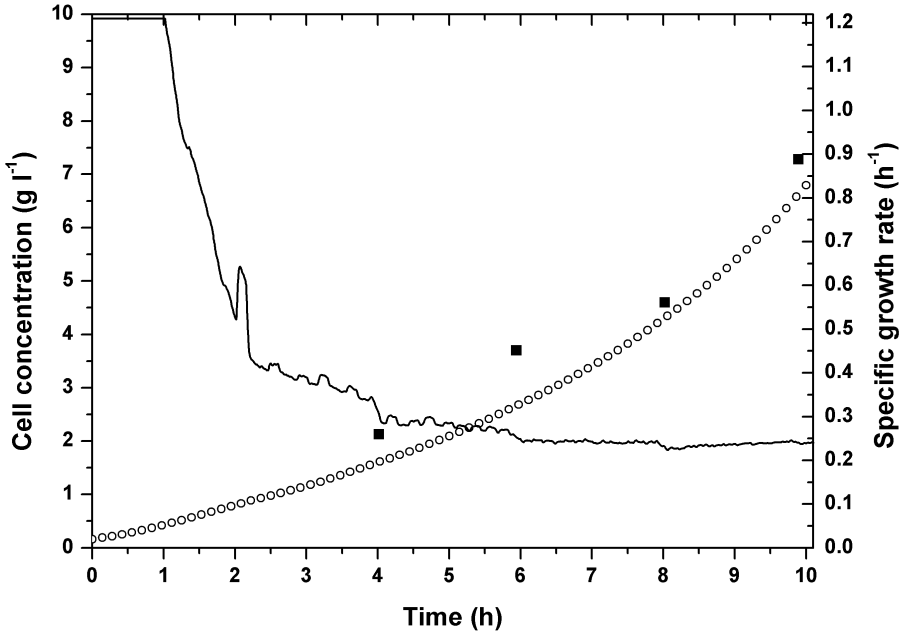

Figure 7.7: Batch culture of *Pichia pastoris*, illustrating the biomass concentration (calculated off-line by DCW measurements (squares) and estimated in real-time (circles)) and the specific growth rate (continuous line) over time.

7.3.5 General applicability

In a very last step, the developed feedback control strategy was applied to cultures of a different yeast strain (*Pichia pastoris*) under different culture conditions, namely in terms of medium composition. During these batch and fed-batch cultures, glycerol was used as a C-source, as described in Table 7.2. Despite this difference, no changes were applied to the experimental set-up. Solely the appropriated values for $Y_{x/s}$ and S_F needed to be entered into the LabVIEW program prior to the experiment.

The potential of biocalorimetry as a tool for monitoring the specific growth rate, as well as biomass is illustrated in Figure 7.7. The values for biomass concentration, predicted in real-time through the estimator described in Equation (7.1), match the off-line measurements obtained through dry cell weight analysis. Furthermore, after initial perturbations in the specific growth rate signal, a constant μ_{max}, typical of the exponential growth phase is observed. During the fed-batch culture of *Pichia pastoris* which was carried out with

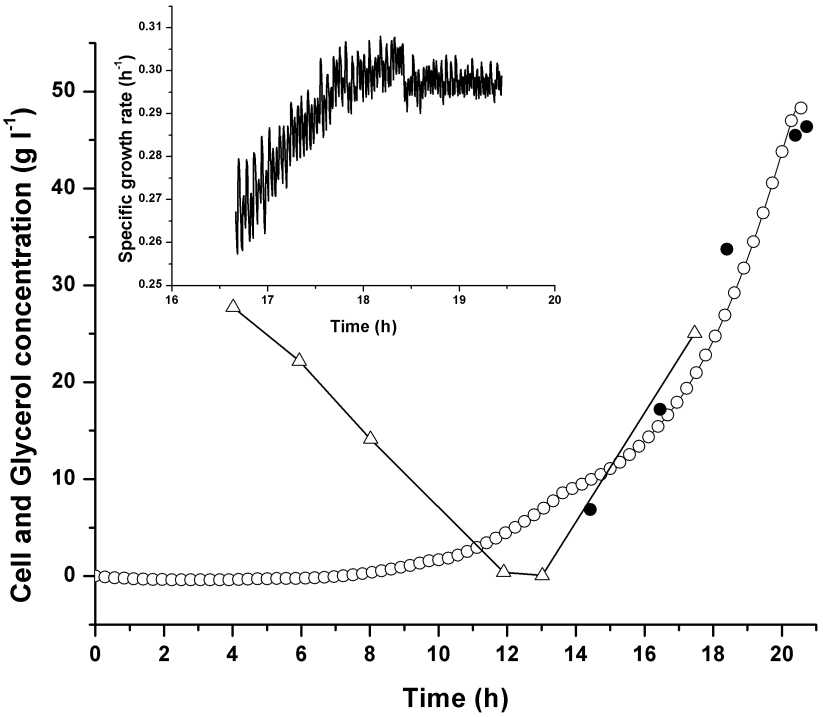

Figure 7.8: Fed-batch culture of *Pichia pastoris* in open-loop control where the specific growth rate (continuous line in window) was set to $0.25h^{-1}$. Biomass concentration was calculated off-line from DCW measurements (black circles) and estimated continuously in real-time (white circles). Glycerol concentration was determined by HPLC method (triangles).

the feed-forward part of the control strategy only, a concordance between off-line measurements and predicted biomass values can once more be observed (Figure 7.8). The specific growth rate (window in Figure 7.8), set to $0.25h^{-1}$, overshoots the setpoint in a first time, decreases slightly and seems to stabilize above μ_{sp}. HPLC measurements of the culture revealed a dramatic increase in the glycerol concentration, due to an inappropriate reaction of the controller at the beginning of the feed phase. The feed-forward part of the control strategy alone is not able to deal with an overshooting in the specific growth rate. The inability of the open-loop control, more commonly used [10], to cope with such situations is strong evidence for the need for a reliable feed-back control strategy, as proposed by this study.

7.4 Conclusion

The present work confirmed the reliability of biocalorimetry as a biomass monitoring device [143] and established the potential of the technique to predict the specific growth rate in real-time. Furthermore, substantial evidence is shown that open-loop control in the form of an exponential feed-forward supply of substrate is not able to cope with a setpoint overshoot, requiring for the development of a robust feedback strategy to control the specific growth rate. Furthermore, the experiments discussed through this study pave the way to the establishment of biocalorimetry as a potential Process Analytical Technology tool and as a basis for a general applicable strategy to control the specific growth rate in microbial fed-batch cultures. Admittedly, an important amount of work and investigation needs to be carried out, especially in terms of controller tuning and data pre-processing, before the proposed feedback strategy might become a robust and flexible PAT platform for microbial culture. Thought needs to be given to an appropriate secondary measurement, as well as to a monitoring enhancement technique, such as data reconciliation, if the developed controller is applied to Crabtree-positive micro-organisms. Furthermore, the sensitivity of the biocalorimeter may require improvements if the device is to be applied to animal cell cultures. Despite these challenges, biocalorimetry has considerable potential, even for industrial application. Furthermore, biocalorimetry provides both real-time information about the biomass and specific growth rate evolution throughout a culture, as well as a considerable insight into the metabolic activity of cells and changes in process conditions, thereby leading to a greater process understanding and a deeper knowledge about the underlying phenomena. Process understanding is a key step in the shift from Quality by Analysis to Quality by Design as recommended by the FDA initiative about industrial PAT, and biocalorimetry, providing a real-time insight into the on-going process, can therefore be considered as a potential Process Analytical Technology tool.

Chapter 8

Summary, conclusion and perspective

Happiness is neither virtue nor pleasure nor this thing nor that but simply growth, we are happy when we are growing. [1]

[1] William Butler Yeats, Irish Poet and Writer (1865-1939)

Table 8.1: Summary of the objectives of this thesis

- Critically analyze existing strategies to monitor and control the specific growth rate in microbial fed-batch cultures
- Develop methods to monitor the specific growth rate in real-time by biocalorimetric and spectroscopic measurements
- Explore ways of achieving control of the specific growth rate by regulating an exponential substrate feed in response to real-time measurements of process variables

8.1 Introduction

The findings, results and conclusions of the different chapters will first be summarized and compared in the next sections, before comparing them to the initially stated objectives, summarized in Table 8.1.

8.2 Summary

The results of Chapter 4 showed that control of the specific growth rate can be achieved by monitoring the biomass evolution by dielectric spectroscopy. Elemental mass and charge balances based on the combined use of off-gas analysis, spectroscopic measurements and data emanating from the monitoring of pH evolution serve as basis for on-line data reconciliation to enhance those biomass measurements [239, 281]. The specific growth rate, estimated in real-time, is controlled at a given setpoint via a novel feedback control strategy that allows for a constant μ through an exponential feed rate without resorting to adaptive control, keeping thereby the control strategy relatively simple. The same strategy can be transferred, without any changes, to control the specific growth rate of Crabtree-positive yeast cell cultures such as *S. cerevisiae* , as shown in Chapter 5. Chapter 6 and Chap-

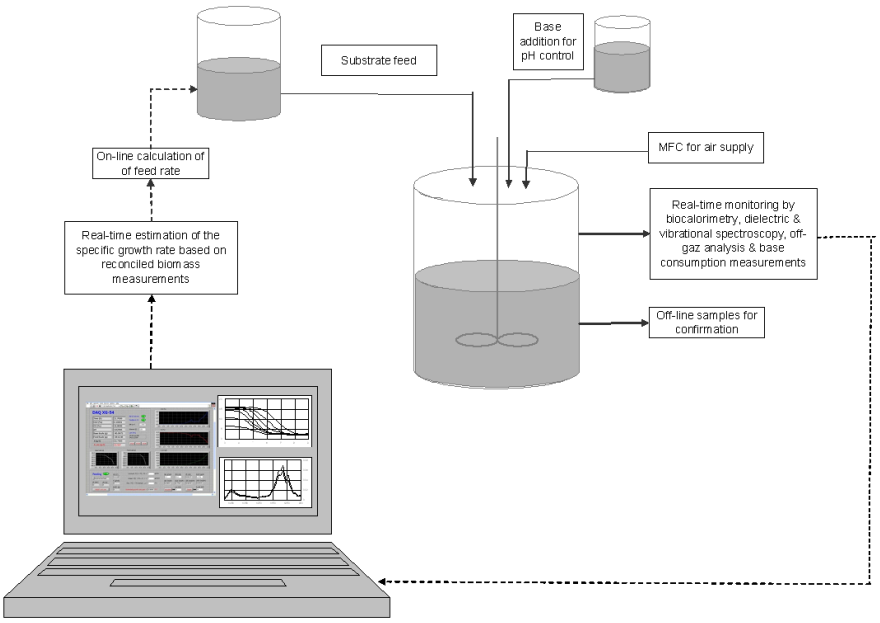

Figure 8.1: Schematic view of the platform for the control of the specific growth rate of microbial fed-batch cultures. (Adapted from [281]).

ter 7 proved that the biomass concentration and the specific growth rate can be estimated in real-time within a bench-scale calorimetric environment and that control of the latter can be achieved by regulating the carbon-source limited substrate feed in the same way then presented in the first two chapters [143, 144]. The developed platform for the control of the specific growth rate in yeast fed-batch cultures is based on simple principles that would allow its applications to different type of cultures (for instance *Escherichia coli* or CHO cell cultures) and maybe even, at one stage, its industrial implementation [72].

Comparison of the spectroscopic and the biocalorimetric approach The optimal control situation would obviously be a constant specific growth rate without any oscillation at all. However, oscillations, even during the batch phase, are common (Section 2.3). When a control strategy such as the one presented within the scope of this thesis is applied, the oscillations tend to be less pronounced than in the batch phase, but do not totally disappear.

The comparison of the two approaches presented here reveals that the oscillations are less pronounced in the biocalorimetric setup where the specific growth rate seems to reach the setpoint $\pm 0.025h^{-1}$ in most cases with optima of control at $\pm 0.0012h^{-1}$. In the case of the spectroscopy based control, the limit was generally at $0.05h^{-1}$. The observed differences in controller limits can have different reasons and the investigation of these reasons can represent a project in itself. A few indications for considerations are discussed in the following paragraph.

Firstly, differences in the averaging of signal values are without doubt partially responsible for the observed variation of controller limits. The approach to averaging signals was kept as similar as possible in both experimental setups, however, differences remain. Secondly, the data acquisition times of the different instruments constituting each setup are not the same, leading to two different general data update times. While all processes variables are updated every two minutes in the spectroscopic setup, the update time is of five minutes for the biocalorimetric setup. However, fewer points are taken for averaging of the specific growth rate in the latter. And last, another origin of the dissimilarity could potentially be seen in the way the feed is delivered. Different pumps as well as different connection needles were used in each setup. Potentially, one particular construction could lead more frequently to small overshootings (bigger drops delivered) or to a more pronounced drift in the feed rate [248]. Exchanging the feed delivering parts of each setup could allow verifying this hypothesis.

Since controlling the specific growth rate in microbial fed-batch cultures at a constant level is not routinely done (Section 2.4), it is not trivial to evaluate if the observed differences should be attributed to the monitoring technique chosen to estimate the specific growth rate, be related to the model for the estimation or simply be considered as being part of the experimental variation. However, papers by Biener and co-workers [6, 7] showed controller errors close to those observed with the biocalorimeteric setups. Their work is also based on heat flow measurement even though differences in the formulation of the feedback part of the controller can be observed since they implemented an adaptive control strategy. The similarity in the controller limit might be an indication that the observed range

of $\pm 0.025 h^{-1}$ is specific to the heat flow based approach of controlling the specific growth rate.

While both approaches lead to satisfactory results in terms of control, each technique has its advantages and limitations. The most obvious difference is without a doubt related to the differences in setup. Even though a bench-scale calorimeter such as the BioRC1 is not a trivial device and required years of development, it does not represent the same complexity than the setup composed of dielectric and mid-infrared spectroscopy, completed with off-gas and base consumption analysis. Moreover, biocalorimetry allows to express the specific growth rate as a rather simple function of the heat evolution rate without particular data modeling, whereas the spectral data acquired by FTIR spectroscopy needs to undergo a certain amount of data treatment. Prediction models for metabolites in this project have been created using a chemometric approach suggested by Brereton [258] and showed to be applicable to all different yeast cultures, providing that the defined culture medium did not change in composition. However, preliminary trials to transfer the developed strategy to bacterial cultures (Figure 8.5) required the creation of a new model, including specific metabolites such as acetate and specificities related to culture conditions such as temperature [259, 260, 205]. In a quest of simplicity, the biocalorimetric approach would be preferred.

The detection limit, different for each technique, might represent an other challenge. Indeed, while the present thesis proved that monitoring and control of the specific growth rate of yeast cells by calorimetry is not a problem, determination of bacterial growth rate might turn out to be more problematic. *Escherichia coli* cultures have been extensively monitored by biocalorimetric methods [153] showing that approximately $1.5 gl^{-1}$ of viable cell mass is necessary to achieve a heat evolution rate that is distinguishable from the baseline using the current BioRC1 setup. Dielectric spectroscopy also suffers from low sensitivity at low cell density, but usually above $0.8 gl^{-1}$ of viable cell mass, the signal is reliable.

Limitations of the presented specific growth rate control strategy The main limitation of the developed strategy to maintain the specific growth rate constant in yeast fed-batch cul-

tures is without a doubt controller disturbance occurring due to oxygen limitation. Indeed, exponential biomass increase can only occur when no additional limitation, for instance oxygen limitation, restricts constant growth. Linear biomass increase associated with decreasing specific growth rate values, in the presence of sufficient carbon and energy source is characteristic for oxygen limitation where the availability of oxygen to cells is determined by the oxygen transfer rate from gas bubbles to the culture broth. During fed-batches carried out within this project, oxygen limitation might have been a substantial problem towards the end of the cultures when higher cell densities were reached. Indeed, dissolved oxygen level was only monitored and not controlled in this project. Maintaining the level of DO high enough throughout all cultures would ensure that the controller for the specific growth rate is not impaired by the physiological response of the cells to oxygen limitation. No particular investigations have been performed during this project to determine the critical lower limit of dissolved oxygen necessary to ensure aerobic growth conditions for each strain. However, initial microscopic investigations of the *Kluyveromyces marxianus* strain showed that below values of 50% of air saturation, only a part of the cell population had the characteristic round shaped yeast form while the totality of cells displayed pseudo-hyphea morphology at dissolved oxygen levels below 20%. Pseudo-hyphea in yeast cells are characteristic for nutrient limitations such as lack of available oxygen [282]. Maintaining a sufficiently high level of oxygen to not limit the controller action is essential if this strategy is to be applied to higher cell density cultures.

Oscillations in the values of the specific growth rate can be observed and represent another limitation of the presented control strategy. Even though those oscillations are not as pronounced and as high in amplitude in controlled fed-batch conditions as they tend to be during the batch phase (Figure 7.5), they need to be taken into account. Especially when working at a critical value of the specific growth rate, it is important to choose a setpoint such that oscillations around the setpoint will still allow for an output within the limits of control. Ways of reducing the amplitude of the oscillations include filtering of the specific growth rate values. In the particular case of the estimation of the specific growth rate based on heat flow measurements, the oscillations can already be reduced by reducing

the raw signal noise further. Indeed, in the current setup, none of the entering streams (air, base and feed) is thermostatted to reaction temperature, creating, when added, unnecessary disturbances in the heat signal.

The last limitation that should be addressed concerns the evaluation of the overall heat transfer coefficient (UA) essential to the estimation of the specific growth rate by biocalorimetry. Indeed, for the present study, the U value was considered being constant over the course of a culture while the term of the overall heat transfer coefficient related to the wetted area of transfer can be estimated from the culture volume. However, if high cell densities are the overall aim in the development and expansion of the present control strategy, then a way of continuously evaluating UA should be considered. Indeed, above $50gl^{-1}$ of microbial biomass, the heat transfer coefficient cannot be considered as constant anymore [7, 161]. Ways to achieve continuous evaluation of UA could include repeated UA evaluation using the internal calibration heater or modeling of the expected changes over culture time applying for instance an artificial neural network to develop such a prediction model.

8.3 Conclusions

When comparing the outcomes and results from Chapters 4, 5, 6 and 7 with the aims stated in Table 8.1, generally speaking the objectives have been reached. However, with each step forward it became clear how much more there could be left to do. Not only can methodology and experiments of the present thesis been improved, but also further expansion of this project in four main directions can be considered and will be discussed in Section 8.4.

In general, when looking at the outcomes of the different chapters, it can be concluded that a firm basis is laid for the development of a novel, yet robust platform that allows controlling the specific growth rate in fed-batch cultures with sufficient reliability. The developed platform (Figure 8.1) could potentially be used to study cell physiology related phenomena and monitor μ-related productivity without resorting to continuous cultures. Most importantly it forms the foundation for automated, dual-stage μ-stat fed-batches where in a first part, cell density could be drastically increase by growing cells close to the critical

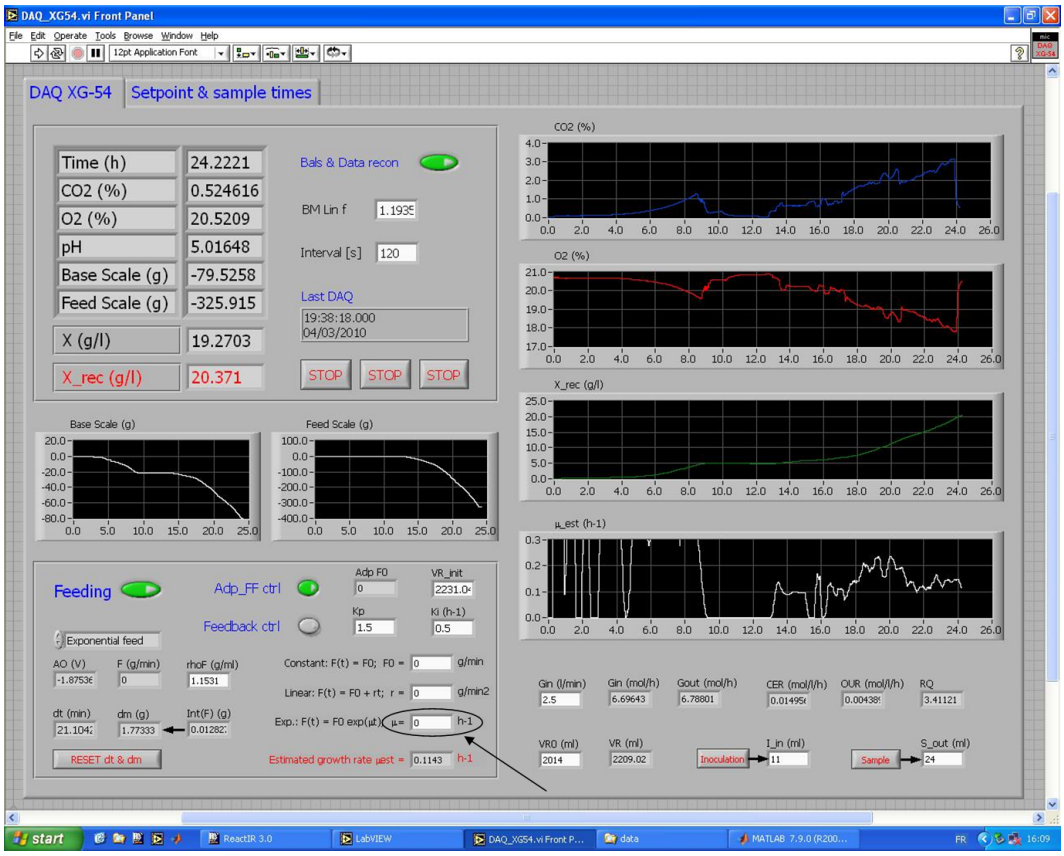

Figure 8.2: Frontpanel of the data acquisition screen connected to the experimental setup based on spectroscopic data (the biocalorimetric one is similar). The setpoint for the specific growth rate can simply be changed by entering the new value into the box marked by an arrow or by pre-programming setpoint changes in the second panel (Setpoint & sample times) allowing thereby a fully automated steering of the process in terms of specific growth rate control.

specific growth rate before setting μ to an optimal, usually rather low value for recombinant protein production. Indeed, in the current setups, the specific growth rate can be changed manually or changes can be programmed time-dependently (Figure 8.2). Only a few parts of code would be necessary to implement additionally a process variable dependent set-point change, where for instance the specific growth rate would be automatically lowered to the required value for optimal recombinant protein production when the culture reaches a certain biomass concentration.

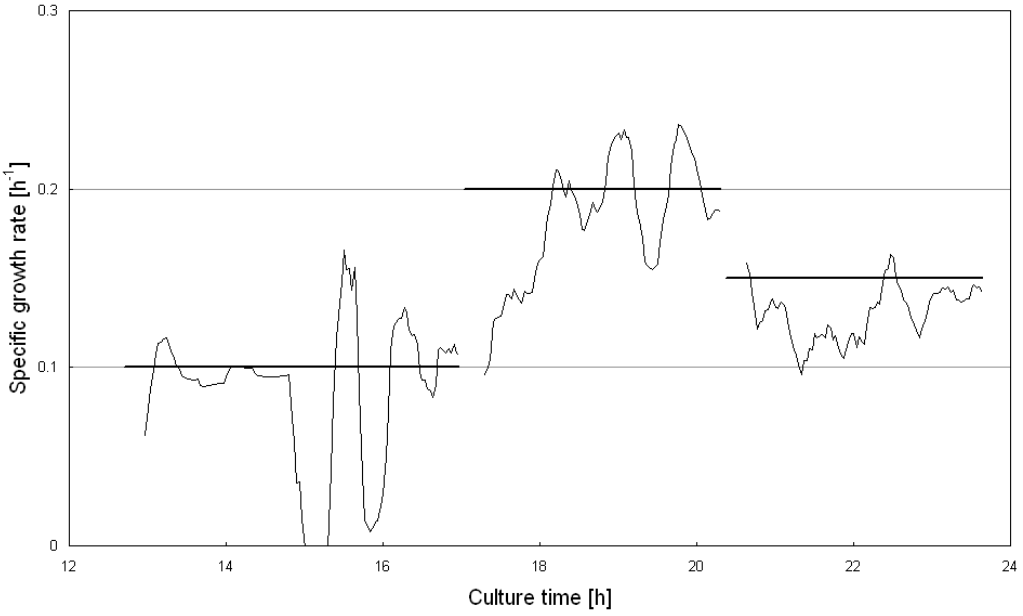

Figure 8.3: Evolution of the specific growth rate over time of a *Kluyveromyces marxianus* culture regulated by a nitrogen limited substrate feed.

8.4 Perspectives

Four main directions could be chosen to expand the present work further:

Alternative limitations of the substrate feed The totality of the presented results within this thesis is based on cultures where a carbon-source limited substrate feed was exponentially fed in order to maintain the specific growth rate at a constant level. However, other types of substrate feeds could be considered. Initial results show that a nitrogen limited substrate feed, under the same control strategy and experimental setup as presented in Chapters 4 and 5, leads to comparable results (Figure 8.3) in terms of specific growth rate control. However, attention should be paid to the metabolic implications of the nature of limitation under consideration.

The implications of lack of carbon-, oxygen- and nitrogen-sources on the metabolism of yeast cells are probably the most studied effects in relation to nutrient limitations [283, 284, 285]. However, additional deficiency of particular ions, such as magnesium, potassium, phosphorus or iron can modulate the impact of the main limitation [286, 284, 287].

For instance a combined nitrogen and iron limitation will lead to a more marked accumulation of unsaturated fatty acids than a nitrogen limitation with sufficient iron available for optimal, iron-dependent, desaturase activity [288] while a dual limitation of nitrogen and phosphorous might lead to enhanced antibiotic production [284].

The aim of a side project, where *Kluyveromyces marxianus* cells were cultured under nitrogen limiting conditions with sufficient carbon-source available, was to prove that the developed control strategy is not only transferable from cell strain to cell strain, but can be applied for maintaining a constant specific growth rate by other means than an exponential glucose or glycerol feed (Figure 8.3). The average controller error during this experiment was 19.8%. This value is certainly above the best average controller errors obtained in the different parts of the present study, but well within the general range of error of the spectroscopic control platform, meaning that the potential of transferability to other type of feeds is guaranteed. It was chosen to start with a nitrogen limited approach since it might be the best studied limitation next to carbon and oxygen limitations. Indeed, nitrogen-limitation is known to lead to a higher protein turnover [283], to glycogen accumulation [289, 290] and to lipid accumulation in oleaginous micro-organisms [288, 291]. The expression of particular sets of genes is also differently regulated in nitrogen limited situations, where an increased expression of ribosomal and glycolytic genes and a decreased expression of genes involved in nitrogen source degradation can be observed [292], while from a phenotypic point of view, nitrogen limitations can lead to changes in morphology in yeasts such as *Yarrowia lipolytica* [293].

While it can be of particular interest to ensure nitrogen limited growth conditions for enhanced production of PHA or PHB production [294], the production of fatty acids by oleaginous micro-organisms [291] or enhanced ethanol production in microaerobic conditions [295], such a limitation can be detrimental in the case of recombinant protein production where a reduced availability of nitrogen sources might impair amino acid and protein synthesis. Also, control of the specific growth rate by the exponential addition of a nitrogen limited substrate feed might lead to an accumulation of carbon source in the culture medium, a condition that could lead again to the production of overflow metabolites. Care-

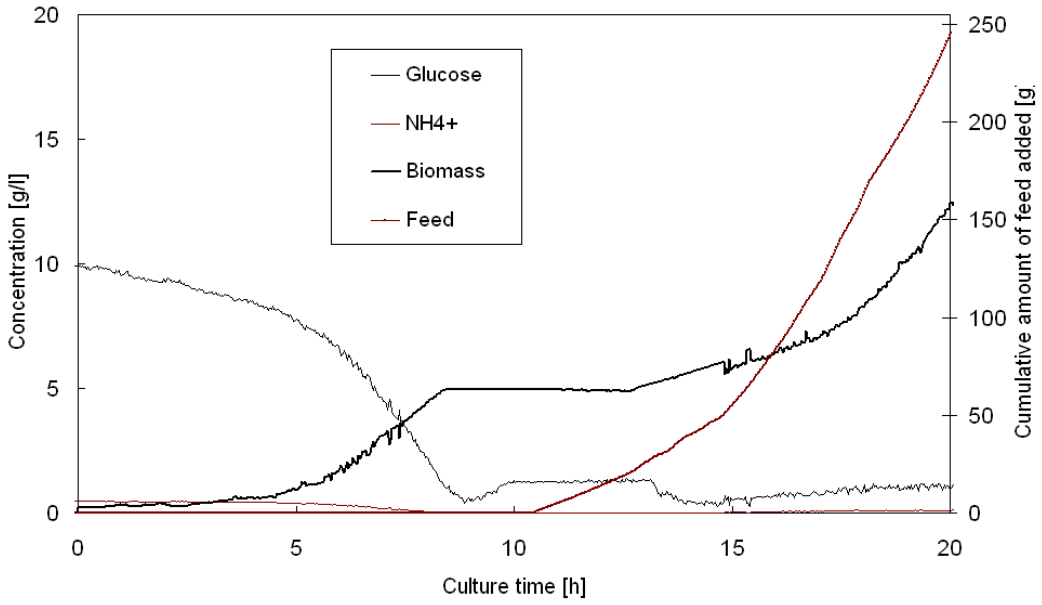

Figure 8.4: Evolution of ammonium, biomass and glucose concentrations over time of a *Kluyveromyces marxianus* culture regulated by a nitrogen limited substrate feed. Metabolite concentrations were monitored by FTIR spectroscopy while biomass concentration values were obtained via dielectric spectroscopy

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ful design of medium composition allows to ensure that the carbon-source, without being limiting, is below the critical concentration (Figure 8.4) at which overflow metabolite production would occur in organisms such as *Saccharomyces cerevisiae* or *Escherichia coli* .

Whatever limiting substrate or combination of substrates is chosen will finally depend on the nature of the process. Nevertheless, as already mentioned in Section 2.5, it can be expected that in a near future, more dual-stage fed-batch processes will be developed, where cells are first grown at a higher specific growth rate to reach a sufficiently high cell density before switching to a particular lower specific growth rate under a specific limitation to produce the product of interest in improved quantities.

Additional control loops An inherent flaw of the presented control strategy is the action of the controller. Indeed, when the specific growth rate is below its setpoint value, the controller action triggers a higher feed rate, since in the initial setup with Crabtree-negative yeast cells, a lower value of μ is in general the result of a lack of carbon and energy source.

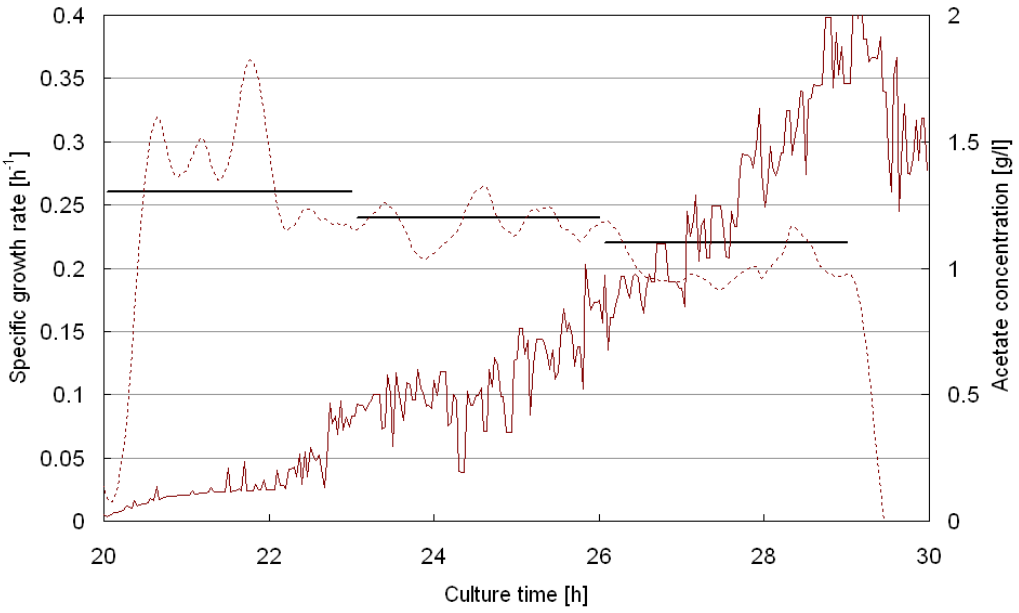

Figure 8.5: Evolution of the specific growth rate and the acetate concentration over the time of a *Escherichia coli* culture. Acetate concentrations were measured via FTIR spectroscopy while the specific growth rate was estimated by Monod's model for exponential growth based on reconciled biomass measurements obtained by dielectric spectroscopy. Towards the end of the culture, the controller is not able to maintain the specific growth rate at the given setpoint anymore. The controller compensates the negative difference between the actual specific growth rate value and the setpoint by increasing the feedrate, leading to the production of acetate as a result of overflow metabolism.

However, in the case of Crabtree-positive organism, a reduced growth rate can also be the consequence of an excess of carbon-source and, as a result, presence of overflow metabolites such as acetate or ethanol. However, the controller is, under the given setup, blind to such considerations, providing the culture with more and more carbon- and energy-source, leading to an out-of-control situation (Figure 8.5). By simply implementing an additional control loop which calculates the required additional substrate concentration based on the carbon-source already present in the medium (measured by FTIR spectroscopy) would improve the situation. In the case of control based on calorimetric measurements, the setup would need to be changed to include a process analyzer in order to have a quantitative information about the amount of carbon-source available. A FTIR spectrometer [203, 241] would obviously be an option, but at-line, real-time HPLC, flow injection analysis (FIA) or enzymatic assays would be an option, as long as the resulting data could be automatically and without important treatment be integrated in the existing LabVIEW environment.

Additionally, in order to ensure controller efficiency, reaction conditions need to be such that no other substrate limitation, in particular no oxygen limitation occurs. Throughout the different projects of this thesis, as highlighted in Section 8.2, the dissolved oxygen level during the bioprocesses was only monitored and not controlled. An independent control loop, commonly and routinely implemented in academia and in industry, to maintain the dissolved oxygen level above at least 20% of saturation by regulating the air flow and possibly also the air composition by blending in pure oxygen would be beneficial to ensure sufficient oxygen supply. Indeed, increasing controller errors towards the end of cultures have been systematically observed in all cultures when the dissolved oxygen level was falling below a critical value of about 20%, depending on the strain under study.

Alternative process monitoring tool A few dozen of process monitoring techniques to gather real-time information about substrate, product or biomass concentrations were presented in Chapter 2 and only three of them were applied throughout this thesis. The control strategy developed is dependent on real-time estimation of the specific growth rate, but independent of the way the current value of the control variable is estimated. Therefore, any

technique that would allow measurements that can be introduced in any specific growth rate model would be applicable. The most promising alternative in my opinion is near-infrared spectroscopy [296, 257, 297]. Indeed, in principle and depending on device specific characteristics, NIR spectroscopy allows simultaneous detection of different substrates and products and biomass [89]. McNeil and co-workers proved that monitoring of glucose, acetate, ammonium and biomass was possible off-line [62, 63]. On-line application should be possible [296]. Preliminary work carried out in our lab [298] showed that biomass detection is not trivial. However, most initial barriers were identified and could potentially be removed. Indeed, as for mid-infrared spectroscopy, careful calibration model preparation, including potentially more data pre-treatment steps compared to MIR spectroscopy, and the use of chemically defined culture media simplifies model development. The use of complex YPG (yeast extract, peptone and glucose) medium was partially responsible for the absence of concluding results in the preliminary study. Indeed, despite at least one group having successfully used complex medium to develop monitoring models [62], it is usually advised to work with chemically defined media [63]. The disparity in analyte concentrations should also be considered. While biomass concentration will increase over the time of culture, simplifying its detection and accurate quantification, carbon-source and by-product concentrations, such as ethanol, are rather low and might furthermore overlap with each other in the terms of spectral region of absorbance. Despite these initial hurdles, NIR could be a technique of choice, leading to the same amount of information as with a combined use of dielectric and MIR spectroscopy, but having less instrumentation connected to the reactor. In the end, the choice of the appropriate tool is dependent on the expected output. If simplicity in measurement and ease in handling is paramount, a single device giving as unspecific measurement, such as heat flow, could be preferred over multiple process information gathered by the combined use of different techniques. On the other hand, even though necessitating some time and cost investment for model development, complex measurement setups might allow for real process understanding, final aim suggested by the PAT initiative of the FDA [3].

Application to mammalian cell cultures As initially stated in Section 1.1.1, the overall aim is to have an integrated bioprocess for optimized production of recombinant proteins. Therefore, the control strategy should be applied to *Escherichia coli* cultures and eventually be transferable to mammalian, namely CHO, cell cultures, preferred expression systems for production of such value-added products (Figure 1.1). While monitoring of mammalian cells by dielectric spectroscopy should not represent a major issue [191, 186], the use of FTIR spectroscopy to measure key metabolites in such cultures can be more complex [205], since important compounds such as glutamine are only present in very small amounts, rendering their monitoring difficult and their control even more complex. Monitoring the evolution of a mammalian cell culture by bench-scale calorimetry, in similar setups as those used throughout this thesis, seems possible (Figure 3.4), even though higher cell densities, achieved for instance by encapsulating the cells [299], would allow for a higher signal. Efforts to decrease the signal noise would need to be deployed when calorimetry is considered as a basis for control of such cultures. Moreover, the control strategy itself should be adapted, in a similar way as proposed in the previous paragraph, in order to take into account all different substances potentially used as carbon- and energy-source by the organisms. Furthermore, it still needs to be determined if a specific growth rate estimator such as the one developed throughout Chapter 6, could be formulated for mammalian cells. Indeed, as mentioned by Prof. von Stockar in his recent plenary lecture at the ISBC 2012 [300], the usual black box approach, extremely useful when describing microbial systems, might reach its limits with mammalian cells. However, the specific growth rate estimator recently proposed by Biener and co-workers for Crabtree-positive organisms [6, 7] allows for the determination of the apparent specific growth rate and might allow, based on a-priori knowledge, to control even mammalian cell cultures. When it comes to control of mammalian systems, it should not be forgotten that the lower cellular activity, responsible for the lower heat signal, also means that the time constant of the controller does not need to be or should not be as high as for fast-growing microbial systems. The question about the range and timeframe of acceptable control, as mentioned in Section 3.4 is of even greater importance and should be thoroughly assessed before transferring the present control plat-

form to mammalian cell cultures. It should also be kept in mind that "feedback control only makes sense where the current value of the controlled variable can be determined accurately and fast enough to determine the deviation from the target set-point" [12]. The rate of change in signals in mammalian cell cultures is not expected to be fast, however, an accurate determination of the value of the signal is essential. Considering the weakly exothermic nature of mammalian cell cultures and the detection limit of bench-scale calorimeters such as those dealt with in the scope of the present thesis, alternatives such as at-line chip microcalorimeters [142, 301] or high sensitivity reaction calorimeters [132] should be thought of when realistically thinking of implementing a heat flow based control strategy for such processes.

8.5 Final conclusion

The monitoring of cultures by biocalorimetry, dielectric spectroscopy or by FTIR spectroscopy is not a new topic, as proven by the different references in Chapter 2 and Chapter 3. However, a systematic evaluation of the specific growth rate in real-time based on these measurement techniques is not yet part of typical bioprocess monitoring routines. In 1949, Jacques Monod stated: "The study of the growth of bacterial cultures does not constitute a specialized subject or branch of research: it is the basic method of microbiology" [39]. Monitoring and controlling the specific growth rate should not constitute a specialized, marginal subject or branch of research: it should be the basic method of advanced microbial bioprocessing.

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Appendix

Publications; The following papers have been published or submitted for publication:

- Schuler, MM; Dabros, M & Marison, IW; Beyond Monitoring and Control; *BIOforum Europe*, 2010, 3, 14-15
- Dabros, M; Schuler, MM & Marison, IW; Simple control of specific growth rate in biotechnological fed-batch processes based on enhanced on-line measurements of biomass; *Bioprocess and Biosystems Engineering*, 2010, 1-10
- Sivaprakasam, S; Schuler, MM; Hama, A; Hughes, KM & Marison, IW; Biocalorimetry as a process analytical technology process analyzer; robust in-line monitoring and control of aerobic fed-batch cultures of Crabtree-negative yeast cells; *Journal of Thermal Analysis and Calorimetry*, 2011, 1-11
- Schuler, MM; Foley, R & Marison, IW; Bridging the Gap; Identifying Challenges in Implementing Scientific Advancements; *Industry G.I.T. Laboratory Journal Europe*, 2011, 15, 26-27
- Schuler, MM; Sivaprakasam, S; Freeland, B; Hama, A; Hughes, KM & Marison, IW; Investigation of the potential of biocalorimetry as a process analytical technology (PAT) tool for monitoring and control of Crabtree-negative yeast cultures *Applied Microbiology and Biotechnology*, 2012, 93, 575-584
- Schuler, MM & Marison, IW; Real-time monitoring and control of microbial bioprocesses with focus on the specific growth rate: current state and perspectives; *Applied Microbiology and Biotechnology*, 2012, 94, 1469-1482
- Marison, IW; Hennessey, S. Foley, R; Schuler, MM; Sivaprakasam, S & Freeland, B; The choice of suitable on-line analytical techniques and data processing for monitoring and control of bioprocesses - Book Chapter M3C, *Springer Book Series* (submitted - under review)
- Schuler, MM; Byrne, K; Foley, R; Freeland, B & Marison, IW; Investigation of the potential of bench-scale biocalorimetry as a monitoring tool for CHO DP12 cell cultures - Intended for submission to *New Biotechnology* at the end of September 2012

Oral presentations; at the following conferences, oral presentations have been given:

- International Society for Biological Calorimetry (ISBC) XVII Conference, Leipzig; *Real-time monitoring and control f the specific growth rate and of temperature shift induced recombinant protein production in Escherichia coli by the use of a modified bench-scale calorimeter*; June (2012)
- Product-to-Process (P2P) Conference, Washington; *Bioprocess Monitoring and Control: From Raw Materials to Final Product* (replacement for Prof. I. W. Marison invited speaker / opening session); October (2011)
- European Conference of Applied Biotechnology (ECAB), Berlin; *Development of a feedback strategy to control the specific growth rate and to prevent overflow metabolism in Crabtree-positive microorganisms*; September (2011)
- 6th Recombinant Protein Production (RPP) Conference, Vienna; *Development of a general applicable control strategy of microbial fed-batch cultures*; February (2011)
- Research Day of School of Biotechnology, Dublin City University, Dublin; *Beyond Control*; January (2011)
- European Society of Biochemical Engineering Sciences (ESBES) Conference, Bologna; *On-line data reconciliation based on energy balances enhances the potential of biocalorimetry as a PAT process analyser*; September (2010)
- Process Analytical Technology (PAT) - Schweizerischer Verband Diplomierter Chemiker FH (SVC) Meeting, Basel; *Fed-batch control of yeast fermentation based on calorimetric & spectroscopic data; a new insight into PAT* ; November (2009)