Diese Dissertation haben begutachtet:

Prof. Christoph Herwig	Prof. Dietmar Haltrich
Vienna University of Technology	University of Natural Resources and Life Sciences, Vienna

TECHNISCHE UNIVERSITÄT WIEN
Vienna University of Technology

Dissertation

Protein expression in *Pichia pastoris* – A novel strategy for fast bioprocess development

ausgeführt zur Erlangung des akademischen Grades eines Doktors der technischen Wissenschaften unter der Leitung von

Prof. DI Dr. Christoph Herwig
Institut 166/4
Institut für Verfahrens-, Umwelttechnik und technische Biowissenschaften

Eingereicht an der Technischen Universität Wien
Fakultät für Technische Chemie

von

DI Christian Dietzsch
Matrikelnummer 0828938
Gymnasiumstrasse 68/13
1190 Wien

Wien, am Unterschrift

Acknowledgements

First, I would like to express my sincere gratitude to my supervisor, Prof. Christoph Herwig. It has been great to be one of his first PhD students, to help setting up the lab environment from the beginning and to experience how the working group has evolved.
I appreciate all his efforts to make my PhD experience productive, stimulating and succesful.

Many thanks to Prof. Dietmar Haltrich for the spontaneous agreement to review this doctoral thesis.

I am very grateful to Dr. Oliver Spadiut. Our extensive discussions, his assistance in preparing manuscripts and his experimental assistance in the lab have been extremely helpful. Thanks for the patience and friendship.

I want to thank all members of the Bioprocess Engineering Group at Vienna Univeristy of Technology. Special thanks to “my colleagues from day one”: Patrick Wechselberger, Daniela Rial, Arne Seifert, Simon Rittmann and Andreas Posch. I am also grateful to the diploma students Josef Schulze and Dénes Zalai and student trainee Christian Hutterer for their excellent assistance. Thanks to Silvia Gluderer for support in the lab and Birgit Wittholm for help in secretarial work.

Finally, I would like to thank Anne for all her love, support and encouragement throughout the last years. And thank you Thea for making me smile each day.

Abstract

The methylotrophic yeast *Pichia pastoris* has evolved to a widely used expression host for the production of recombinant proteins in the past few years. However, most strategies in process development for increased process understanding and optimization are based on empiricism. Such strategies are, caused by product or host specific interactions, generally not transferable to other production systems.

In the present study, a novel physiologically based process development strategy for the fast and scalable determination of key parameter sets for fed batch production processes with *P. pastoris* strains was developed. The strategy was applied to various phenotypes expressing different recombinant products. Certain strain specific parameters were reliably quantified in batch pulse experiments and successfully transferred into fed batch feeding regimes. The method based on the specific substrate uptake rate q_s as a key parameter for process control and optimization, was applied in single as well as in mixed feed substrate systems.

Increased process understanding regarding the correlation of q_s to product generation was achieved and physiological changes caused by genetic modifications of the methanol utilization pathway were quantified reliably. Additionally, a novel on-line device for fast process monitoring of physiological relevant components as well as product quantity and quality was introduced and supported the process development strategy to increase process understanding by real time data extraction.

With the present strategy, enhanced bioprocess information for recombinant protein expression in *P. pastoris* was achieved within a short time. The strategy represents a valuable and transferable tool for fast early process development in academic as well as in industrial environments, where several strains have to be quantitatively screened for their potential use in later production processes.

Table of contents

1. Motivation

In recent decades the use of biotechnologically produced compounds, like proteins, has increased due to very high efficient production processes. The major challenge to develop novel products, which are produced in biological systems, is the interdisciplinary link of biological and process engineering aspects for reliable process development [1].

The development strategy of a production process is divided into different phases, where an early and late phase of the development is distinguished (Figure 1-1).

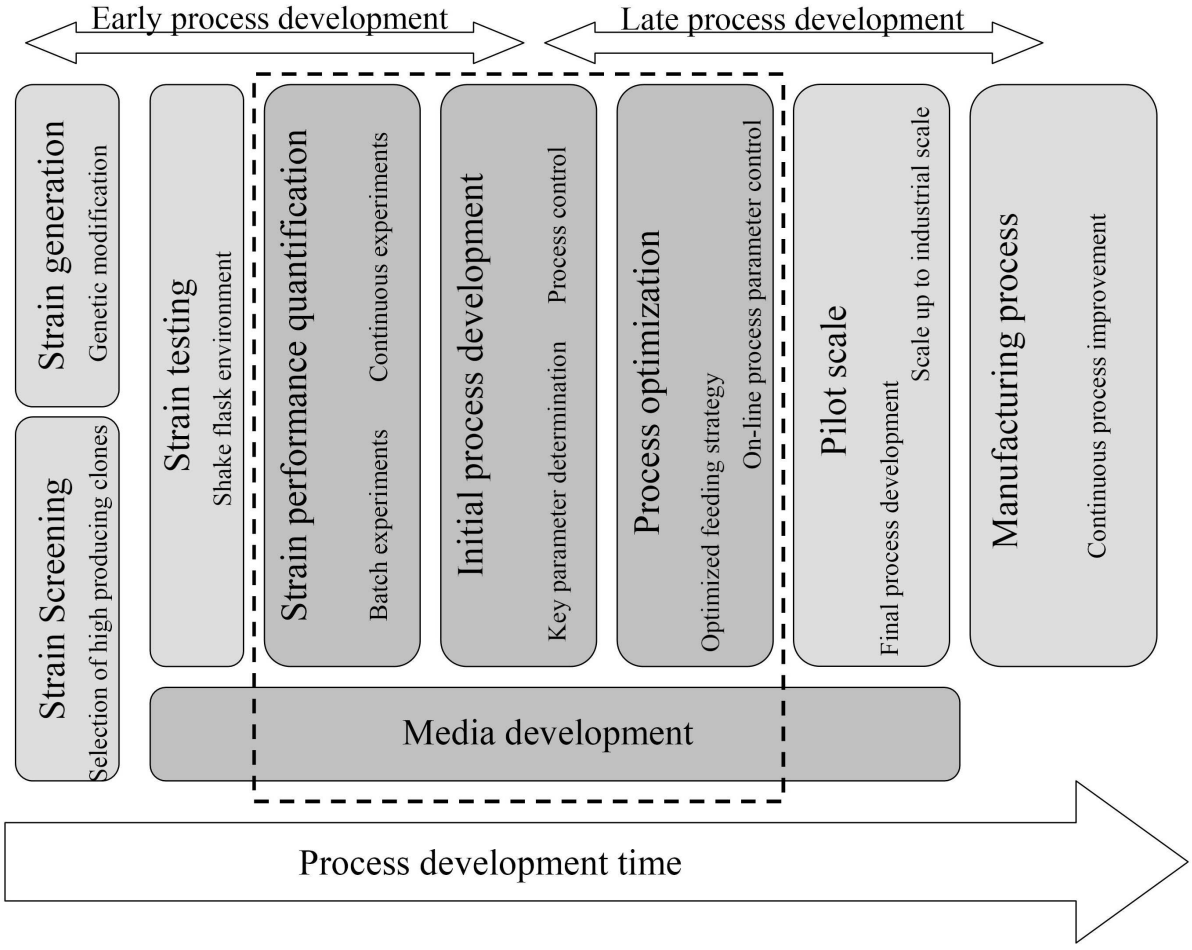


Figure 1-1 Scheme of a process development strategy of a biotechnological product including all steps from initial process development to the manufacturing process. The dashed line indicates steps, which were investigated in more detail in the present study.

Initially, a variety of biological hosts and strains have to be screened for high producing clones regarding the target product [2]. Thereby, the number of potential candidates has to be reduced dramatically in order to save time and cost for further process development steps. Criteria for the selection of potential candidates are e.g. pathogenesis of the host, control of gene expression, biological activity of the product, post-translational modification or glycosylation structures of the product [1]. The identified clones are then transferred to a

larger scale for general strain performance quantification, which is mostly carried out in lab scale environments using initial batch [3] and long lasting continuous cultures [4-5]. These experiments are directed to determine key parameters which are potentially related to product yields ($g \cdot g^{-1}$), volumetric productivities ($g \cdot l^{-1} \cdot h^{-1}$) or final titres ($g \cdot l^{-1}$) [1]. Such key parameters either represent process variables like feeding rates or inducer concentrations, but also can reflect physiological information, like the specific growth rate, which can be used for further process control regimes. The most promising clones are subsequently transferred into next development steps where, based on the defined control parameters, an optimization of the process regarding maximum product release within a short time is envisioned. This can either be carried out by genetically based strain improvements regarding substrate conversion or modified pathways [1], or by the variation of process technology parameters, like pH or temperature [6].

Due to advances in genetic modification of biological hosts, the number of potential clones is increasing continuously. The initial screening step is commonly carried out by the use of automated multi-well systems, which can perform the screening of hundreds of clones in parallel. Nevertheless, for the strain performance quantification a more sophisticated bioreactor environment is needed to detect physiological parameters of the strains in a reliable way. An inherent drawback is the quantification time required for each strain and its potential in further process development. Therefore, a fast methodology is needed to determine strain specific characteristics in order to set up control strategies for further process optimization during the following development steps. With the help of such a fast methodology, more promising strains could be quantified in a short time.

An important prerequisite for the key parameters identified during the strain performance quantification is the scalability of these parameter sets. Scalable control parameters have to be easily transferrable into all following development steps and scales to allow a general valid control strategy for the manufacturing process.

The different process development steps are supplemented by parallel media development to optimize strain performance and to generate defined media, which ensure all physiological requirements at minimum cost level [1]. The implementation of on-line monitoring devices at early steps of process development, leads to accelerated data generation, which can be consequently used to increase process information for media development and process control.

The scope of the present study was to combine several process development steps, where the main focus was addressed to the strain performance quantification, the initial process

development, process optimization as well as media development of a recombinant protein production system and is highlighted with a dashed line in Figure 1-1.

2. Goal

Recombinant protein expression in *Pichia pastoris* was studied extensively during the last years [7-9]. Basically, different approaches can be tried to find novel process strategies, not only motivated by the goal of increasing the productivity, but also for the implementation of novel automated control techniques. Strain performance quantification is either carried out in long lasting continuous cultivations [4] and several fed batch experiments [10-11], or solely based on empirical knowledge [12]. Due to the use of different *P. pastoris* phenotypes producing different products, the direct transformation of process settings from one to another system arises to be difficult.

To facilitate process development, the present study focused on the establishment of a novel process development strategy, which describes a physiologically based method for the fast and scalable determination of key parameter sets for a fed batch production process with recombinant *P. pastoris* strains.

Advances in genetic manipulation leads to the generation of a high number of potential expression hosts in a short time. Therefore, a generic approach for the quantitative characterisation of different strains expressing different products in a fast way is of utmost interest. In addition, a potential on-line execution of the derived methodologies would speed up the gathering of process knowledge and thus lead to improved product yields in a short time. The prerequisite for a novel quantification methodology is the scalability as well as the general applicability of the derived key parameters. Therefore, the power of the method has to be checked by its execution on different strains and hosts in different process modes, like single and mixed substrate environments in order to meet industrial requirements.

Different aspects have to be considered to reach the goal of the study. The proposal of a novel tool for process development was divided into several subunits, which are interacting with each other (Figure 2-1). The subunit “method for strain quantification” describes a novel experimental approach for strain performance characterisation using batch experiments and the consequent data transfer into a fed batch environment (Chapters 4.1.1 and 4.1.2). Novel strain specific parameters, like the specific substrate uptake rate, were addressed as a basis for process control in fed batch cultivations in single (Chapter 4.1.1) as well as in mixed substrate regimes (Chapter 4.2.1). Therefore, a detailed analysis of the metabolic interactions during protein expression in these process modes is needed and arranged within the subunit “process understanding” (Chapters 4.2.1 and 4.2.2). In addition, “on-line process monitoring” for the identification of substrates, metabolites as well as the recombinant product is addressed in chapters 4.3.1 and 4.3.2 by the use of a novel on-line monitoring device. The target of this

subunit is dedicated to highlight the potential of an on-line executable methodology for the increase of process understanding during process development.

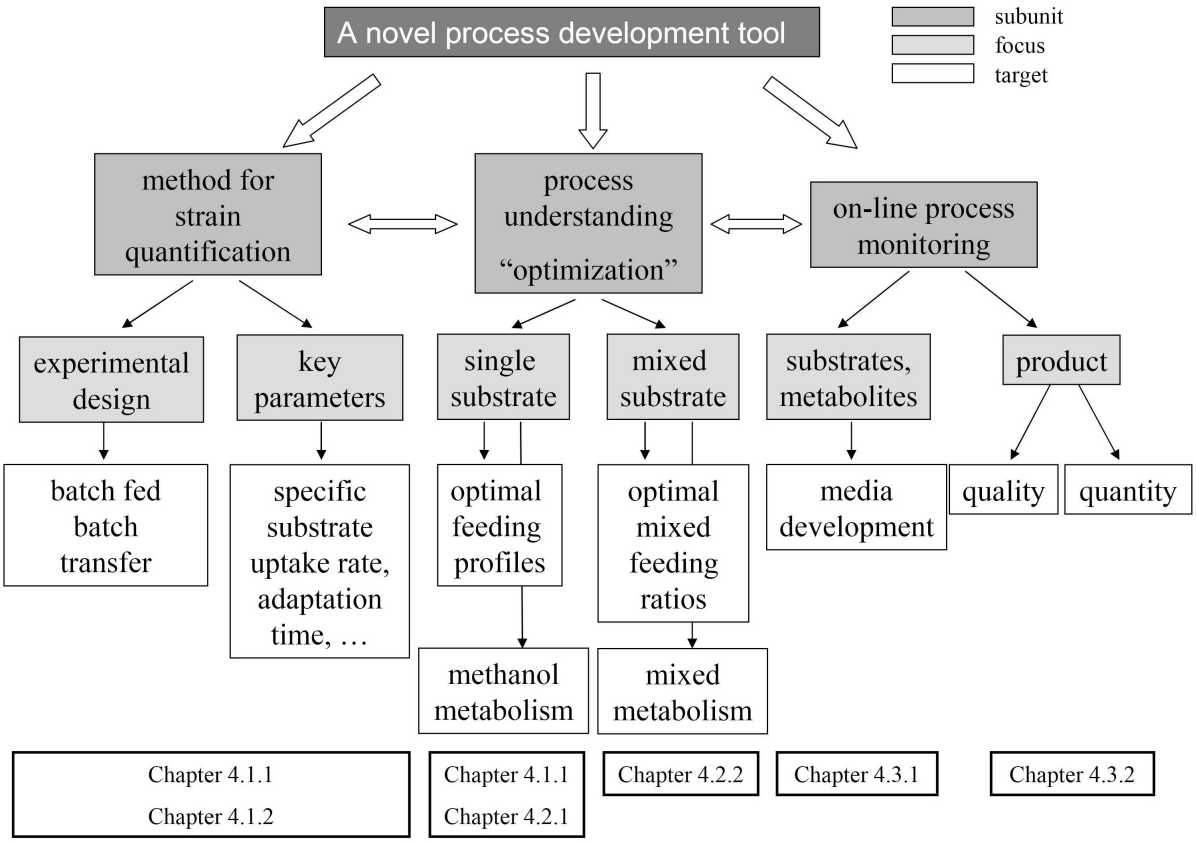


Figure 2-1 Overview of the approaches and targets of the present study classified into respective subunits of a novel process development tool.

Reliable data extraction out of batch experiments and the subsequent transfer into fed batch production environments is the major challenge and novelty of the conducted study. In contrast to time consuming continuous cultivations, a fast determination of scalable strain characteristic parameters leads to a quick and successful production process set up using fed batch experiments. Most of the published studies using *P. pastoris* were carried out with a wild type strain (Methanol utilization plus, Mut^{+}), whereas the present study describes a large and detailed work for the alternative phenotype methanol utilization slow (Mut^{S}).

The use of a novel on-line monitoring device for media development and also product quantification increases process understanding and therefore enables the early process development to focus to Process Analytical Technology, where quality attributes of the process are addressed.

3. Background

3.1. The methylotrophic yeast *Pichia pastoris*

Forty years ago, Koichi Ogata described for the first time the ability of several yeasts to utilize methanol as a sole carbon source [13]. Initially, methylotrophic microorganisms were used as a potential single cell protein source marketed as protein animal feed, which was initiated by the petrochemical industry [7]. In the 1970s, the Phillips Petroleum Company developed a first cultivation medium and protocol for *P. pastoris* growing on methanol. In continuous cultivation already high cell densities of up to 130 $g \cdot l^{-1}$ were achieved [14]. Alternative sources for animal feed production, like soybeans, presented a low cost alternative to methane because of rising costs during the oil crisis in 1970s [7]. Thus, in the following decades the focus on the use of *P. pastoris* was more directed in recombinant product production, especially since the researchers of the Salk Institute Biotechnology/Industrial Associates, Inc. (SIBIA) isolated the gene and the promoter of the alcohol oxidase (*p*AOX) and generated first vectors for molecular genetic manipulation. Hence, a huge variety of recombinant products were successfully produced in *P. pastoris* [7, 15].

3.1.1. Metabolism

3.1.1.1 The alcohol oxidase promoter

The facultative methylotrophic yeast species that belong to the four genera *Hansenula, Pichia, Candida* and *Torulopsis* share a specific methanol utilization pathway (MUT pathway) [16]. Hence, *P. pastoris* has the capability to utilize methanol as a sole carbon source. The methanol metabolism is regulated by two genes that code for alcohol oxidase, *aox1* and *aox2*, which are induced by methanol. The AOX1 promoter (*p*AOX1) regulates 85 % of the alcohol oxidase activity in the cell, while *p*AOX2 is less active.

The regulation of the MUT pathway is strongly affected by promoter repression and de-repression effects of several substrates. Especially the enzyme alcohol oxidase (AOX) is present at high levels in cells growing on methanol but is not detectable in cells grown on other substrates, like glucose, glycerol or ethanol [7]. A high induction of the *aox* gene was reported, where up to 30 % of the total soluble protein was found to be AOX for fermentation cultures growing on methanol at limiting conditions [17-18].

In general, three different phenotypes regarding the methanol utilization of *P. pastoris* are described. The methanol utilization plus phenotype (Mut^+) contains both genes (*aox1* and *aox2*) in an active state and is dedicated as a wild type growth strain. For the methanol utilization slow phenotype (Mut^S) the *aox1* gene is knocked out and therefore the consumption of methanol is slowed down. In the third phenotype, methanol utilization minus (Mut^-), both genes are knocked out and subsequently no growth on the substrate methanol is possible [7]. However, also the Mut^- strain retains the potential to be inducible by methanol [19].

The benefits of using the different phenotypes Mut^S, Mut^+ or Mut^- was discussed controversially in the literature. In general, Mut^+ strains are characterized by a higher growth rate than Mut^S strains and have also been reported to show higher productivities [20-23], which is why the majority of research so far has been performed with this phenotype; to date 7 times more studies can be found in PubMed dealing with recombinant protein expression in *P. pastoris* Mut^+ strains than in Mut^S strains. This is why the main focus in this work was addressed to Mut^S to further increase bioprocess information for this recombinant expression phenotype.

3.1.1.2 Methanol metabolism

The enzyme alcohol oxidase (AOX) catalyses the first step in the dissimilation of methanol, where methanol is oxidized to formaldehyde and hydrogen peroxide using molecular oxygen. This reaction takes place within specialized organelles called peroxisomes (Figure 3.1.1-1) in order to avoid the toxic effect of hydrogen peroxide [24]. Peroxisomal catalase (CAT) ensures the degradation of hydrogen peroxide to water and molecular oxygen.

Formaldehyde forms a complex with reduced glutathione and enters the cytosol where it is oxidized to carbon dioxide by two subsequent dehydrogenase (FLD, formaldehyde dehydrogenase and FDH, formate dehydrogenase) reactions. Besides the generation of energy in the form of NADH, this dissimilatory oxidation pathway of formaldehyde is thought to play an important role in the detoxification of formaldehyde in methylotrophic yeasts [25].

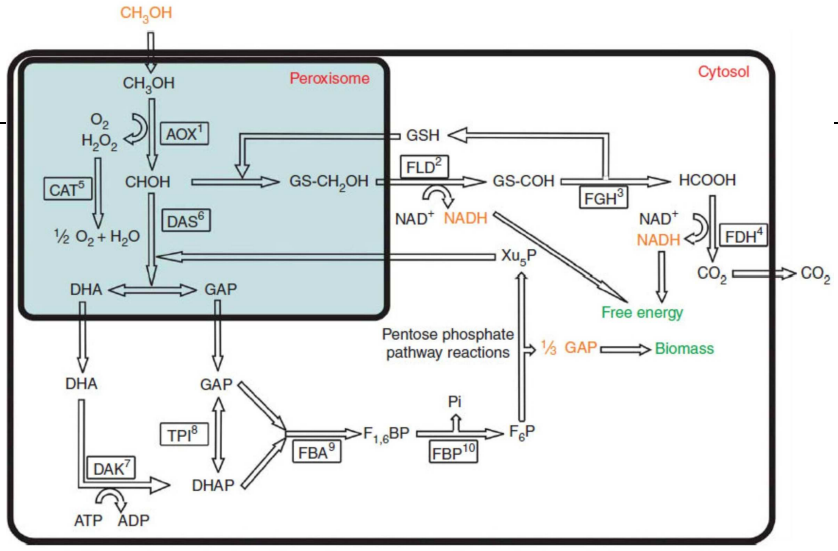


Figure 3.1.1-1 Different cellular locations where methanol utilization takes place; scheme taken from [26].

The remaining formaldehyde reacts in a transketolase reaction catalysed by dihydroxiacetone synthase (DAS) with xylulose-5-phosphate (Xu_5P) to yield dihydroxyacetone (DHA) and glyceraldehyde-3-phosphate (GAP). The formed C3 compounds are further assimilated within the cytosol and serve as building blocks for biomass synthesis. DHA is phosphorylated by a dihydroxyacetone kinase, and subsequently, in an aldolase reaction with GAP, it forms fructose-1,6-bisphosphate ($F_{1,6}$ BP), which is then converted to fructose-6-phosphate (F_6P) by a phosphatase. F_6P enters the pentose phosphate pathway to regenerate xylulose-5-phosphate. Every three cycles, one net molecule of GAP is produced and utilized in order to form biomass by standard reactions of gluconeogenesis.

There are contradictory results reported on the role of the dissimilatory pathway in methylotrophic yeasts. Some studies report the formaldehyde dehydrogenase and formate dehydrogenase enzymes to be responsible for the protection of the cell from toxic levels of formaldehyde caused by a high residual methanol concentration in the medium [27-28]. In contrast, Jahic *et al.* described this pathway as the main energy source for the cells when growing solely on methanol [29]. It is also not clear, how formaldehyde is distributed between the assimilatory and the dissimilatory pathways in the cell.

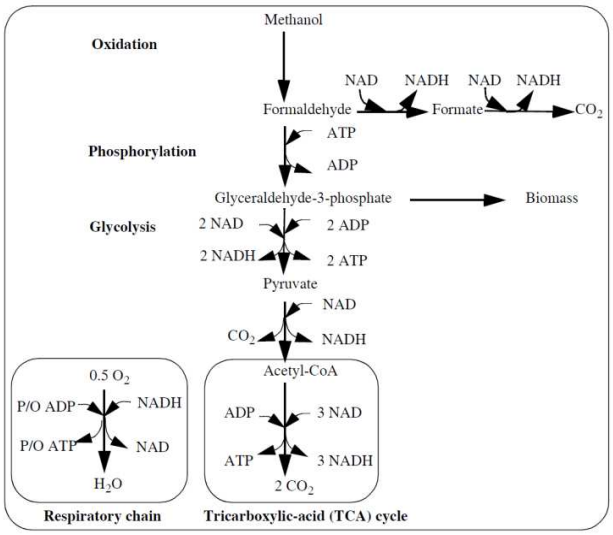


Figure 3.1.1-2 Metabolic pathway for methanol in *P. pastoris;* scheme taken and adapted from [10].

3.1.1.3 Glycerol metabolism

Glycerol is phosphorylated by a glycerol kinase, which leads to glycerol 3-phosphate, followed by oxidation to dihydroxyacetone phosphate by a FAD-dependent glycerol-3-phosphate dehydrogenase located on the outer surface of the mitochondrial inner membrane. The dihydroxyacetone phosphate formed enters the glycolytic pathway [10]. Pyruvate is formed as the outcome of glycolysis and is further oxidized to acetyl-CoA, via pyruvate dehydrogenase. Acetyl-CoA enters the tricarboxylic acid (TCA) cycle, where numerous metabolites are produced and used for the synthesis of cellular constituents such as amino acids, nucleic acids and cell wall components. In the TCA cycle, most of the energy is conserved in the form of NADH and subsequently used for cell growth and maintenance.

P. pastoris is a Crabtree-negative yeast [30]. According to Ren *et al.*, the residual ethanol concentration measured during growth on glycerol is negligible [10]. Hence, ethanol formation can be neglected when oxygen is not limiting. The use of glucose as carbon source is usually avoided because of higher amounts of ethanol produced as a metabolite on glucose [31]. Residual ethanol concentrations repress the *p*AOX, even at very low levels of around 10-50 $mg \cdot l^{-1}$ [32]. However, glucose depicts a low cost substrate for biomass growth prior induction.

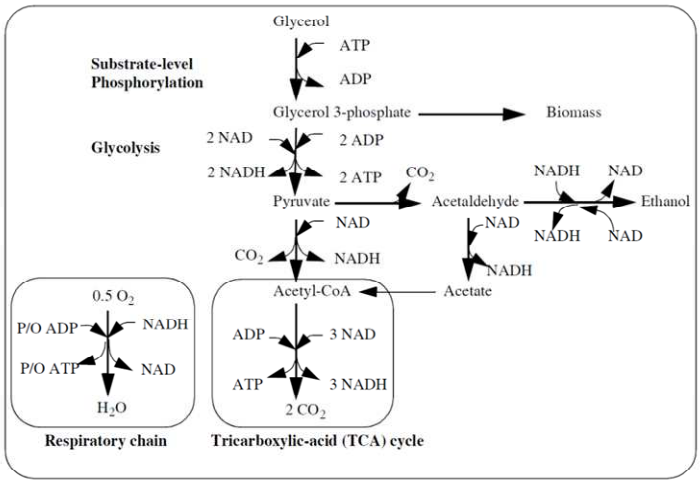


Figure 3.1.1-3 Metabolic pathway for glycerol in *P. pastoris*; scheme taken and adapted from [10].

3.1.1.4 Mixed substrate metabolism

The metabolism of glycerol and methanol in a mixed substrate environment was investigated in batch [3, 33], carbon-limited fed batch as well as in continuous cultivation systems [4, 34-35]. Under glycerol excess the *p*AOX is repressed and methanol is not taken up. Hence, in a multiple substrate batch environment, diauxic growth was reported. However, in a carbon-limited environment, *P. pastoris* takes up glycerol and methanol in parallel. Mixed feed studies using *P. pastoris* are mostly designed to obtain high productivities caused by increased biomass yields on glycerol. Interestingly, the metabolism in a mixed substrate environment is still not entirely understood.

A clear advantage of a mixed feeding strategy compared to single methanol feed is the reduced heat production of the culture [34] caused by the enthalpies of combustion of methanol and glycerol, which are -727 kJ·C-mol^{-1} and -549.5 kJ·C-mol^{-1}, respectively [36]. In addition, oxygen uptake is also reduced in a mixed feeding strategy because of a lower oxygen demand for glycerol oxidation compared to methanol. The reduction of oxygen consumption rate and heat production rate is very advantageous in high cell density cultures with recombinant *P. pastoris* strains, especially at large industrial scales.

3.1.2. Recombinant protein expression with *Pichia pastoris*

Several advantages of using *P. pastoris* for recombinant protein production were reported. In contrast to the baker's yeast *Saccharomyces cerevisiae*, *P. pastoris* is dedicated as Crabtree negative yeast [30] and does not produce significant amounts of potentially inhibiting fermentative by-products like ethanol [37]. *P. pastoris* simply turns the carbon source into biomass and no significant change of the biomass yield is expected, which enables a straight

forward and easy process regime for recombinant protein production with *P. pastoris*. Protocols for fed batch and continuous cultivations, which are based on defined and inexpensive media [37-38], are described. Since *P. pastoris* is mostly cultivated at relatively low pH and in the presence of methanol, only a minimum risk of contamination by other microorganisms exists.

3.1.2.1 Expression vectors

The construction of recombinant *P. pastoris* expression strains is divided in different steps. A foreign gene has to be inserted into an expression vector and introduced into the genome of *P. pastoris*. Afterwards, the generated strains have to be examined for potential foreign gene expression capability [7]. Besides the prominent AOX promoter, other promoter systems like GAP, FLD1 and others were described and consequently used for recombinant protein expression [7]. The GAP promoter provides a constitutive expression of the foreign gene on glucose at comparable levels to that with the AOX promoter. The major advantage is that methanol is not required for induction. The FLD1 gene encodes the formaldehyde dehydrogenase enzyme and the promoter can be induced either by methanol or by methylamine as nitrogen source combined with glucose as carbon source [7].

All expression vectors can be designed as *E. coli/P. pastoris* shuttle vectors and contain markers for one or even both microorganisms. The foreign gene is inserted into the expression cassette between the promoter and the terminator sequences.

3.1.2.2 Secretion signals and posttranslational modifications

Foreign proteins can be produced in *P. pastoris* either intracellular or, combined with a secretion signal, released to the supernatant. For secretion of a recombinant product, vectors are available where in-frame fusions of foreign proteins and the secretion signal can be generated. The most prominent secretion signals are the *P. pastoris* acid phosphatase (PHO1) or the α-mating factor (α-MF) from *S. cerevisiae* [7].

For secretion processes, the vast majority of the total protein in the supernatant represents the heterologous one due to the low level secretion of endogenous proteins by *P. pastoris* [7]. Another major advantage of *P. pastoris* over other expression systems (e.g. bacterial hosts) is the potential for post-translational modifications. Therefore, recombinant proteins derived from fungi, plants and even from humans were expressed successfully in *P. pastoris* [7, 15]. *Pichia* is at present the most frequently used yeast species for the production of heterologous proteins. Due to relatively low efforts needed for process development and with respect to its secretion efficiency, it was dedicated to be superior to other yeasts [15].

3.1.2.3 The model enzyme horseradish peroxidase

In the presented study, the enzyme horseradish peroxidase was exemplarily chosen as a model enzyme for recombinant protein production in *P. pastoris.*

Horseradish peroxidase (HRP) is a member of the plant peroxidase super family [39] and catalyzes the oxidation of many substrates using hydrogen peroxide, resulting in oxidized products and in the formation of two molecules of water per molecule of hydrogen peroxide [40-42] (Eq.3.1).

$$H_2O_2 + 2AH_2 \rightarrow 2H_2O + 2AH^{\bullet} \qquad \text{(Equation 3.1)}$$

AH_2 represents the reduced substrate and $AH^{\bullet}$ its radical product. Typical substrates are aromatic phenols, phenolic acids, indoles, amines and sulfonates.

HRP is a heme-containing glycoprotein with a molecular weight of approximately 44 kDa that has been studied for more than 100 years [42] and gained more and more industrial relevance in the past few years; it is used in waste water treatment [43-44], analytical diagnostics [45] and for the elimination of H_2O_2 from food and industrial wastewater [46].

In nature, the enzyme HRP can be found in horseradish (*Armoracia rusticana*) and comprises between 18 to 22 % carbohydrates. HRP expressed in *P. pastoris* showed higher amounts of carbohydrates caused by higher glycosylation with mannose-residues [47]. The molecular mass of HRP, produced in *P. pastoris* was described at around 62 to 66 kDa [47]. Several different isoenzymes exist of which the isoenzyme C is the most abundant and characterized one.

The isoenzyme Horseradish Peroxidase C comprises a single polypeptide of 308 amino acid residues and the sequence was determined by Welinder in 1976 [39]. Four disulfide bridges between cysteine residues and one buried salt bridge between an Aspargine and an Arginin residue were found. Furthermore, nine potential N- glycosylation sites were recognised [42].

Two different types of metal centres are described (iron(III) protoporphyrin IX = heme group and two calcium atoms), which are essential for the structural and functional integrity of the enzyme. A loss of calcium results in lower thermal and enzyme stability as well as in slight changes in the heme environment [42].

The three dimensional structure of horseradish peroxidase isoenzyme C was described by Gajhede *et al.* [48] and by Veitch [42] (Figure 3.1.2-1).

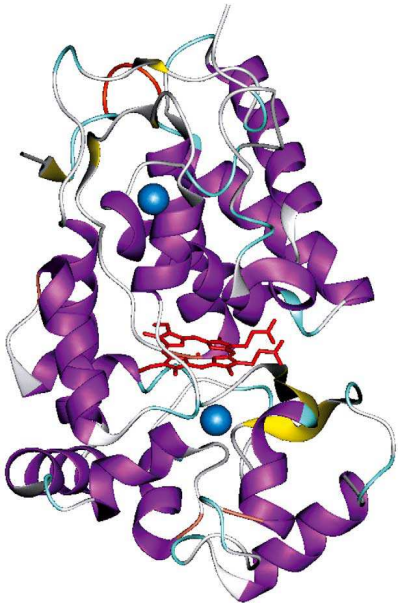


Figure 3.1.2-1 Three-dimensional representation of the X-ray crystal structure of horseradish peroxidase isoenzyme C from Veitch 2004 [42]. The heme group (coloured in red) is located between the distal and proximal domains, which contain one calcium atom each (shown as blue spheres). α-Helical and β-sheet regions of the enzyme are shown in purple and yellow, respectively.

3.2. Bioprocess development for recombinant protein expression in *Pichia pastoris*

3.2.1. Steps of process development

3.2.1.1 Strain screening experiments

The initial step of a development process is a first screening of potential candidates [1]. The target of this step is to select the most promising candidates out of a pool of available strains. Here, shake flasks or multi well microtitre plates are used and the output of the screening is mainly based on qualitative statements based on empiric results. Normally, these systems are rather uncontrolled and especially changes in pH evoke the use of strong buffer systems which are not scalable up to larger process sizes [49]. In general, the highest producers are selected and transferred into next development steps. Within this procedure it is possible that potentially high producers are rejected due to not optimal process conditions for the respective strains. Therefore, novel tools for fast and semi-quantitative experimental screening procedures were developed during the last decade and have been reviewed elsewhere [2]. Improvements regarding process control and scalability for critical parameters, like oxygen transfer, compared to other experimental systems were achieved.

3.2.1.2 Strain performance quantification and initial process development

The selected strains from the screening procedures are subsequently transferred to the next development step. During strain performance quantification, detailed information about characteristic parameters of the respective strain is obtained for initial process development. In order to launch a successful protein production process, key parameter sets, which combine biological as well as process technological knowledge, are needed to keep the strains in a defined physiological state. This knowledge is basically derived by raw data generation and subsequent data exploitation to compile conversion rates and specific rates out of the process. The use of specific rates, like e.g. specific growth rate, ensures scale independency and easy transferability into further development steps. The potential of such key parameters to act as operational control variables can be seen in Figure 3.2.1-1, where a general strategy for data transfer to information and knowledge is shown [49].

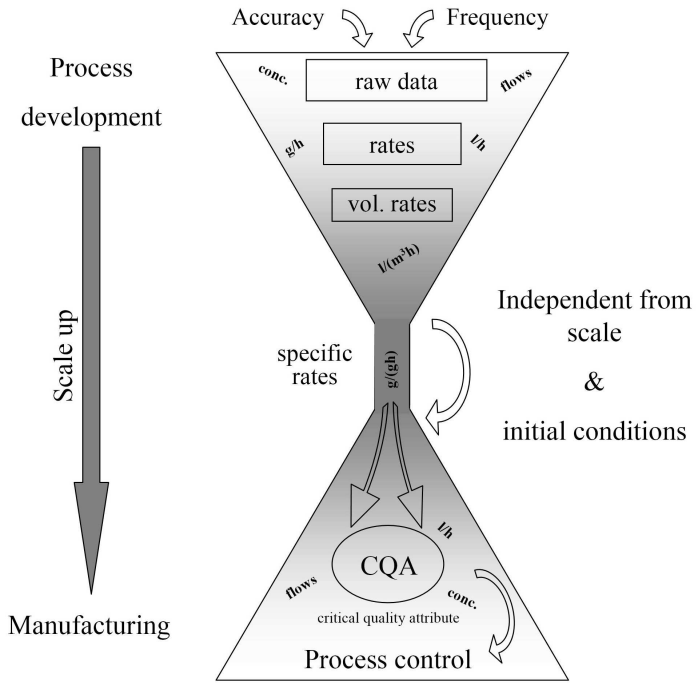


Figure 3.2.1-1 General strategy for transfer of data to knowledge from process development to manufacturing.

Strain characteristic parameters are estimated in different cultivation modes during strain performance quantification. So far, empirical driven approaches as well as studies based on continuous cultivation systems have been conducted to investigate the performance of different *P. pastoris* strains. Time intensive experiments are used to define optimal parameters, like the specific growth rate in respective to the highest productivity [4-5]. In contrast to continuous cultivations, another approach used several consecutive fed batch cultivations with changed parameter sets in order to find an optimal specific growth rate for

maximum productivity [10-11]. Both of the experimental approaches are time-consuming and therefore not suitable for numerous strain quantifications in a short time.

The fact that still a high number of strains are selected for strain quantification highlights the necessity of a reliable and fast methodology for performance quantification. Exemplarily, parallel bioreactor systems have been established to quantify selected strains in lab-scale environment of up to 2 litres working volume [50].

3.2.1.3 Process optimization and process control

Based on the findings of the initial process development, the next step is to optimize the process and to establish a reliable control automatism in order to scale up the production system to industrial scales. The approaches for optimization are very interdisciplinary, as in principle all biological and process parameters might interact with each other and therefore might have a possible impact on productivity. This development phase is characterized by quantitative analysis of product quality related parameters, identified as Critical Process Parameters (CPP) and their relationship to the quality of the product, defined as Critical Quality attributes (CQA) [49] (see also Figure 3.2.1-1). In that sense the concept of "design space" has been proposed in the ICH Q8 guidelines [51] and was exemplarily applied to a *P. pastoris* system by Harms *et. al.* Numerous process variables like pH, dissolved oxygen, temperature as well as feeding rates were used for process characterization and optimal parameter identification with a Design of Experiment (DoE) approach [6]. The final goal of the process optimization phase is the gathering of process understanding. A process is generally considered well understood when, i) all critical sources of variability are identified and explained; ii) variability is managed by the process; and iii) product quality attributes can be accurately and reliably predicted over the design space [52]. A structured process development on a small scale, using experimental design, can provide increased insight and understanding for process development, optimization, scale-up and control [52].

After optimization, the final development is carried out in pilot scale to check for parameter consistency at industrial scales and to proof the validity of the developed control strategy.

3.2.2. Key parameters for process control strategies

3.2.2.1 Getting started – Pichia pastoris *fermentation guidelines*

The wide use of *P. pastoris* for protein production is mainly caused by a multitude of advantages like substrate variety, phenotypic differences and easy genetic manipulation. In 1993, Phillips Petroleum sold the *P. pastoris* expression system to Research Corporation

Technologies (Tucson, AZ, US), which is the current patent holder [7]. The company Invitrogen in contract with Research Corporation Technologies established further protocols for the easy set up of *P. pastoris* , namely the "*Pichia* Fermentation Process Guidelines" [12]. In general, the production process of proteins using *P. pastoris* is divided into several steps (Figure 3.2.2-1). Initially, a batch on a carbon source like glycerol or glucose is conducted for biomass generation. Afterwards, further increase of biomass concentration is achieved by a fed batch phase with high concentrated feeds of glycerol or glucose, either with linear or exponential feeding profiles. Subsequently, methanol is used to induce the cells for recombinant protein production under the control of *p*AOX. The experimental strategies suggested in the fermentation guideline from Invitrogen describe a constant volumetric flow of methanol for a certain adaptation time and is then incrementally increased to a higher value and further kept constant until the end of induction. Due to concomitant biomass growth on the inducer methanol, the biomass concentration is increasing which leads to a decrease in the specific growth and specific substrate uptake rate during induction. Therefore, the physiological state of the cells is rather uncontrolled and obviously not aiming at optimal productivity. For all periods (batch, fed batch and induction), the experimental strategy is based on empirical knowledge and the transfer from one strain to another is difficult due to potential different characteristics.

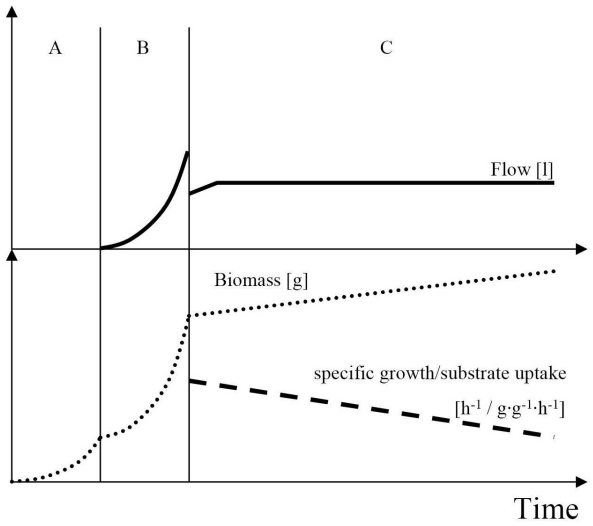


Figure 3.2.2-1 A protein production experiment carried out with *P. pastoris* in different phases based on the fermentation guidelines from Invitrogen; A, batch on glycerol or glucose; B, fed batch on glycerol of glucose; C, induction period on methanol for recombinant protein production.

3.2.2.2 Methanol excess conditions

During the last decades, high efforts were made to optimize recombinant protein production in *P. pastoris*. Based on the fermentation guidelines developed by Invitrogen [12], different improvements regarding optimal feeding strategies during induction were done. In some cases protein expression was carried out under methanol excess and systems for the automatic control of methanol concentrations during induction were developed [53-55]. According to the controlled methanol concentration in the culture broth and to the Monod kinetic of the methanol assimilation, the cells are most probably growing closely to the maximum specific growth rate. Therefore, the physiological state of the cells is rather undefined, e.g. due to different phenotypes, and thus not transferable from one strain to another.

3.2.2.3 Methanol limited conditions

In contrast, it was reported that a higher induction of the *p*AOX is achieved under methanol limited conditions [56] and several studies focused on easy to do feed forward regimes based on the specific growth rate [29, 53, 57-59].

The conducted research on the relationship between the process parameter specific growth rate μ on the inducer methanol and specific productivity was reported diverse; growth associated productivity was reported once [60], whereas a few studies showed no correlation between μ and the productivity of the cells [54, 57-58, 61].

Based on these contradictory results, other parameters were tested for their possible correlation with the productivity in *P. pastoris*. Parameters, like the specific substrate uptake rate q_s and its relationship to the specific productivity were analyzed in the past years [62-64]. In summary, all studies showed higher productivity at lower specific substrate uptake rates and q_s was stated as the most important induction parameter. The control parameter q_s in methanol based production systems reflects a physiological variable, which is directly correlated to the induction of recombinant protein. Due to the parallel usage of the *p*AOX for substrate assimilation on the one hand and protein induction at the other hand, possible interactions might be correlated to the physiological status of the cells.

A biological burden caused by protein expression was reported in the literature and changing biomass yields caused by stress conditions [65-67] and higher maintenance effects [68] were described for *E. coli*. Therefore, a control regime based on the specific substrate uptake rate could handle such physiological changes in biomass yields and describes a promising tool for process control in recombinant protein expression systems. However, an approach based on q_s for process control of feeding profiles has not been tested yet.

3.2.2.4 Mixed feed conditions

Beside the single substrate induction strategy where methanol presents the carbon and energy source as well as the inducer to the cells, several studies are described using a second substrate to integrate also biomass growth into the protein expression phase in *P. pastoris* [4, 8, 34, 69-71]. Technical benefits, like lower oxygen consumption and lower heat production [34], as well as increased volumetric productivities caused by accelerated biomass generation due to higher biomass yields on the second substrate, are reported [8]. For supporting cell growth, glycerol is a prominent C-source for mixed feed approaches, although it was several times reported to repress *p*AOX, even if fed in limiting amounts [69-70]. Thus, the glycerol feeding rate is a versatile parameter to optimize a mixed feed strategy [71]. Different approaches were carried out to identify optimal feeding regimes in a mixed feed environment. On the one hand methanol concentrations were maintained at 1-5 $g \cdot l^{-1}$ in the broth and variations of the glycerol feeding rate were carried out in continuous [4] as well as in fed batch approaches [72-73]. In contrast to that, defined ratios of the two substrates were mixed and fed together in limiting amounts in other studies [34-35, 74]. To investigate the effect of different substrate mixing ratios, again time-consuming continuous cultures or several fed batch experiments were performed. In summary, all these studies comprised the disadvantage that the substrate feeding rates were not clearly independent and therefore the estimation of any related effect of either of the substrates on the productivity and physiology of the cells was not possible [34-35, 74].

A novel approach based on the individual control of both of the substrates would probably reveal additional information about physiological and productivity interactions of *P. pastoris*. Detailed information of the correlation between the metabolism of the two substrates, like physiologic repression effects or the differentiation of energy and building block distribution, would additionally increase process understanding and subsequently improve productivity. Open questions concerning the stoichiometric interaction, as e.g. whether the physiological substrate uptake rate is a sum of the two individual rates or whether it can be even boosted in mixed environments, are still remaining. Therefore, a process regime based on the specific uptake rates as control parameters is a promising tool for mixed feed protein production systems on *P. pastoris.*

3.2.3. On-line monitoring for process development

During the last decades more and more effort was put in the development of robust and reliable devices for on-line monitoring of processes. Motivated by the Process Analytical Technology (PAT) initiative, which was suggested by the US Food and Drug Administration (FDA) [51-52], the development of novel techniques for process parameter determination in real time is more and more implemented within process development. PAT is defined to be a system for designing, analyzing and controlling manufacturing through timely measurements of critical quality and performance attributes of raw and in-process materials and processes, with the final goal of ensuring final product quality [52]. The term “analyzing” includes chemical, physical, microbiological, mathematical, and risk analysis conducted in an integrated manner.

The motivation of implementing on-line monitoring devices in early process development is mainly driven by media development issues and control parameter identification. The fast and reliable determination of substrates and products by these dedicated PAT tools enhances process understanding during development phases and additionally facilitates potential real time CQA estimation in manufacturing at industrial scales.

3.2.3.1 On-line monitoring for substrates and metabolites

The monitoring of substrates and metabolites in bioprocesses is mainly motivated to enhance bioprocess understanding and bioprocess knowledge in order to take action and interfere with the process, while the process is still being carried out. A broad portfolio of different analytical devices based on different analytical principles were described to extract important information out of bioprocesses and the field has been reviewed intensively in the last two decades [75-77].

One of the main goals of the research group of Biochemical Engineering at the Vienna University of Technology (VUT) is the establishment of novel tools and methods for reliable on-line monitoring of bioprocesses and a real time quantification of physiological reactions. Therefore, studies were conducted to use on-line available signals as input variables for kinetic modelling and balancing (elemental and mass balances) for the real time quantification of bioprocess key parameters like specific growth and substrate uptake rates [78]. In addition, a theoretical study on the needed data quality to reliably extract quantitative information out of on-line measurements was carried out to indentify prerequisites regarding the signal to noise ratio for potential novel on-line monitoring principles [79].

The on-line monitoring aspect for substrate and metabolite analysis focused within the present study was addressed to media development and the real time extraction of key parameters during strain performance quantification experiments.

3.2.3.2 On-line monitoring for product determination

Another focus area of on-line monitoring in recombinant protein expression systems is the determination of the target product quantity and/or quality. In case of a recombinantly expressed enzyme, analysis regarding the protein content and the activity of the enzyme are of interest. Interestingly, only few approaches for the on-line determination of proteins are described in literature. In principle, quantification methods are either based on the direct measurement of the protein, like HPLC [80-81], enzymatic assays [82], as well as fluorescence sensors [83-84] or on the reliable determination of compounds, like metabolites or by-products, which are directly related to the product expression [85-87].

In the presented study, a novel on-line monitoring tool was chosen which combined the capability to determine substrates, metabolites as well as the target protein HRP. Currently, the research group Biochemical Engineering at VUT is focusing in parallel on the reliable and fast determination of target proteins with automated HPLC systems.

3.3. References

1. Nielsen J, Villadsen J, Liden G: *Bioreaction Engineering Principles.* 2nd edn; 2002.
2. Duetz WA: **Microtiter plates as mini-bioreactors: miniaturization of fermentation methods.** *Trends Microbiol* 2007, **15:**469-475.
3. Orman MA, Calik P, Ozdamar TH: **The influence of carbon sources on recombinant-human-growth-hormone production by Pichia pastoris is dependent on phenotype: a comparison of Muts and Mut+ strains.** *Biotechnology and Applied Biochemistry* 2009, **52:**245-255.
4. d'Anjou MC, Daugulis AJ: **A rational approach to improving productivity in recombinant Pichia pastoris fermentation.** *Biotechnology and bioengineering* 2001, **72:**1-11.
5. Curvers S, Linnemann J, Klauser T, Wandrey C, Takors R: **Recombinant protein production with Pichia pastoris in continuous fermentation - kinetic analysis of growth and product formation.** *Engineering in Life Sciences* 2002, **2:**229-235.
6. Harms: **Defining Process Design Space for Biotech Products: Case Study of Pichia pastoris Fermentation.** *Biotechnology Progress* 2008.
7. Cereghino JL, Cregg JM: **Heterologous protein expression in the methylotrophic yeast Pichia pastoris.** *FEMS Microbiol Rev* 2000, **24:**45-66.
8. Cos O, Ramon R, Montesinos JL, Valero F: **Operational strategies, monitoring and control of heterologous protein production in the methylotrophic yeast Pichia pastoris under different promoters: a review.** *Microbial Cell Factories* 2006, **5:**1-20.
9. Li P, Anumanthan A, Gao X-G, Ilangovan K, Suzara VV, Duzgunes N, Renugopalakrishnan V: **Expression of recombinant proteins in Pichia pastoris.** *Applied Biochemistry and Biotechnology* 2007, **142:**105-124.
10. Ren HT, Yuan JQ, Bellgardt KH: **Macrokinetic model for methylotrophic Pichia pastoris based on stoichiometric balance.** *Journal of Biotechnology* 2003, **106:**53-68.
11. Ren H, Yuan J: **Model-based specific growth rate control for Pichia pastoris to improve recombinant protein production.** *Journal of Chemical Technology and Biotechnology* 2005, **80:**1268-1272.
12. Invitrogen: **Pichia Fermentation Process Guidelines.** 2002.
13. Ogata K, Nishikaw.H, Ohsugi M: **A Yeast Capable of Utilizing Methanol.** *Agricultural and Biological Chemistry* 1969, **33:**1519-&.
14. Wegner GH: **Emerging Applications of the Methylotrophic Yeasts.** *FEMS microbiology reviews* 1990, **87:**279-283.
15. Schmidt FR: **Recombinant expression systems in the pharmaceutical industry.** *Applied Microbiology and Biotechnology* 2004, **65:**363-372.
16. Faber KN, Harder W, Ab G, Veenhuis M: **Methylotrophic yeasts as factories for the production of foreign proteins.** *Yeast* 1995, **11:**1331-1344.
17. Couderc R, Baratti J: **Oxidation of methanol by the yeast, Pichia pastoris. Purification and properties of the alcohol oxidase.** *Agricultural and Biological Chemistry* 1980, **44:**2279-2289.
18. Roggenkamp R, Hansen H, Eckart M, Janowicz Z, Hollenberg CP: **Transformation of the methylotrophic yeast Hansenula polymorpha by autonomous replication and integration vectors.** *Molecular and General Genetics* 1986, **202:**302-308.
19. Chiruvolu V, Cregg JM, Meagher MM: **Recombinant protein production in an alcohol oxidase-defective strain of Pichia pastoris in fedbatch fermentations.** *Enzyme and Microbial Technology* 1997, **21:**277-283.
20. Romanos MA, Scorer CA, Clare JJ: **Foreign Gene-Expression in Yeast - a Review.** *Yeast* 1992, **8:**423-488.
21. van den Burg HA, de Wit PJGM, Vervoort J: **Efficient C-13/N-15 double labeling of the avirulence protein AVR4 in a methanol-utilizing strain (Mut(+)) of Pichia pastoris.** *Journal of Biomolecular Nmr* 2001, **20:**251-261.
22. Kim SJ, Lee JA, Kim YH, Song BK: **Optimization of the Functional Expression of Coprinus cinereus Peroxidase in Pichia pastoris by Varying the Host and Promoter.** *Journal of Microbiology and Biotechnology* 2009, **19:**966-971.
23. Chiruvolu V, Cregg JM, Meagher MM: **Recombinant protein production in an alcohol oxidase-defective strain of Pichia pastoris in fedbatch fermentations.** *Enzyme and Microbial Technology* 1997, **21:**277-283.
24. Zhang W, Inan M, Meagher MM: **Fermentation strategies for recombinant protein expression in the methylotrophic yeast Pichia pastoris.** *Biotechnology and Bioprocess Engineering* 2000, **5:**275-287.

25. Lee B, Yurimoto H, Sakai Y, Kato N: **Physiological role of the glutathione-dependent formaldehyde dehydrogenase in the methylotrophic yeast Candida boidinii.** *Microbiology* 2002, **148:**2697-2704.
26. De Schutter K, Lin Y-C, Tiels P, Van Hecke A, Glinka S, Weber-Lehmann J, Rouze P, Van de Peer Y, Callewaert N: **Genome sequence of the recombinant protein production host Pichia pastoris.** *Nat Biotech* 2009, **27:**561-566.
27. Sibirny AA, Titorenko VI, Gonchar MV, Ubiyvovk VM, Ksheminskaya GP, Vitvitskaya OP: **Genetic control of methanol utilization in yeasts.** *Journal of Basic Microbiology* 1988, **28:**293-319.
28. Jones JG, Bellion E: **Methylamine metabolism in Hansenula polymorpha: an in vivo carbon-13 and phosphorus-31 nuclear magnetic resonance study.** *Journal of Bacteriology* 1991, **173:**4959-4969.
29. Jahic M, Rotticci-Mulder JC, Martinelle M, Hult K, Enfors SO: **Modeling of growth and energy metabolism of Pichia pastoris producing a fusion protein.** *Bioprocess and Biosystems Engineering* 2002, **24:**385-393.
30. Porro D, Sauer M, Branduardi P, Mattanovich D: **Recombinant protein production in yeasts.** *Molecular Biotechnology* 2005, **31:**245-260.
31. Macauley-Patrick S, Fazenda ML, McNeil B, Harvey LM: **Heterologous protein production using the Pichia pastoris expression system.** *Yeast* 2005, **22:**249-270.
32. Inan M, Meagher MM: **The effect of ethanol and acetate on protein expression in Pichia pastoris.** *J Biosci Bioeng* 2001, **92:**337-341.
33. Jungo C, Marison I, Von Stockar U: **Regulation of alcohol oxidase of a recombinant Pichia pastoris Mut+ strain in transient continuous cultures.** *Journal of Biotechnology* 2007, **130:**236-246.
34. Jungo C, Marison I, von Stockar U: **Mixed feeds of glycerol and methanol can improve the performance of Pichia pastoris cultures: A quantitative study based on concentration gradients in transient continuous cultures.** *Journal of Biotechnology* 2007, **128:**824-837.
35. Sola A, Jouhten P, Maaheimo H, Sanchez-Ferrando F, Szyperski T, Ferrer P: **Metabolic flux profiling of Pichia pastoris grown on glycerol/methanol mixtures in chemostat cultures at low and high dilution rates.** *Microbiology (Reading, United Kingdom)* 2007, **153:**281-290.
36. STOCKAR V, #160, U., GUSTAFSSON, L., LARSSON, C., MARISON, I., TISSOT, et al: *Thermodynamic considerations in constructing energy balances for cellular growth.* Amsterdam, PAYS-BAS: Elsevier; 1993.
37. Cregg JM: **Pichia Protocols, Second Edition.** In *Methods Mol Biol* Edited by Cregg JM. Totowa, NJ, U. S.; 2007: 268 pp
38. Stratton J, Chiruvolu V, Meagher M: **High cell-density fermentation.** *Methods in Molecular Biology (Totowa, New Jersey)* 1998, **103:**107-120.
39. Welinder KG: **Amino acid sequence studies of horseradish peroxidase. 4. Amino and carboxyl termini, cyanogen bromide and tryptic fragments, the complete sequence, and some structural characteristics of horseradish peroxidase C.** *European Journal of Biochemistry* 1979, **96:**483-502.
40. Gajhede M: **Horseradish peroxidase.** In *Handbook of Metalloproteins. Volume* 1; 2001: 195-210
41. Rodriguez-Lopez JN, Lowe DJ, Hernandez-Ruiz J, Hiner ANP, Garcia-Canovas F, Thorneley RNF: **Mechanism of reaction of hydrogen peroxide with horseradish peroxidase: Identification of intermediates in the catalytic cycle.** *Journal of the American Chemical Society* 2001, **123:**11838-11847.
42. Veitch NC: **Horseradish peroxidase: a modern view of a classic enzyme.** *Phytochemistry (Elsevier)* 2004, **65:**249-259.
43. Lai Y-C, Lin S-C: **Application of immobilized horseradish peroxidase for the removal of p-chlorophenol from aqueous solution.** *Process Biochemistry (Oxford, United Kingdom)* 2004, **40:**1167-1174.
44. Dalal S, Gupta MN: **Treatment of phenolic wastewater by horseradish peroxidase immobilized by bioaffinity layering.** *Chemosphere* 2007, **67:**741-747.
45. Azevedo AM, Martins VC, Prazeres DMF, Vojinovic V, Cabral JMS, Fonseca LP: **Horseradish peroxidase: a valuable tool in biotechnology.** *Biotechnology Annual Review* 2003, **9:**199-247.
46. Hamid M, Khalil ur R: **Potential applications of peroxidases.** *Food Chemistry* 2009, **115:**1177-1186.
47. Morawski B, Lin Z, Cirino P, Joo H, Bandara G, Arnold FH: **Functional expression of horseradish peroxidase in Saccharomyces cerevisiae and Pichia pastoris.** *Protein Engineering* 2000, **13:**377-384.
48. Gajhede M, Schuller DJ, Henriksen A, Smith AT, Poulos TL: **Crystal structure of horseradish peroxidase C at 2.15 A resolution.** *Nature structural biology* 1997, **4:**1032-1038.
49. Herwig C: **The Analytical Challenge in QbD- from Data to Information and to Knowledge-from (Bioprocess) Development to Manufacturing.** In *Quality by design - Putting Theory into Practice.* Edited by Schmitt S: DHI / PDA; 2011: 163-194
50. Weuster-Botz D: **Parallel reactor systems for bioprocess development.** *Advances in Biochemical Engineering/Biotechnology* 2005, **92:**125-143.
51. ICH: **Q8, Pharmaceutical Development (R1).** *wwwichorg* 2008.

52. FDA: **Guidance for Industry PAT-A framework for Innovative Pharmaceutical Development, Manufacturing, and Quality Assurance.** *wwwfdagov* 2003.
53. Trinh LB, Phue JN, Shiloach J: **Effect of methanol feeding strategies on production and yield of recombinant mouse endostatin from Pichia pastoris.** *Biotechnology and Bioengineering* 2003, **82:**438-444.
54. Zhang W, Sinha J, Smith LA, Inan M, Meagher MM: **Maximization of Production of Secreted Recombinant Proteins in Pichia pastoris Fed-Batch Fermentation.** *Biotechnology Progress* 2005, **21:**386-393.
55. Plantz BA, Sinha J, Villarete L, Nickerson KW, Schlegel VL: **Pichia pastoris fermentation optimization: energy state and testing a growth-associated model.** *Applied Microbiology and Biotechnology* 2006, **72:**297-305.
56. Cregg JM: **Expression in the methylotrophic yeast Pichia pastoris.** *Gene Expression Systems* 1999**:**157-191.
57. Potgieter TI, Kersey SD, Mallem MR, Nylen AC, d'Anjou M: **Antibody expression kinetics in glycoengineered Pichia pastoris.** *Biotechnology and Bioengineering* 2010, **106:**918-927.
58. Kobayashi K, Kuwae S, Ohya T, Ohda T, Ohyama M, Tomomitsu K: **High level secretion of recombinant human serum albumin by fed-batch fermentation of the methylotrophic yeast, Pichia pastoris, based on optimal methanol feeding strategy.** *Journal of Bioscience and Bioengineering* 2000, **90:**280-288.
59. Jacobs P, Inan M, Festjens N, Haustraete J, Van Hecke A, Meagher M, Contreras R, Callewaert N: **Fed-batch fermentation of GM-CSF-producing glycoengineered Pichia pastoris under controlled specific growth rate.** *Microbial Cell Factories* 2010, **9:**93.
60. Ohya T, Ohyama M, Kobayashi K: **Optimization of human serum albumin production in methylotrophic yeast Pichia pastoris by repeated fed-batch fermentation.** *Biotechnology and Bioengineering* 2005, **90:**876-887.
61. Sinha J, Plantz BA, Zhang W, Gouthro M, Schlegel V, Liu C-P, Meagher MM: **Improved production of recombinant ovine interferon-t by Mut+ strain of Pichia pastoris using an optimized methanol feed profile.** *Biotechnology Progress* 2003, **19:**794-802.
62. Khatri NK, Hoffmann F: **Oxygen-limited control of methanol uptake for improved production of a single-chain antibody fragment with recombinant Pichia pastoris.** *Applied Microbiology and Biotechnology* 2006, **72:**492-498.
63. Khatri NK, Hoffmann F: **Impact of methanol concentration on secreted protein production in oxygen-limited cultures of recombinant Pichia pastoris.** *Biotechnology and Bioengineering* 2006, **93:**871-879.
64. Cunha AE, Clemente JJ, Gomes R, Pinto F, Thomaz M, Miranda S, Pinto R, Moosmayer D, Donner P, Carrondo MJT: **Methanol induction optimization for scFv antibody fragment production in Pichia pastoris.** *Biotechnology and bioengineering* 2004, **86:**458-467.
65. Bylund F, Collet E, Enfors SO, Larsson G: **Substrate gradient formation in the large-scale bioreactor lowers cell yield and increases by-product formation.** *Bioprocess Engineering* 1998, **18:**171-180.
66. Neubauer P, Lin HY, Mathiszik B: **Metabolic load of recombinant protein production: inhibition of cellular capacities for glucose uptake and respiration after induction of a heterologous gene in Escherichia coli.** *Biotechnology and bioengineering* 2003, **83:**53-64.
67. Strandberg L, Andersson L, Enfors SO: **The Use of Fed-Batch Cultivation for Achieving High Cell Densities in the Production of a Recombinant Protein in Escherichia-Coli.** *FEMS microbiology reviews* 1994, **14:**53-56.
68. Bylund F, Castan A, Mikkola R, Veide A, Larsson G: **Influence of scale-up on the quality of recombinant human growth hormone.** *Biotechnology and bioengineering* 2000, **69:**119-128.
69. Hellwig S, Emde F, Raven NPG, Henke M, Van der Logt P, Fischer R: **Analysis of single-chain antibody production in Pichia pastoris using on-line methanol control in fed-batch and mixed-feed fermentations.** *Biotechnology and Bioengineering* 2001, **74:**344-352.
70. Files D, Ogawa M, Scaman CH, Baldwin SA: **A Pichia pastoris fermentation process for producing high-levels of recombinant human cystatin-C.** *Enzyme and Microbial Technology* 2001, **29:**335-340.
71. Arnau C, Casas C, Valero F: **The effect of glycerol mixed substrate on the heterologous production of a Rhizopus oryzae lipase in Pichia pastoris system.** *Biochemical Engineering Journal* 2011, **57:**30-37.
72. Xie J, Zhang L, Ye Q, Zhou Q, Xin L, Du P, Gan R: **Angiostatin production in cultivation of recombinant <i>Pichia pastoris</i> fed with mixed carbon sources.** *Biotechnology Letters* 2003, **25:**173-177.
73. Katakura Y, Zhang W, Zhuang G, Omasa T, Kishimoto M, Goto Y, Suga K-I: **Effect of methanol concentration on the production of human beta 2-glycoprotein I domain V by a recombinant**

Pichia pastoris: a simple system for the control of methanol concentration using a semiconductor gas sensor. *Journal of Fermentation and Bioengineering* 1998, **86:**482-487.

74. Brierley RA, Bussineau C, Kosson R, Melton A, Siegel RS: **Fermentation development of recombinant Pichia pastoris expressing the heterologous gene: bovine lysozyme.** *Annals of the New York Academy of Sciences* 1990, **589:**350-362.
75. Schugerl K: **Progress in monitoring, modeling and control of bioprocesses during the last 20 years.** *Journal of Biotechnology* 2001, **85:**149-173.
76. Vojinovic V, Cabral JMS, Fonseca LP: **Ex situ bioprocess monitoring techniques.** *Chemical Industry & Chemical Engineering Quarterly* 2007, **13:**103-116.
77. Rehbock C, Beutel S, Brueckerhoff T, Hitzmann B, Riechers D, Rudolph G, Stahl F, Scheper T, Friehs K: **Bioprocess analysis.** *Chemie Ingenieur Technik* 2008, **80:**267-286.
78. Wechselberger P, Seifert A, Herwig C: **PAT method to gather bioprocess parameters in real time using simple input variables and first principle relationships.** *Chem Eng Sc* 2010, **65:**5734–5746.
79. Wechselberger P, Herwig C: **Model-based analysis on the relationship of signal quality to real-time extraction of information in bioprocesses.** *Biotechnology Progress* 2011, **accepted for publication**.
80. Rathore AS, Parr L, Dermawan S, Lawson K, Lu Y: **Large scale demonstration of a process analytical technology application in bioprocessing: Use of on-line high performance liquid chromatography for making real time pooling decisions for process chromatography.** *Biotechnology Progress* 2010, **26:**448-457.
81. Rathore AS, Yu M, Yeboah S, Sharma A: **Case study and application of process analytical technology (PAT) towards bioprocessing: Use of on-line high-performance liquid chromatography (HPLC) for making real-time pooling decisions for process chromatography.** *Biotechnology and bioengineering* 2008, **100:**306-316.
82. Brown R, O'Kennedy RD, Helwigh J, Madden E, Hoare M: **Accelerated prediction of recombinant protein production in Saccharomyces cerevisiae by using rapid monitoring techniques.** *Enzyme and Microbial Technology* 2000, **26:**801-807.
83. Surribas A, Resina D, Ferrer P, Valero F: **Riboflavin may interfere with on-line monitoring of secreted green fluorescence protein fusion proteins in Pichia pastoris.** *Microbial Cell Factories* 2007, **6:**No pp given.
84. Jones JJ, Bridges AM, Fosberry AP, Gardner S, Lowers RR, Newby RR, James PJ, Hall RM, Jenkins O: **Potential of real-time measurement of GFP-fusion proteins.** *Journal of Biotechnology* 2004, **109:**201-211.
85. Turner C, Gregory ME, Thornhill NF: **Closed-loop control of fed-batch cultures of recombinant Escherichia coli using on-line HPLC.** *Biotechnology and Bioengineering* 1994, **44:**819-829.
86. VanDusen WJ, Fu J, Bailey FJ, Burke CJ, Herber WK, George HA: **Adenine quantitation in yeast extracts and fermentation media and its relationship to protein expression and cell growth in adenine auxotrophs of Saccharomyces cerevisiae.** *Biotechnology Progress* 1997, **13:**1-7.
87. Looser V, Hammes F, Keller M, Berney M, Kovar K, Egli T: **Flow-cytometric detection of changes in the physiological state of E. coli expressing a heterologous membrane protein during carbon-limited fed-batch cultivation.** *Biotechnology and Bioengineering* 2005, **92:**69-78.

4. Results and Discussion

4.1. A novel experimental strategy for fast strain performance quantification

4.1.1. A dynamic method based on the specific substrate uptake rate to set up a feeding strategy for *Pichia pastoris*

Christian Dietzsch, Oliver Spadiut, Christoph Herwig[§]

Vienna University of Technology, Institute of Chemical Engineering, Research Area Biochemical Engineering, Vienna, Austria

[§]Corresponding Author: Christoph Herwig, Vienna University of Technology, Institute of Chemical Engineering, Research Area Biochemical Engineering, Gumpendorfer Strasse 1a, A-1060 Vienna, Austria. Tel: +43 1 85501 166400, christoph.herwig@tuwien.ac.at

Manuscript published:

Dietzsch C, Spadiut O, Herwig C: **A dynamic method based on the specific substrate uptake rate to set up a feeding strategy for Pichia pastoris.** *Microbial Cell Factories* 2011, **10:**14.

Abstract

Background

Pichia pastoris is one of the most important host organisms for the recombinant production of proteins in industrial biotechnology. To date, strain specific parameters, which are needed to set up feeding profiles for fed batch cultivations, are determined by time-consuming continuous cultures or consecutive fed batch cultivations, operated at different parameter sets.

Results

Here, we developed a novel approach based on fast and easy to do batch cultivations with methanol pulses enabling a more rapid determination of the strain specific parameters specific substrate uptake rate q_s, specific productivity q_p and the adaption time ($\Delta time_{adapt}$) of the culture to methanol. Based on q_s, an innovative feeding strategy to increase the productivity of a recombinant *Pichia pastoris* strain was developed. Higher specific substrate uptake rates resulted in increased specific productivity, which also showed a time dependent trajectory. A dynamic feeding strategy, where the setpoints for q_s were increased stepwise until a $q_{s\ max}$ of 2.0 $mmol \cdot g^{-1} \cdot h^{-1}$ resulted in the highest specific productivity of 11 $U \cdot g^{-1} \cdot h^{-1}$.

Conclusions

Our strategy describes a novel and fast approach to determine strain specific parameters of a recombinant *Pichia pastoris* strain to set up feeding profiles solely based on the specific substrate uptake rate. This approach is generic and will allow application to other products and other hosts.

Keywords: recombinant protein expression, *Pichia pastoris*, Mut^S, specific substrate uptake rate, specific productivity, batch cultivation, methanol pulse, dynamic feeding profile

Background

Recombinant protein expression with biological hosts is one of the most examined key processes in the pharmaceutical industry. Numerous products like organic acids, antibiotics, enzymes and amino acids are produced heterologously by recombinant microorganisms. The methylotrophic yeast *Pichia pastoris* is one of the most important host organisms for this purpose.

Several of the published fermentation strategies for *P. pastoris* to date are based on the Invitrogen protocol (http://tools.invitrogen.com). This protocol suggests constant feeding profiles for fed batch cultivations, but does not aim to improve production efficiency regarding time and yield or substrate consumption. Based on this protocol, different process strategies were developed to optimize recombinant protein production with *P. pastoris* in the past few years. A commonly used feeding strategy describes a feed forward regime based on a constant specific growth rate μ [1-5]. This strategy results in an exponential feeding profile and does not require complex instrumentation, but μ is also not controlled, and since the cells capacity may change over time, the feeding profiles consider a large safety margin. Another feeding strategy is based on a controlled μ and requires laborious continuous culture investigations and an effective computer controlled operation, based on established growth models and a feedback algorithm requiring expensive online measurement sensors for methanol [2, 6-8]. Employing these strategies, the outcome regarding specific productivity and specific growth rate was diverse; some studies showed that the maximal specific productivity did not relate to the maximal specific growth rate [1, 3, 6-7], whereas another study showed a more or less growth associated productivity [9].

Due to these controversial findings, other parameters were analyzed for their possible correlation with the productivity in the past few years. Khatri and Hoffmann analyzed the specific substrate uptake rate (q_s) and its association with the specific productivity (q_p) in fed-batch cultivations of *P. pastoris* and showed that lower q_s resulted in higher q_p [10-11]. In another study, Cunha *et al.* used a constant feeding rate of methanol during several fed-batch cultivations with different initial biomass contents to monitor q_s over time [12]. Interestingly, Cunha *et al.* also showed increased q_p at lower q_s and clearly stated that q_s was the most important induction parameter. However, despite the obvious effect of q_s on q_p, feeding profiles based on q_s have not been tested yet.

Regardless of which control parameter is chosen to set up feeding profiles for fed batch cultivations, strain specific parameters have to be determined. This can either be done by continuous cultures [13] or by several, consecutive fed batch cultivations, which are operated

at different parameter sets [14-15]. These methods have the disadvantage of being very time-consuming and labor-intensive. A robust method to determine these strain specific data and to develop a suitable feeding strategy in a shorter time period is of high interest in biotechnology to speed up process development and to quantitatively screen industrial relevant strains.

In this study, we developed a new and fast method to determine the strain specific data q_s and q_p, as well as the adaptation time of the culture to the substrate methanol, based on easy to do batch cultivations with methanol pulses. The host *P. pastoris* and the product horseradish peroxidase (HRP) were used as a model system, for which we subsequently developed a novel feeding strategy solely based on the determined q_s (Figure 4.1.1-1). Various fed batch cultivations, employing different q_s feeding profiles, were carried out to characterize the strain, hence, to determine yields ($Y_{X/S}$, $Y_{CO2/S}$) and the specific productivity (q_p) to evaluate and improve the feeding strategy. To our knowledge this is the first time that different dynamic feeding profiles only based on q_s were tested to develop a feeding strategy for *P. pastoris* aiming at increased productivity.

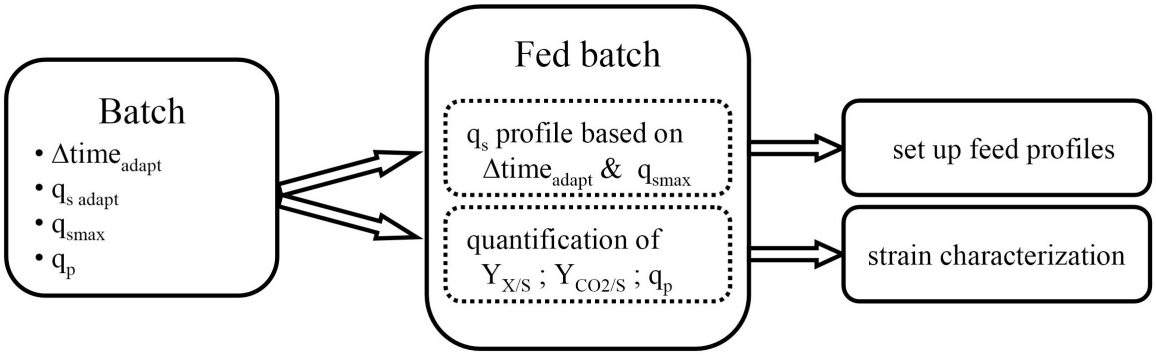


Figure 4.1.1-1 Experimental strategy for the determination of relevant strain specific parameters in a batch cultivation with methanol pulses. These strain specific data (Δtimeadapt - adaptation time; q_s adapt – specific substrate uptake rate during the adaptation period; q_s max – maximum specific substrate uptake rate) are then used to set up a feeding profile for P. pastoris in fed batch cultivations. The calculated yields ($Y_{X/S}$, $Y_{CO2/S}$) and the specific productivity (q_p) can consequently be used for strain characterization.

Material and Methods

Microorganism and recombinant protein

The *Pichia pastoris* strain KM71H (*arg4 aox1:arg4)* was transformed with a plasmid containing the gene for the horseradish peroxidase isoenzyme C1A (HRP) and was gratefully provided by Prof. Anton Glieder (Graz University of Technology, Austria). The phenotype of

the strain corresponded to an AOX1–deficient clone which is characterized as Mut^S (methanol utilization slow) and HRP was secreted into the fermentation broth.

Horseradish peroxidase is a member of the plant peroxidase super family [16] and catalyzes the oxidation of many substrates using hydrogen peroxide, resulting in oxidized products and in the formation of two molecules of water per molecule of hydrogen peroxide [17-19]. It is a heme-containing glycoprotein with a molecular weight of approximately 44 kDa that has been studied for more than 100 years [19]. Horseradish peroxidase gains more and more industrial relevance in the past few years; it is used in waste water treatment [20-21], analytical diagnostics [22] and for the elimination of H_2O_2 from food and industrial wastewater [23].

Stability of the enzyme horseradish peroxidase in the presence of methanol

To check whether certain concentrations of methanol were affecting the stability of the enzyme horseradish peroxidase, HRP was diluted either in water or in BSM medium to a concentration of 1 U/mL and incubated in a waterbath at 28°C in the presence of up to 20 mg/mL methanol (which corresponds to 2.5 % v/v) overnight. At several time points samples were taken and analyzed for catalytic activity and protein content.

Culture Media

Preculture: Yeast nitrogen base media (YNBM), per liter: potassium phosphate buffer (pH 6.0), 0.1 M; YNB w/o Amino acids and Ammonia Sulfate (Difco™), 3.4 g; $(NH_4)_2SO_4$, 10 g; biotin, 400 mg; glucose, 20 g.

Batch/fed batch: Basal salt media (BSM) [24], per liter: 85% phosphoric acid, 26.7 mL; $CaSO_4 \cdot 2H_20$, 1.17 g; K_2SO_4, 18.2 g; $MgSO_4 \cdot 7H_2O$, 14.9 g; KOH, 4.13 g; $C_6H_{12}O_6 \cdot H_2O$, 44 g, Antifoam Struktol J650, 0.2 mL; PTM1, 4.35 mL; NH_4OH as N-source (see experimental procedure). Trace element solution (PTM1), per litre: $CuSO_4 \cdot 5H_2O$, 6.0 g; NaI 0.08 g; $MnSO_4 \cdot H_2O$, 3.0 g; $Na_2MoO_4 \cdot 2H_2O$, 0.2 g; H_3BO_3, 0.02 g; $CoCl_2$, 0.5 g; $ZnCl_2$, 20.0 g; $FeSO_4 \cdot 7H_2O$, 65.0 g; biotin, 0.2 g, H_2SO_4, 5 mL.

Feed glucose, per liter: glucose, 250 g; PTM1, 12 mL, Struktol J650, 0.3 mL.

Feed methanol, per liter: methanol, 300 g; PTM1, 4 mL; Struktol J650, 0.3 mL, induction period was carried out in presence of δ-Aminolevulinic acid (δ-ALA), 1 mM.

Base: NH_4OH, concentration was determined by titration with 0.25 M potassium hydrogen phthalate (KHP).

Experimental Procedure

Preculture

Frozen stocks (-80 °C) were pre-cultivated in 100 mL of YNBM in 1000 mL shake flasks at 28 °C and 200 rpm for max. 24 hours. Then, the preculture was transferred aseptically to the respective culture vessel. The inoculation volume was approximately 10 % of the final starting volume.

Batch cultivation and determination of q_s

Batch cultivations were carried out in a 1 L working volume glass bioreactor (Applikon, Netherlands). Basal salt media was sterilized in the bioreactor and pH was adjusted to pH 5.0 by using concentrated ammonia solution after autoclaving. Sterile filtered trace elements were transferred to the reactor aseptically. Dissolved oxygen (dO_2) was measured with a sterilizable polarographic dissolved oxygen electrode (Mettler Toledo, Switzerland). The pH was measured with a sterilizable electrode (Mettler Toledo, Switzerland) and maintained constant with a step controller using ammonia solution (1 to 2 M). Base consumption was determined gravimetrically. Cultivation temperature was set to 28 °C and agitation was fixed to 1200 rpm. The culture was aerated with 1.25 vvm dried air and off-gas of the culture was measured by using an infrared cell for CO_2 and a paramagnetic cell for O_2 concentration (Servomax, Switzerland). Temperature, pH, dO_2, agitation as well as CO_2 and O_2 in the off-gas were measured online and logged in a process information management system (PIMS; Lucullus, Biospectra, Switzerland).

After the complete consumption of the substrate glucose, which was indicated by an increase of dissolved oxygen and a drop in off-gas activity, the first methanol pulse of a final concentration of 0.5 % (v/v) was conducted with pure methanol (supplemented with PTM1, 12 mL/L of methanol). Following pulses were performed with 1 % (v/v), before a last pulse with 2 % (v/v) final concentration of methanol was carried out. To investigate the metabolic activity during methanol excess and also the dynamic behavior of the cell metabolism after methanol limitation for several hours, the pulse experiments were performed like this: after methanol was depleted after the “first” pulse (followed by off-gas analysis), an immediate “second” methanol pulse with the same concentration was conducted per day. After methanol depletion, methanol starvation was carried out for several hours before another so-called “first” pulse was applied (Figure 4.1.1-2).

For each pulse, at least two samples were taken to determine the concentrations of substrate and product as well as dry cell weight and OD_{600} to calculate the specific substrate uptake rate q_s.

Fed batch cultivations

Fed batch cultures were carried out in a 7.5 L (5 L working volume) glass bioreactor (Infors, Switzerland). Concentrated BSM medium (2-fold concentrated to supply necessary salts for high cell densities) was sterilized in the bioreactor and pH was adjusted to pH 5.0 by using concentrated ammonia solution after autoclaving. The initial volume was set to 1.5 L. Trace elements were filter sterilized and transferred to the reactor aseptically. Dissolved oxygen was measured with a sterilizable polarographic dissolved oxygen electrode (Hamilton, Switzerland). The pH was measured with a sterilizable electrode (Hamilton, Switzerland) and maintained constant using ammonia solution (3 to 5 M). Agitation was set to 1500 rpm. The culture was aerated with at least 1 vvm to avoid oxygen limitation. The dissolved oxygen signal was used to adjust air-in flow manually to keep levels >30 % dO_2 at all time points. In case air flow was limited, pure oxygen was added. CO_2 and O_2 were measured as described above. Base consumption and reactor weight were measured gravimetrically. The fed batch feed was measured and controlled using a gravimetrically based PID flow controller.

At several time points during fed batch cultivations, samples were taken and analyzed for accumulated methanol, biomass concentration (dry cell weight and optical density OD_{600}), protein content and enzymatic activity. Based on the total biomass content, feed rates were adjusted manually corresponding to the defined q_s setpoint.

Three different fed batch strategies were tested: fed batch A, where methanol was adjusted to a constant flow during the whole induction phase; fed batch B, where after the adaption time with a $q_{s\ adapt}$, a shift to a high substrate uptake rate of ~90 % of $q_{s\ max}$ was done and adjustments to the very same q_s set point during cultivation were performed repeatedly; and fed batches C1 and C2, where after the adaptation period with $q_{s\ adapt}$, the methanol flow was stepwise increased up to $q_{s\ max}$. Values for $q_{s\ adapt}$ and $q_{s\ max}$ had been determined in batch experiments (*vide supra*). An overview of the fed batch cultivations and the corresponding settings is given in Table 4.1.1-1.

Table 4.1.1-1 Description of feeding strategies for fed batch cultivations based on the specific substrate uptake rate qs.

Fed batch name	Symbol	Description of the strategy
Fed batch A	▲	conventional feeding strategy: long adaptation time and initially adjusted, constant flow rate
Fed batch B	♦	short adaptation time (out of batch exp.) and adjustment to high uptake rate (90 % of $q_{s\,max}$) with repeated readjustments
Fed batch C1	▼	short adaptation time (out of batch exp.) and stepwise adjustment of q_s until $q_{s\,max}$ with repeated readjustments
Fed batch C2	●	

Analysis of growth- and expression-parameters

Dry cell weight (DCW) was determined by centrifugation of 5 mL culture broth (5000 rpm, 4 °C, 10 min) in a laboratory centrifuge (Sigma 4K15, rotor 11156), washing the pellet with 5 mL deionized water and subsequent drying at 105°C to a constant weight in an oven. Optical density of the culture broth was measured using a spectrophotometer (U-1100 Hitachi, Japan) at a wavelength of 600 nm (OD_{600}). Correlation between dry cell weight measurement and OD_{600} showed a coefficient of regression of R^2=0.997 over the full concentration range (data not shown) and could therefore be used for q_s adaptation.

The activity of HRP was determined using a CuBiAn XC enzymatic robot (Innovatis, Germany). Cell free samples (10 µl) were added to 140 µl of 1 mM ABTS (2.2' azino bis 3-ethylbenzthiazoline-6-sulphonic acid) prepared in 50 mM NaOAc buffer (pH 4.5). The reaction mixture was incubated at 37 °C and was started by the addition of 20 µl of 0.075 % H_2O_2. Changes of absorbance at 415 nm were measured for 80 seconds and rates were calculated. Calibration was done using commercially available horseradish peroxidase (Type VI-A, Sigma-Aldrich, P6782, Lot# 118K76703) as standard at six different concentrations (0.02; 0.05; 0.1; 0.25; 0.5 and 1.0 U/mL). Samples with high enzymatic activity were automatically diluted by the system. Protein concentrations were determined at 595 nm by the Bradford assay [25] using the BioRad Protein Assay Kit with BSA as standard.

Substrate concentrations

Concentrations of methanol were determined in cell free samples by HPLC (Agilent Technologies, USA) equipped with a Supelcoguard column, a Supelcogel C-610H ion-exchange column (Sigma-Aldrich, USA) and a refractive index detector (Agilent

Technologies, USA). The mobile phase was 0.1 % H_3PO_4 with a constant flow rate of 0.5 mL/min and the system was run isocratic. Calibration was done by measuring standard points in the range of 0.1 to 10 g/L methanol.

Concentrations of glucose were determined in cell free samples by a commercial enzymatic assay kit using the CuBiAN XC enzymatic robot (Innovatis, Germany). Calibration was done with 4 standard points in the range from 0 to 3 g/L glucose. Samples with higher glucose concentration were diluted automatically by the system.

Data analysis

Measurements of biomass concentration, product concentration and substrate concentration were executed in duplicates: along the observed standard deviation for the single measurement, the error was propagated to the specific rates q_s and q_p as well as to the yield coefficients. The error of determination of the specific rates and the yields was therefore set to 10 % and 5 %, respectively.

Electrophoresis

To check the purity of the excreted HRP, electrophoresis was done with aliquots of supernatants obtained at different time points during the cultivation of *P. pastoris* expressing the *hrp* gene extracellularly as described by Laemmli *et al*. [26]. SDS-PAGE was performed using a 5% stacking gel and a 10% separating gel in 1x Tris-glycine buffer. Gels were run in the vertical electrophoresis Mini-PROTEAN Tetra Cell apparatus (Biorad; Vienna, Austria) at 150 V for about 2 h. Gels were stained with Coomassie blue. The protein mass standard used was the PageRuler Prestained Ladder (Fermentas; Vienna, Austria).

Results and Discussion

Stability of the enzyme horseradish peroxidase in the presence of methanol

To check whether the enzyme horseradish peroxidase denatures at certain concentrations of methanol, HRP was incubated in the presence of methanol concentrations of up to 2.5 % (v/v) at 28 °C overnight. No loss in catalytic activity and protein content was detected after this incubation, which is why methanol pulses in batch experiments with concentrations of up to 2.5 % (v/v) were theoretically possible without any denaturing effects on HRP.

Determination of strain specific parameters by easy to do batch cultivations with methanol pulses

After depletion of glucose in batch cultivations, a first methanol adaption pulse with a final concentration of 0.5 % (v/v) was applied. The adaptation time to the new substrate methanol ($\Delta time_{adapt}$) was determined with 7 hours and was defined by the detection of a maximum in off-gas activity (Figure 4.1.1-2A).

The calculated carbon dioxide evolution rate (CER), signifying metabolic activity, allowed to distinguish different states within the methanol pulses with local minima and maxima (Figure 4.1.1-2). This metabolic behavior of the cells results from inhibition and regulation events in transient conditions, caused by intracellular components like e.g. produced H_2O_2, and transport actions, and has been described for various systems in literature before [27-29].

The high frequent determination of biomass, methanol and product concentrations allowed specific rate calculations for methanol uptake q_s and productivity q_p during the methanol pulses. Specific substrate uptake rates were calculated with 0.8 ± 0.08 $mmol \cdot g^{-1} \cdot h^{-1}$ in the adaptation period ($q_{s\ adapt}$) and with around 2 ± 0.20 $mmol \cdot g^{-1} \cdot h^{-1}$ as a maximum during pulses ($q_{s\ max}$). No difference in the calculated q_s between the “first” and the subsequent “second” pulse on a respective day was observed (Figure 4.1.1-2A). In contrast, the calculated values for q_p (Figure 4.1.1-2B) were very different between the first and the subsequent second pulse. During the first pulse specific productivities of maximum 1.3 ± 0.13 $U \cdot g^{-1} \cdot h^{-1}$ were observed, whereas during the second pulse an increased specific productivity of up to 2.5 ± 0.25 $U \cdot g^{-1} \cdot h^{-1}$ was measured. This increased productivity during the second methanol pulse may be due to the fact that no adaption of the cells to methanol was necessary because all metabolic key functions for methanol assimilation remained in an active state. Thus, recombinant protein expression could start directly and energy was used more efficiently for product formation. This result shows clearly that *Pichia* cultures should be kept induced at all time to obtain maximal productivity. Interestingly, q_p also increased over time for first and second pulses, respectively (Figure 4.1.1-2B). Methanol pulses, where the same final concentration of methanol was used, but which were conducted in the later phase of the batch cultivation, showed higher specific productivities compared to pulses before. Obviously, the culture exhibited a “memory effect” and thus a time-dependence of q_p could be observed in the batch experiment.

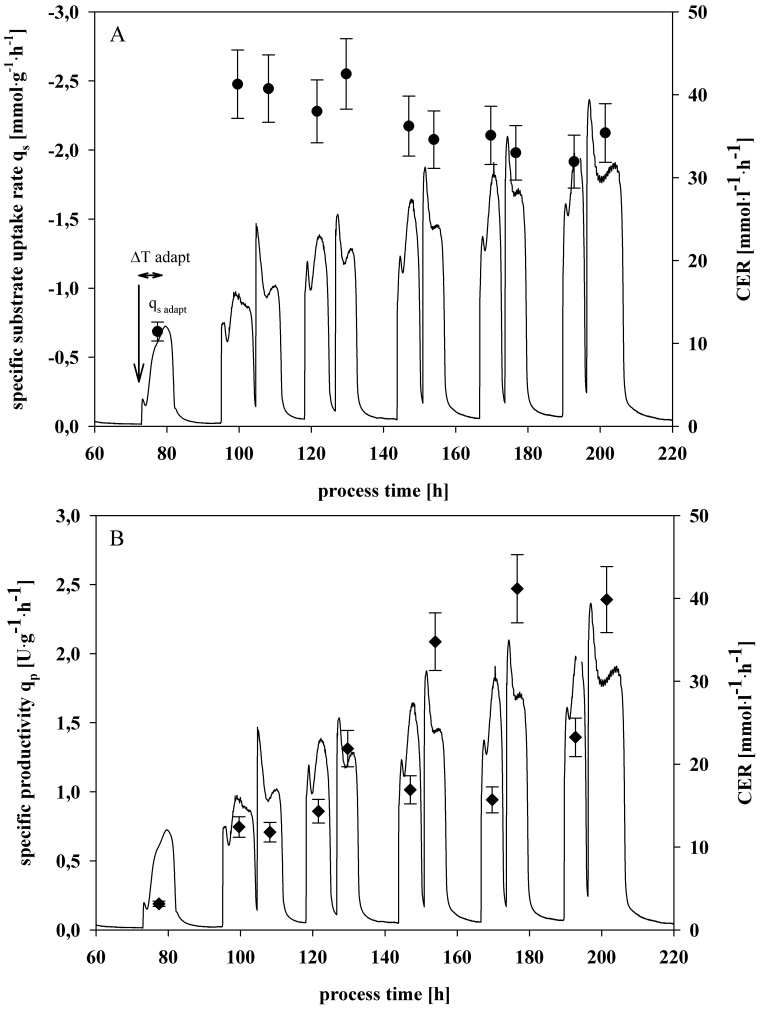


Figure 4.1.1-2 Batch cultivation with methanol pulses of 0.5, 1 and 2 % (v/v). A, calculated specific substrate uptake rate q_s (circle) and carbon dioxide evolution rate (CER); B, specific production rate q_p (diamond) and carbon dioxide evolution rate (CER).

As we show here, batch cultivations with methanol pulses allowed a fast identification of strain specific parameters, which are crucial for subsequent fed batch cultivations. The determined maximum specific substrate uptake rate $q_{s\ max}$, represents the upper end of the feed profile respective to q_s. This novel method has the advantage of being less time-consuming and labor-intensive compared to the traditional methods, like continuous cultivations, and additionally allows a free choice of substrate, like e.g. the 2 to 3-fold cheaper glucose instead of glycerol.

Fed batch feeding strategy based on q_s

Based on the batch results, we performed several fed batch cultivations with different feeding profiles based on the specific substrate uptake rate q_s, to find a feeding strategy for a recombinant *P. pastoris* strain.

Feeding profiles

After a batch phase on glucose as substrate (volume 1.5 L), an exponential fed batch cultivation with glucose yielded in biomass concentrations of up to 70 g/L in a volume of 2.5 L. At the end of this fed batch phase, a sample was taken to determine the current biomass concentration by measuring the OD_{600} and the DCW.

Based on the calculated specific methanol uptake rate during adaptation ($q_{s\ adapt}$) from the batch experiment, which was around 0.8 ± 0.08 mmol·g^{-1}·h^{-1}, we used a little bit lower $q_{s\ adapt}$ of 0.5 mmol·g^{-1}·h^{-1} methanol for the fed batch cultivations during the adaption time. After $\Delta time_{adapt}$, three different feeding strategies (fed batches A, B and C1/C2) were tested and compared (Figure 4.1.1-3, Table 4.1).

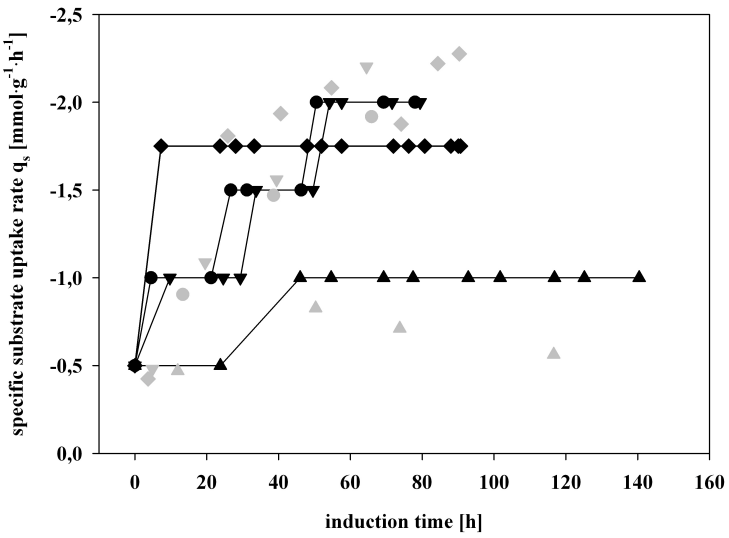


Figure 4.1.1-3 Specific substrate uptake rate profiles which were used for different fed batch cultivations. Theoretical q_s fed batch A (black triangle up), offline determined q_s fed batch A (grey triangle up); theoretical q_s fed batch B (black diamond), offline determined q_s fed batch B (grey diamond); theoretical q_s fed batch C1 (black triangle down), offline determined q_s fed batch C1 (grey triangle down); theoretical q_s fed batch C2 (black circle), offline determined q_s fed batch C2 (grey circle). The error of determination for offline q_s (10 %) is not shown for better readability.

For fed batch A, which described a conventional feeding strategy as in the Invitrogen protocol, the methanol feed was adjusted to a substrate uptake rate of 1.0 mmol·g^{-1}·h^{-1}. The adjustment just happened at the beginning and no further readjustments of the feeding rate

were performed during the cultivation. Consequently, as biomass still increased, a drop down of q_s over time was observed (Figure 4.1.1-3).

In the other fed batch experiments B, C1 and C2 adjustments of q_s were done after each sampling, based on OD_{600} measurements. As shown in Figure 3, the effective q_s, determined by off-line sampling, and the designated q_s in these fed batch cultivations were very similar. Thus, a frequent determination of the actual biomass concentration in the reactor vessel is necessary, to be able to adjust the feeding rate to the chosen q_s. The development of robust measurement devices for the online determination of the biomass concentration, as e.g. FTIR, capacity probes or soft sensors, could allow an online adjustment of the feeding rate to q_s by an automatic control system in the future, and thus the labor-intensive, frequent sampling procedure would be unnecessary.

Maintenance metabolism vs. cell growth

The relationship between q_s and the specific growth rate was tested and found to be linearly correlated (data not shown). Based on this, the maintenance coefficient for this *P. pastoris* strain could be determined with around 0.5 $mmol \cdot g^{-1} \cdot h^{-1}$ (which equals 0.016 $g \cdot g^{-1} \cdot h^{-1}$). Very similar results were reported by Jahic *et al.* for fed batch cultures using *P. pastoris* expressing a fusion protein [5] with a maintenance coefficient of 0.013 $g \cdot g^{-1} \cdot h^{-1}$. When we adjusted q_s to values higher than 1.0 $mmol \cdot g^{-1} \cdot h^{-1}$, we observed a significant increase in $Y_{X/S}$ and a corresponding decrease in $Y_{CO2/S}$ (Figure 4.1.1-4). That means that at q_s of <1.0 $mmol \cdot g^{-1} \cdot h^{-1}$ most of the energy is obviously used for maintenance metabolism of the cells and thus for the production of CO_2 and not for cell growth or protein production. Interestingly, the same effect on biomass yield was observed by d'Anjou *et al.* using a continuous culture system with a mixed feed strategy [13]. However, we were able to extract this information from time-efficient, dynamic experiments equivalently. Consequently, we employed specific substrate uptake rates of >1.0 $mmol \cdot g^{-1} \cdot h^{-1}$ to guarantee a sufficient amount of energy for both, maintenance and cell growth.

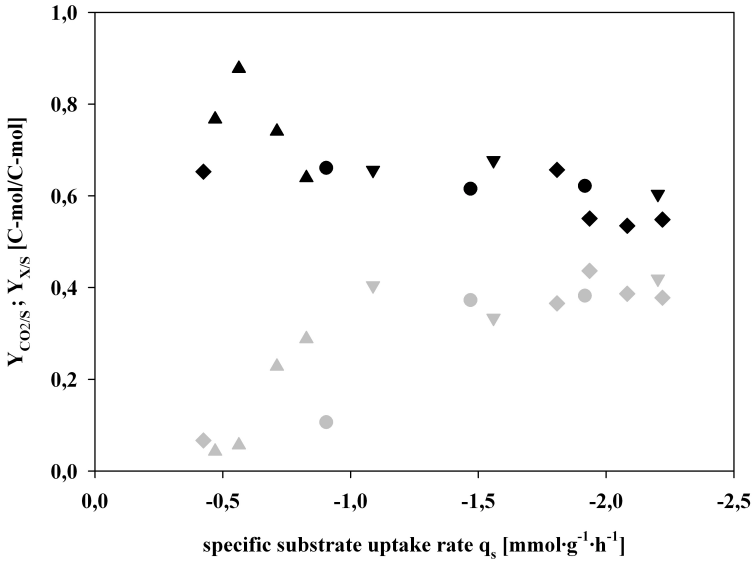


Figure 4.1.1-4 Carbon dioxide yield ($Y_{CO2/S}$) and biomass yield ($Y_{X/S}$) at different specific substrate uptake rates in fed batch cultivations. $Y_{CO2/S}$ fed batch A (black triangle up), $Y_{X/S}$ fed batch A (grey triangle up); $Y_{CO2/S}$ fed batch B (black diamond), $Y_{X/S}$ fed batch B (grey diamond); $Y_{CO2/S}$ fed batch C1 (black triangle down), $Y_{X/S}$ fed batch C1 (grey triangle down); $Y_{CO2/S}$ fed batch C2 (black circle); $Y_{X/S}$ fed batch C2 (grey circle). The error of determined yields (5 %) is not shown for better readability.

Required methanol concentration for induction – a principle question

When producing recombinant proteins with *P. pastoris*, the principle question is how much methanol has to be fed to guarantee a fully induced AOX promoter. A lot of different studies have examined this topic, with different results. D'Anjou *et al.*, for example, reported that a methanol concentration between 1 and 2 g/L should be maintained in the culture to guarantee fully induced heterologous protein production [13]. Another study of the methanol concentration and its effect on *P. pastoris* Mut^S strains was done by Kupcsulik and Sevella, who showed that the specific productivity of a recombinant human serum albumin expression system showed a maximum at 0.45 g/L of methanol [30]. In contrast to those studies, Cregg reported an even stronger induction of the AOX promoter in limited conditions [31], which was the basis for several following studies applying a limited methanol supply for induction [1-3].

For all described fed batch strategies in our study, no significant methanol accumulation was detected (detection limit 0.1 g/L). We clearly show that high residual concentrations of methanol during the production phase are not required for the induction of the *P. pastoris* Mut^S strain, as even higher specific productivities were obtained in limited fed batches with constant substrate uptake rates, compared to the batch cultivations with high methanol concentrations applied in the pulse-experiments (Table 4.1.1-2).

Table 4.1.1-2 Comparison of the specific substrate uptake rates qs and determined maximum specific productivities qp.

Experiment[a]	**Methanol concentration**	**Specific substrate uptake rate qs** [mmol·g^{-1}·h^{-1}]	**Specific productivity qp** [U·g^{-1}·h^{-1}]
Batch with methanol pulses	from 0 to 16 g/L	~ 2	2.5 ± 0.25
Fed batch A ▲	limited	<1	2.0 ± 0.20
Fed batch B ♦	limited	1.75	5.0 ± 0.50
Fed batch C1 ▼	limited	stepwise up to 2	10 ± 0.10
Fed batch C2 ●	limited	stepwise up to 2	11 ± 0.11

[a] all experiments were performed in the presence of different concentrations of the inducer methanol

Extracellular protein production and specific productivity q_p

To follow the formation of excreted horseradish peroxidase (HRP) during the induction phase, SDS-PAGE analyses with cell-free supernatants were performed. SDS-PAGE analysis (Coomassie staining, Figure 4.1.1-5) of the cultivation broth taken at different time points did not show a distinct band for HRP, but rather a smear between 60 – 65 kDa. The difference between the reported molecular weight of around 44 kDa for HRP and the observed molecular weight in this study results from the high degree of glycosylation of the recombinant protein expressed in *P. pastoris* with mannose-type oligosaccharides, as described previously for HRP by Morawski *et al.* [32].

The protein content in the cultivation broth increased over time from 0.052 mg/mL after the fed-batch phase with glucose to 0.243 mg/mL at the end of the cultivation, which were around 91 hours of induction. As shown in Figure 4.1.1-5, the increase of the protein content in the cultivation broth is ascribed to an increased amount of HRP, which constituted the majority of secreted proteins in the cultivation broth.

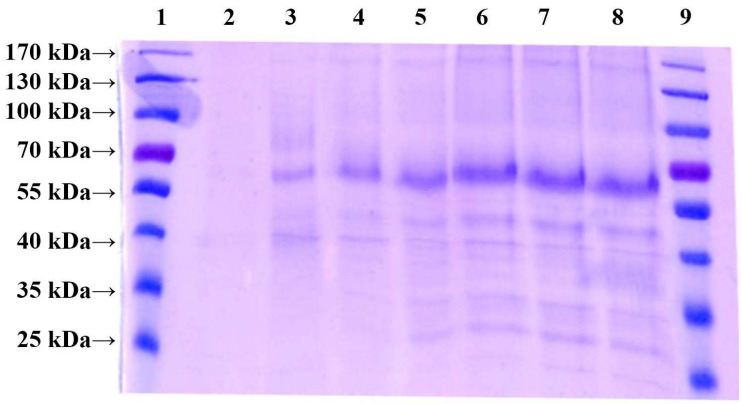


Figure 4.1.1-5 SDS-PAGE analysis of recombinantly produced HRP in fed batch B. Aliquots of the supernatant obtained at different time points during the cultivation of *P. pastoris* expressing the hrp gene extracellularly at 28 °C were loaded onto the gel. lanes 1 and 9, molecular mass standard; lane 3, not induced (after fed-batch phase with glucose); lane 3-8, 7 h; 33 h; 58 h; 76 h; 88 h; 91 h of induction.

Based on the determined biomass content and the enzymatic activity, the specific productivity q_p during the different fed batch cultivations was calculated. The lowest q_p was obtained in fed batch A, which described a conventional feeding strategy, where also the lowest q_s was used and was not adapted over time (Figure 4.1.1-6). In fed batch B, which considered the actual biomass concentration and where the q_s of 1.75 $mmol \cdot g^{-1} \cdot h^{-1}$ was adapted regularly over time and thus the cells were kept in a certain physiological state, a 2-fold increase in q_p was observed. Clearly, a higher q_s resulted in a higher q_p, which disagrees with the results obtained by Khatri and Hoffmann and Cunha *et al.* [10-11], who stated that lower q_s resulted in higher protein production.

However, a dynamic, stepwise feeding strategy resulted in an even higher productivity compared to the other strategies tested (Figure 4.1.1-6). This dynamic feeding strategy considered $q_{s\,max}$, which had been determined in the batch experiment, as the highest possible substrate uptake rate of the cells, as well as the yield coefficients and the maintenance coefficient as the lower end of efficient energy usage. The feed profile was set up in a way to head off as quickly as possible from the maintenance state of the cells (hence q_s was set to values ≥ 1.0 $mmol \cdot g^{-1} \cdot h^{-1}$), before q_s was dynamically adapted in steps to $q_{s\,max}$. In fed batch cultivations C1 and C2, where this dynamic feeding strategy was applied, a q_p of around 11 ± 0.11 $U \cdot g^{-1} \cdot h^{-1}$ was determined, which represents a 5.5-fold increase compared to fed batch A.

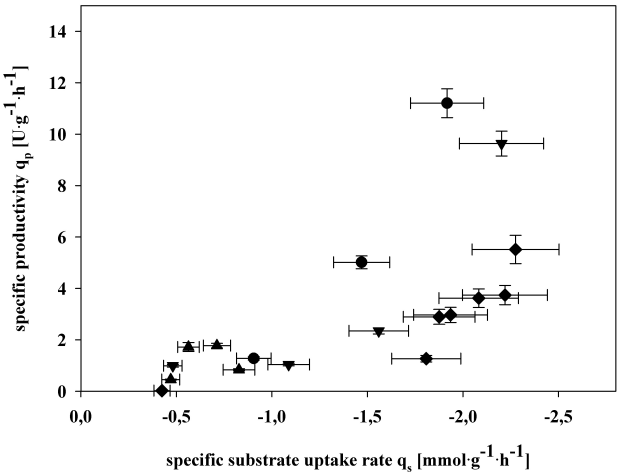


Figure 4.1.1-6 Specific productivity (q_p) plotted against the specific substrate uptake rate (q_s). Fed batch A (triangle up); fed batch B (diamond); fed batch C1 (triangle down); fed batch C2 (circle).

Time adaptation

The fact that there was still a more than 2-fold difference in q_p between fed batches C1/C2, where q_s was increased stepwise, and fed batch B, where immediately after the adaption period a high q_s of 1.75 mmol·g^{-1}·h^{-1} was applied, indicated another factor being crucial for the specific productivity besides q_s. Therefore, the specific productivity q_p was plotted against the induction time, to analyze possible time-dependent effects (Figure 4.1.1-7). Fed batch A and B were characterized by a linear increase of q_p over time (fed batch B was characterized by a significant steeper slope), whereas fed batches C1 and C2 even showed an exponential increase of the specific productivity. It became obvious that the productivity was directly correlated to the induction time for all experiments conducted in this study, even in the batch experiments (Figure 4.1.1-2). Plantz *et al.* have recently reported an influence of the induction time on q_p as well. They showed increasing product yields for the recombinant production of an interferon with *P. pastoris* in the first period of induction, but a subsequent decrease in product formation and a shift of the energy transfer towards biomass growth during the later phase of the cultivation [8]. In contrast to the study of Plantz *et al.*, we clearly showed an increase of q_p over the whole induction time for all experiments in this study (Figure 4.1.1-7).

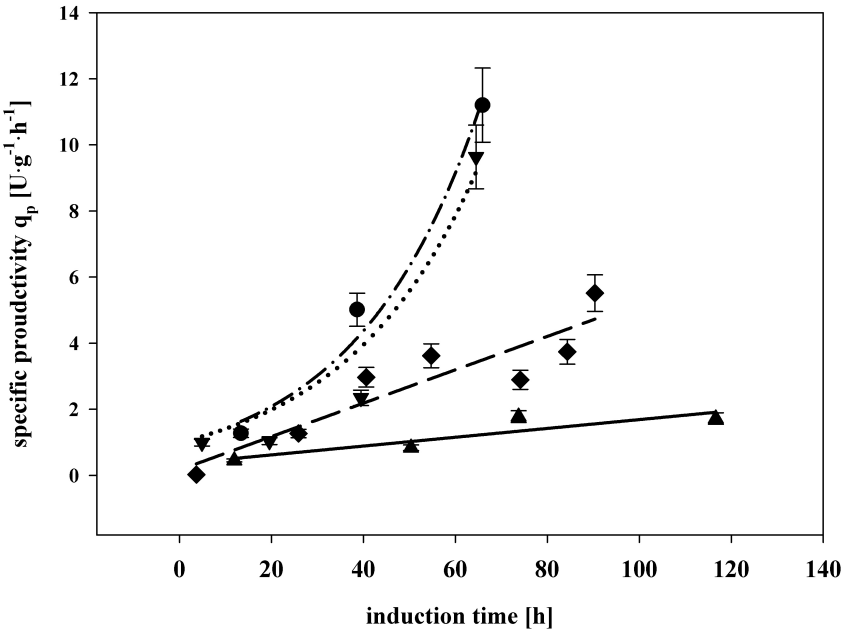


Figure 4.1.1-7 Specific productivity (q_p) in fed batch cultures over the induction time. Fed batch A (triangle up); fed batch B (diamond); fed batch C1 (triangle down); fed batch C2 (circle). Regression lines: linear regression for fed batch A (continuous line); linear regression for fed batch B (discontinuous line); exponential regression for fed batch C1 (dotted line); exponential regression for fed batch C2 (dashed-dotted line).

Furthermore, fed batches C1 and C2 were characterized by an exponential increase of q_p over time, in contrast to a linear increase for fed batches A and B (Figure 4.1.1-7). Obviously, a feeding strategy starting with a lower q_s at the beginning of the induction phase, followed by a subsequent increase with a stepwise, dynamic feeding profile until $q_{s\ max}$ (fed batch C1 and C2), was superior to a feeding strategy, where immediately after the $\Delta time_{adapt}$ a high q_s was applied and kept constant (fed batch B). When the cells had the time to undergo a dynamic, physiological adaptation to the current culture condition before q_s was increased, a boost in q_p was observed, which probably cannot be triggered by constant or exponential feeding regimes (Table 4.1.1-2).

Summarizing, we showed a clear time dependent trajectory for specific product formation, as q_p increased over induction time for all experiments in this study. A tool for the early identification of this effect is of utmost importance in order to consistently compare different feeding profiles.

Conclusions

In this study, we developed a fast and easy to do method based on batch cultivations with methanol pulses to determine strain specific parameters of a *P. pastoris* Mut^S strain expressing the enzyme horseradish peroxidase. A subsequently developed dynamic feeding strategy solely based on q_s, where the cells on the one hand had time to adapt to culture conditions, but were then challenged again repeatedly by a stepwise increase of q_s up to $q_{s\,max}$, resulted in the highest q_p compared to the other strategies tested. Hence, dynamic feeding profiles turned out to be a valuable method to boost the specific productivity. This calls for increased use of dynamic process conditions even for industrial feed profiles. We strongly believe that the strategy presented here can be successfully applied on other microbial expression systems, which is why we are currently testing the applicability of our novel, dynamic approach on other expression systems, like *E. coli*, and other products.

List of Abbreviations

$\Delta time_{adapt}$ time for adaptation of the culture to the new substrate (methanol) [h]; μ, specific growth rate [h^{-1}]; CER, carbon dioxide evolution rate [$mmol \cdot L^{-1} \cdot h^{-1}$]; HRP, horseradish peroxidase; Mut^S, methanol utilization slow phenotype; PID, proportional-integrative-derivative controller; q_p, specific productivity of horseradish peroxidase [$U \cdot g^{-1} \cdot h^{-1}$]; q_s, specific substrate uptake rate [$mmol \cdot g^{-1} \cdot h^{-1}$]; $q_{s\,adapt}$, specific substrate uptake rate during adaptation [$mmol \cdot g^{-1} \cdot h^{-1}$]; $q_{s\,max}$, maximum specific substrate uptake rate [$mmol \cdot g^{-1} \cdot h^{-1}$], rpm, rounds per minute, vvm, volume gas flow per volume liquid per minute, $Y_{CO2/S}$, yield coefficient of carbon dioxide respective to methanol [$C\text{-}mol \cdot C\text{-}mol^{-1}$], $Y_{X/S}$, yield coefficient of biomass respective to methanol [$C\text{-}mol \cdot C\text{-}mol^{-1}$]

Competing interests

The authors declare that they have no competing interests.

Author contributions

CD designed and performed the experiments and analyzed data. OS performed some experiments. CD and OS wrote the paper, CH conceived the study and supervised research. All authors read and approved the final manuscript.

Acknowledgements

The authors are very grateful to Prof. Anton Glieder and his workgroup for providing the *P. pastoris* Mut^S strain expressing the enzyme horseradish peroxidase. Additionally, the authors express their gratitude to D. V. Rial (Consejo Nacional de Investigaciones Científicas y Técnicas CONICET, Argentina), who supported the experimental work in the lab.

References

1. Potgieter TI, Kersey SD, Mallem MR, Nylen AC, d'Anjou M: **Antibody expression kinetics in glycoengineered Pichia pastoris.** *Biotechnology and Bioengineering* 2010**, 106:**918-927.
2. Trinh LB, Phue JN, Shiloach J: **Effect of methanol feeding strategies on production and yield of recombinant mouse endostatin from Pichia pastoris.** *Biotechnology and Bioengineering* 2003, **82:**438-444.
3. Kobayashi K, Kuwae S, Ohya T, Ohda T, Ohyama M, Tomomitsu K: **High level secretion of recombinant human serum albumin by fed-batch fermentation of the methylotrophic yeast, Pichia pastoris, based on optimal methanol feeding strategy.** *Journal of Bioscience and Bioengineering* 2000, **90:**280-288.
4. Jacobs P, Inan M, Festjens N, Haustraete J, Van Hecke A, Meagher M, Contreras R, Callewaert N: **Fed-batch fermentation of GM-CSF-producing glycoengineered Pichia pastoris under controlled specific growth rate.** *Microbial Cell Factories* 2010, **9:**93.
5. Jahic M, Rotticci-Mulder JC, Martinelle M, Hult K, Enfors SO: **Modeling of growth and energy metabolism of Pichia pastoris producing a fusion protein.** *Bioprocess and Biosystems Engineering* 2002, **24:**385-393.
6. Zhang W, Sinha J, Smith LA, Inan M, Meagher MM: **Maximization of Production of Secreted Recombinant Proteins in Pichia pastoris Fed-Batch Fermentation.** *Biotechnology Progress* 2005, **21:**386-393.
7. Sinha J, Plantz BA, Zhang W, Gouthro M, Schlegel V, Liu C-P, Meagher MM: **Improved production of recombinant ovine interferon-t by Mut+ strain of Pichia pastoris using an optimized methanol feed profile.** *Biotechnology Progress* 2003, **19:**794-802.
8. Plantz BA, Sinha J, Villarete L, Nickerson KW, Schlegel VL: **Pichia pastoris fermentation optimization: energy state and testing a growth-associated model.** *Applied Microbiology and Biotechnology* 2006, **72:**297-305.
9. Ohya T, Ohyama M, Kobayashi K: **Optimization of human serum albumin production in methylotrophic yeast Pichia pastoris by repeated fed-batch fermentation.** *Biotechnology and Bioengineering* 2005, **90:**876-887.
10. Khatri NK, Hoffmann F: **Oxygen-limited control of methanol uptake for improved production of a single-chain antibody fragment with recombinant Pichia pastoris.** *Applied Microbiology and Biotechnology* 2006, **72:**492-498.
11. Khatri NK, Hoffmann F: **Impact of methanol concentration on secreted protein production in oxygen-limited cultures of recombinant Pichia pastoris.** *Biotechnology and Bioengineering* 2006, **93:**871-879.
12. Cunha AE, Clemente JJ, Gomes R, Pinto F, Thomaz M, Miranda S, Pinto R, Moosmayer D, Donner P, Carrondo MJT: **Methanol induction optimization for scFv antibody fragment production in Pichia pastoris.** *Biotechnology and bioengineering* 2004, **86:**458-467.
13. d'Anjou MC, Daugulis AJ: **A rational approach to improving productivity in recombinant Pichia pastoris fermentation.** *Biotechnology and bioengineering* 2001, **72:**1-11.
14. Ren H, Yuan J: **Model-based specific growth rate control for Pichia pastoris to improve recombinant protein production.** *Journal of Chemical Technology and Biotechnology* 2005, **80:**1268-1272.
15. Ren HT, Yuan JQ, Bellgardt KH: **Macrokinetic model for methylotrophic Pichia pastoris based on stoichiometric balance.** *Journal of Biotechnology* 2003, **106:**53-68.

16. Welinder KG: **Amino acid sequence studies of horseradish peroxidase. 4. Amino and carboxyl termini, cyanogen bromide and tryptic fragments, the complete sequence, and some structural characteristics of horseradish peroxidase C.** *European Journal of Biochemistry* 1979, **96:**483-502.
17. Gajhede M: **Horseradish peroxidase.** In *Handbook of Metalloproteins. Volume* 1; 2001: 195-210
18. Rodriguez-Lopez JN, Lowe DJ, Hernandez-Ruiz J, Hiner ANP, Garcia-Canovas F, Thorneley RNF: **Mechanism of reaction of hydrogen peroxide with horseradish peroxidase: Identification of intermediates in the catalytic cycle.** *Journal of the American Chemical Society* 2001, **123:**11838-11847.
19. Veitch NC: **Horseradish peroxidase: a modern view of a classic enzyme.** *Phytochemistry (Elsevier)* 2004, **65:**249-259.
20. Lai Y-C, Lin S-C: **Application of immobilized horseradish peroxidase for the removal of p-chlorophenol from aqueous solution.** *Process Biochemistry (Oxford, United Kingdom)* 2004, **40:**1167-1174.
21. Dalal S, Gupta MN: **Treatment of phenolic wastewater by horseradish peroxidase immobilized by bioaffinity layering.** *Chemosphere* 2007, **67:**741-747.
22. Azevedo AM, Martins VC, Prazeres DMF, Vojinovic V, Cabral JMS, Fonseca LP: **Horseradish peroxidase: a valuable tool in biotechnology.** *Biotechnology Annual Review* 2003, **9:**199-247.
23. Hamid M, Khalil ur R: **Potential applications of peroxidases.** *Food Chemistry* 2009, **115:**1177-1186.
24. Cregg JM, Editor: *Pichia Protocols, Second Edition. [In: Methods Mol. Biol. (Totowa, NJ, U. S.)], 2007; 389]*. 2007.
25. Bradford MM: **A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding.** *Analytical Biochemistry* 1976, **72:**248-254.
26. Laemmli UK: **Cleavage of structural proteins during the assembly of the head of bacteriophage T4.** *Nature (London, United Kingdom)* 1970, **227:**680-685.
27. Herwig C, von Stockar U: **A small metabolic flux model to identify transient metabolic regulations in Saccharomyces cerevisiae.** *Bioprocess and Biosystems Engineering* 2002, **24:**395-403.
28. Diderich JA, Schepper M, van Hoek P, Luttik MA, van Dijken JP, Pronk JT, Klaassen P, Boelens HF, de Mattos MJ, van Dam K, Kruckeberg AL: **Glucose uptake kinetics and transcription of HXT genes in chemostat cultures of Saccharomyces cerevisiae.** *The Journal of biological chemistry* 1999, **274:**15350-15359.
29. Rizzi M, Theobald U, Querfurth E, Rohrhirsch T, Baltes M, Reuss M: **In vivo investigations of glucose transport in Saccharomyces cerevisiae.** *Biotechnology and Bioengineering* 1996, **49:**316-327.
30. Kupcsulik B, Sevella B: **Effects of methanol concentration on the recombinant Pichia pastoris MutS fermentation.** *Periodica Polytechnica, Chemical Engineering* 2004, **48:**73-87.
31. Cregg JM: **Expression in the methylotrophic yeast Pichia pastoris.** *Gene Expression Systems* 1999**:**157-191.
32. Morawski B, Lin ZL, Cirino PC, Joo H, Bandara G, Arnold FH: **Functional expression of horseradish peroxidase in Saccharomyces cerevisiae and Pichia pastoris.** *Protein Engineering* 2000, **13:**377-384.

4.1.2. A fast approach to determine a fed batch feeding profile for recombinant *Pichia pastoris* strains

Christian Dietzsch, Oliver Spadiut, Christoph Herwig*

Vienna University of Technology, Institute of Chemical Engineering, Research Area Biochemical Engineering, Vienna, Austria

*Corresponding Author: Christoph Herwig, Vienna University of Technology, Institute of Chemical Engineering, Research Area Biochemical Engineering, Gumpendorfer Strasse 1a, A-1060 Vienna, Austria. Tel: +43 1 85501 166400, christoph.herwig@tuwien.ac.at

Manuscript published:
Dietzsch, C.; Spadiut, O.; Herwig, C., **A fast approach to determine a fed batch feeding profile for recombinant Pichia pastoris strains.** *Microb. Cell Fact.* 2011, 10, (1), 85.

Abstract

Background

The microorganism *Pichia pastoris* is a commonly used microbial host for the expression of recombinant proteins in biotechnology and biopharmaceutical industry. To speed up process development, a fast methodology to determine strain characteristic parameters, which are needed to subsequently set up fed batch feeding profiles, is required.

Results

Here, we show the general applicability of a novel approach to quantify a certain minimal set of bioprocess-relevant parameters, *i.e.* the adaptation time of the culture to methanol, the specific substrate uptake rate during the adaptation phase and the maximum specific substrate uptake rate, based on fast and easy-to-do batch cultivations with repeated methanol pulses in a batch culture. A detailed analysis of the adaptation of different *P. pastoris* strains to methanol was conducted and revealed that each strain showed very different characteristics during adaptation, illustrating the need of individual screenings for an optimal parameter definition during this phase. Based on the results obtained in batch cultivations, dynamic feeding profiles based on the specific substrate uptake rate were employed for different *P. pastoris* strains. In these experiments the maximum specific substrate uptake rate, which had been defined in batch experiments, also represented the upper limit of methanol uptake, underlining the validity of the determined process-relevant parameters and the overall experimental strategy.

Conclusion

In this study, we show that a fast approach to determine a minimal set of strain characteristic parameters based on easy-to-do batch cultivations with methanol pulses is generally applicable for different *P. pastoris* strains and that dynamic fed batch strategies can be designed on the specific substrate uptake rate without running the risk of methanol accumulation.

Keywords: *Pichia pastoris*, strain characterization, specific substrate uptake rate, batch cultivation, methanol pulse, dynamic feeding profile

Background

Advances in molecular biology, cloning techniques and strain improvement allowed an increasing use of recombinant organisms for the industrial production of a variety of substances like organic acids, antibiotics, enzymes and amino acids. In this context the methylotrophic yeast *Pichia pastoris* is one of the most important host organisms for the expression of recombinant proteins. To meet industrial demands, a fast and easy-to-do characterization of recombinant *Pichia* strains to extract bioprocess-relevant strain characteristic parameters for the subsequent set-up of production processes is essential to speed up process development. Normally, this strain characterization procedure is done by time-consuming experiments, which require complex and costly equipment, like continuous cultures [1-2] or several, consecutive fed batch cultivations, operated at different conditions [3-4]. Parameters, which have to be extracted out of these experiments, describe the best operating conditions for each strain as well as the optimal condition for the adaptation of the culture from the growth substrate (e.g. glucose or glycerol) to the inducer methanol. To date, different strategies are employed regarding the adaptation of *Pichia* to methanol; two prominent examples are: 1) after a fed batch on glycerol or glucose a certain low concentration or flow of methanol is applied to the culture which is then increased to a pre-defined maximum and constantly maintained throughout the whole cultivation time [5-6], and 2) the glycerol flow in the reactor is decreased following a linear function during a concomitant addition of methanol, a period which is called transition phase, to slowly adapt the culture to methanol [7-10]. These methods are often based on specific experiences with a certain strain, but are nevertheless often used as a general approach for different *Pichia* strains in following studies, without taking into account the specific requirements of the single strains during adaptation.

After adaptation of the culture to methanol, different feeding strategies can be employed for recombinant protein production with *P. pastoris*. Besides the two common strategies of either using a feed forward regime or a controlled specific growth rate (μ) [6-7, 9-14], a few studies have also described the importance of the specific substrate uptake rate (q_s) on recombinant protein production [15-16]. A direct correlation between q_s and the specific productivity (q_p) was shown [15-16], and it was clearly stated, that q_s was the most important induction parameter in these experiments [17]. Based on those findings and motivated by problems which occur, when more traditional feeding strategies are applied (e.g. possible accumulation of methanol caused by changing cell capacities during cultivation or the need of expensive monitoring equipment to allow μ-controlled feeding [9, 14]), we have focused our research on

the specific substrate uptake rate (q_s) and have recently shown optimization potential using dynamic feeding profiles based on this parameter [18].

In our previous study we also developed a fast approach based on batch experiments with methanol pulses to extract a minimal set of strain characteristic parameters (*i.e.* $\Delta time_{adapt}$ – time for adaptation, $q_{s\ adapt}$ – specific substrate uptake rate during adaptation, $q_{s\ max}$ – maximum specific substrate uptake rate), which are required to set up a subsequent feeding regime based on q_s. However, our previous study dealt with the development and the application of this approach for only one recombinant *P. pastoris* Mut^S strain [18]. In the present work, we characterized various *P. pastoris* strains with different phenotypes (Mut^s and Mut^+) expressing different target enzymes using the above mentioned strategy. We analyzed the required time for adaptation to methanol of each strain in detail and could reliably derive certain strain characteristic parameters from pulse experiments to fed batch cultivations. With the variety of used strains in this study, we demonstrate that this approach is generally applicable for different *P. pastoris* strains and is thus a valuable tool for fast process development, which is especially interesting in an industrial environment.

Material and Methods

The experiments conducted in the present study were performed according to our previous study [18], and are thus only described briefly here.

Microorganisms and recombinant proteins

Different *P. pastoris* strains with different phenotypes expressing different target enzymes were used in this study to prove the general applicability of our strategy. A list of the various strains is given in Table 4.1.2-1.

Table 4.1.2-1 Different *P. pastoris* strains used in this study

strain	phenotype	expressed enzyme	in this study designated as
KM71H	Mut^S	-	KM71H
KM71H	Mut^S	HRP	KM71H HRP
KM71H	Mut^S	PDI and HRP	KM71H PDI HRP
CBS7435	Mut^S	HRP	CBS7435 HRP
SMD1168H	Mut^+	GalOX	SMD1168H GalOX

All recombinant genes used in this study were under the control of the AOX1 promoter. The expressed HRP gene coded for the isoenzyme HRP C1A. The strain KM71H PDI HRP concomitantly expressed HRP and the chaperone protein disulfide isomerase (PDI), which was under the control of a modified AOX1 promoter [19]. The strains KM71H, KM71H HRP, KM71H PDI HRP and CBS7435 HRP were gratefully provided by Prof. Anton Glieder (Graz University of Technology, Austria). The strain SMD1168H GalOX was constructed by Spadiut *et al.*, as described elsewhere [20].

Culture Media

Precultures were performed in complex yeast nitrogen base media (YNBM), whereas batch and fed batch cultivations were done in defined basal salt media (BSM; [21]). The glucose feed was prepared with glucose (250 $g \cdot l^{-1}$), trace element solution PTM1 (12 $ml \cdot l^{-1}$) and antifoam Struktol J650 (0.3 ml/l). The methanol feed was composed of methanol (300 $g \cdot l^{-1}$), PTM1 (4 $ml \cdot l^{-1}$) and Struktol J650 (0.3 $ml \cdot l^{-1}$). The induction period for HRP expression was carried out in the presence of δ-Aminolevulinic acid (δ-ALA) in a final concentration of 1 mM. The concentration of the base NH_4OH was determined by titration with 0.25 M potassium hydrogen phthalate (KHP).

Experimental Procedure

Preculture

Frozen stocks (-80 °C) were precultivated in 100 ml of YNBM in 1000 ml shake flasks at 28 °C and 230 rpm for max. 24 hours.

Batch cultivation with methanol pulses

Batch cultivations were carried out in a 5 l working volume glass bioreactor (Infors, Switzerland) at 28 °C and a fixed agitation speed of 1200 rpm. The culture was aerated with 1 vvm dried air and off-gas was measured by using an infrared cell for CO_2 and a paramagnetic cell for O_2 concentration (Servomex, Switzerland). Process parameters were recorded and logged in a process information management system (PIMS; Lucullus, Biospectra, Switzerland). After the complete consumption of glucose, which was indicated by an increase of dissolved oxygen and a drop in off-gas activity, the first methanol pulse (adaptation pulse) with a final concentration of 0.5 % (v/v) was conducted with pure methanol (supplemented with PTM1, 12 $ml \cdot l^{-1}$ of methanol). Following pulses were performed with

1 % (v/v) concentration of methanol. For all strains, several pulses were conducted after the adaptation pulse to generate consistent data for each strain. For each methanol-pulse, at least two samples were taken to determine the concentrations of substrate and product as well as dry cell weight and OD_{600} to calculate the specific substrate uptake rate q_s.

Fed batch cultivations

Fed batch cultivations were carried out in a 5 l working volume glass bioreactor (Infors, Switzerland) in 2-fold concentrated BSM medium at 28 °C and 1500 rpm. The culture was aerated with at least 1 vvm to keep dissolved oxygen levels > 30 %. In case, air flow was limited, pure oxygen was added. The fed batch feed was measured and controlled using a gravimetrically based PID flow controller. At several time points during fed batch cultivations, samples were taken and analyzed for accumulated methanol, biomass concentration (dry cell weight and optical density OD_{600}) and, if applicable, enzymatic activity. Based on the total biomass content, feeding rates were adjusted manually corresponding to the defined q_s set point. All fed batches described in this study were conducted in the same way: after an adaptation period at q_s of 0.5 $mmol \cdot g^{-1} \cdot h^{-1}$, a stepwise increase of q_s up to $q_{s\,max}$ of the respective strain was carried out with step times of 24 hours.

Analysis of growth- and expression-parameters

Dry cell weight (DCW), OD_{600}, substrate concentrations as well as the catalytic activity of HRP were determined as described before [18]. However, in this study the ABTS solution for HRP activity measurements was prepared in 50 mM KH_2PO_4-buffer at pH 6.5 and the calibration range was expanded to 2.0 $U \cdot ml^{-1}$. Also GalOX activity was measured with an ABTS assay; *i.e.* a sample of diluted enzyme (10 µl) was added to 990 µl of assay buffer containing horseradish peroxidase (222 U) (Type VI-A, Sigma-Aldrich, P6782), ABTS (17.7 mg), KH_2PO_4-buffer (50 mM, pH 6.5) and D-galactose (300 mM). The absorbance change at 420 nm (ε_{420} = 42.3 $mM^{-1} \cdot cm^{-1}$) was recorded at 30 °C for 180 seconds. One Unit of GalOX activity was defined as the amount of enzyme necessary for the oxidation of 2 µmol of ABTS per min, corresponding to the consumption of 1 µmol of O_2 per min. An additional post-translational activation of GalOX by adding $CuSO_4$ to the samples in the presence of oxygen before activity measurements, as described elsewhere [20], was not executed.

Specific rate calculations

Batch cultivations

To obtain the specific rates for substrate uptake and productivity, samples were taken at certain time points during the methanol pulses (*i.e.* beginning of pulse, maximum off-gas of pulse, end of pulse) and analyzed offline for biomass content, methanol concentration and, if applicable, enzymatic activity. Determined values at the beginning and the end of the respective pulse were used to calculate an average rate of the specific substrate uptake, which was corrected for stripping using Antoine's equation, and the specific productivity. Errors for specific rates were set to 10 %, according to our previous study [18]. Online calculated carbon dioxide evolution rate (CER) was divided by actual biomass concentrations to obtain the specific carbon dioxide production rate (qCO_2). In addition, a time derivative of the qCO_2 signal (*i.e.* qCO_2') was calculated using a time window of 30 minutes (15 minutes before and 15 minutes after the actual time point).

Fed batch cultures

During different cultivation periods, representing defined q_s set points, several samples were taken and OD_{600} measurements were used to calculate the actual total biomass content, which allowed adjustments of the methanol feed flow to the actual q_s set point. Specific rates were calculated using DCW and the amount of consumed methanol, which was determined gravimetrically. Presented results correspond to an average value over the respective q_s set point period. Again, errors for specific rates were set to 10 %.

Results and Discussion

A fast approach to derive a minimal set of strain characteristic parameters relevant for bioprocess development

Each *P. pastoris* strain was cultivated in an easy-to-do batch system with methanol pulses to obtain certain strain characteristic parameters during the adaptation period ($\Delta time_{adapt}$ – time for adaptation of the culture to methanol, $q_{s\ adapt}$ – specific substrate uptake rate during the adaptation pulse) and the maximum specific substrate uptake rate ($q_{s\ max}$). These parameters were extracted and consecutively transformed into a feeding profile for fed batch operations based on q_s.

Adaptation of the culture to methanol

After depletion of glucose in batch cultivation, a methanol adaptation pulse with a final concentration of 0.5 % (v/v) was applied. The time required to develop a maximum in off-gas activity was used to define $\Delta time_{adapt}$, according to our previous study [18], and is shown here as the specific carbon dioxide production rate (qCO_2; Figure 4.1.2-1).

The used strains showed very different metabolic characteristics during the adaptation to methanol. The shortest $\Delta time_{adapt}$ of 3.5 h was detected for the KM71H strain, which was a Mut^S strain, not carrying a recombinant gene for heterologous protein expression. The other Mut^S strains KM71H HRP, KM71H PDI HRP and CBS7435 HRP (Figure 4.1.2-1B, C and D, respectively) showed 2-fold longer adaptation times compared to the KM71H strain (see also Table 4.1.2-2). These results clearly show that recombinant *Pichia* strains, which heterologously produce proteins upon the presence of the inducer methanol, carry an additional biological burden which significantly changes their metabolism and slows down their adaptation to methanol.

Surprisingly, the *P. pastoris* Mut^+ strain SMD1168H GalOX showed the longest adaptation time of 14 h (Figure 4.1.2-1E). However, this maximum was just detected as a result of methanol depletion and thus Monod kinetics, rather than representing the real time point of full methanol adaptation. As shown in Figure 4.1.2-1, the Mut^+ strain SMD1168H GalOX showed a very different metabolic behaviour with a flat slope for qCO_2 compared to the qCO_2 curves of the Mut^S strains, which might be due to intracellular regulation and inhibition events, caused by produced H_2O_2 and the absence of sufficient catalases in the cells. Regulation events like this have been described in various systems before [22-24]. Thus, the determined $\Delta time_{adapt}$ of 14 h for the Mut^+ strain is questionable and the cells had probably been adapted to methanol before.

Consequently, the usefulness of the strain characteristic parameter $\Delta time_{adapt}$, especially with regard to the Mut^+ strain SMD1168H GalOX, was checked by introducing a time derivative of the qCO_2 signal (qCO_2'). Since qCO_2 and the specific growth rate μ are linearly related, the maximum of qCO_2' represents the time point of adaptation of the culture to the new substrate methanol. At the maximum qCO_2' the cells should be already fit for further assimilation to methanol, and thus this point represents a good starting point for consecutive fed batch cultivations without running the risk of methanol accumulation.

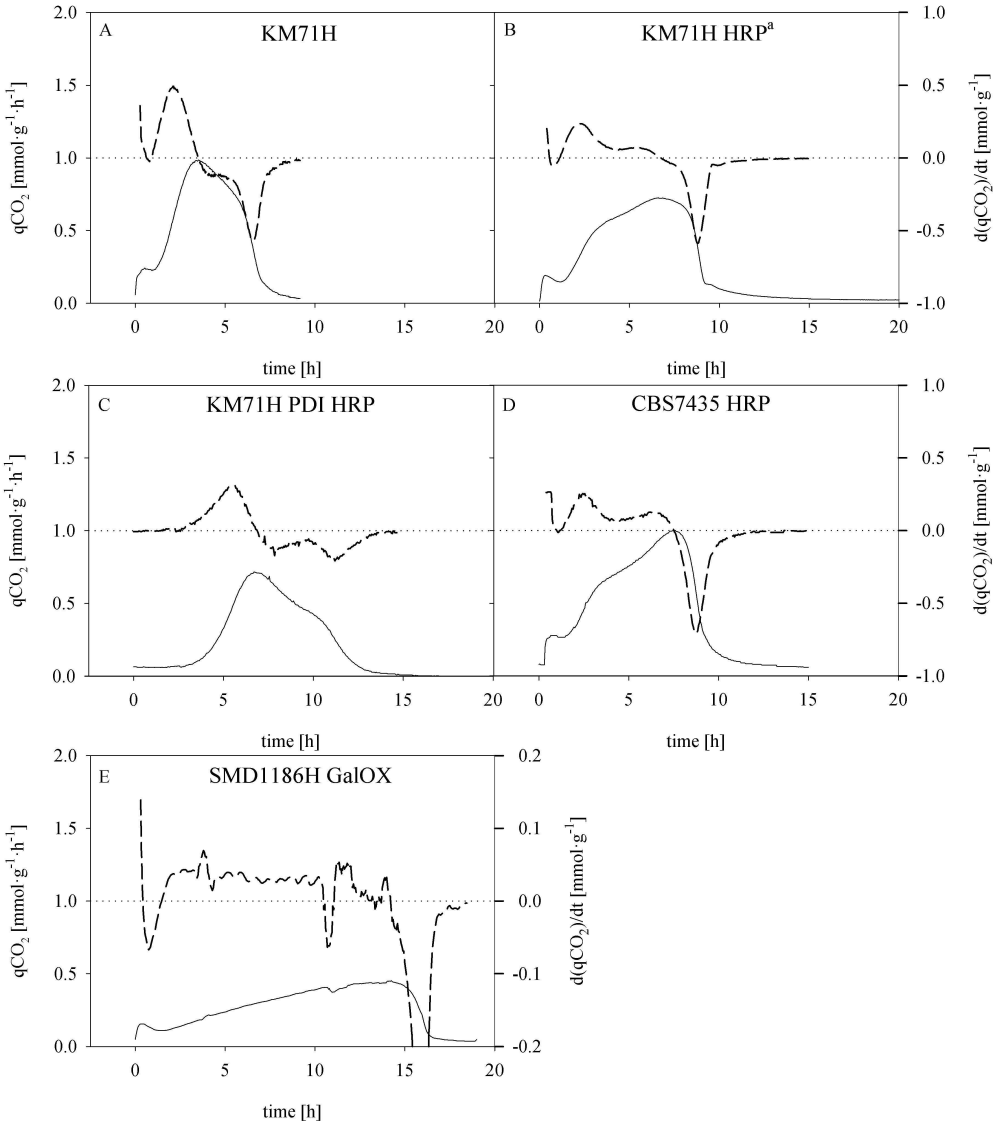


Figure 4.1.2-1 Adaptation pulses with 0.5 % (v/v) methanol after glucose depletion for different *P. pastoris* strains. Straight line, specific carbon dioxide production rate qCO_2; dashed line, time-derivative of the specific carbon dioxide production rate qCO_2' ($d(qCO_2)/dt$); A, KM71H; B, KM71H HRP[a]; C, KM71H PDI HRP; D, CBS7435 HRP; E, SMD1168H GalOX.

[a]data taken from [18]

A good example for this is shown in Figure 4.1.2-1A for the strain KM71H, where a rising slope is shown with a single qCO_2' maximum after 2.1 h. However, the other Mut^S strains tested (KM71H HRP, KM71H PDI HRP and CBS7435 HRP) were characterized by a more bumpy qCO_2 curve, resulting in a qCO_2' signal with several shoulders (Figure 4.1.2-1B, C and D, respectively). Apparently, the adaptation of these strains to methanol did not happen as straight-forward as for the strain KM71H, but with local minima and maxima of the metabolic capacity probably caused by regulatory events upon an excess of methanol. For the Mut^+

strain SMD1168H GalOX the specific carbon dioxide production was rather low compared to the Mut^S strains. The maximum in qCO_2' was determined already after 2.7 h, which was by far sooner than the observed $\Delta time_{adapt}$ of 14 h. The quite constant qCO_2' signal over time (Figure 4.1.2-1E), further supports the fact that the cell capacity was already adapted to its maximum after this short time of 2.7 h and that the cells had been fully adapted to methanol much sooner than the $\Delta time_{adapt}$ of 14 h.

In general, the more detailed analysis of the different strains in their adaptation to methanol revealed three different patterns in the qCO_2' signal: 1) with a single maximum, 2) with several shoulders and 3) a quite constant signal over time. This underlines the necessity for individual analyses of different strains in order to quantitatively characterize them during the adaptation phase in the presence of methanol excess. Of course, the observed maximum in off-gas activity is dependent on the affinity of the cells to the substrate methanol. The maximum in qCO_2 could also be reached in terms of substrate limitation and a consequent drop in the qCO_2 signal due to Monod kinetics rather than by the maximum metabolic adaptation to methanol, which in this study can clearly be seen for the Mut^+ strain SMD1186H GalOX (Figure 4.1.2-1E). However, for all strains tested in this study, maximum values of qCO_2' were reached before the maximum off-gas activity (Figure 4.1.2-1and Table 4.1.2-2), demonstrating that the applied concentration of methanol in the adaptation pulse was high enough to guarantee that the maximum qCO_2' was reached independent of Monod kinetic effects. The validity of qCO_2' as a reliable signal to detect the adaptation of the culture to methanol is further underlined when analyzing the respiratory quotient (RQ) during the adaptation pulse, which is exemplarily shown for the strain KM71H in Figure 4.1.2-2. During the adaptation pulse, RQ fluctuates with local minima and maxima until the signal becomes rather constant indicating the adaptation of the culture to methanol, which actually coincides with the maximum of qCO_2'. Bespoken fluctuations of RQ at the beginning of the adaptation pulse represent the differences in catabolic and anabolic activity of the adapting cells. Similar effects have been observed for *Saccharomyces cerevisiae* [22, 25-26]. During the following pulses, RQ shows a rather constant signal indicating that the cells had already been adapted to the new substrate. These findings validate the parameter qCO_2' as a reliable indicator for methanol adaptation.

Table 4.1.2-2 Batch experiments with methanol pulses to determine strain specific parameters of different *P. pastoris* strains.

	KM71H	KM71H HRP[a]	KM71H PDI HRP	CBS7435 HRP	SMD1168H GalOX
$\Delta time_{adapt\ Batch}$ [h]	3.5	7	7	7.5	14
max. qCO_2' [h]	2.1	2.3	5.7	2.5	2.7
$q_{s\ adapt}$ [$mmol \cdot g^{-1} \cdot h^{-1}$]	0.96 ± 0.10	0.80 ± 0.08	0.56 ± 0.06	0.77 ± 0.08	0.48 ± 0.05
$q_{s\ max}$[b] [$mmol \cdot g^{-1} \cdot h^{-1}$]	1.94 ± 0.19	2.00 ± 0.20	1.08 ± 0.10	1.54 ± 0.15	2.62 ± 0.26
$q_{p\ max}$[b] [$U \cdot g^{-1} \cdot h^{-1}$]	-	2.5 ± 0.25	6.3 ± 0.63	4.25 ± 0.43	200.8 ± 20.1

[a] data taken from [18]

[b] representing the maximum value determined out of all pulses

However, despite the advantage of describing the time point of adaptation of the culture to methanol more accurately, the use of qCO_2' as a parameter to determine the starting point of the following fed batch could be risky because of the described fluctuations in qCO_2 (Figure 4.1.2-1). On the other hand, the maximum off-gas activity ($\Delta time_{adapt}$) is a parameter which safely describes methanol adaptation. Another advantage of using $\Delta time_{adapt}$ instead of qCO_2' is the fact that due to no significant biomass increase during the adaptation pulses, the carbon dioxide evolution rate (CER), which can easily be derived in online mode, can be used to determine $\Delta time_{adapt}$, as also shown in our previous study [18], and thus describes a valuable online tool for process monitoring and control. Consequently, we stuck to $\Delta time_{adapt}$ as a minimum and safe parameter for complete methanol adaptation, as we have done previously [18], while the maximum of qCO_2' should be regarded as a possible minimum prerequisite to start the fed batch feed.

Determination of the specific substrate uptake rates ($q_{s\ adapt}$ and $q_{s\ max}$) using batch cultivations with repeated methanol pulses

The frequent determination of biomass and methanol concentrations allowed specific rate calculations for methanol uptake (q_s) during the methanol pulses. Adaptation pulses of 0.5 % (v/v) methanol were used to determine the parameter $q_{s\ adapt}$ (specific substrate uptake rate during the adaptation pulse) for each strain (Table 4.1.2-2). The knowledge of $q_{s\ adapt}$

allows the operator to adjust a specific, optimal flow of methanol during the adaptation for each single strain, thus preventing methanol accumulation.

After the methanol of the adaptation pulse was depleted, several pulses with 1 % (v/v) methanol were conducted to determine the maximum specific substrate uptake rate ($q_{s\ max}$) for each *P. pastoris* strain. In Figure 4.1.2-2, this strategy is exemplarily shown for the Mut^S strain KM71H, for which the specific substrate uptake rate was calculated with 0.96 ± 0.09 mmol·g^{-1}·h^{-1} in the adaptation period ($q_{s\ adapt}$) and with 1.94 ± 0.19 mmol·g^{-1}·h^{-1} as a maximum during pulses (Figure 4.1.2-2 and Table 4.1.2-2).

All *P. pastoris* strains were characterized with the above mentioned strategy and results are summarized in Table 4.1.2-2. Very different values for the single strain characteristic parameters of the different *P. pastoris* strains were determined and it becomes evident, that *P. pastoris* strains require specific conditions for an optimal adaptation to methanol and that the maximum levels of methanol uptake differ significantly between the single strains.

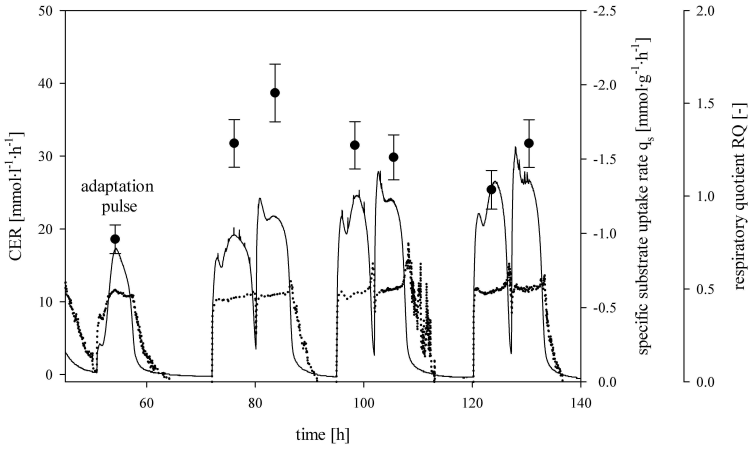


Figure 4.1.2-2 Batch cultivation of the strain KM71H with a 0.5 % (v/v) methanol pulse for adaptation and 1 % (v/v) methanol pulses for qs max determination. Straight line, calculated carbon dioxide evolution rate (CER); dotted line, respiratory quotient RQ (CER/OUR); black dot, specific substrate uptake rate (q_s) for each pulse.

Summing up, our results underline the importance of analyzing different *P. pastoris* strains in more detail during methanol adaptation and regarding the maximum substrate uptake rate. By a more detailed analysis of each strain, which can be done in a fast way by the strategy described here, the bioprocess-relevant strain characteristic parameters $\Delta time_{adapt}$, $q_{s\ adapt}$ and $q_{s\ max}$ can be easily extracted and used for setting up a consecutive fed batch experiment based on q_s. Additionally, the more detailed individual analysis of each strain during the adaptation phase delivers important information for an early process development.

Fed batch design based on batch cultivations with methanol pulses

After characterization experiments in batch cultivations, fed batch experiments using q_s based feeding profiles were carried out, according to our previous study [18]. Besides proving the general applicability of this feeding strategy on different *P. pastoris* strains, we wanted to check for parameter consistency, *i.e.* whether $q_{s\,max}$, which had been determined in batch experiments before, could be reached in fed batch cultivations without observable methanol accumulation.

After a batch phase on glucose as substrate (volume 1.5 l), an exponential fed batch cultivation with glucose yielded in biomass concentrations of up to 70 g/l in a final volume of 2.5 l. As soon as glucose was depleted, a sample was taken to determine the current biomass concentration by measuring the OD_{600} and the DCW. Afterwards, all cultures were induced with a flow corresponding to a q_s below $q_{s\,adapt}$ to guarantee a certain safety margin. The observed adaptation times during the fed batch cultivations for all strains are summarized in Table 4.1.2-3. All the Mut^S strains were characterized by slightly longer adaptation times in fed-batches compared to the batch cultivations, which might be due to the mode of providing methanol, *i.e.* in batch experiments methanol was pulsed into the reactor, resulting in a temporal excess of methanol, whereas in fed batch cultivations methanol was slowly fed into the bioreactor according to the actual biomass content, which is why it took the cells longer to fully adapt to the new substrate methanol.

However, the Mut^+ strain SMD1168H GalOX showed a completely different behaviour: the adaptation time in the fed batch was much shorter than the one observed in the batch experiment, which might be due to the fact that the sudden excess of methanol in the batch-pulse experiment resulted in inhibition events caused by produced H_2O_2 and thus $\Delta time_{adapt}$, which had been determined in the batch experiment, did not really describe the time point of adaptation of the culture to methanol, but rather qCO_2' (*vide supra*).

However, as soon as the maximum in off-gas activity was reached in the fed batch cultivations, the feed was increased to 1.0 $mmol \cdot g^{-1} \cdot h^{-1}$ and then stepwise (0.5 $mmol \cdot g^{-1} \cdot h^{-1}$/step) every 24 hours up to $q_{s\,max}$. Since $q_{s\,max}$ for the strain KM71H HRP PDI had been determined with just 1.08 $mmol \cdot g^{-1} \cdot h^{-1}$, smaller increases of 0.25 $mmol \cdot g^{-1} \cdot h^{-1}$ in the q_s steps were performed. This strategy, describing a stepwise increase of q_s to $q_{s\,max}$, was chosen to allow the detection of possible dependencies between q_s and q_p. At several time points during each step, samples were taken and, based on the apparent biomass content (estimated by OD_{600} measurements) feeding rates were adjusted manually corresponding to the defined q_s set point. Since these regular adjustments of q_s to the actual biomass content

were performed, the feeding profile actually represented an accelerated exponential feeding profile, which has proven to result in higher specific productivities compared to other feeding profiles tested [18].

Table 4.1.2-3 Results of dynamic fed batch cultivations of different *P. pastoris* strains based on q_s.

	KM71H	KM71H HRP[a]	KM71H PDI HRP	CBS7435 HRP	SMD1168H GalOx
$\Delta time_{adapt\ Fedbatch}$ [h]	5.5	9.7	10.4	9.2	5.3
$q_{s\ max}$ determined in batch experiments [mmol·g^{-1}·h^{-1}]	1.94 ± 0.19	2.00 ± 0.21	1.08 ± 0.10	1.54 ± 0.15	2.62 ± 0.26
q_s reached without methanol accumulation [mmol·g^{-1}·h^{-1}]	1.79 ± 0.18	1.92 ± 0.19	1.22 ± 0.12	1.64 ± 0.16	2.44 ± 0.24
$q_{p\ max}$[b] [U·g^{-1}·h^{-1}]	-	11.0 ± 1.10	7.09 ± 0.10	6.48 ± 0.65	139 ± 13.9

[a] data taken from [18]

[b] representing the maximum value determined out of all q_s periods

In Figure 4.1.2-3, this fed batch strategy, which was applied for all *P. pastoris* strains in this study, is exemplarily shown for the Mut^s strain KM71H and the Mut$^+$ strain SMD1186H GalOX. The q_s set points were increased stepwise to a value of 2.00 mmol·g^{-1}·h^{-1} for the KM71H strain, and, as shown in Figure 4.1.2-3A, methanol accumulation was only observed when the feeding rate exceeded values above the respective $q_{s\ max}$ of 1.94 ± 0.19 mmol·g^{-1}·h^{-1}. When we stopped feeding after ~ 70 h, the accumulated methanol was consumed immediately. For the strain SMD1168H GalOX, the q_s set point was stepwise increased to 2.5 mmol·g^{-1}·h^{-1} ($q_{s\ max}$ = 2.62 mmol·g^{-1}·h^{-1}) and no methanol accumulation was detected when these q_s steps were conducted (Figure 4.1.2-3B). The same dynamic feeding strategy was applied to the other *P. pastoris* strains and all essential results are summarized in Table 4.1.2-3.

As shown in Table 4.1.2-3, values for $q_{s\ max}$, which had been determined in batch pulsing experiments before, were reached in fed batch experiments without methanol accumulation. However, when q_s set points were further increased, methanol accumulation was observed.

This proves that the values for $q_{s\,max}$ from batch experiments can be found again in consecutive fed batch experiments for different *P. pastoris* strains and thus shows the great potential of this approach: by determining $q_{s\,max}$ in fast and easy-to-do batch experiments, the operator does not run the risk of overfeeding methanol in consecutive fed batch cultivations. However, we recommend a maximum feed flow below $q_{s\,max}$ to guarantee a certain safety margin.

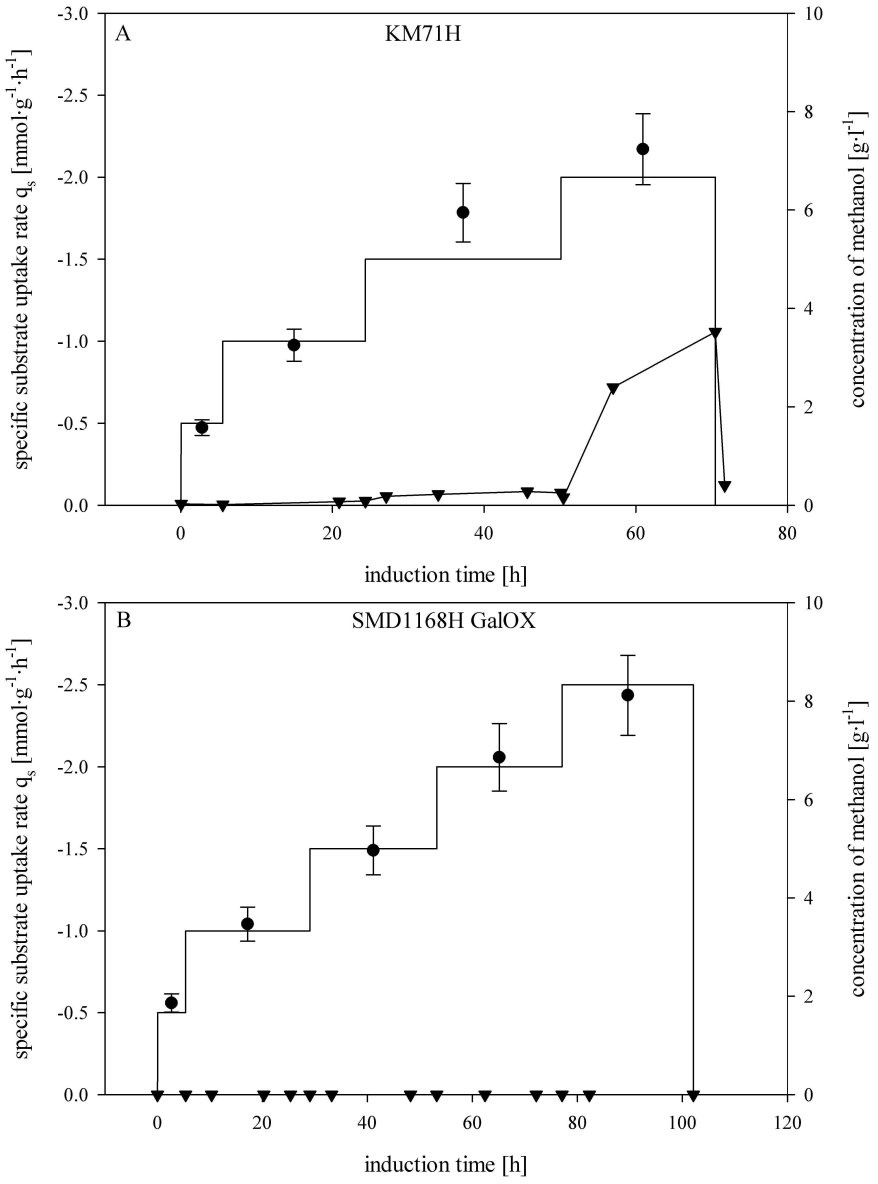


Figure 4.1.2-3 Fed batch cultivations of different *P. pastoris* strains on methanol with a stepwise increase of q_s to $q_{s\,max}$. A, *P. pastoris* Mut^S strain KM71H; B, *P. pastoris* Mut$^+$ strain SMD1186H GalOX; straight line, set point for q_s; black dot, calculated q_s values; black triangle, methanol concentration in the supernatant.

Regarding the productivity, for all the strains, except for SMD1168H GalOX, same or even higher specific productivities were obtained in fed batch cultures compared to the batch

experiments, signifying that volumetric productivities were higher in fed batch cultivations compared to batch experiments (Table 4.1.2-2 and Table 4.1.2-3). This shows that our approach employing dynamic feeding profiles [18] can be successfully applied on different *P. pastoris* strains. The lower $q_{p\ max}$ of the strain SMD1168H GalOX in fed batch cultivation probably resulted from copper-limitation [20], but was not investigated any further, since it was not goal of this study to optimize the production of recombinant enzymes of the single *P. pastoris* strains.

Summing up, in this study we show the general applicability of a fast approach to determine certain strain characteristic parameters, which were extracted out of batch experiments and were verified in subsequent fed batch cultures of different *P. pastoris* strains, making this approach a valuable tool for fast bioprocess development.

Conclusions

In the present study we prove that the fast approach to determine bioprocess-relevant strain characteristic parameters and the novel dynamic feeding strategy based on q_s, which we have described recently for one recombinant *P. pastoris* strain [18], are applicable for a variety of *P. pastoris* strains with different phenotypes producing different recombinant proteins. This underlines the great potential of this strategy as a fast and simple tool to quantify a minimal set of parameters needed to set up consecutive fed batch regimes, which is particularly important for industry, where a fast process development is essential.

Our strategy describes:

1) a batch experiment with

• a 0.5 % (v/v) methanol adaptation pulse to determine $\Delta time_{adapt}$ and $q_{s\ adapt}$

• at least 4 consecutive 1.0 % (v/v) methanol pulses to determine $q_{s\ max}$

2) a dynamic fed batch feeding strategy based on q_s, where after the adaptation of the culture to methanol (described by a maximum in off-gas activity), q_s set points can be increased to $q_{s\ max}$ without observable methanol accumulation

We further show that a detailed analysis of the adaptation to methanol reveals a variability of adaptation characteristics of the different strains, highlighting that an individual analysis of potentially new strains in this respect is required to allow quantitative strain characterization and to derive parameters necessary for a consecutive fed batch set-up. The parameter $\Delta time_{adapt}$ safely describes the transition condition during methanol adaptation. Since also the carbon dioxide evolution rate (CER), as well as the oxygen uptake rate (OUR), can be used to determine $\Delta time_{adapt}$, it further describes an online available data source allowing real-time

monitoring and controlling of bioprocesses, which is essential under the aspect of Process Analytical Technology (PAT).

In this study we show that easy-to-do batch experiments with methanol pulses delivered valid and safe strain characteristic parameters which were consistent and precise enough to set up fed batch feeding profiles based on the specific substrate uptake rate. Our strategy is faster than the usually used continuous cultures or consecutive fed batch cultivations, and therefore allows faster process development. Besides, the strategy described here can be carried out using standard equipment without the need of cost-intensive tools; only a standard bioreactor connected to an off-gas analysis system and an HPLC or GC to determine methanol concentrations are required to carry out the experiments.

Acknowledgements

The authors are very grateful to Prof. Anton Glieder and his workgroup for providing all *P. pastoris* Mut^S strains expressing the enzyme horseradish peroxidase. Additionally, the authors express their gratitude to Josef Schulze, who supported the experimental work in the lab.

Competing interests

The authors declare that they have no competing interests.

Author contributions

CD designed and performed the majority of the experiments, analyzed the data and drafted the manuscript. OS designed and performed some experiments and finalized the manuscript. CH conceived the study and supervised research. All authors read and approved the final manuscript.

List of Abbreviations

$\Delta time_{adapt}$ - mtime for adaptation of the culture to the new substrate (methanol) [h],
μ - specific growth rate [h^{-1}], ABTS - 2,2' azino bis 3-ethylbenzthiazoline-6-sulphonic acid CER- carbon dioxide evolution rate [$mmol \cdot l^{-1} \cdot h^{-1}$], $d(qCO_2)/dt$ - derivative of qCO_2, DCW - dry cell weight, HPLC - high performance liquid chromatography, HRP - horseradish peroxidise, GalOX - galactose 6-oxidase, Mut^+ - methanol utilization phenotype, wildtype, Mut^S - methanol utilization slow phenotype, OD_{600} - optical density at 600 nm [AU], PID - proportional-integrative-derivative controller, qCO_2 - specific carbon dioxide production rate [$mmol \cdot g^{-1} \cdot h^{-1}$], qCO_2' - derivative of qCO_2, q_p - specific productivity [$U \cdot g^{-1} \cdot h^{-1}$], $q_{s\ adapt}$ -

specific substrate uptake rate during adaptation [mmol·g^{-1}·h^{-1}], $q_{s\,max}$ - maximum specific substrate uptake rate [mmol·g^{-1}·h^{-1}], q_s - specific substrate uptake rate [mmol·g^{-1}·h^{-1}], rpm - rounds per minute, RQ - respiratory quotient, Vvm - volume gas flow per volume liquid per minute

References

1. d'Anjou MC, Daugulis AJ: **A rational approach to improving productivity in recombinant Pichia pastoris fermentation.** *Biotechnology and bioengineering* 2001, **72:**1-11.
2. Curvers S, Linnemann J, Klauser T, Wandrey C, Takors R: **Recombinant protein production with Pichia pastoris in continuous fermentation - kinetic analysis of growth and product formation.** *Engineering in Life Sciences* 2002, **2:**229-235.
3. Ren H, Yuan J: **Model-based specific growth rate control for Pichia pastoris to improve recombinant protein production.** *Journal of Chemical Technology and Biotechnology* 2005, **80:**1268-1272.
4. Ren HT, Yuan JQ, Bellgardt KH: **Macrokinetic model for methylotrophic Pichia pastoris based on stoichiometric balance.** *Journal of Biotechnology* 2003, **106:**53-68.
5. Invitrogen: **Pichia Fermentation Process Guidelines.** 2002.
6. Jahic M, Rotticci-Mulder JC, Martinelle M, Hult K, Enfors SO: **Modeling of growth and energy metabolism of Pichia pastoris producing a fusion protein.** *Bioprocess and Biosystems Engineering* 2002, **24:**385-393.
7. Trinh LB, Phue JN, Shiloach J: **Effect of methanol feeding strategies on production and yield of recombinant mouse endostatin from Pichia pastoris.** *Biotechnology and Bioengineering* 2003, **82:**438-444.
8. Zhang W, Bevins MA, Plantz BA, Smith LA, Meagher MM: **Modeling Pichia pastoris growth on methanol and optimizing the production of a recombinant protein, the heavy-chain fragment C of botulinum neurotoxin, serotype A.** *Biotechnology and Bioengineering* 2000, **70:**1-8.
9. Zhang W, Sinha J, Smith LA, Inan M, Meagher MM: **Maximization of Production of Secreted Recombinant Proteins in Pichia pastoris Fed-Batch Fermentation.** *Biotechnology Progress* 2005, **21:**386-393.
10. Plantz BA, Sinha J, Villarete L, Nickerson KW, Schlegel VL: **Pichia pastoris fermentation optimization: energy state and testing a growth-associated model.** *Applied Microbiology and Biotechnology* 2006, **72:**297-305.
11. Potgieter TI, Kersey SD, Mallem MR, Nylen AC, d'Anjou M: **Antibody expression kinetics in glycoengineered Pichia pastoris.** *Biotechnology and Bioengineering* 2010, **106:**918-927.
12. Kobayashi K, Kuwae S, Ohya T, Ohda T, Ohyama M, Tomomitsu K: **High level secretion of recombinant human serum albumin by fed-batch fermentation of the methylotrophic yeast, Pichia pastoris, based on optimal methanol feeding strategy.** *Journal of Bioscience and Bioengineering* 2000, **90:**280-288.
13. Jacobs P, Inan M, Festjens N, Haustraete J, Van Hecke A, Meagher M, Contreras R, Callewaert N: **Fed-batch fermentation of GM-CSF-producing glycoengineered Pichia pastoris under controlled specific growth rate.** *Microbial Cell Factories* 2010, **9:**93.
14. Sinha J, Plantz BA, Zhang W, Gouthro M, Schlegel V, Liu C-P, Meagher MM: **Improved production of recombinant ovine interferon-t by Mut+ strain of Pichia pastoris using an optimized methanol feed profile.** *Biotechnology Progress* 2003, **19:**794-802.
15. Khatri NK, Hoffmann F: **Oxygen-limited control of methanol uptake for improved production of a single-chain antibody fragment with recombinant Pichia pastoris.** *Applied Microbiology and Biotechnology* 2006, **72:**492-498.

16. Khatri NK, Hoffmann F: **Impact of methanol concentration on secreted protein production in oxygen-limited cultures of recombinant Pichia pastoris.** *Biotechnology and Bioengineering* 2006, **93:**871-879.
17. Cunha AE, Clemente JJ, Gomes R, Pinto F, Thomaz M, Miranda S, Pinto R, Moosmayer D, Donner P, Carrondo MJT: **Methanol induction optimization for scFv antibody fragment production in Pichia pastoris.** *Biotechnology and bioengineering* 2004, **86:**458-467.
18. Dietzsch C, Spadiut O, Herwig C: **A dynamic method based on the specific substrate uptake rate to set up a feeding strategy for Pichia pastoris.** *Microbial Cell Factories* 2011, **10:**14.
19. Hartner FS, Ruth C, Langenegger D, Johnson SN, Hyka P, Lin-Cereghino GP, Lin-Cereghino J, Kovar K, Cregg JM, Glieder A: **Promoter library designed for fine-tuned gene expression in Pichia pastoris.** *Nucleic Acids Research* 2008, **36:**e76/71-e76/15.
20. Spadiut O, Olsson L, Brumer H, 3rd: **A comparative summary of expression systems for the recombinant production of galactose oxidase.** *Microb Cell Fact* 2010, **9:**68.
21. Cregg JM: **Pichia Protocols, Second Edition.** In *Methods Mol Biol* Edited by Cregg JM. Totowa, NJ, U. S.; 2007: 268 pp
22. Herwig C, von Stockar U: **A small metabolic flux model to identify transient metabolic regulations in Saccharomyces cerevisiae.** *Bioprocess and Biosystems Engineering* 2002, **24:**395-403.
23. Diderich JA, Schepper M, van Hoek P, Luttik MA, van Dijken JP, Pronk JT, Klaassen P, Boelens HF, de Mattos MJ, van Dam K, Kruckeberg AL: **Glucose uptake kinetics and transcription of HXT genes in chemostat cultures of Saccharomyces cerevisiae.** *The Journal of biological chemistry* 1999, **274:**15350-15359.
24. Rizzi M, Theobald U, Querfurth E, Rohrhirsch T, Baltes M, Reuss M: **In vivo investigations of glucose transport in Saccharomyces cerevisiae.** *Biotechnology and Bioengineering* 1996, **49:**316-327.
25. Duboc P, Von Stockar U, Villadsen J: **Simple generic model for dynamic experiments with Saccharomyces cerevisiae in continuous culture: decoupling between anabolism and catabolism.** *Biotechnology and Bioengineering* 1998, **60:**180-189.
26. Duboc P, Cascao-Pereira LG, von Stockar U: **Identification and control of oxidative metabolism in Saccharomyces cerevisiae during transient growth using calorimetric measurements.** *Biotechnology and Bioengineering* 1998, **57:**610-619.

4.2. Physiological evaluation of recombinant *Pichia pastoris* strains

4.2.1. A dynamic fed batch strategy for a *Pichia pastoris* mixed feed system to increase process understanding

Dénes Zalai, Christian Dietzsch, Christoph Herwig, Oliver Spadiut*

Vienna University of Technology, Institute of Chemical Engineering, Research Area Biochemical Engineering, Vienna, Austria

*Corresponding Author: Oliver Spadiut, Vienna University of Technology, Institute of Chemical Engineering, Research Area Biochemical Engineering, Gumpendorfer Strasse 1a, A-1060 Vienna, Austria. Tel: +43 1 85501 166473, email: oliver.spadiut@tuwien.ac.at

Manuscript submitted to Biotechnology Progress.

Abstract

Mixed substrate feeding strategies are frequently investigated to enhance the productivity of recombinant *Pichia pastoris* processes. For this purpose, numerous fed batch experiments or time-consuming continuous cultivations are required to optimize control parameters such as the substrate mixing ratio or the applied methanol concentration.

In this study, we decoupled the feeding of methanol and glycerol in a mixed substrate fed batch environment to gain process understanding for a recombinant *P. pastoris* Mut^S strain producing the model enzyme horseradish peroxidase. Specific substrate uptake rates (q_s) were controlled separately, and a stepwise increased q_{Gly}-control scheme was applied in order to investigate the effect of various substrate fluxes on the culture. The q_s-controlled strategy allowed a parallel characterization of the metabolism and the recombinant protein expression in a fed batch environment. A critical specific glycerol uptake rate was determined, where a decline of the specific productivity occurred, and a time dependent acceleration of protein expression was characterized with the dynamic fed batch approach.

Based on the observations on recombinant protein expression, propositions for an optimal feeding design to target maximal productivities were stated. Thus, the dynamic fed batch strategy was found to be a valuable tool for both process understanding and optimization of product formation for *P. pastoris* in a mixed substrate environment.

Introduction

The major objective of bioprocess development is the identification and optimization of process parameters in order to achieve the highest concentration and quality of the desired product. In this respect, recombinant protein production is mainly affected by the process mode during induction. Parameters like the substrate feeding rate or the inducer concentration are known to play key roles regarding the productivity of recombinant microorganisms [1].

The methylotrophic yeast *Pichia pastoris* can be designed in a way to use methanol as a substrate as well as an inducer for recombinant protein expression. In such a case, the recombinant gene is inserted either at the locus of the endogenous alcohol oxidase gene *aox*1 or *aox*2. Depending on the locus, three phenotypes of recombinant *P. pastoris* strains can be defined: methanol utilization plus (Mut^+), methanol utilization slow (Mut^S) and methanol utilization minus (Mut^-) [2]. All three phenotypes express the recombinant gene under the control of the AOX promoter (*p*AOX). This promoter is induced by methanol and repressed by several other carbon sources [3]. Thus, commonly used fermentation strategies for *P. pastoris* describe two different phases: growth on a carbon source, like glycerol or glucose, to get a high biomass yield, followed by the induction of recombinant protein production by methanol [4].

To integrate and combine growth and induction of protein expression, different mixed feed approaches were investigated [1, 5-9]. A mixed feed strategy gives different technical benefits, like lower oxygen consumption and lower heat production [5], and also facilitates biomass growth due to higher biomass yields on the second substrate [10]. Increased cell densities result in increased volumetric productivity, the most emphasized benefit of a mixed feed system [1]. A prominent C-source for these approaches is glycerol although it was reported to repress *p*AOX, even if fed in limiting amounts [6-7]. Thus, an important parameter in such a mixed feed strategy turned out to be the glycerol feeding rate [8].

To investigate a mixed feed environment, both continuous [9] and fed batch approaches [11-12] were conducted, where methanol concentrations of 1-5 $g \cdot l^{-1}$ were maintained in the broth, while the feeding rate of glycerol was varied. However, this strategy did not allow a tight control of the methanol uptake of the culture and thus to reliably show dependencies of the productivity on certain substrate uptake rates. Another study described enhanced AOX expression under methanol-limited conditions [10], which is why a controlled methanol uptake at a low level could boost the productivity of recombinant *P. pastoris* strains. To analyze such effects, defined ratios of the two substrates glycerol and methanol were mixed and fed together in limiting amounts, either based on the specific growth rate [5, 13] or on

constant volumetric flows [14]. However, time-consuming continuous cultivations or several fed batch experiments had to be performed in these studies in order to investigate the effect of different substrate mixing ratios on the culture. A step towards faster process understanding would be to control the feeding rates of the two substrates separately, as this allows the investigation of several substrate mixing ratios in just a single fed batch experiment.

A common way to control limiting substrate fluxes in a fed batch environment is to increase the respective feeding rate exponentially in order to maintain a defined specific growth rate (μ) during the induction phase [15-16]. However, the different biomass yields on methanol and glycerol can lead to deviations from the targeted μ in a mixed substrate environment [17]. Moreover, the results on the relationship between growth and recombinant protein production are contradictory, even for *P. pastoris* processes on the single substrate methanol [10, 18]. Thus, it is desirable to use other process parameters than μ to control the feeding rates.

We recently reported an experimental approach which enabled both a fast strain characterization and the enhancement of recombinant protein production of a recombinant *P. pastoris* Mut^S strain in a single substrate system [19-20]. We determined certain strain specific parameters in fast and easy to do batch cultivations with methanol pulses and used the specific methanol uptake rate (q_s) as a control parameter for the development of a dynamic fed batch strategy [19-20]. We could prove that q_s was a valuable and powerful alternative control parameter to μ for bioprocess design.

In the present study, we aimed at enhancing process understanding for a mixed feed regime using a recombinant *P. pastoris* Mut^S strain. Specific substrate uptake rates (q_s) were used as a strain specific process parameter to control methanol and glycerol feeding rates. The decoupled feeding of the two substrates allowed the individual adjustment of q_{Gly} and q_{MeOH}. With this experimental approach, the effect of different substrate mixing ratios on the metabolism and the productivity of the culture was investigated in just a few dynamic fed batch experiments.

Material and Methods

Microorganism and recombinant protein

The *P. pastoris* strain CBS7435 (*arg4 aox1:arg4*) was transformed with a plasmid carrying the gene of the horseradish peroxidase isoenzyme C1A (HRP) and was gratefully provided by Prof. Anton Glieder (Graz University of Technology, Austria). The phenotype of the strain was Mut^S and HRP was secreted into the fermentation broth. The stability of HRP in the presence of methanol was checked and reported in our previous study [19].

Culture Media

Preculture: Yeast nitrogen base medium (YNBM), per liter: potassium phosphate buffer (pH 6.0), 0.1 M; YNB w/o Amino acids and Ammonia Sulfate (Difco™), 3.4 g; $(NH_4)_2SO_4$, 10 g; biotin, 400 mg; glucose, 20 g.

Batch/fed batch: Basal salt medium (BSM) [21], per liter: 85% phosphoric acid, 26.7 ml; $CaSO_4 \cdot 2H_20$, 1.17 g; K_2SO_4, 18.2 g; $MgSO_4 \cdot 7H_2O$, 14.9 g; KOH, 4.13 g; $C_6H_{12}O_6 \cdot H_2O$, 44 g, Antifoam Struktol J650, 0.2 ml; PTM1, 4.35 ml; NH_4OH as N-source. Trace element solution (PTM1), per litre: $CuSO_4 \cdot 5H_2O$, 6.0 g; NaI 0.08 g; $MnSO_4 \cdot H_2O$, 3.0 g; $Na_2MoO_4 \cdot 2H_2O$, 0.2 g; H_3BO_3, 0.02 g; $CoCl_2$, 0.5 g; $ZnCl_2$, 20.0 g; $FeSO_4 \cdot 7H_2O$, 65.0 g; biotin, 0.2 g, H_2SO_4, 5 ml.

Feed glycerol (exponential fed batch phase), per liter: glycerol, 250 g; PTM1, 12 ml; Struktol J650, 0.3 ml.

Feed glycerol (induction phase), per liter: glycerol, 200 g; PTM1, 12 ml; Struktol J650, 0.3 ml.

Feed methanol (induction phase), per liter: methanol, 300 g; PTM1, 4 ml; Struktol J650, 0.3 ml, induction was carried out in presence of d-Aminolevulinic acid (δ-ALA), 1 mM.

Base: NH_4OH, concentration was determined by titration with 0.25 M potassium hydrogen phthalate (KHP).

Experimental Procedure

Preculture

Frozen stocks (-80 °C) were pre-cultivated in 100 ml of YNBM in 1000 ml shake flasks at 28 °C and 230 rpm for max. 24 hours. Then, the preculture was transferred aseptically to the respective culture vessel. The inoculation volume was approximately 10 % of the final starting volume.

Batch cultivations

Batch cultivations were carried out in a 3 l (2 l working volume) glass bioreactor (Infors, Switzerland). Basal salt medium (BSM) was sterilized in the bioreactor and pH was adjusted to pH 5.0 by using concentrated ammonia solution after autoclaving. The cultivations were carried out with initial substrate concentrations of 20 $g \cdot l^{-1}$for both glycerol and methanol. Glycerol was added to the basal salt medium before sterilization. Calculated amount of sterile filtered methanol was transferred into the reactor aseptically with sterile filtered trace elements and δ-ALA. The pH was measured with a sterilizable electrode (Hamilton, Switzerland) and maintained constant with a PID controller using ammonia solution (approximately 4 M). Cultivation temperature was set to 28 °C and agitation was fixed to 1200 rpm. Dissolved oxygen was measured with a sterilizable optical dissolved oxygen electrode (Hamilton, Switzerland). The culture was aerated with 1.5 vvm dried air and off-gas of the culture was measured by using an infrared cell for CO_2 and a paramagnetic cell for O_2 concentration (Servomax, Switzerland).

Samples were taken frequently to determine substrate and product concentrations as well as biomass dry cell weight (DCW) to calculate strain specific parameters. The calculated methanol uptake rates were corrected for stripping using Antoine's equation, as described previously [20].

Fed batch cultivations

Fed batch cultivations were carried out in a 7.5 l (5 l working volume) glass bioreactor (Infors, Switzerland). 1.5 l concentrated BSM (2-fold concentrated to supply required salts for high cell densities) containing 40 $g \cdot l^{-1}$ glycerol was prepared. Agitation was set to 1500 rpm. pH, dissolved oxygen, CO_2 and O_2 were measured as described above. The culture was aerated with at least 1 vvm to avoid oxygen limitation. The dissolved oxygen signal was used to adjust air-in flow manually to keep levels > 30 % dO_2. In case air flow was limited, pure oxygen was added. Methanol and glycerol feeds were measured and controlled separately using gravimetrically based PID flow controllers and were added via a submerged tube to the fermentation broth. The setup of the fed batch bioreactor with separately controlled feeds is shown in Figure 4.2.1-1.

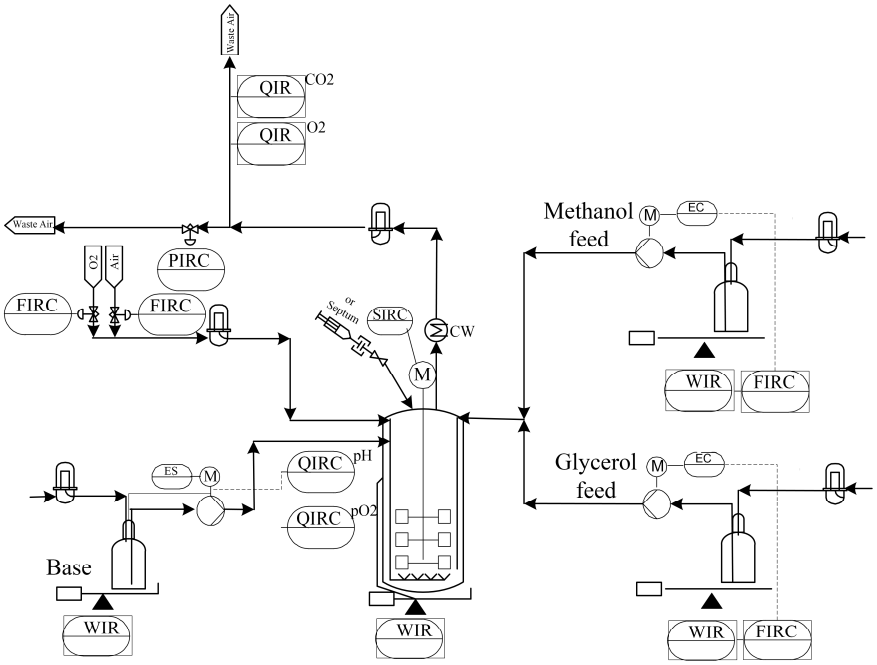


Figure 4.2.1-1 Process and Instrumentation Diagram of the experimental set-up to adjust substrate flow rates separately in fed batch experiments. Nomenclature is according to DIN EN ISO 10628.

The fed batch experiments were conducted as following: after a batch phase on 40 $g \cdot l^{-1}$ glycerol, an exponential fed batch phase was implemented with a controlled specific growth rate of μ=0.15 h^{-1}. The exponential glycerol feed was terminated as the volume in the bioreactor reached 2.5 l. Then, the induction phase was started with a methanol feed corresponding to a q_{MeOH} setpoint of 0.5 C-mmol$\cdot g^{-1} \cdot h^{-1}$ to ensure the adaptation of the culture to methanol. After an adaptation period of 12 hours, the glycerol feed was started. We used slightly different values for the adaptation time ($\Delta time_{adapt}$) and $q_{s\ adapt}$ compared to the values, which we had determined for this strain before (*i.e.* $q_{s\ adapt}$ = 0.77 C-mmol$\cdot g^{-1} \cdot h^{-1}$and $\Delta time_{adapt}$ = 9.2 h [20]) to guarantee a certain safety margin.

Samples were taken at several time points of the induction phase and analyzed for accumulated methanol, biomass concentration (DCW and optical density OD_{600}) and enzymatic activity. Based on the total biomass content, feeding rates were adjusted manually corresponding to the current conditions of the culture and the defined q_s setpoint. Accurate $q_{s\ real}$ values were calculated based on the online measurement of feed balances, reactor volume and off-line DCW.

Temperature, pH, dO_2, agitation, the weight of the reactor, base and feeds, as well as CO_2 and O_2 in the off-gas were measured online and logged in a process information management system (PIMS; Lucullus, Biospectra, Switzerland).

Analysis of growth- and expression-parameters

Biomass DCW was determined by centrifugation of 5 ml culture broth (5000 rpm, 4 °C, 10 min) in a laboratory centrifuge (Sigma 4K15, rotor 11156), washing the pellet with 5 ml deionized water and subsequent drying at 105°C to a constant weight in an oven. Optical density of the culture broth was measured using a spectrophotometer (U-1100 Hitachi, Japan) at a wavelength of 600 nm (OD_{600}). Correlation between DCW and OD_{600} showed a coefficient of regression of R^2=0.997 over the full concentration range and could therefore be used for q_s adjustment. The activity of HRP was determined according to our previous study [20].

Substrate concentrations

Concentrations of methanol and glycerol were determined in cell free samples by HPLC (Agilent Technologies, USA) equipped with a Supelcoguard column, a Supelcogel C-610H ion-exchange column (Sigma-Aldrich, USA) and a refractive index detector (Agilent Technologies, USA). The mobile phase was 0.1 % H_3PO_4 with a constant flow rate of 0.5 $ml \cdot min^{-1}$ and the system was run isocratic. Calibration was done by measuring standard points in the range of 0.2 to 20 $g \cdot l^{-1}$ for each substrate.

Data analysis

Errors

Measurements of biomass and substrate concentration as well as HRP activity were executed in duplicates. According to our previous study, the observed standard deviation for the single measurement was propagated to an error of 10 % for the specific rates q_s and q_p and an error of 5 % for the yield coefficients [19].

Calculation of theoretical respiration quotients

Respiration quotients (RQ) were calculated for the individual substrates based on the biomass yields determined in the dual substrate batch experiments. Then, theoretical mixed feed respiration quotients were determined to show dependencies of RQ on different substrate uptake ratios according to equation 4.2.1.1.

$$RQ_{mixedfeed} = \left(RQ_{MeOH} \cdot \frac{q_{MeOH}}{q_{total}} \right) + \left(RQ_{Gly} \cdot \frac{q_{Gly}}{q_{total}} \right) \qquad \text{(Eq. 4.2.1.1)}$$

Check of data consistency

Carbon- and degree of reduction balances were calculated for each q_s period. The acceptance criterion was set to be ± 10 % of closing balances.

Results and Discussion

Strain specific parameters determined in dual substrate batch experiments

In order to quantify important strain specific parameters of the recombinant CBS7435 Mut^S strain, dual substrate batch cultivations were performed. Methanol was only consumed, when glycerol was exhausted (data not shown). This typical diauxic behaviour was in accordance to previous studies with *P. pastoris* [22-23]. Recombinant HRP production was only detected in the phase of methanol uptake. Samples were taken with high frequency for offline measurements of substrate concentrations and biomass DCW, and strain specific parameters were calculated (Table 4.2.1-1). The maximal specific substrate uptake rate for glycerol ($q_{Gly, max}$) and methanol ($q_{MeOH, max}$) were subsequently extracted into the design of fed batch experiments, where they represented the upper limit for the q_s-based feed control. The calculated biomass yields ($Y_{X/S, Gly}$ and $Y_{X/S, MeOH}$) were used to estimate cell growth in the mixed substrate fed batch environment.

Table 4.2.1-1 Strain specific parameters determined in dual substrate batch experiments.

parameter	result	
$q_{Gly, max}$	-12.21 ± 0.15	C-mmol·g^{-1}·h^{-1}
$q_{MeOH, max}$	-1.44 ± 0.33	C-mmol·g^{-1}·h^{-1}
$Y_{X/S, Gly}$	0.65 ± 0.06	C-mol·C-mol^{-1}
$Y_{X/S, MeOH}$	0.41 ± 0.09	C-mol·C-mol^{-1}

Mixed feed fed batch design based on the specific substrate uptake rate q_s

In order to ensure the complete derepression of *p*AOX and to reach high cell densities, a limiting exponential glycerol fed batch phase was implemented after the batch on glycerol. Afterwards, the q_s-controlled induction phase was initiated. First, methanol was fed as a single substrate to ensure the full induction of the methanol-utilization pathway in the cells. During this adaptation phase, methanol accumulated (Figure 4.2.1-2), but was taken up completely as soon as the cells were fully adapted.

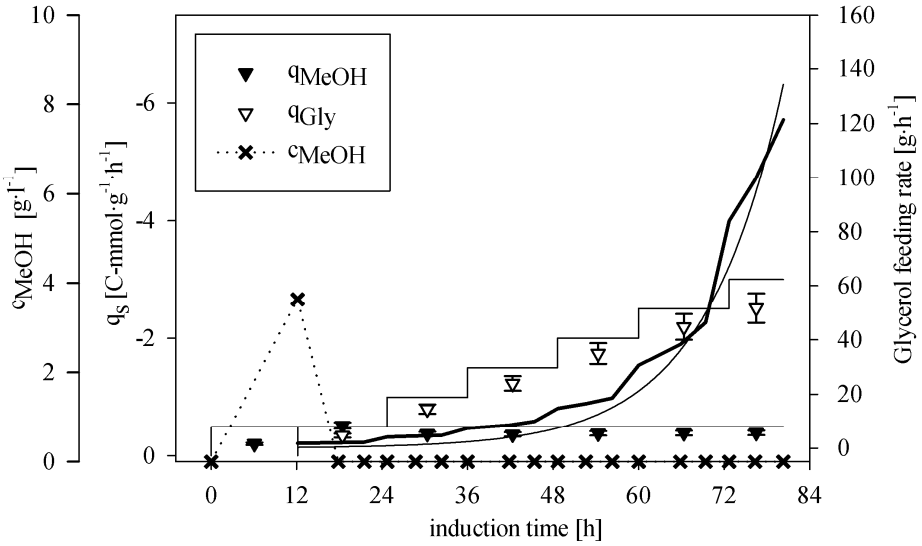


Figure 4.2.1-2 Design of a fed batch experiment with a dynamically increasing specific glycerol uptake rate. The thick solid line F_{Gly} represents the periodically adjusted glycerol feeding rate. The thin solid line F_{Gly} is a fitted accelerated exponential curve following the equation $F = F_0 \cdot e^{((k+a \cdot t) \cdot t)}$. Solid lines, setpoint for respective uptake rates; triangles, calculated values for respective uptake rates; cross, methanol concentration.

During the mixed feed induction phase, the q_{MeOH} setpoint was kept at a constant low value of 0.5 C-mmol·g^{-1}·h^{-1} to avoid the use of high amounts of methanol, but to assure continuous induction of *p*AOX. In order to investigate the behaviour of the culture at defined q_{Gly}/q_{MeOH} ratios, the q_{Gly} setpoint was kept constant for 12 hour periods before it was stepwise increased. Samples were taken with high frequency and feeding rates were adjusted according to the apparent biomass content in the bioreactor to keep the offset between the $q_{s,\ setpoint}$ and $q_{s,\ real}$ values low. To ensure constant q_{MeOH} in the course of the experiment, methanol feeding rate values described an exponential curve due to cell growth, whereas the dynamic increase of the q_{Gly} resulted in an accelerated exponential function (Figure 4.2.1-2).

Three dynamic fed batch cultivations with different parameter settings were done according to the strategy shown in Figure 4.2.1-2 in order to investigate the effects of certain substrate ratios on the metabolism and the productivity of a recombinant *P. pastoris* Mut^S strain. The basic settings of each fed batch experiment and the respective objective are summarized in Table 4.2.1-2.

Table 4.2.1-2 Summary of the experimental strategies for the conducted fed batch (FB) cultivations. Specific substrate uptake rate setpoints are given in [C-mmol·g^{-1}·h^{-1}].

experiment	$q_{Gly\ setpoint}$	$q_{MeOH\ setpoint}$	major objective
FB1	0.5-3.0	0.5	investigate the culture at various q_{Gly}/q_{MeOH} ratios
FB2	3.0-7.0	0.5	extend the q_{Gly} range of experiment FB1
FB3	ascending + descending	1.0	analyze the effect of a higher q_{MeOH} on the repression of productivity

Decoupled substrate feeds for the characterization of metabolic behaviour

In order to prove the applicability of the experimental approach based on q_s, we decoupled the two substrates glycerol and methanol and controlled the feeding rates separately according to the respective q_s setpoint. The glycerol feed was started after the cells were adapted to methanol, and subsequently the two substrates were taken up simultaneously in the carbon-limited mixed feed environment.

Predictable metabolism of the Mut^S strain on mixed substrates

The ability to predict metabolic attributes like oxygen consumption or biomass yield facilitates process understanding and scale up in early process development. Therefore, we calculated various yields in order to investigate possible changes in the metabolic activity of the culture at different q_{Gly}/q_{MeOH} ratios.

The q_{Gly}/q_{MeOH} ratio had a significant impact on the respiration of the culture. $Y_{O2/S}$ values decreased as the q_{Gly}/q_{MeOH} ratio increased (Figure 4.2.1-3). This is in coincidence with the fact that less oxygen is necessary for the oxidation of glycerol than methanol [5], a clear advantage of mixed feed processes over single methanol feed processes. Also the $Y_{CO2/S}$ yield decreased at increasing q_{Gly} values. Biomass yields were between 0.41 and 0.65 C-mol·C-mol^{-1}, which was in good agreement with the values calculated in the dual substrate batch experiments for the individual substrates (Table 4.2.1-1). Carbon balances were calculated close to 100 % (error less than 10%, data not shown) and as the product formation was expected to have a negligible effect on the carbon balance due the low amount of recombinant protein expressed, the decrease in the $Y_{CO2/S}$ yield was predicted to happen in concomitance with a proportional increase in the $Y_{X/S}$ yield. However, no significant increase of the calculated $Y_{X/S}$ values was detected with increasing q_{Gly}/q_{MeOH} ratios (Figure 4.2.1-3), which might be due to deviations in biomass weight measurements and subsequent error propagation.

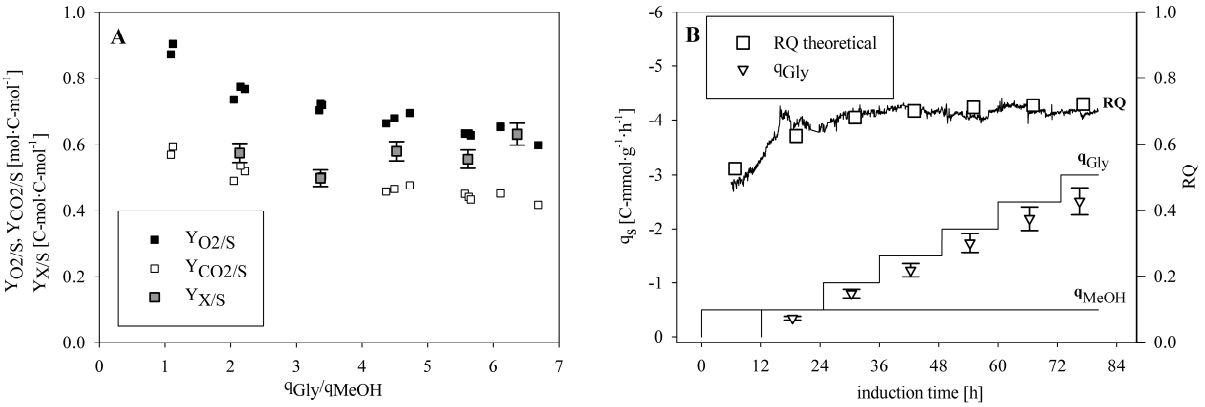


Figure 4.2.1-3 Changes in the metabolism of the culture at increasing qGly/qMeOH ratios in experiment FB1. A) Calculated yields based on consumed mols of the two substrates glycerol and methanol. The error of determined $Y_{CO2/S}$ and $Y_{O2/S}$ yields (<5%) is not shown for better reading. B) On-line calculated respiration quotients compared to theoretically calculated respiration quotients and the q_s profile.

To validate this assumption, online measured carbon dioxide evolution rate (CER) and oxygen uptake rate (OUR) values were used to calculate the respiration quotient. Moreover, the theoretical respiration quotient ($RQ_{theoretical}$) was calculated based on the biomass yields on the individual substrates and the q_{Gly}/q_{MeOH} ratio. A good correlation between the theoretically calculated values and the online determined values for RQ was shown (Figure 4.2.1-3). Thus, the biomass yield could be predicted as the weighted mean of the biomass yields on the two individual substrates. This observation is in accordance with other studies reporting biomass yields on mixed substrates in continuous cultivations [5, 13]. However, the q_s-controlled feeding regime in the present study allowed us to quantify the change in the biomass yield at different substrate mixing ratios in a less time consuming fed batch environment.

Metabolic interactions at high glycerol fluxes

In order to investigate the metabolic behaviour of the culture at higher q_{Gly} rates, experiment FB2 was designed to extend the q_{Gly} range of experiment FB1 (see Table 4.2.1-2). Therefore, the glycerol feeding rate was set to a q_{Gly} of 3.0 C-mmol·g^{-1}·h^{-1} immediately after the adaptation phase on methanol, and was then stepwise increased up to 7.0 C-mmol·g^{-1}·h^{-1} (Figure 4.2.1-4A). Above q_{Gly} values of 5.0 C-mmol·g^{-1}·h^{-1}, methanol accumulation was observed in the culture, indicating that the cells could not take up methanol at the rate of 0.5 C-mmol·g^{-1}·h^{-1} anymore. The continuous decrease of the calculated q_{MeOH} value (Figure 4.2.1-4B) indicated that the culture kept losing its methanol assimilating capacity, most probably due to the repression of *p*AOX.

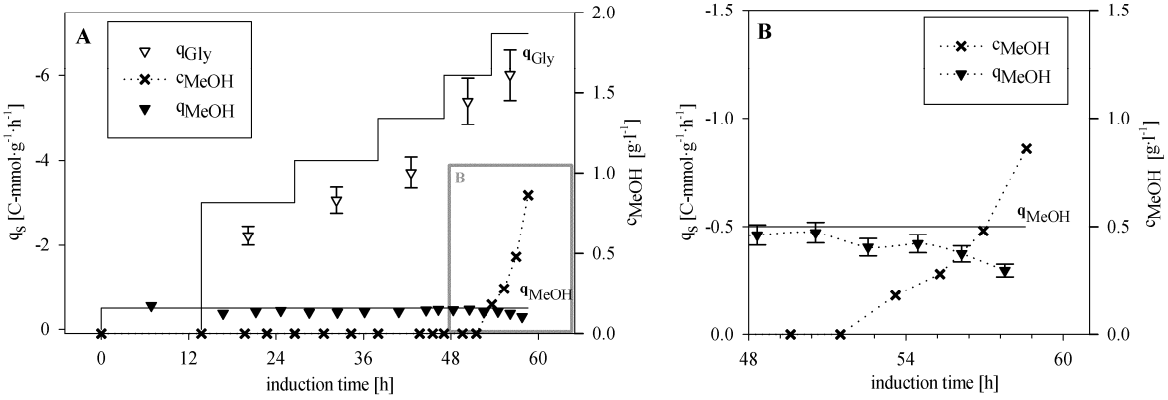


Figure 4.2.1-4. Methanol accumulation at high q_{Gly} setpoints in experiment FB2. A) Experimental strategy and calculated specific uptake rates. B) Zoom in of the last phase of the experiment - marked with a gray box in figure A - where methanol accumulated.

As most of the studies on mixed feed fermentations with *P. pastoris* were conducted at low glycerol feeding rates and with a controlled methanol concentration of 1-5 $g \cdot l^{-1}$ so far [6, 8, 11-12], there is only little reported on methanol accumulation in a carbon-limited environment. Sola *et al.* observed residual methanol concentrations in continuous mixed feed cultivations of a *P. pastoris* Mut^+ strain at dilution rates, which were higher than the respective μ_{max} on methanol [13]. However, the reason for methanol accumulation was not clear, as it could have been caused either by repression effects due to high glycerol fluxes or by methanol feeding rates which were higher than the maximal q_{MeOH} of the strain. As the two substrates were mixed in specified ratios and fed together, the reason could not be investigated accurately by the authors. In contrast, with the separately controlled feeding regime based on q_s used in the present study, exact q_{Gly} values, where the methanol uptake of the culture started to be repressed, could be determined.

Summarizing, in only two dynamic fed batch experiments (FB1 and FB2), we could estimate the effect of the q_{Gly}/q_{MeOH} ratio on the metabolic activity and determine a q_{Gly}, where methanol uptake was repressed. These findings are important steps towards process understanding and facilitate process design. Based on this knowledge, the most important metabolic attributes, like biomass growth or oxygen demand, can be predicted for different specific substrate uptake rate ratios in a mixed feed environment. Moreover, besides the characterization of metabolism in the mixed feed environment, we could gain valuable knowledge on recombinant protein expression in the two fed batch experiments.

Recombinant protein production in the dynamically changed mixed feed environment

Recombinant HRP production was induced in the adaptation phase of experiment FB1 and the specific productivity increased in parallel to the dynamically changed q_{Gly}, especially in the phases where q_{Gly} values reached 1-2 C-mmol·g^{-1}·h^{-1} (indicated in Figure 4.2.1-5). However, above a q_{Gly} of approximately 2.5 C-mmol·g^{-1}·h^{-1}, the specific productivity did not increase to the same extent as before. To analyze this trend in more detail, the effect of higher q_{Gly} rates on recombinant protein production was investigated in experiment FB2.

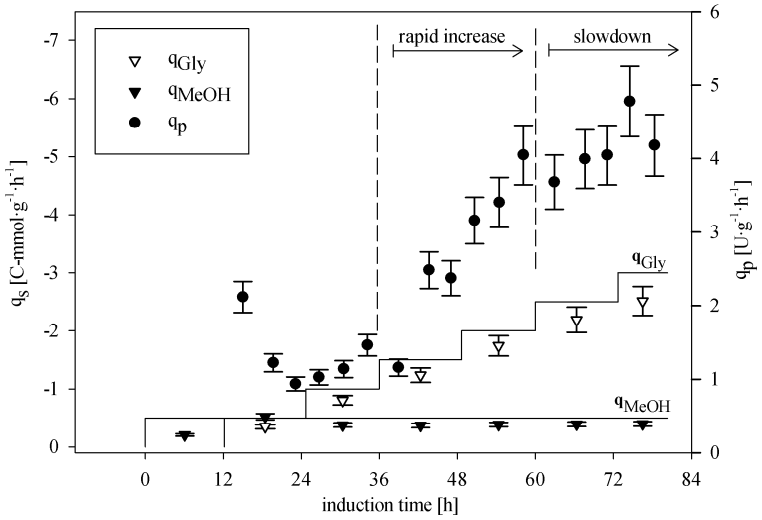


Figure 4.2.1-5 Increase of the specific productivity in the dynamic mixed feed environment of experiment FB1. The rapid increase in q_p observed in the first phase of induction slowed down at higher specific glycerol uptake rates.

Repression of recombinant protein production at high specific glycerol uptake rates

In experiment FB2 the q_{Gly} setpoint was set to 3.0 C-mmol·g^{-1}·h^{-1} immediately after the adaptation phase on methanol. Specific HRP productivity increased only slightly after the first step, and as q_{Gly} reached levels higher than 3.0 C-mmol·g^{-1}·h^{-1}, a significant drop in q_p occurred and further decreased with increasing q_{Gly} (Figure 4.2.1-6).

According to Monod kinetics, residual glycerol concentrations can be present at a high specific glycerol uptake rate, which could cause the observed decrease in q_p due to repression effects. Nevertheless, no glycerol could be detected in offline samples with the HPLC method used in this study.

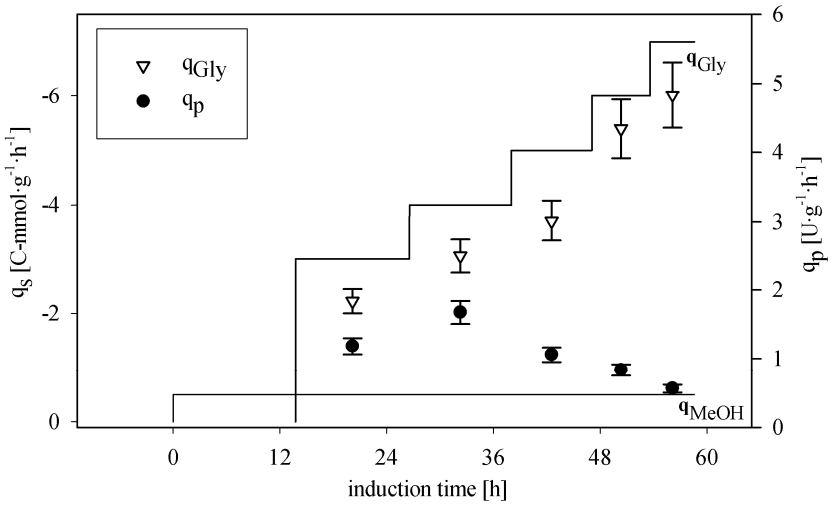


Figure 4.2.1-6 Recombinant protein expression in experiment FB2. A significant drop in recombinant protein expression was observed above a specific glycerol uptake rate of 3.0 C-mmol·g^{-1}·h^{-1}.

However, by using q_{Gly} as a process variable, we could determine the critical glycerol flux, where the culture exhibited a decline in recombinant protein expression. Therefore, we postulate here to use substrate fluxes instead of residual concentrations for the identification of repression effects in dynamic experiments. This was also suggested by Herwig *et al.* for dynamic experiments on metabolic shifts in continuous cultures with *Saccharomyces cerevisiae* [24]. Recently, Zhang *et al.* also demonstrated the existence of a carbon-flux dependent mechanism responsible for the repression of *aox* expression at a transcriptional level [25]. They reported a direct relationship between the PpHxt1 hexose transporter protein and *aox*1 repression and validated their observation by creating a Δhxt1 strain, which effectively expressed *aox* by non-limiting glucose concentrations. Interestingly, PpHxt was found to be responsible for hexose transport only at high substrate fluxes. We propose that a similar repression mechanism could lead to the decline of recombinant protein expression, as well as to the change in methanol assimilation at high glycerol fluxes, in the present study.

Interestingly, lower specific productivities were observed in experiment FB2 compared to FB1. This indicates that a feeding strategy with high initial q_{Gly} leads to low productivity (experiment FB2), whereas a more dynamic gradually increasing q_{Gly} turns out to be beneficial (experiment FB1). These observations are in coincidence with our previous study on the single substrate methanol, where we reported a stepwise increased q_s ramp to be superior to a strategy, which ensured high q_s values right from the beginning of the induction phase, in terms of productivity [19].

Repression of recombinant protein production at a higher specific methanol uptake rate

After we indicated the repression of product formation to be dependent on the glycerol flux in the mixed feed environment, we also wanted to investigate a possible effect of the methanol flux on this phenomenon. We conducted another fed batch experiment (FB3) with a higher q_{MeOH} to determine any changes in the critical q_{Gly}, at which the decline in q_p occurs. Thus, after the adaptation phase at a q_{MeOH} of 0.5 C-mmol·g^{-1}·h^{-1}, q_{MeOH} was shifted to 1.0 C-mmol·g^{-1}·h^{-1} after 12 hours (Figure 4.2.1-7A).

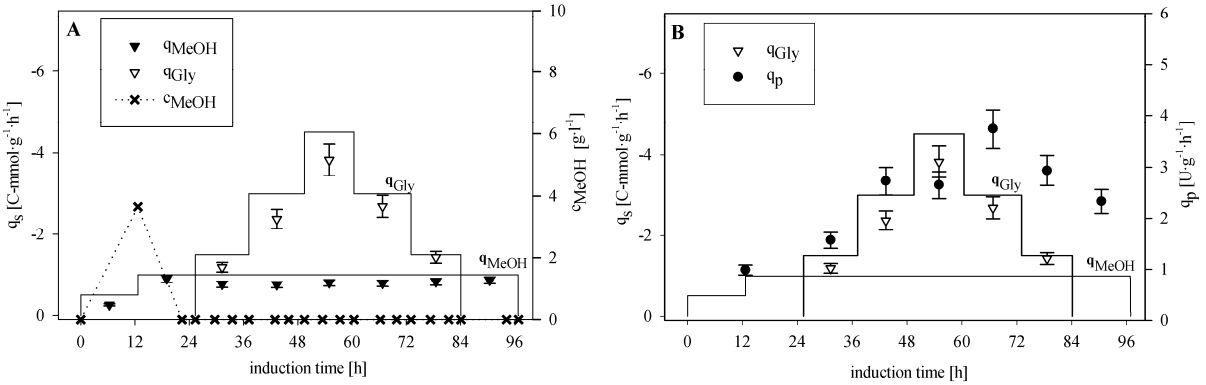


Figure 4.2.1-7 Time dependent increase of the specific productivity in experiment FB3. A) Experimental strategy and calculated specific uptake rates. B) Specific productivity in the ascending and descending phases of the glycerol feed.

After the initiation of the mixed feed phase, we observed a dynamic increase in the specific productivity in parallel to the increase of q_{Gly} (Figure 4.2.1-7B). No further increase in the specific productivity was determined as q_{Gly} reached values higher than 3.0 C-mmol·g^{-1}·h^{-1}, which was in coincidence with the results of FB2 (Figure 4.2.1-6). We concluded that an increased q_{MeOH} did not significantly change the critical q_{Gly}, where repression of productivity occurs.

In order to find out whether the culture can be released from the repression effect at high glycerol fluxes, we decreased the q_{Gly} setpoint stepwise. Interestingly, a higher q_p value (3.73 U·g^{-1}·h^{-1}) was observed in the descending phase compared to the same q_{Gly} setpoint in the ascending phase of the experiment (2.72 U·g^{-1}·h^{-1}). This observation indicates the existence of a time-dependent adaptation effect for recombinant protein expression. Hence, recombinant *P. pastoris* cells apparently need a certain induction time to reach the maximal capacity for recombinant protein expression. We have described a similar effect for a *P. pastoris* Mut^S culture in a dynamic fed batch system on the single substrate methanol before [19].

Results of ongoing experiments in our group suggest that this time-dependent effect is not detectable in chemostat cultures due to the continuous exchange of cells. The results described by d´Anjou *et al.* indicate a similar effect, as the authors observed a significantly higher specific productivity in a fed batch culture as predicted from continuous experiments [9]. Thus, a feeding strategy which is designed based on parameters from chemostat experiments would fail to consider such a time-dependent increase in recombinant protein production and deviations from the expected productivity in the fed batch environment would occur.

The effect of q_{Gly} on q_p

A decrease in recombinant protein production was observed in all experiments carried out at high q_{Gly} rates. To visualise this effect, the specific productivities determined in FB1, FB2 and FB3 were plotted against the specific uptake rate of glycerol (Figure 4.2.1-8). The maximal productivity was determined at a specific glycerol uptake rate of 3.0 C-mmol·g^{-1}·h^{-1}. Above this q_{Gly} value, repression of recombinant protein expression gets dominant.

Other mixed feed studies defined critical ratios of feeding rates [8] or specific growth rates [17], where productivity was maximal. In contrast, the individual control of specific substrate uptake rates in this study allowed the determination of a single q_{Gly} value, where the system exhibits maximal specific productivity.

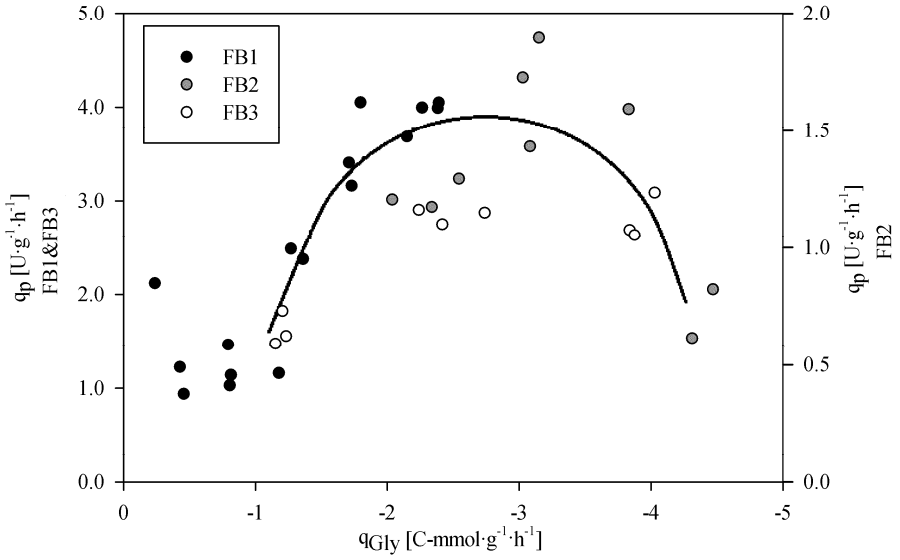


Figure 4.2.1-8 Specific productivities plotted against the specific glycerol uptake rate. The right Y axis was scaled considering the maximum specific productivity value of experiment FB2.

Conclusions

In this study, we applied a specific substrate uptake rate controlled fed batch strategy to characterize a recombinant *P. pastoris* Mut^S strain on mixed substrates. Individually adjusted feeding rates for methanol and glycerol allowed the fast investigation of the metabolism and recombinant protein expression by different limiting substrate fluxes in a fed batch environment. Specific glycerol uptake rates were identified, where repression effects for methanol uptake and productivity occurred.

Based on the observed relationship between q_{Gly} as a controlled process parameter and q_p as the critical quality attribute, following proposals for a process design targeting maximal productivities can be withdrawn:

A dynamically increasing q_{Gly} regime with a low initial q_{Gly} setpoint has beneficial effects on specific productivity in the mixed feed system. This observation highlights the use of dynamic q_s feeding regimes for production purposes.

The highest specific productivity of the used *P. pastoris* strain was observed at a specific glycerol uptake rate of 3.0 C-mmol·g^{-1}·h^{-1}. This should subsequently define the maximal value of the dynamic q_{Gly} control scheme.

A time-dependent factor, which accelerates recombinant protein production during induction, was detected with the dynamic fed batch approach. Based on this knowledge, a q_s-controlled fed batch strategy can be designed to reach the maximal q_s in correspondence with the adaptation pattern of the culture. We are currently working on the development of an optimal q_{Gly} ramp for maximal productivity.

The characteristics of the recombinant *P. pastoris* Mut^S strain were investigated in this study as a function of the specific substrate uptake rate. By using q_s as a process control parameter, a fast increase of strain- and process understanding was achieved in a few batch and fed batch cultivations. Moreover, it was possible to determine a time-dependent acceleration of the productivity in a fed batch environment, whereas continuous experiments with the same strain in our laboratory failed to predict this phenomenon.

The reported results clearly legitimate the use of the specific substrate uptake rate for process control and optimization. The q_s-controlled fed batch strategy is not only an effective method for process understanding, but also a ready-to-use optimization tool for bioprocess development.

Acknowledgements

The authors are very grateful to Prof. Anton Glieder (University of Technology, Graz, Austria) and his working group for providing the recombinant *P. pastoris* Mut^S strain expressing the horseradish peroxidase enzyme.

Abbreviations

q_s	specific substrate uptake rate [$mmol \cdot g^{-1} \cdot h^{-1}$]
$q_{s,\,max}$	maximum specific substrate uptake rate [$mmol \cdot g^{-1} \cdot h^{-1}$]
Mut^S	methanol utilization slow phenotype
AOX	alcohol oxidase
μ	specific growth rate [h^{-1}]
HRP	horseradish peroxidase
PID	proportional-integrative-derivative controller
q_p	specific productivity of horseradish peroxidase [$U \cdot g^{-1} \cdot h^{-1}$]
RQ	respiration quotient
rpm	rounds per minute
vvm	volume gas flow per volume liquid per minute
$Y_{CO2/S}$	yield coefficient of carbon dioxide respective to consumed mols of substrate [$mol \cdot C\text{-}mol^{-1}$]
$Y_{O2/S}$	yield coefficient of oxygen respective to consumed mols of substrate [$mol \cdot C\text{-}mol^{-1}$]
$Y_{X/S}$	yield coefficient of biomass respective to consumed mols of substrate [$C\text{-}mol \cdot C\text{-}mol^{-1}$]
c_{MeOH}	methanol concentration in the broth [$g \cdot l^{-1}$]

References

1. Cos O, Ramon R, Montesinos JL, Valero F: **Operational strategies, monitoring and control of heterologous protein production in the methylotrophic yeast Pichia pastoris under different promoters: a review.** *Microbial Cell Factories* 2006, **5:**1-20.
2. Zhang W, Inan M, Meagher MM: **Fermentation strategies for recombinant protein expression in the methylotrophic yeast Pichia pastoris.** *Biotechnology and Bioprocess Engineering* 2000, **5:**275-287.
3. Inan M, Meagher MM: **Non-repressing carbon sources for alcohol oxidase (AOX1) promoter of Pichia pastoris.** *Journal of Bioscience and Bioengineering* 2001, **92:**585-589.
4. Potvin G, Ahmad A, Zhang Z: **Bioprocess engineering aspects of heterologous protein production in Pichia pastoris: A review.** *Biochemical Engineering Journal*.
5. Jungo C, Marison I, von Stockar U: **Mixed feeds of glycerol and methanol can improve the performance of Pichia pastoris cultures: A quantitative study based on concentration gradients in transient continuous cultures.** *Journal of Biotechnology* 2007, **128:**824-837.
6. Hellwig S, Emde F, Raven NPG, Henke M, Van der Logt P, Fischer R: **Analysis of single-chain antibody production in Pichia pastoris using on-line methanol control in fed-batch and mixed-feed fermentations.** *Biotechnology and Bioengineering* 2001, **74:**344-352.
7. Files D, Ogawa M, Scaman CH, Baldwin SA: **A Pichia pastoris fermentation process for producing high-levels of recombinant human cystatin-C.** *Enzyme and Microbial Technology* 2001, **29:**335-340.
8. Arnau C, Casas C, Valero F: **The effect of glycerol mixed substrate on the heterologous production of a Rhizopus oryzae lipase in Pichia pastoris system.** *Biochemical Engineering Journal* 2011, **57:**30-37.
9. d'Anjou MC, Daugulis AJ: **A rational approach to improving productivity in recombinant Pichia pastoris fermentation.** *Biotechnology and bioengineering* 2001, **72:**1-11.
10. Jungo C, Rerat C, Marison IW, von Stockar U: **Quantitative characterization of the regulation of the synthesis of alcohol oxidase and of the expression of recombinant avidin in a Pichia pastoris Mut+ strain.** *Enzyme and Microbial Technology* 2006, **39:**936-944.
11. Xie J, Zhang L, Ye Q, Zhou Q, Xin L, Du P, Gan R: **Angiostatin production in cultivation of recombinant <i>Pichia pastoris</i> fed with mixed carbon sources.** *Biotechnology Letters* 2003, **25:**173-177.
12. Katakura Y, Zhang W, Zhuang G, Omasa T, Kishimoto M, Goto Y, Suga K-I: **Effect of methanol concentration on the production of human beta 2-glycoprotein I domain V by a recombinant Pichia pastoris: a simple system for the control of methanol concentration using a semiconductor gas sensor.** *Journal of Fermentation and Bioengineering* 1998, **86:**482-487.
13. Sola A, Jouhten P, Maaheimo H, Sanchez-Ferrando F, Szyperski T, Ferrer P: **Metabolic flux profiling of Pichia pastoris grown on glycerol/methanol mixtures in chemostat cultures at low and high dilution rates.** *Microbiology (Reading, United Kingdom)* 2007, **153:**281-290.
14. Brierley RA, Bussineau C, Kosson R, Melton A, Siegel RS: **Fermentation development of recombinant Pichia pastoris expressing the heterologous gene: bovine lysozyme.** *Annals of the New York Academy of Sciences* 1990, **589:**350-362.
15. Kobayashi K, Kuwae S, Ohya T, Ohda T, Ohyama M, Tomomitsu K: **High level secretion of recombinant human serum albumin by fed-batch fermentation of the**

methylotrophic yeast, Pichia pastoris, based on optimal methanol feeding strategy. *Journal of Bioscience and Bioengineering* 2000, **90:**280-288.

16. Trinh LB, Phue JN, Shiloach J: **Effect of methanol feeding strategies on production and yield of recombinant mouse endostatin from Pichia pastoris.** *Biotechnology and Bioengineering* 2003, **82:**438-444.
17. Zhang W, Hywood Potter KJ, Plantz BA, Schlegel VL, Smith LA, Meagher MM: **Pichia pastoris fermentation with mixed-feeds of glycerol and methanol: growth kinetics and production improvement.** *Journal of Industrial Microbiology & Biotechnology* 2003, **30:**210-215.
18. Zhang W, Sinha J, Smith LA, Inan M, Meagher MM: **Maximization of Production of Secreted Recombinant Proteins in Pichia pastoris Fed-Batch Fermentation.** *Biotechnology Progress* 2005, **21:**386-393.
19. Dietzsch C, Spadiut O, Herwig C: **A dynamic method based on the specific substrate uptake rate to set up a feeding strategy for Pichia pastoris.** *Microbial Cell Factories* 2011, **10:**14.
20. Dietzsch C, Spadiut O, Herwig C: **A fast approach to determine a fed batch feeding profile for recombinant Pichia pastoris strains.** *Microbial Cell Factories* 2011, **10:**85.
21. Cregg JM: **Pichia Protocols, Second Edition.** In *Methods Mol Biol* Edited by Cregg JM. Totowa, NJ, U. S.; 2007: 268 pp
22. Jungo C, Marison I, Von Stockar U: **Regulation of alcohol oxidase of a recombinant Pichia pastoris Mut+ strain in transient continuous cultures.** *Journal of Biotechnology* 2007, **130:**236-246.
23. Orman MA, Calik P, Ozdamar TH: **The influence of carbon sources on recombinant-human-growth-hormone production by Pichia pastoris is dependent on phenotype: a comparison of Muts and Mut+ strains.** *Biotechnology and Applied Biochemistry* 2009, **52:**245-255.
24. Herwig C, Marison I, Von Stockar U: **On-line stoichiometry and identification of metabolic state under dynamic process conditions.** *Biotechnology and Bioengineering* 2001, **75:**345-354.
25. Zhang P, Zhang W, Zhou X, Bai P, Cregg JM, Zhang Y: **Catabolite Repression of Aox in Pichia pastoris Is Dependent on Hexose Transporter PpHxt1 and Pexophagy.** *Applied and Environmental Microbiology* 2010, **76:**6108-6118.

4.2.2. Recombinant protein expression in *Pichia pastoris* strains with an engineered methanol utilization pathway

Florian W. Krainer[1]*, Christian Dietzsch[2]*, Tanja Hajek[1], Christoph Herwig[2], Oliver Spadiut[2†], Anton Glieder[3]

[1] Graz University of Technology, Institute of Molecular Biotechnology, Graz, Austria

[2] Vienna University of Technology, Institute of Chemical Engineering, Research Area Biochemical Engineering, Vienna, Austria

[3] Austrian Centre of Industrial Biotechnology (ACIB GmbH), Graz, Austria

* these authors contributed equally to this work

† corresponding author

Corresponding author: Oliver Spadiut, Vienna University of Technology, Institute of Chemical Engineering, Research Area Biochemical Engineering, Gumpendorfer Strasse 1a, A-1060 Vienna, Austria; oliver.spadiut@tuwien.ac.at; Tel: +43 (0)1 58801 166473

Manuscript submitted in revised form to Microbial Cell Factories.

Abstract

Background

The methylotrophic yeast *Pichia pastoris* has become an important host organism for recombinant protein production and is able to use methanol as a sole carbon source. The methanol utilization pathway describes all the catalytic reactions, which happen during methanol metabolism. Despite the importance of certain key enzymes in this pathway, so far very little is known about possible effects of overexpressing either of these key enzymes on the overall energetic behavior, the productivity and the substrate uptake rate in *P. pastoris* strains.

Results

A fast and easy-to-do approach based on batch cultivations with methanol pulses was used to characterize different *P. pastoris* strains. A strain with Mut^S phenotype was found to be superior over a strain with Mut^+ phenotype in both the volumetric productivity and the efficiency in expressing recombinant horseradish peroxidase C1A. Consequently, either of the enzymes dihydroxyacetone synthase, transketolase or formaldehyde dehydrogenase, which play key roles in the methanol utilization pathway, was co-overexpressed in Mut^S strains harboring either of the reporter enzymes horseradish peroxidase or *Candida antarctica* lipase B. Although the co-overexpression of these enzymes did not change the stoichiometric yields of the recombinant Mut^S strains, significant changes in the specific growth rate, the specific substrate uptake rate and the specific productivity were observed. Co-overexpression of dihydroxyacetone synthase yielded a 2- to 3-fold more efficient conversion of the substrate methanol into product, but also resulted in a reduced volumetric productivity. Co-overexpression of formaldehyde dehydrogenase resulted in a 2-fold more efficient conversion of the substrate into product and at least similar volumetric productivities compared to strains without an engineered methanol utilization pathway, and thus turned out to be a valuable strategy to improve recombinant protein production.

Conclusions

Co-overexpressing enzymes of the methanol utilization pathway significantly affected the specific growth rate, the methanol uptake and the specific productivity of recombinant *P. pastoris* Mut^S strains. A recently developed methodology to determine strain specific parameters based on dynamic batch cultivations proved to be a valuable tool for fast strain characterization and thus early process development.

Background

The methylotrophic yeast *Pichia pastoris* has become an important host organism for the high level production of recombinant proteins (e.g. [1-3]). It can be grown to high cell densities, is characterized by an efficient secretory system and many tools for molecular manipulation are available. Thus, *P. pastoris* has become an interesting and important alternative to bacterial expression systems such as *E. coli*, especially when it comes to the production of complex proteins which require typical eukaryotic post-translational modifications or contain disulfide bridges [4].

The ability of *P. pastoris* to utilize methanol as the sole carbon source is a crucial aspect of its metabolism. The enzyme alcohol oxidase (AOX, EC 1.1.3.13) catalyzes the first step in the recently described methanol utilization pathway (MUT pathway) [5]. The genome of *P. pastoris* contains two genes, *AOX*1 and *AOX*2, encoding two enzymes with AOX activity [2]. AOX1 can comprise up to 30 % of the total soluble protein in extracts of *P. pastoris* grown solely on methanol [5-7], showing the outstanding strength of the AOX1 promoter (*p*AOX1). On the contrary, the second alcohol oxidase AOX2 is controlled by a much weaker promoter (*p*AOX2) and thus accounts for just 15 % of the overall AOX activity in the cell [8]. The tight regulation of these promoters [9], the strong inducibility of *p*AOX1 and differently regulated *p*AOX1 promoter variants [10] as well as the alternative weaker AOX2-mediated methanol oxidation allow the design of different expression strains with specific properties. Three phenotypes of *P. pastoris* with regard to methanol utilization are currently available: I, Mut^+ (methanol utilization plus), where both *AOX* genes are intact and active; II, Mut^S (methanol utilization slow), where *AOX*1 is knocked out; and III, Mut^- (methanol utilization minus), which is unable to grow on methanol as the sole carbon source due to a knock-out of both *AOX* genes [2]. In Mut^+ and Mut^S strains the transcription of MUT pathway genes is repressed when grown in the presence of sufficiently high concentrations of glucose or glycerol. Employing either of the two natural AOX promoters, protein expression at high levels can be induced by methanol in media lacking such fermentable carbon sources [11]. De-repression at low concentrations of glucose or glycerol can be used for strong induction, if AOX1 promoter variants are used [10].

In general, Mut^+ strains are characterized by a higher growth rate than Mut^S strains and have also been reported to show higher productivities [12-15]. In addition, if antibiotics are used for selection of transformants, there is no direct need to employ Mut^S strains, but wildtype strains such as *P. pastoris* CBS7435 (which is identical to *P. pastoris* NRRL Y-11430 [16]) or *P. pastoris* X-33 can be used as hosts. This is why the majority of research so far has been

performed with this phenotype. However, Mut^+ strains are very sensitive to transient high methanol concentrations, rendering the scale up of bioprocesses more difficult [15, 17, 18]. Methanol oxidation is also linked to hydrogen peroxide formation as a by-product which is known to cause cellular stress and to induce cell death [19]. The combustion of methanol (-727 $kJ \cdot C\text{-}mol^{-1}$) results in the production of heat, which might also constitute a problem in large scale processes. Another important issue is the high demand for oxygen in high cell density cultures of *P. pastoris* with Mut^+ phenotype [20, 21]. By using *P.* pastoris strains with Mut^S phenotype these problems can be bypassed due to the lower methanol consumption rate. However, the Mut^S phenotype also leads to long induction times and reduced growth rates. Mixed feed strategies (e.g. glycerol and methanol) are commonly employed for the fermentation induction phase when using Mut^S strains. Hereby the cells are not dependent on the slow metabolization of methanol, which then primarily functions as an inducer, and can use the alternative C-source for growth [20, 22]. Moreover, the strong production of AOX1 in Mut^+ strains during growth on methanol may compete with the production of recombinant proteins [15]. Other advantages and disadvantages of using the AOX1 promoter system in *P. pastoris* Mut^+ strains have been summarized by Macauley-Patrick *et al.* recently [7]. Interestingly, Mut^S strains have been reported to be advantageous over Mut^+ strains for the production of some recombinant proteins [23-25], which currently drives the ongoing discussion in the scientific community about the most favorable *P. pastoris* phenotype for recombinant protein production.

Regardless of which phenotype is used, AOX catalyzes the first reaction in the MUT pathway, oxidizing methanol to formaldehyde while reducing O_2 to H_2O_2 (Figure 4.2.2-1A). Formaldehyde is then either oxidized to CO_2 in the dissimilative branch of the MUT pathway giving 2 NADH molecules per molecule formaldehyde, or is condensed with xylulose-5-phosphate and subsequently converted to dihydroxyacetone and glyceraldehyde-3-phosphate in the assimilative branch of the MUT pathway (Figure 4.2.2-1A; see also [5]).

Knock-out studies of the genes encoding formaldehyde dehydrogenase (FLD, EC 1.2.1.1) and formate dehydrogenase (FDH; EC 1.2.1.2) revealed FLD, hereafter called FLD1, to be the key enzyme in the dissimilative branch of the MUT pathway [26, 27]. FLD1 is encoded by the *FLD1* gene and catalyzes the NAD^+-dependent oxidation of S-hydroxymethylglutathione to S-formylglutathion (Figure 4.2.2-1B). Consequently, FLD1 has been studied in more detail in terms of being a promising enzyme for improved cofactor regeneration recently [28].

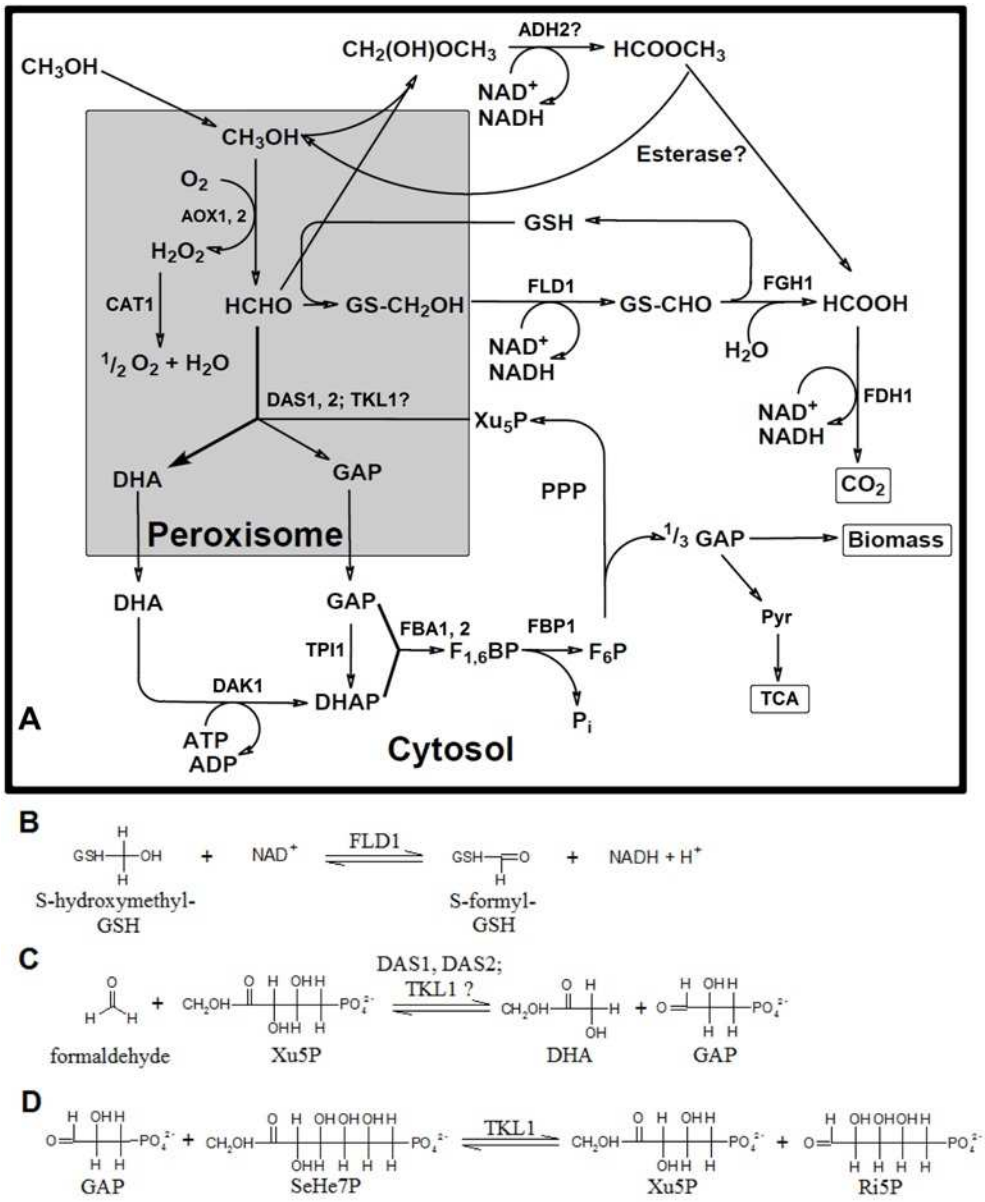


Figure 4.2.2-1 Methanol utilization (MUT) pathway in *P. pastoris*. A, MUT pathway overview (adapted from [5] and [16]); B, catalytic reaction of DAS1, DAS2 and hypothetically TKL1; C, catalytic reaction of FLD1; D, catalytic reaction of TKL1 in the pentose phosphate pathway. ADH: methylformate synthase. AOX: alcohol oxidase. CAT: catalase. DAK: dihydroxyacetone kinase. DHA: dihydroxyacetone. DHAP: dihydroxyacetone phosphate. $F_{1,6}BP$: fructose-1,6-bisphosphate. F_6P: fructose-6-phosphate. FBA: fructose-1,6-bisphosphate aldolase. FBP: fructose-1,6-bisphosphatase. FLD: formaldehyde dehydrogenase. FDH: formate dehydrogenase. FGH: S-formylglutathione hydrolase. GAP: glyceraldehyde-3-phosphate. GSH: glutathione. Pyr: pyruvate. PPP: pentose phosphate pathway. Ri5P: ribose-5-phosphate. SeHe7P: sedoheptulose-7-phosphate. TCA: tricarboxylic acid cycle. Xu_5P: xylulose-5-phosphate.

The key enzyme in the assimilative branch of the MUT pathway is dihydroxyacetone synthase (DAS; EC 2.2.1.3). In *P. pastoris*, two isoforms of DAS are encoded by the two genes *DAS1* and *DAS2* which are 91 % identical [5, 16]. Due to the high sequence identity, wrong sequences for these two genes were described before, which were artefacts from wrong

assemblies during sequencing. Both DAS1 and DAS2 catalyze the conversion of xylulose-5-phosphate and formaldehyde to dihydroxyacetone and glyceraldehyde-3-phosphate (Figure 4.2.2-1C; [5, 16]). Despite the importance of this enzyme, no analysis has been reported so far, describing whether the transcription of the two genes *DAS1* and *DAS2* is equally high induced upon methanol induction or if differences in transcription, such as for *AOX1* and *AOX2*, occur.

Another important enzyme in the assimilative branch is the transketolase (TKL; EC 2.2.1.1) [16], hereafter called TKL1, which catalyzes the reaction between xylulose-5-phosphate and ribose-5-phosphate to form glyceraldehydes-3-phosphate and sedoheptulose-7-phosphate and *vice versa* (Figure 4.2.2-1D). In *Saccharomyces cerevisiae* transketolase activity has been shown to constitute the rate-limiting factor in the non-oxidative part of the pentose phosphate pathway [29], demonstrating the importance of this enzyme in yeast metabolism. In *P. pastoris* TKL1 has recently been assigned a hypothetical dihydroxyacetone synthase activity (Figure 4.2.2-1C; [16]). A highly conserved domain structure of the enzymes DAS1, DAS2 and TKL1 and the fact that a double knock-out strain of *DAS1* and *DAS2* is still able to grow on the substrate methanol (personal communication with Prof. Anton Glieder) actually underline this hypothesis (Supplementary Figure 4.2.2-1).

In this study, we determined and compared the specific substrate uptake rate (q_s) and the specific productivity (q_p) of a recombinant *P. pastoris* CBS7435 Mut^+ strain with a Mut^S strain, both overexpressing the reporter enzyme horseradish peroxidase (HRP), to determine the more efficient phenotype for recombinant protein expression. Based on our findings, we co-overexpressed either of the enzymes DAS1, TKL1 or FLD1, which all play key roles in the MUT pathway, in recombinant *P. pastoris* Mut^S strains harboring either of the reporter enzymes horseradish peroxidase (HRP) or *Candida antarctica* lipase B (CalB), to check for possible influences on stoichiometric yields, the growth rate, the methanol uptake and the production of the respective reporter enzyme. We used a novel and fast approach based on batch cultivations with repeated methanol pulses, which has proven to be a valuable alternative to the traditionally more often used strategies of either employing continuous cultures or repetitive fed-batch cultivations at different parameter sets [33, 34], to determine these strain specific parameters.

Material and Methods

Chemicals

Enzymes were purchased from Fermentas GmbH, Germany. 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) diammonium salt (ABTS) was obtained from Sigma-Aldrich Handels GmbH, Austria. Difco™ yeast nitrogen base w/o amino acids (YNB), Bacto™ tryptone and Bacto™ yeast extract were obtained from Becton Dickinson and Company, Austria. Zeocin™ was obtained from InvivoGen-Eubio, Austria. D-Biotin and p-nitrophenyl butyrate (p-NPB) were obtained from Fluka Chemia AG, Switzerland. All other chemicals were purchased from Carl Roth GmbH & Co. KG, Germany.

Strains and vectors

The strain *P. pastoris* Mut^S (Δ*aox*1::*FRT*) was engineered by Näätsaari *et al*. (manuscript in preparation) at Graz University of Technology, Institute of Molecular Biotechnology, based on the *P. pastoris* wildtype strain CBS7435. These two strains were used as starting strains for the corresponding overexpression studies. The shuttle vectors pPpT4_S and pPpKan_S, derivatives of pPpT2 [38] with two point mutations in the EM72 promoter and a *Smi*I restriction site instead of *Bgl*II, were used for cloning. pPpKan_S contains a kanamycin/geneticin resistance gene instead of a zeocin resistance gene [39, 40]. pPpT4_S was used for harboring either the HRP isoenzyme C1A or CalB, which both were codon-optimized for high-level expression in *P. pastoris*. The codon table described in [38] was applied for codon optimization. Overexpression of *DAS*1, *FLD*1, *TKL*1 and the genes encoding HRP and CalB was under the control of *p*AOX1. Secretion of HRP and CalB to the cultivation supernatant was facilitated by a N-terminally fused prepro-signal sequence of the *S. cerevisiae* alpha-factor. pPpKan_S was used as shuttle vector harboring *DAS1*, *FLD1* or *TKL1*, which were amplified from chromosomal DNA of *P. pastoris* CBS7435 using the cloning primers depicted in Table 1A with Phusion™ high fidelity DNA-polymerase (Finnzymes Oy, Finland) and GC buffer according to the manufacturer's protocol.

Strain construction and screening procedure

Transformation of approximately 2 µg *Smi*I-linearized pPpT4_S or pPpKan_S plasmid DNA harboring the respective gene of interest into *P. pastoris* was done as described by Lin-Cereghino *et al*. [41]. Selection of successfully transformed clones was performed on YPD agar plates containing 25 mg·l^{-1} zeocin (for HRP/CalB strains) or 25 mg·l^{-1} zeocin plus 300 mg·l^{-1} geneticin (for HRP/CalB DAS1/FLD1/TKL1 strains). Screening of randomly

chosen transformants and rescreening of promising clones (in quadruplicates) were done as micro-scale cultivations in 96-deep well plates similar to the protocol described by Weis *et al*. [42]: cells were cultivated in 250 µl BMD1% (1 % α-D(+)-glucose monohydrate, 6.0 $g \cdot l^{-1}$ K_2HPO_4, 23.6 $g \cdot l^{-1}$ KH_2PO_4, 13.4 $g \cdot l^{-1}$ YNB, 0.4 mg/l D-biotin) for approximately 60 h, followed by addition of 250 µl BMM2 (1 % methanol, 6.0 $g \cdot l^{-1}$ K_2HPO_4, 23.6 $g \cdot l^{-1}$ KH_2PO_4, 13.4 $g \cdot l^{-1}$ YNB, 0.4 $mg \cdot l^{-1}$ D-biotin) and 50 µl BMM10 (5 % methanol, 6.0 $g \cdot l^{-1}$ K_2HPO_4, 23.6 $g \cdot l^{-1}$ KH_2PO_4, 13.4 $g \cdot l^{-1}$ YNB, 0.4 $mg \cdot l^{-1}$ D-biotin) per well 12 h, 24 h and 36 h after the first addition of BMM2.

HRP activity was verified by using an ABTS assay: after centrifugation of the induced transformants (3000 g, 10 min), 15 µl of the cultivation supernatant were mixed with 140 µl assay solution (0.5 mM ABTS in 50 mM NaOAc, pH 4.5, 2.9 mM H_2O_2) in a 96-well PS-microplate [35]. The increase in absorbance at 405 nm was followed in a Spectramax Plus 384 platereader (Molecular Devices, Germany) at room temperature for 5 min. CalB activity was evaluated by an esterase activity assay similar to the assay described by Zhang *et al.* [43]: 20 µl cultivation supernatant were mixed with 180 µl fresh assay solution (4 mM p-NPB in 300 mM Tris-HCl, pH 7.4, 1 % dimethyl sulfoxid). The increase in absorbance at 405 nm was followed in a Spectramax Plus 384 platereader at room temperature for 5 min.

Evaluation of the transcription of *DAS1* and *DAS2* upon methanol induction was performed by quantitative real-time PCR (qPCR). For this purpose, RNA was isolated from the corresponding strains before and 5 h after methanol induction using the ZR Fungal/Bacterial RNA MicroPrep™ kit from Zymo Research Corporation (Irvine, CA, USA). In-column DNase digestion was performed according to the manufacturer's recommendations using RNase-free DNase I from Zymo Research. RNA was eluted in 20 µl DNase/RNase-free water (plus 1 µl RNaseOUT™ Recombinant Ribonuclease Inhibitor from Invitrogen Corporation, CA, USA) and used directly for qPCR using the SuperScript® III Platinum® SYBR® Green One-Step qRT-PCR Kit (Invitrogen). The qPCR was performed in the ABI PRISM 7500 Real Time PCR System (Applied Biosystems, CA, USA) according to the manufacturer's recommendations employing the primers listed in Table 4.2.2-1B. For visualization of the magnitude of transcription induction, the determined cycle threshold (Ct) signals were transformed to 2^{-Ct} and normalized to the corresponding *ARG4* signals (which is supposed to be constitutively transcribed). The extent of induction of a target transcript was calculated as the ratio of the normalized 2^{-Ct} signal of the target transcript at 5 h over the normalized signal at 0 h (*i.e.* before induction). The time point 0 h represented the de-repression state of the *P. pastoris* culture, since the C-source glucose was depleted before the addition of methanol.

Analogically, the overexpression of genes encoding MUT pathway enzymes was verified via qPCR with the primers listed in Table 4.2.2-1C. The transcript levels were shown as 2^{-Ct} signals, normalized to the corresponding *ARG4* signals.

To determine the copy number of the transformed genes, a qPCR based method was used as described by Abad *et al*. [44] using the Power SYBR® Green Master Mix (Applied Biosystems) with the ABI PRISM 7500 Real Time PCR System. Hereto, genomic DNA was isolated from *P. pastoris* according to the protocol by Hoffman *et al*. [45]. The primers (Table 4.2.2-1D) were used at concentrations of 200 nM per primer with 0.66 ng of genomic DNA as template. The copy number of the genes encoding the target enzymes was determined indirectly via verification of the copy number of the Zeocin™ resistance-mediating gene Zeo^R. Conditions were 10 min at 95°C, 40 cycles of 15 s at 95°C and 60 s at 60°C followed by a final dissociation step.

Table 4.2.2-1 Oligonucleotide-primers used for A, amplification of *DAS1*/*FLD1*/*TKL1* from chromosomal DNA; B, evaluation of *DAS1*/*DAS2* transcription induction; C, the analysis of *DAS1*/*FLD1*/*TKL1* overexpression; D, copy number determination of the reporter genes (via verification of the Zeo^R copy number).

A.	***target***	***orientation***	***sequence 5'-3'***
	DAS1	forward	AAAAGGCGCGCCGAAACGATGGCTAGAATTCCCAAAGCAG
		reverse	TTTGCGGCCGCTTACAACTTGTCATGCTTTGGTTTTC
	FLD1	forward	AACACTAGTATGTCTACCGAAGGTCAA
		reverse	AACGCGGCCGCTTAGTGCATAGTAATCAC
	TKL1	forward	AGAGAATTCGAAACGATGTCTGATCTCTTAGCTATCAACAC
		reverse	AGAGCGGCCGCCTACGCATGAACAGACTCAAAAG

B.	***target***	***orientation***	***sequence 5'-3'***
	ARG4	forward	TGCTGGCTACAGATCTTGCCGACT
		reverse	CTCGGCTTGTCTGACACATTCACCAG
	AOX2	forward	ATACTCATCCGAGGCCAGAGCTTACG
		reverse	ACCGTGAGCAAGACCAGCAGTCAA
	DAS1	forward	CTGAGAAACCAGCTAAAGGTGACGAGT
		reverse	TCTTGTCCCTCACGAGGGTACTCT
	DAS2	forward	CTGAAAAACCAGCCGAGGGTGATC
		reverse	TTCCTCACCTTCTTGAGGATAGTTCTTAACG

C.	***target***	***orientation***	***sequence 5'-3'***
	ARG4	forward	TGCTGGCTACAGATCTTGCCGACT
		reverse	CTCGGCTTGTCTGACACATTCACCAG
	AOX2	forward	ATACTCATCCGAGGCCAGAGCTTAC
		reverse	ACCGTGAGCAAGACCAGCAGTCAA
	DAS1	forward	CTGAGAAACCAGCTAAAGGTGACGAGT

		reverse	TCTTGTCCCTCACGAGGGTACTCT
	FLD1	forward	TGGATTATCTGTCATCCAAGGTGCAGTTTC
		reverse	GTCCGCCCATGCCTTCTTTGAATC
	TKL1	forward	GTCGCTACACATGACTCGATTGGTC
		reverse	CATGAGGTTTGGAAGAGCTCTCAAGTG

D.	***target***	***orientation***	***sequence 5'-3'***
	Zeo^R	forward	GACTCGGTTTCTCCCGTGACT
		reverse	CTGCGGAGATGAACAGGGTAA
	ARG4	forward	TCCTCCGGTGGCAGTTCTT
		reverse	TCCATTGACTCCCGTTTTGAG

Strain characterization in bioreactors

Culture Media

Precultures were done in yeast nitrogen base medium (YNBM; 0.1 M potassium phosphate buffer pH 6.0, 3.4 $g \cdot l^{-1}$ YNB w/o amino acids and ammonia sulfate, 10 $g \cdot l^{-1}$ $(NH_4)_2SO_4$, 400 $mg \cdot l^{-1}$ biotin, 20 $g \cdot l^{-1}$ glucose). Batch cultivations were performed in basal salt medium (26.7 $ml \cdot l^{-1}$ 85 % phosphoric acid, 1.17 $g \cdot l^{-1}$ $CaSO_4 \cdot 2H_2O$, 18.2 $g \cdot l^{-1}$ K_2SO_4, 14.9 $g \cdot l^{-1}$ $MgSO_4 \cdot 7H_2O$, 4.13 $g \cdot l^{-1}$ KOH, 44 $g \cdot l^{-1}$ $C_6H_{12}O_6 \cdot H_2O$, 0.2 $ml \cdot l^{-1}$ Antifoam Struktol J650, 4.35 $ml \cdot l^{-1}$ PTM1, NH_4OH as N-source). Trace element solution (PTM1) was made of 6.0 $g \cdot l^{-1}$ $CuSO_4 \cdot 5H_2O$, 0.08 $g \cdot l^{-1}$ NaI, 3.0 $g \cdot l^{-1}$ $MnSO_4 \cdot H_2O$, 0.2 $g \cdot l^{-1}$ $Na_2MoO_4 \cdot 2H_2O$, 0.02 $g \cdot l^{-1}$ H_3BO_3, 0.5 $g \cdot l^{-1}$ $CoCl_2$, 20.0 $g \cdot l^{-1}$ $ZnCl_2$, 65.0 $g \cdot l^{-1}$ $FeSO_4 \cdot 7H_2O$, 0.2 $g \cdot l^{-1}$ biotin, 5 $ml \cdot l^{-1}$ H_2SO_4. Induction was carried out in presence of 1 mM δ-aminolevulinic acid for HRP overexpressing strains. The concentration of the base NH_4OH was determined by titration with 0.25 M potassium hydrogen phthalate.

Experimental Procedure

Preculture

Frozen stocks (-80°C) were pre-cultivated in 100 ml YNBM in 1000 ml shake flasks at 28°C and 230 rpm for max. 24 h. The grown preculture was transferred aseptically to the respective culture vessel. The inoculation volume was approximately 10 % of the final starting volume.

Batch cultivation

Batch cultivations were carried out in either a 3 l or a 5 l working volume glass bioreactor (Infors, Switzerland). Basal salt medium was sterilized in the bioreactor and pH was adjusted to pH 5.0 by using concentrated NH_4OH solution after autoclaving. Sterile filtered trace elements were transferred to the reactor aseptically. Dissolved oxygen (dO_2) was measured with a sterilizable dO_2 electrode (Visiferm™, Hamilton, Switzerland). The pH was measured with a sterilizable electrode (Easyferm™, Hamilton, Switzerland) and maintained constant with a PID controller using NH_4OH solution (1 to 3 M). Base consumption was determined gravimetrically. Cultivation temperature was set to 28°C and agitation was fixed to 1200 rpm. The culture was aerated with 1.0 vvm dried air and off-gas of the culture was measured by using an infrared cell for CO_2 and a paramagnetic cell for O_2 concentration (Servomax, Switzerland). Temperature, pH, dO_2, agitation as well as CO_2 and O_2 in the off-gas were measured online and logged in a process information management system (PIMS; Lucullus, Biospectra, Switzerland).

After the complete consumption of the substrate glucose, indicated by an increase of dO_2 and a drop in off-gas activity, the first methanol pulse of a final concentration of 0.5 % (v/v) was conducted with methanol (supplemented with 12 $ml \cdot l^{-1}$ PTM1). Following pulses were performed with 1 % methanol (v/v). All pulses were conducted directly after exhaustion of the substrate without lack phases. For each pulse, at least two samples were taken to determine the concentrations of substrate and product, as well as dry cell weight to calculate specific rates and yields.

Analysis of growth- and expression-parameters

Dry cell weight was determined by centrifugation of 5 ml culture broth (5000 rpm, 4°C, 10 min) in a laboratory centrifuge (Sigma 4K15, rotor 11156), washing the pellet with 5 ml deionized water and subsequent drying at 105°C to a constant weight in an oven.

The enzymatic activity of HRP was measured using an ABTS assay in a CuBiAn XC enzymatic robot (Innovatis, Germany). Ten µl of sample were mixed with 140 µl 1 mM ABTS solution (50 mM KH_2PO_4, pH 6.5). The reaction mixture was incubated at 37°C for 5 min before the reaction was started by the addition of 20 µl 0.078% H_2O_2 (v/v). Changes in absorbance at 415 nm were measured for 80 seconds and rates were calculated. The standard curve was prepared using a commercially available HRP preparation (Type VI-A, Sigma-Aldrich, USA) in the range from 0.02 to 2.0 $U \cdot ml^{-1}$.

The enzymatic activity of CalB was measured by determining the esterase activity of CalB. A stock solution was generated by mixing 42 µl p-NPB with 458 µl DMSO and stored at -20°C. The assay reagent was prepared freshly by addition of 100 µl stock solution to 10 ml TrisHCl (300 mM, pH 7.5). Color development at 405 nm was followed in a UV-1601 spectrophotometer (Shimadzu GmbH; Austria) at 30°C for 5 min. One Unit of CalB was defined as the formation of 1 µmol p-nitrophenol·min^{-1}.

Substrate concentrations

Concentrations of methanol were determined in cell free samples by HPLC (Agilent Technologies, USA) equipped with an ion-exclusion column (Supelcogel C-610H Sigma-Aldrich, USA) and a refractive index detector (Agilent Technologies, USA). The mobile phase was 0.1 % H_3PO_4 with a constant flow rate of 0.5 ml·min^{-1} and the system was run isocratically. Calibration was done by measuring standard points in the range from 0.1 to 10 g·l^{-1} methanol.

Data analysis

Measurements of biomass concentration, product concentration and substrate concentration were executed in duplicates. Along the observed standard deviation for the single measurements, the error was propagated to the specific rates q_s and q_p as well as to the yield coefficients. The error for the volumetric rate of product formation was set to 5 %.

Data reconciliation

The Mut^S phenotype caused a slow biomass growth during conducted methanol pulses. Thus, the determined increase of biomass during one pulse was close to the standard deviation observed for one duplicate measurement and could therefore not be regarded as accurate. Hence, the biomass rate was calculated using the MATLAB software (MathWorks, USA), using the carbon balance and the degree of reduction balance, made of the determined volumetric rates for the substrate uptake, the oxygen consumption and the carbon dioxide evolution. As the system of equations consisted of two elemental balances and only one rate to be calculated, the degree of freedom was 1. Consequently, the statistical test value h had to be lower than 3.84 not to reject the null hypothesis, that there were no errors in the measurements at a 95 % confidence level for each data point. Including error margins of 5 % for off-gas measurements and substrate determinations, a χ^2 (chi-square distribution) test was

conducted for each data point and gave the corresponding h-values. This method was described in detail elsewhere [37].

Results and Discussion

Mut$^+$ vs. Mut^S for recombinant protein production

To date, the majority of research has been performed with *P. pastoris* Mut$^+$ strains, since they have been reported to grow faster on methanol and thus to produce more recombinant protein (e.g. [14]). However, several other studies have shown Mut^S strains to be superior over Mut$^+$ strains in terms of recombinant protein production (e.g. [23]). To shed more light on this controversial topic, we designed *P. pastoris* strains overexpressing the reporter enzyme HRP in both phenotypes Mut$^+$ and Mut^S, and compared these two strains in terms of specific substrate uptake rate, specific productivity and volumetric productivity. This strain characterization was performed using a recently reported strategy employing fast and easy-to-do batch cultivations with methanol pulses [33, 34] and is shown for the Mut^S and for the Mut$^+$ phenotype in Supplementary Figure 4.2.2-2 and Supplementary Figure 4.2.2-3, respectively.

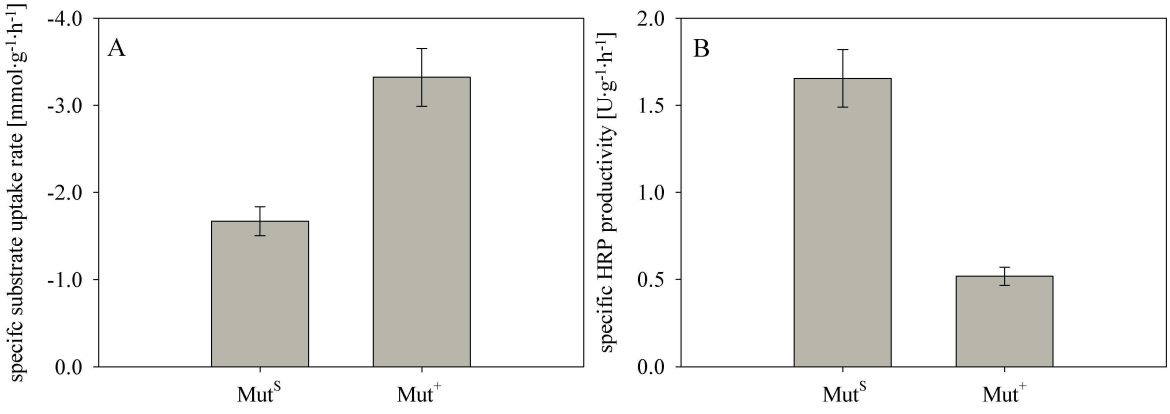


Figure 4.2.2-2 Average values for specific rates obtained in pulse experiments with *P. pastoris* Mut^S and Mut$^+$ strains overexpressing HRP. A, specific substrate uptake rate for methanol; B, specific HRP productivity.

The frequent determination of biomass, methanol and product concentration allowed specific rate calculations during methanol pulses. Average values for the specific rates were calculated out of several pulses to be able to compare the two strains on the basis of a reliable set of data. The specific growth rate of the Mut$^+$ strain was calculated to be approximately 1.5-fold higher than for the Mut^S strain (data not shown). As shown in Figure 4.2.2-2, the Mut$^+$ strain was also characterized by a nearly 2-fold higher specific uptake rate for methanol (q_s), whereas the specific productivity (q_p) was 3-fold lower compared to the Mut^S strain.

To combine the observed effect on q_s and q_p in just one parameter and thus put the productivity of the strains in direct relation to the consumed substrate, we introduced the efficiency factor η (equation 4.2.2.1), which is in fact the product yield respective to the substrate methanol.

$$\eta = \left| \frac{q_p}{q_s} \right| \text{ [U/mmol]} \qquad \text{(equation 4.2.2.1)}$$

In Figure 4.2.2-3A, the benefit of using a Mut^S strain for the recombinant production of HRP is highlighted, as the substrate methanol was converted 7-fold more efficient into product compared to the Mut^+ strain.

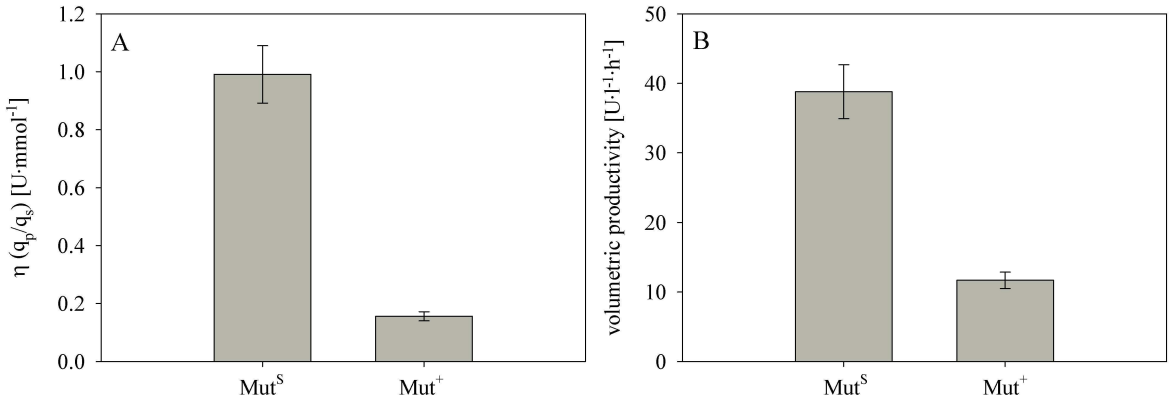


Figure 4.2.2-3 Comparison of a *P. pastoris* Mut^S and Mut^+ strain overexpressing HRP. A, efficiency factor η (relationship between q_p and q_s); B, volumetric productivity.

Additionally, the volumetric productivity was calculated to compare the two Mut phenotypes in terms of product formation per volume and time. As shown in Figure 4.2.2-3B, the volumetric productivity of the Mut^S strain was more than 3-fold higher than for the Mut^+ strain, underlining the usefulness of this phenotype for the production of recombinant proteins. Interestingly, Morawski *et al.* reported up to 3-fold higher HRP activity levels using the *P. pastoris* wildtype strain X-33 with Mut^+ phenotype compared to the strain KM71 with Mut^S phenotype (*his4*, *aox1::ARG4*) [35]. An explanation for this contradictory result might be a difference in the gene copy number of HRP in the Mut^+ and Mut^S strains studied by Morawski *et al*. and/or differences in the operational conditions. Since the strains used in the present study both were found to have a single copy integration of the HRP encoding gene, and since the batch cultivations were performed in the same way in the same bioreactors, we can exclude such influences. Consequently, the *P. pastoris* Mut^S strain was chosen as the basis for subsequent overexpression studies.

Transcription of the genes *DAS1* and *DAS2* upon methanol induction

In order to study a possible difference between *DAS1* and *DAS2* transcription upon methanol induction, as known for the *AOX1* and *AOX2* genes [36], we determined the respective transcript levels in a *P. pastoris* Mut^S strain and calculated the increase in transcription after 5 h upon induction. The transcription of *DAS1* and *DAS2* was found to be equally high induced upon methanol induction, as *DAS1* transcription was 98.1-fold and *DAS2* was 99.1-fold induced. Methanol induction of the *DAS1* and *DAS2* genes was also much stronger than for *AOX2* (about 30-fold). Considering the high sequence similarity of *DAS1* and *DAS2* and the equivalent increase in transcript levels upon methanol induction, we focused on the co-overexpression of *DAS1* to subsequently study the influence of emphasized dihydroxyacetone synthase activity on recombinant protein expression.

Co-overexpression of the MUT pathway enzymes DAS1/FLD1/TKL1

We co-overexpressed three key enzymes of the MUT pathway, *i.e.* dihydroxyacetone synthase 1 (DAS1), formaldehyde dehydrogenase 1 (FLD1) and transketolase 1 (TKL1), to analyze possible effects on stoichiometric yields and on the strain specific parameters q_s and q_p. We used a Mut^S strain for this study, since it had been shown to be superior to the Mut^+ phenotype in terms of recombinant protein production (*vide supra*). To be able to draw more general valid conclusions from these experiments, we used two recombinant *P. pastoris* Mut^S strains overexpressing either the reporter enzyme HRP or CalB, hereafter called benchmark strains, as a platform for co-overexpression studies with the above mentioned MUT pathway enzymes. We characterized all strains in terms of gene copy numbers of the respective reporter enzyme to exclude variations in the measured enzyme activity levels due to copy number rearrangements, such as duplications or deletions. All the generated strains had a single copy integration of the gene encoding the respective reporter enzyme. Thus, all observed variations in enzymatic activity were considered to be due to the respective co-overexpressed MUT pathway enzyme. In order to verify the successful co-overexpression of the MUT pathway enzymes, the mRNA levels of *DAS1*, *FLD1* and *TKL1* were quantified relatively to the transcript levels of the constitutively transcribed *ARG4* gene via qPCR analysis (Figure 4.2.2-4).

In all strains the transcript level of *AOX2* increased upon methanol addition, indicating successful induction with methanol. We also observed elevated transcript levels of the respective mRNAs of *DAS1*, *FLD1* and *TKL1* upon methanol induction in all co-overexpression strains compared to the benchmark strains (Figure 4.2.2-4), proving the

successful co-overexpression of the MUT pathway enzymes in these strains. However, transcript levels varied between HRP and CalB strains: the transcript level of *FLD1* in the CalB FLD1 strain was 2.5-fold higher than in the HRP FLD1 strain, whereas *DAS1* and *TKL1* were 1.5-fold and 2.5-fold higher transcribed in HRP DAS1/TKL1 strains, respectively. This could be due to two reasons: despite the fact that the same amount of DNA was used for each transformation, different copy numbers of the respective genes might have integrated. Also, the genes might have integrated at different loci in the chromosome, which might influence the accessibility of the transcription machinery to the transformed gene and thus the extent of transcription. Regardless of these variations, all MUT pathway enzymes were successfully co-overexpressed, which is why observed effects on stoichiometric yields, the specific substrate uptake rate and the specific productivity of the recombinant *P. pastoris* Mut^S strains were ascribed to these enzymes.

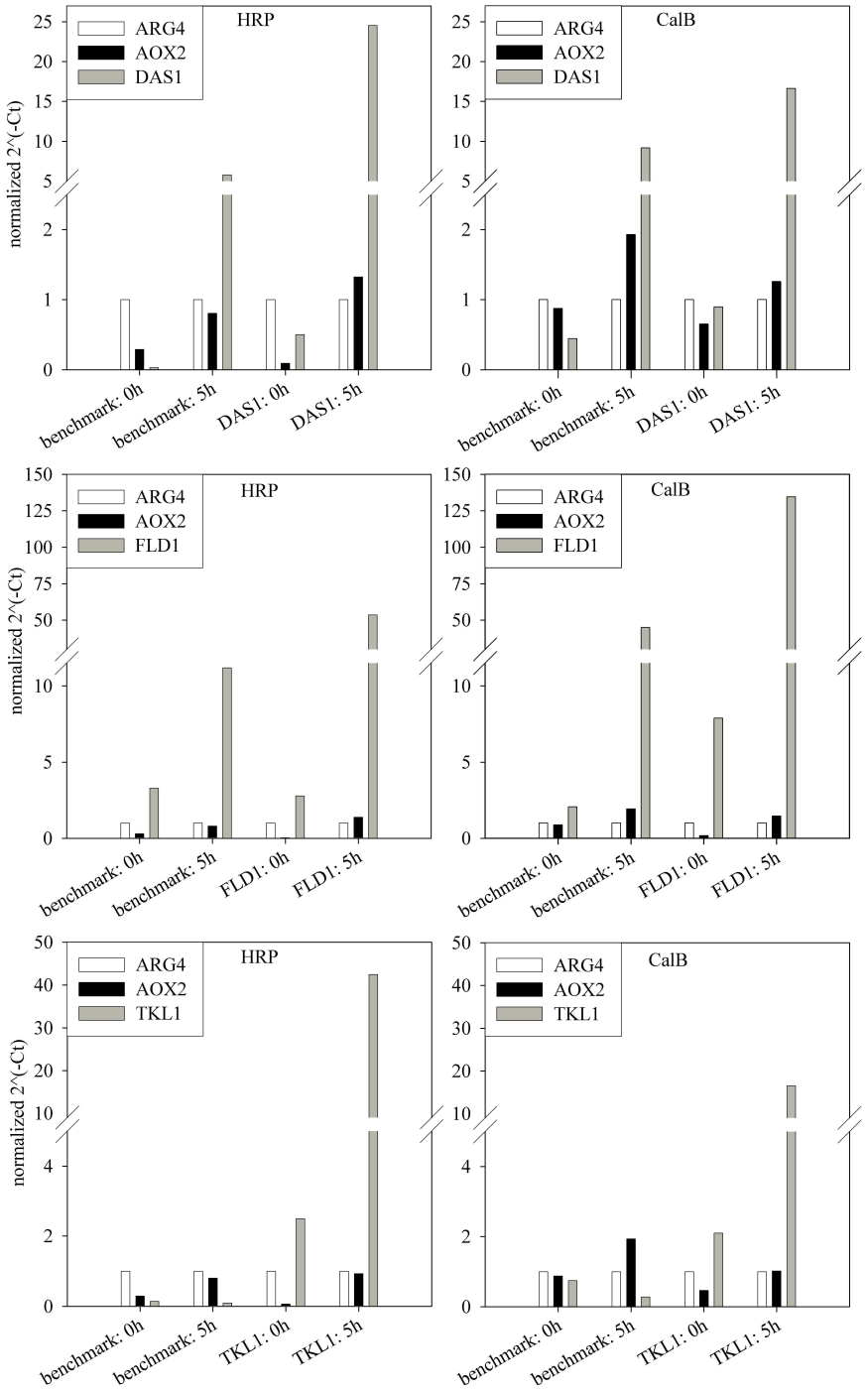


Figure 4.2.2-4 Transcription analysis of the co-overexpressed MUT pathway genes *DAS1*/*FLD1*/*TKL1* in HRP/CalB overexpressing strains. The transcript levels were normalized to the corresponding transcript levels of *ARG4*. The increase in *AOX2* transcript levels indicated successful induction with methanol. All co-overexpression strains showed elevated transcript levels of the respective target mRNAs compared to the benchmark strains.

Characterization of *P. pastoris* strains in the bioreactor

We characterized the *P. pastoris* Mut^S strains HRP FLD1/TKL1/DAS1 and CalB FLD1/TKL1/DAS1 using a fast and dynamic approach based on batch cultivations with repeated methanol pulses, as described in detail before [33, 34].

Growth rate and stoichiometric yields of the different P. pastoris *strains*

Due to the very low biomass growth rate of all *P. pastoris* strains in the pulsed batch experiments, the error of the dry cell weight duplicate measurements yielded standard deviations close to the determined biomass increase within one pulse (for an example see Supplementary Figure 4.2.2-2 and Supplementary Figure 4.2.2-3). Thus, the biomass growth rate had to be calculated using the carbon balance and the degree of reduction balance to get more accurate values. All the calculated h-values for a χ^2 test were lower than 3.84, implying that all determined values were within error margins. Furthermore, the respiratory quotients (RQ) of the strains were calculated using the online measured off-gas data and were compared with the theoretical values for RQ, which had been estimated based on the reconciled biomass yields. In general, calculated and theoretical RQ were very similar (data not shown), signifying that the reconciled biomass rates were accurate.

The specific growth rate was reduced 2- to 3-fold for strains which co-overexpressed either of the MUT pathway enzymes, compared to the benchmark strains. Apparently, the overexpression of yet another enzyme described an additional metabolic burden for the host cells and/or down-regulated the transcription of *AOX2*, as indicated by the transcription analysis (Figure 4.2.2-4), thus reducing the specific growth rate.

In analogy to the determination of the specific growth rates, we studied the influence of the co-overexpressed MUT pathway enzymes on the stoichiometric yields of the recombinant *P. pastoris* Mut^S strains. The yields of biomass on substrate ($Y_{X/methanol}$), CO_2 on substrate ($Y_{CO2/methanol}$) and O_2 on substrate ($Y_{O2/methanol}$) were calculated in a way to close both the carbon balance and the degree of reduction balance to 100 % [37]. No significant differences between the Mut^S strains could be observed as equal yields were detected for all the strains (*i.e.* $Y_{X/Methanol} = 0.42$ C-mol·C-mol^{-1}, $Y_{CO2/Methanol} = 0.57$ C-mol·C-mol^{-1} and $Y_{O2/Methanol} = 1.06$ C-mol·C-mol^{-1}). Apparently, the energetic behavior of the recombinant strains was not affected by co-overexpressing either of the three MUT pathway enzymes. This fact was further underlined by the analysis of the respiratory quotient (RQ), since no significant differences in RQ between the different strains were detected (Table 4.2.2-2).

Table 4.2.2-2 Summary Table. The respiratory quotient (RQ), the specific substrate uptake rate (q_s), the specific productivity (q_p), the efficiency factor (η) and the volumetric productivity of the benchmark strains (overexpressing either HRP or CalB) and the respective benchmark strains co-overexpressing either of the MUT pathway enzymes DAS1, FLD1 or TKL1 were determined.

reporter enzyme	MUT enzyme	RQ	q_s [mmol·g^{-1}·h^{-1}]	q_p [U·g^{-1}·h^{-1}]	η [U·mmol^{-1}]	vol. productivity [U·l^{-1}·h^{-1}]
HRP	benchmark	0.54	- 1.67	1.66	0.99	38.79
	DAS1	0.56	- 0.51	1.56	3.06	28.35
	FLD1	0.57	- 1.22	2.01	1.65	49.11
	TKL1	0.57	- 0.88	1.96	2.22	39.87
CalB	benchmark	0.57	- 1.32	1.39	1.05	30.27
	DAS1	0.57	- 0.36	0.78	2.15	15.23
	FLD1	0.57	- 0.70	1.49	2.13	31.27
	TKL1	0.57	- 0.35	0.12	0.34	2.07

We believe that the fact that Mut^S strains metabolize methanol at a lower rate, and thus produce less of the intermediate formaldehyde, compared to Mut$^+$ strains, describes the metabolic bottleneck responsible for this outcome. The situation might be different for the Mut$^+$ phenotype, which has been found to be faster in methanol oxidation (*vide supra*). Thus, possible effects of overexpressing either of the MUT pathway enzymes, e.g. an increased formation of CO_2 by overexpression of FLD1, have to be determined independently for Mut$^+$ strains.

Specific substrate uptake rate and specific productivity

The specific substrate uptake rate (q_s) and the specific productivity (q_p) of all the strains were calculated as average values from all the conducted pulses after data reconciliation and are summarized in Table 4.2.2-2.

Regarding q_s, all strains co-overexpressing either of the MUT pathway enzymes consumed less methanol per biomass and time than the benchmark strains (Table 4.2.2-2). Co-overexpression of DAS1 decreased q_s even up to 3-fold compared to the benchmark strains. Although the total numbers differ slightly the same trend for HRP strains and CalB strains can be seen (Table 4.2.2-2). The lowered q_s might be explained by a general deceleration of the metabolism of the cells which have to deal with the overexpression of recombinant proteins

(*i.e.* either of the reporter enzymes HRP or CalB and additionally either of the MUT pathway enzymes).

Interestingly, co-overexpression of FLD1 increased q_p of both the HRP FLD1 strain and the CalB FLD1 strain compared to the benchmark strains, whereas co-overexpression of DAS1 decreased q_p (Table 4.2.2-2).

Co-overexpression of TKL1 led to controversial results with regard to q_p, as a slightly higher q_p for HRP could be observed, whereas q_p for CalB was even lower than for the benchmark strain. Since the strains were checked for the copy number of the integrated reporter enzyme and for the successful overexpression of the MUT pathway enzymes on transcript level (*vide supra*), and since the bioreactor cultivations of the TKL1 co-overexpressing strains were conducted repeatedly, giving the same results, we currently have no evident explanation for this outcome. We hypothesize that product specific effects might be the reason for the observed difference in q_p for TKL1 co-overexpressing strains.

Due to the unaltered stoichiometric yields of the co-overexpression strains compared to the respective benchmark strains, we can currently not explain on which metabolic level the co-overexpressed MUT pathway enzymes affected the production of the recombinant reporter enzymes. However, this outcome underlines the importance and necessity of having a fast and easy-to-do methodology to characterize recombinant *P. pastoris* strains, as certain changes in the metabolism apparently might not have the same effect on different Mut^S strains harboring different target enzymes.

Efficiency factor η

Co-overexpression of DAS1 resulted in the most efficient conversion of the substrate methanol into product, regardless of which reporter enzyme was produced; for HRP 3-fold more product was obtained per substrate, and for CalB a more than 2-fold increase could be observed (Table 4.2.2-2). Also co-overexpression of FLD1 led to an approximately 2-fold increased efficiency for both reporter enzymes (Table 4.2.2-2). Interestingly, we found that the calculated efficiency factors correlated with the observed transcription levels of the co-overexpressed MUT pathway genes (Figure 4.2.2-4): *DAS1* transcript levels were higher in the HRP strain than in the CalB strain, whereas *FLD1* transcript levels were higher in the CalB strain than in the HRP strain. This trend can also be seen in the respective efficiency factors (Table 4.2.2-2). Thus, increasing the copy number of the respective MUT pathway gene in the genome of *P. pastoris* might intensify the beneficial effects on recombinant protein production and could be an interesting target for future studies.

For the recombinant strains co-overexpressing TKL1 different results for HRP strains and CalB strains were observed. While the co-overexpression of TKL1 resulted in a 2-fold more efficient conversion of methanol into the product HRP, a lower η than for the benchmark strain was detected for the production of CalB due to the very low q_p (Table 4.2.2-2).
Summarizing, all strains which co-overexpressed enzymes of the MUT pathway, excluding the CalB TKL1 strain, showed a more efficient conversion of the substrate methanol into the respective product. In particular, an expression platform, where the MUT pathway enzyme DAS1 is overexpressed, could be interesting for industrial large-scale protein production processes due to significantly lower substrate consumption and consequently a reduced risk management compared to strains without an engineered MUT pathway.

Volumetric productivity

Besides the importance of reducing the required amount of methanol and the associated reduction in cost and risk management, the volumetric productivity, *i.e.* the amount of product per volume and time, is an important factor in industrial production processes. The determined average volumetric productivities of the different recombinant *P. pastoris* strains are summarized in Table 4.2.2-2.
In terms of volumetric productivity only FLD1 co-overexpressing strains showed slightly higher or similar volumetric productivities compared to the benchmark strains, whereas the DAS1/TKL1 co-overexpressing strains produced less product per volume and time. The beneficial effect of co-overexpressing the MUT pathway enzyme DAS1, which resulted in a much better conversion of substrate to product (Table 4.2.2-2), obviously came at cost of process time.
Taking into account all the different strain specific parameters, an expression platform where the MUT pathway enzyme FLD1 is overexpressed turned out to be a very interesting tool for the industrial production of proteins, since not only methanol can be converted into product 2-fold more efficiently, but also the volumetric productivity is at least equally high compared to benchmark strains.
In summary, several aspects have to be considered when designing and choosing a production strain, *i.e.* if the main goal is a significant reduction in cost and risk management due to a more efficient conversion of substrate into product or a fast production process due to a high volumetric productivity. With the methodology described here, these strain characteristics can be determined in a fast and easy way, which significantly speeds up bioprocess development.

Conclusions

In this study a fast and easy-to-do method based on batch cultivations with methanol pulses was used to characterize different recombinant *P. pastoris* strains. The results can be summarized as follows:

• a direct comparison of a Mut^S and a Mut^+ strain, both overexpressing the recombinant enzyme HRP, revealed the Mut^S strain to be 7-fold more efficient in the conversion of substrate into product and to have a 3-fold higher volumetric productivity.

• the transcription of the genes *DAS1* and *DAS2*, which encode the key enzymes of the assimilative branch of the MUT pathway, was equally high induced upon MeOH addition.

• co-overexpression of either of the MUT pathway enzymes DAS1, TKL1 or FLD1 in *P. pastoris* Mut^S strains overexpressing either HRP or CalB did not cause any changes in the energetic behavior of the strains, as calculated yields and observed respiratory quotients were equal, but significantly reduced the growth rate.

• co-overexpressing MUT pathway enzymes affected the specific substrate uptake rate as well as the specific productivity of recombinant Mut^S strains. The co-overexpression of DAS1 resulted in a 2- to 3-fold more efficient conversion of methanol into product, but came at cost of process time.

• co-overexpression of FLD1 resulted in an approximately 2-fold increased efficiency in the conversion of methanol into product and showed at least equally high volumetric productivities compared to benchmark strains, making this expression platform highly interesting for industrial production processes.

• since product specific effects might influence certain strain specific parameters, as shown for TKL1 overexpressing strains in this study, the necessity of having a fast methodology to characterize recombinant strains before going into industrial production processes was highlighted. The methodology described here provides such a tool and has great potential for the use in early process development in an industrial environment.

Outlook

The present study shows the effect of co-overexpressing MUT pathway enzymes on strain specific parameters of different recombinant *P. pastoris* Mut^S strains and revealed interesting targets for strain engineering. However, this study was performed using only a Mut^S strain as expression platform since, it showed significantly better properties compared to the Mut^+ strain (without co-overexpression of MUT pathway genes). Thus, effects on some strain specific parameters might be more pronounced in Mut^+ strains and have to be evaluated

independently. Moreover, the molecular mechanisms that cause the beneficial influence of the studied MUT pathway enzymes on the efficiency in recombinant protein production remain to be elucidated in more detail. Increasing the copy number of the respective MUT pathway genes in the chromosome might further intensify their effects and should be considered when designing a specific *P. pastoris* expression host in future studies. We further recommend to analyze interesting co-overexpressing strains in fed-batch cultivations, which can be easily set up with the parameters extracted out of the described batch method with methanol pulses (see also [33, 34]), to test their long term stability in production processes.

List of abbreviations

ADH, methylformate synthase; AOX, alcohol oxidase; CalB, *Candida antarctica* lipase B; CAT, catalase; CER, carbon dioxide emission rate; C-mol, molarity of component based on one carbon atom; DAK, dihydroxyacetone kinase; DAS1, dehydroxyacetone synthase 1; DHA, dihydroxyacetone; DHAP, dihydroxyacetone phosphate; $F_{1,6}BP$, fructose-1,6-bisphosphate; F_6P, fructose-6-phosphate; FBA, fructose-1,6-bisphosphate aldolase; FBP, fructose-1,6-bisphosphatase; FDH, formate dehydrogenase; FGH, S-formylglutathione hydrolase; FLD1, formaldehyde dehydrogenase; GAP, glyceraldehyde 3-phosphate; GSH, glutathione; HRP, horseradish peroxidase; MUT, methonal utilization pathway; Mut^+, methanol utilization positive (*AOX*1 intact); Mut^-, methanol utilization minus (knock-out of *AOX1* and *AOX2*); Mut^S, methanol utilization slow (knock-out of *AOX*1); OUR, oxygen uptake rate; PPP, pentose phosphate pathway; Pyr, pyruvate; q_p, specific productivity; q_s, specific substrate uptake rate; Ri5P, ribose-5-phosphate; SeHe7P, sedoheptulose-7-phosphate; TCA, tricarboxylic acid cycle; TKL1, transketolase; Xu_5P, xylulose-5-phosphate; η, efficiency factor

Competing interests

The authors declare that they have no competing interests.

Author's contributions

FK, CD, TH and OS designed and performed the experiments, analyzed and interpreted the data. FK, CD and OS wrote the manuscript. AG, CH and OS conceived the study and supervised the research. All authors read and approved the final manuscript.

Acknowledgements

The authors would like to thank Anna Hatzl for technical assistance in the lab. Financial support by ACIB GmbH is gratefully acknowledged.

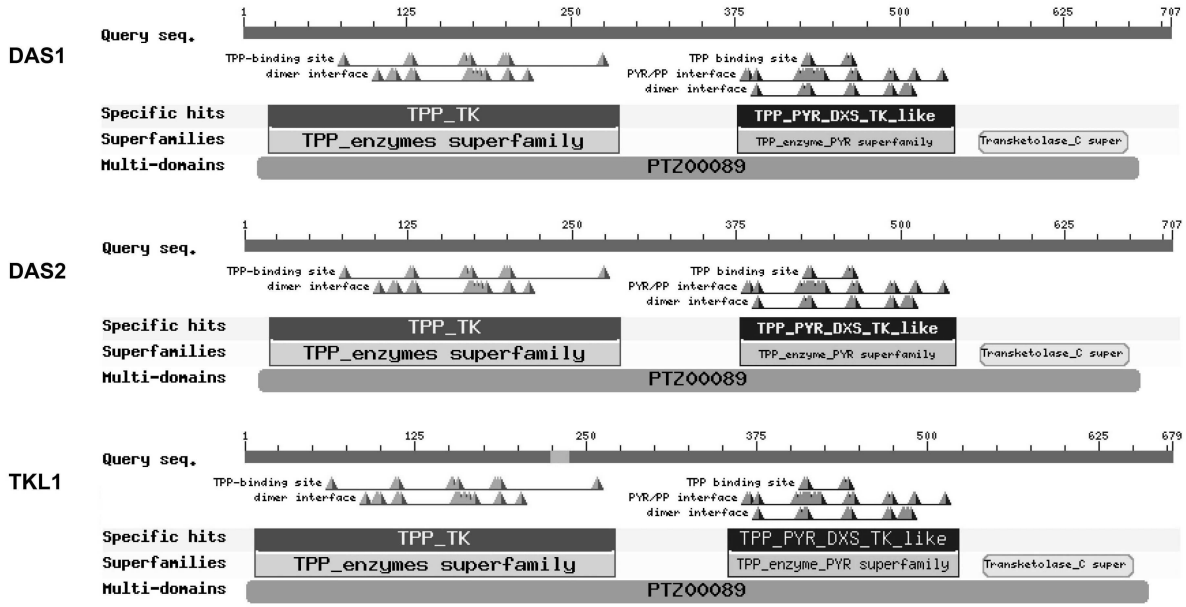
References

1. Abad S, Nahalka J, Winkler M, Bergler G, Speight R, Glieder A, Nidetzky B: **High-level expression of Rhodotorula gracilis d-amino acid oxidase in Pichia pastoris.** *Biotechnology Letters* 2011, **33:**557-563.
2. Cereghino JL, Cregg JM: **Heterologous protein expression in the methylotrophic yeast Pichia pastoris.** *Fems Microbiology Reviews* 2000, **24:**45-66.
3. Cregg JM, Cereghino JL, Shi JY, Higgins DR: **Recombinant protein expression in Pichia pastoris.** *Molecular Biotechnology* 2000, **16:**23-52.
4. Cereghino GPL, Cereghino JL, Ilgen C, Cregg JM: **Production of recombinant proteins in fermenter cultures of the yeast Pichia pastoris.** *Current Opinion in Biotechnology* 2002, **13:**329-332.
5. Hartner FS, Glieder A: **Regulation of methanol utilisation pathway genes in yeasts.** *Microbial Cell Factories* 2006, **5**.
6. Couderc R, Baratti J: **Oxidation of Methanol by the Yeast, Pichia-Pastoris - Purification and Properties of the Alcohol Oxidase.** *Agricultural and Biological Chemistry* 1980, **44:**2279-2289.
7. Macauley-Patrick S, Fazenda ML, McNeil B, Harvey LM: **Heterologous protein production using the Pichia pastoris expression system.** *Yeast* 2005, **22:**249-270.
8. Cregg JM, Madden KR, Barringer KJ, Thill GP, Stillman CA: **Functional-Characterization of the 2 Alcohol Oxidase Genes from the Yeast Pichia-Pastoris.** *Molecular and Cellular Biology* 1989, **9:**1316-1323.
9. Brierley RA, Bussineau C, Kosson R, Melton A, Siegel RS: **Fermentation Development of Recombinant Pichia-Pastoris Expressing the Heterologous Gene - Bovine Lysozyme.** *Annals of the New York Academy of Sciences* 1990, **589:**350-362.
10. Hartner FS, Ruth C, Langenegger D, Johnson SN, Hyka P, Lin-Cereghino GP, Lin-Cereghino J, Kovar K, Cregg JM, Glieder A: **Promoter library designed for fine-tuned gene expression in Pichia pastoris.** *Nucleic Acids Res* 2008, **36:**e76.
11. Cregg JM, Tschopp JF, Stillman C, Siegel R, Akong M, Craig WS, Buckholz RG, Madden KR, Kellaris PA, Davis GR, et al: **High-Level Expression and Efficient Assembly of Hepatitis-B Surface-Antigen in the Methylotrophic Yeast, Pichia-Pastoris.** *Bio-Technology* 1987, **5:**479-485.
12. Romanos MA, Scorer CA, Clare JJ: **Foreign Gene-Expression in Yeast - a Review.** *Yeast* 1992, **8:**423-488.
13. van den Burg HA, de Wit PJGM, Vervoort J: **Efficient C-13/N-15 double labeling of the avirulence protein AVR4 in a methanol-utilizing strain (Mut(+)) of Pichia pastoris.** *Journal of Biomolecular Nmr* 2001, **20:**251-261.
14. Kim SJ, Lee JA, Kim YH, Song BK: **Optimization of the Functional Expression of Coprinus cinereus Peroxidase in Pichia pastoris by Varying the Host and Promoter.** *Journal of Microbiology and Biotechnology* 2009, **19:**966-971.
15. Chiruvolu V, Cregg JM, Meagher MM: **Recombinant protein production in an alcohol oxidase-defective strain of Pichia pastoris in fedbatch fermentations.** *Enzyme and Microbial Technology* 1997, **21:**277-283.
16. Kuberl A, Schneider J, Thallinger GG, Anderl I, Wibberg D, Hajek T, Jaenicke S, Brinkrolf K, Goesmann A, Szczepanowski R, et al: **High-quality genome sequence of Pichia pastoris CBS7435.** *Journal of Biotechnology* 2011, **154:**312-320.
17. Swartz JR, Cooney CL: **Methanol Inhibition in Continuous Culture of Hansenula-Polymorpha.** *Applied and Environmental Microbiology* 1981, **41:**1206-1213.
18. Hazeu W, Donker RA: **A Continuous Culture Study of Methanol and Formate Utilization by the Yeast Pichia-Pastoris.** *Biotechnology Letters* 1983, **5:**399-404.

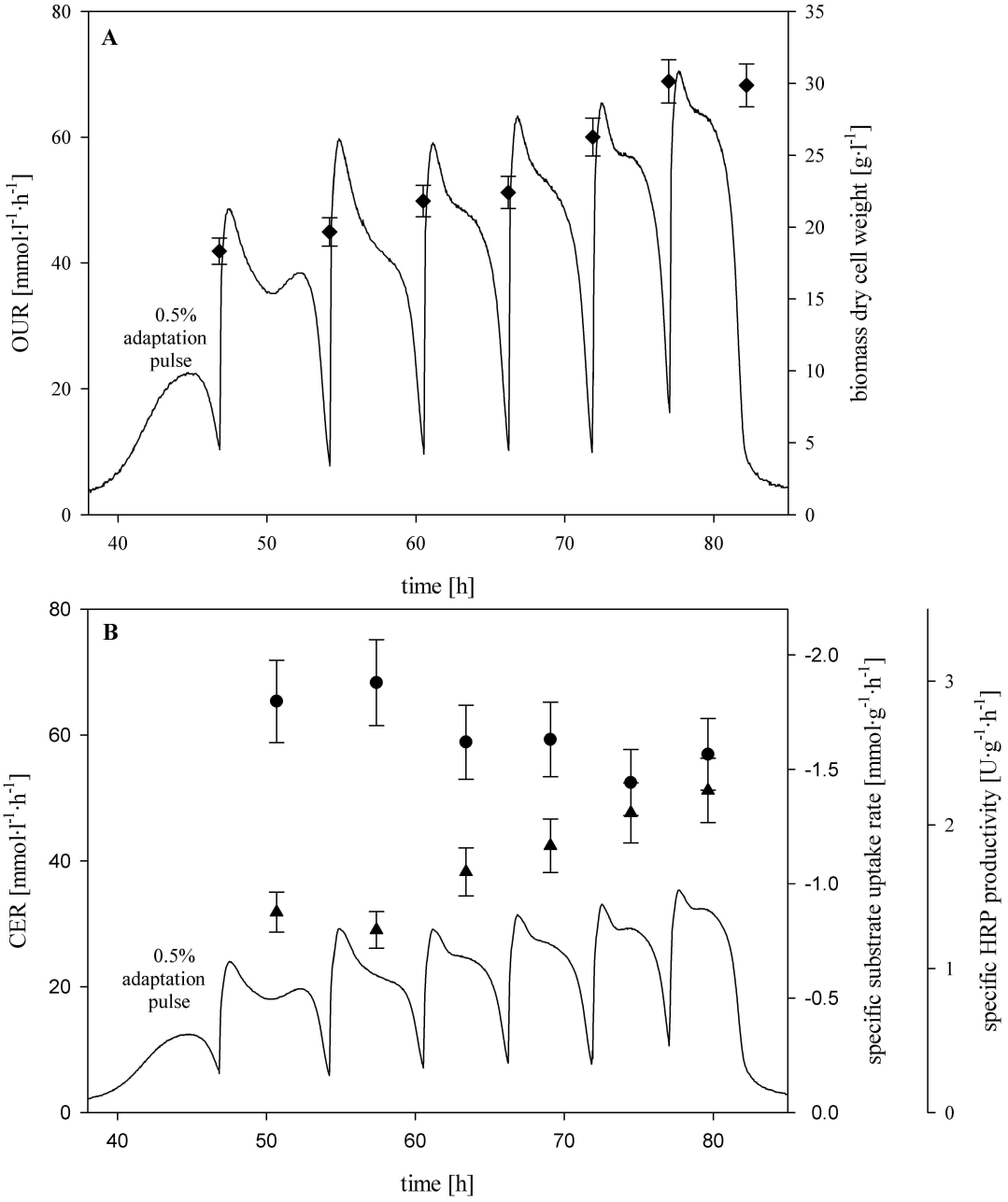
19. Kern A, Hartner FS, Freigassner M, Spielhofer J, Rumpf C, Leitner L, Frohlich KU, Glieder A: **Pichia pastoris "just in time" alternative respiration.** *Microbiology* 2007, **153:**1250-1260.
20. Arnau C, Casas C, Valero F: **The effect of glycerol mixed substrate on the heterologous production of a Rhizopus oryzae lipase in Pichia pastoris system.** *Biochemical Engineering Journal* 2011, **57:**30-37.
21. Jungo C, Marison I, von Stockar U: **Mixed feeds of glycerol and methanol can improve the performance of Pichia pastoris cultures: A quantitative study based on concentration gradients in transient continuous cultures.** *Journal of Biotechnology* 2007, **128:**824-837.
22. Zhang WH, Potter KJH, Plantz BA, Schlegel VL, Smith LA, Meagher MM: **Pichia pastoris fermentation with mixed-feeds of glycerol and methanol: growth kinetics and production improvement.** *Journal of Industrial Microbiology & Biotechnology* 2003, **30:**210-215.
23. Orman MA, Calik P, Ozdamar TH: **The influence of carbon sources on recombinant-human-growth-hormone production by Pichia pastoris is dependent on phenotype: a comparison of Mut(s) and Mut(+) strains.** *Biotechnology and Applied Biochemistry* 2009, **52:**245-255.
24. Ascacio-Martinez JA, Barrera-Saldana HA: **Production and secretion of biologically active recombinant canine growth hormone by Pichia pastoris.** *Gene* 2004, **340:**261-266.
25. Pla IA, Damasceno LM, Vannelli T, Ritter G, Batt CA, Shuler ML: **Evaluation of Mut(+) and Mut(S) Pichia pastoris phenotypes for high level extracellular scFv expression under feedback control of the methanol concentration.** *Biotechnology Progress* 2006, **22:**881-888.
26. Lee B, Yurimoto H, Sakai Y, Kato N: **Physiological role of the glutathione-dependent formaldehyde dehydrogenase in the methylotrophic yeast Candida boidinii.** *Microbiology-Sgm* 2002, **148:**2697-2704.
27. Nakagawa T, Ito T, Fujimura S, Chikui M, Mizumura T, Miyaji T, Yurimoto H, Kato N, Sakai Y, Tomizuka N: **Molecular characterization of the glutathione-dependent formaldehyde dehydrogenase gene FLD1 from the methylotrophic yeast Pichia methanolica.** *Yeast* 2004, **21:**445-453.
28. Schroer K, Luef KP, Hartner FS, Glieder A, Pscheidt B: **Engineering the Pichia pastoris methanol oxidation pathway for improved NADH regeneration during whole-cell biotransformation.** *Metabolic Engineering* 2010, **12:**8-17.
29. Slekar KH, Kosman DJ, Culotta VC: **The yeast copper zinc superoxide dismutase and the pentose phosphate pathway play overlapping roles in oxidative stress protection.** *Journal of Biological Chemistry* 1996, **271:**28831-28836.
30. Marchler-Bauer A, Bryant SH: **CD-Search: protein domain annotations on the fly.** *Nucleic Acids Research* 2004, **32:**W327-W331.
31. Marchler-Bauer A, Anderson JB, Chitsaz F, Derbyshire MK, DeWeese-Scott C, Fong JH, Geer LY, Geer RC, Gonzales NR, Gwadz M, et al: **CDD: specific functional annotation with the Conserved Domain Database.** *Nucleic Acids Research* 2009, **37:**D205-D210.
32. Marchler-Bauer A, Lu SN, Anderson JB, Chitsaz F, Derbyshire MK, DeWeese-Scott C, Fong JH, Geer LY, Geer RC, Gonzales NR, et al: **CDD: a Conserved Domain Database for the functional annotation of proteins.** *Nucleic Acids Research* 2011, **39:**D225-D229.
33. Dietzsch C, Spadiut O, Herwig C: **A dynamic method based on the specific substrate uptake rate to set up a feeding strategy for Pichia pastoris.** *Microbial Cell Factories* 2011, **10**.

34. Dietzsch C, Spadiut O, Herwig C: **A fast approach to determine a fed batch feeding profile for recombinant Pichia pastoris strains.** *Microbial Cell Factories,* **10:**85.
35. Morawski B, Lin ZL, Cirino PC, Joo H, Bandara G, Arnold FH: **Functional expression of horseradish peroxidase in Saccharomyces cerevisiae and Pichia pastoris.** *Protein Engineering* 2000, **13:**377-384.
36. Ohi H, Miura M, Hiramatsu R, Ohmura T: **The Positive and Negative Cis-Acting Elements for Methanol Regulation in the Pichia-Pastoris Aox2 Gene.** *Molecular & General Genetics* 1994, **243:**489-499.
37. van der Heijden RTJM, Romein B, Heijnen JJ, Hellinga C, Luyben KCAM: **Linear constraint relations in biochemical reaction systems: II. Diagnosis and estimation of gross errors.** *Biotechnol Bioeng* 1994, **43:**11 - 20.
38. Abad S, Nahalka J, Bergler G, Arnold SA, Speight R, Fotheringham I, Nidetzky B, Glieder A: **Stepwise engineering of a Pichia pastoris D-amino acid oxidase whole cell catalyst.** *Microbial Cell Factories* 2010, **9**.
39. Oka Y, Ishida H, Morioka M, Numasaki Y, Yamafuji T, Osono T, Umezawa H: **Combimicins, New Kanamycin Derivatives Bioconverted by Some Micromonosporas.** *Journal of Antibiotics* 1981, **34:**777-781.
40. Wach A, Brachat A, Pohlmann R, Philippsen P: **New Heterologous Modules for Classical or Pcr-Based Gene Disruptions in Saccharomyces-Cerevisiae.** *Yeast* 1994, **10:**1793-1808.
41. Lin-Cereghino J, Wong WW, Xiong S, Giang W, Luong LT, Vu J, Johnson SD, Lin-Cereghino GP: **Condensed protocol for competent cell preparation and transformation of the methylotrophic yeast Pichia pastoris.** *Biotechniques* 2005, **38:**44-+.
42. Weis R, Luiten R, Skranc W, Schwab H, Wubbolts M, Glieder A: **Reliable high-throughput screening with Pichia pastoris by limiting yeast cell death phenomena.** *Fems Yeast Research* 2004, **5:**179-189.
43. Zhang NY, Suen WC, Windsor W, Xiao L, Madison V, Zaks A: **Improving tolerance of Candida antarctica lipase B towards irreversible thermal inactivation through directed evolution.** *Protein Engineering* 2003, **16:**599-605.
44. Abad S, Kitz K, Hormann A, Schreiner U, Hartner FS, Glieder A: **Real-time PCR-based determination of gene copy numbers in Pichia pastoris.** *Biotechnology Journal* 2010, **5:**413-420.
45. Hoffmann K, Onodera S, Noiva R: **Expression of recombinant human protein disulfide isomerase in the yeast Pichia pastoris.** *Faseb Journal* 1996, **10:**677-677.

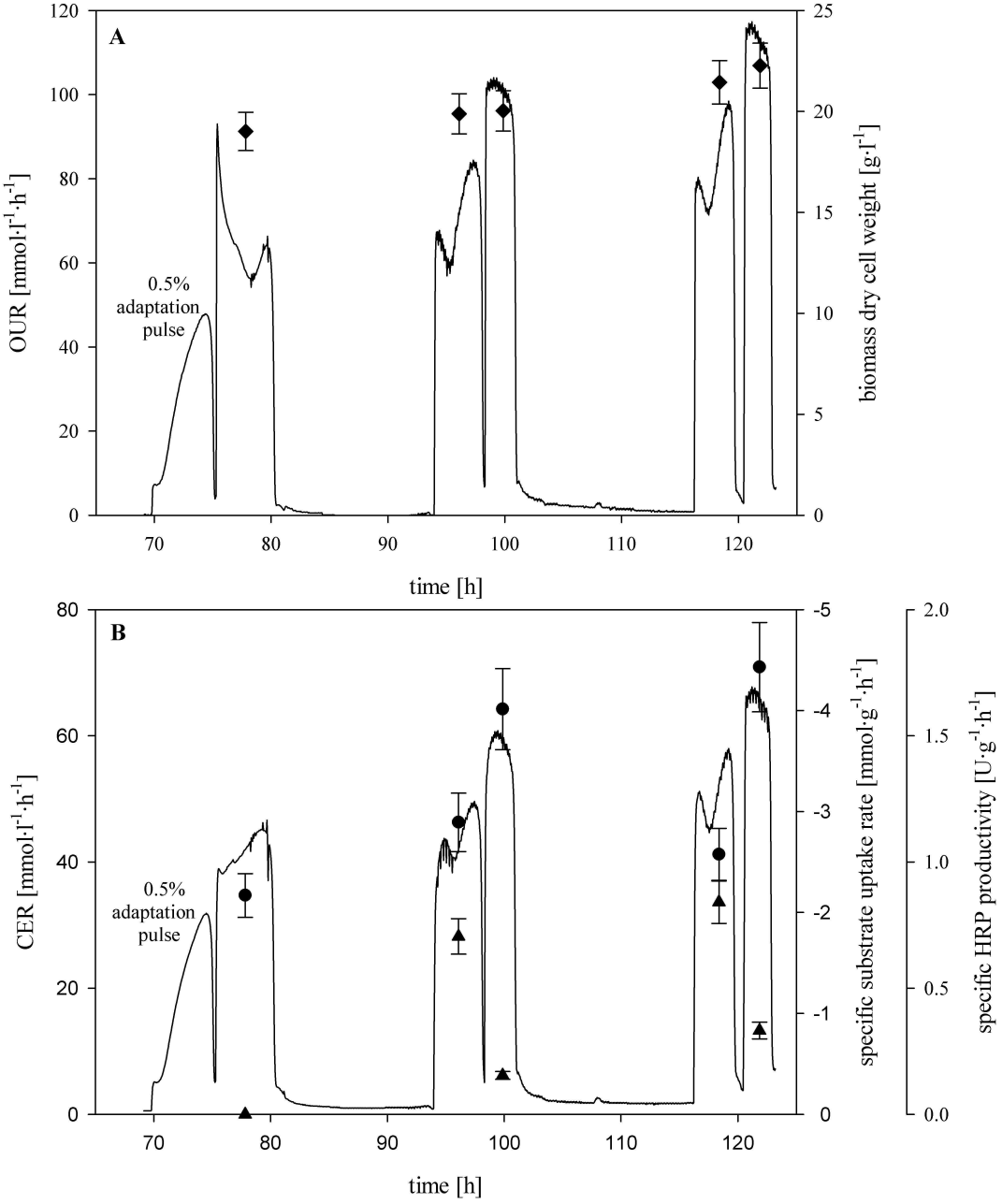
Supplementary Figures



Supplementary Figure 4.2.2-1 Domain structure of DAS1, DAS2 and TKL1 (conserved domain prediction via CD-search tool [30-32]). All three enzymes belong to I, a thiamine pyrophosphate (TPP)-enzyme superfamily [NCBI CDD:cl01629]; II, a superfamily of TPP-depending enzymes containing a pyrimidine binding domain [NCBI CDD:cl11410]; and III, a superfamily of enzymes with a transketolase C-terminal domain [NCBI CDD:cl08363]. Amino acid sequences of *P. pastoris* strain CBS7435 from [16].



Supplementary Figure 4.2.2-2 Experimental strategy for the fast determination of strain specific parameters of the *P. pastoris* Mut^S HRP strain using a batch experiment with methanol pulses of 0.5 % and 1 % (v/v). A, (continuous line), oxygen uptake rate OUR; (diamond) biomass dry cell weight concentration; B, (continuous line), carbon dioxide emission rate CER; (circle), calculated specific substrate uptake rate q_s; (triangle up), calculated specific HRP productivity q_p.



Supplementary Figure 4.2.2-3 Experimental strategy for the fast determination of strain specific parameters of the *P. pastoris* Mut$^+$ HRP strain using a batch experiment with methanol pulses of 0.5 % and 1 % (v/v). A, (continuous line), oxygen uptake rate OUR; (diamond) biomass dry cell weight concentration; B, (continuous line), carbon dioxide emission rate CER; (circle), calculated specific substrate uptake rate q_s; (triangle up), calculated specific HRP productivity q_p.

4.3. A novel on-line monitoring device for early process development

4.3.1. On-line multiple component analysis for efficient quantitative bioprocess development

Christian Dietzsch, Oliver Spadiut, Christoph Herwig[§]

Vienna University of Technology, Institute of Chemical Engineering, Research Area Biochemical Engineering, Vienna, Austria

[§]Corresponding Author: Christoph Herwig, Vienna University of Technology, Institute of Chemical Engineering, Research Area Biochemical Engineering, Gumpendorfer Strasse 1a, A-1060 Vienna, Austria. tel.: +43 1 85501 166400, fax: +43 1 58801 166980
e-mail: christoph.herwig@tuwien.ac.at

Manuscript submitted to Journal of Biotechnology

Abstract

On-line monitoring devices for the precise determination of a multitude of components are a prerequisite for fast bioprocess quantification. On-line measured values have to be checked for quality and consistency, in order to extract quantitative information from these data.

In the present study we characterized a novel on-line sampling and analysis device comprising an automatic photometric robot. We connected this on-line device to a bioreactor and concomitantly measured six components (*i.e.* glucose, glycerol, ethanol, acetate, phosphate and ammonium) during different batch cultivations of *Pichia pastoris*. The on-line measured data did not show significant deviations from off-line taken samples and were consequently used for incremental rate and yield calculations. In this respect we highlighted the importance of data quality and discussed the phenomenon of error propagation. On-line calculated rates and yields depicted the physiological responses of the *Pichia pastoris* cells in unlimited and limited cultures. A more detailed analysis of the physiological state was possible by considering the off-line determined biomass dry weight and the calculation of specific rates.

Here we present a novel device for on-line monitoring of bioprocesses, which ensures high data quality in real-time and therefore refers to a valuable tool for Process Analytical Technology (PAT).

Introduction

Recombinant proteins and an increasing number of different other products are currently produced in biological systems using bioprocess technology. To speed up process development and process understanding, a fast and reliable determination of several components (substrates, metabolites, etc.) is a prerequisite for the quantitative estimation of process interactions. Monitoring of certain process variables in real-time enables the operator to take action and interfere with the process, while the process is still being carried out. Consequently, processes can be characterized faster and carried out more efficiently, resulting in higher productivity and enhanced quality control. All these facts are implemented in the Process Analytical Technology (PAT[1]) initiative, which was proposed by the Food and Drug Administration recently [1-2]. Thus, a lot of research has been focused on the development of reliable and robust on-line monitoring devices for bioprocesses [3-5].

In general, monitoring devices for bioprocesses can be roughly divided into two groups: either the sensor is integrated inside the bioreactor (*in situ*) or samples are taken from the bioreactor and analyzed on-line (*ex situ*) [6]. In-line *in situ* sensors are directly interfacing with the process, which causes several problems like long-term stability, selectivity, detection limits and a difficult re-calibration [7]. Regarding *ex situ* on-line sampling systems several aspects have to be considered: any contamination of the bioprocess by the sampling process must be omitted, a representative sample should be delivered to the measuring device and the sample composition must not change during the transport. Therefore, on-line sampling systems have to be thoroughly characterized to guarantee reliable results, which was exemplarily shown by Christensen *et al.* for a polypropylene sampling membrane in 1991 [8].

On-line analytical devices based on proven quantification principles such as gas chromatography [9-10], HPLC [11-12] or biosensors, *e.g.* via Flow Injection Analysis (FIA) [10, 13-16], are the systems of choice to date. Commercially available FIA systems, like YSI Biochemistry Analyzer (YSI Life Sciences, US) or BioProfile (Nova Biomedical, US), are

[1]Abbreviations: BDW, biomass dry weight; CER, carbon dioxide evolution rate; DoE, Degree of Reduction; FDA, Food and Drug Administration; FIA, Flow Injection Analysis; HPLC, High performance liquid chromatography; ICH, International Conference on Harmonisation; NH4, ammonia (NH_4^+); OUR, oxygen uptake rate; PAT, Process Analytical Technology; PIMS, Process Information Management System; PO4, phosphate (PO_4^{3-}); $q_{i,}$ specific rate for component i; r_i, volumetric rate for component i; vvm, volume air per volume liquid per minute; $Y_{i/j,}$ conversion yield of component i out of component j;

increasingly used in scientific and industrial environments, especially for mammalian cell culture processes [17]. However, inherent drawbacks of these systems are on the one hand the use of buffer solutions to maintain the sensor units, which causes high maintenance costs and a quite cumbersome handling, as well as the limited long term stability of the sensor unit itself.

When generating on-line data for bioprocess quantification, not just the equipment, but also signal sensitivity and selectivity have to be taken into account. Recently, we have conducted a theoretical study to reveal the relationship of signal quality and the real-time extraction of information [18]. We showed that error propagation was dependent on the signal-to-noise ratio, the absolute signal value and the variations between two measured data points and analysed the impact of the sampling frequency on the consistency of calculated elemental balances.

One goal of our research is the utilization of a robust on-line monitoring device to concomitantly quantify a multitude of components allowing the real-time exploitation of bioprocesses. In the present study, we describe the implementation and characterization of a novel on-line sampling system. We successfully used this system to determine the concentration of different carbon sources, metabolites and other substrates (nitrogen, phosphate) during several batch cultivations of *Pichia pastoris*. We further motivate to analyse the quality of the data by the extraction of scale independent information. Hence, we aim to demonstrate the applicability of the used system for discussion of physiological responses of microbial cultures in limitation events solely based on on-line data and thus providing process understanding within the PAT context.

Material and Methods

Microorganism

A recombinant *Pichia pastoris* CBS7435 Mut^S strain was used as a model strain in this study and was gratefully provided by Prof. Anton Glieder (Graz University of Technology, Austria).

Culture Media

Preculture: Yeast nitrogen base medium (YNBM), per liter: potassium phosphate buffer (pH 6.0), 0.1 M; YNB w/o Amino acids and Ammonia Sulfate (Difco™), 3.4 g; $(NH_4)_2SO_4$, 10 g; biotin, 400 mg; glucose, 20 g.

Trace element solution (PTM1), per litre: $CuSO_4 \cdot 5H_2O$, 6.0 g; NaI 0.08 g; $MnSO_4 \cdot H_2O$, 3.0 g; $Na_2MoO_4 \cdot 2H_2O$, 0.2 g; H_3BO_3, 0.02 g; $CoCl_2$, 0.91 g; $ZnCl_2$, 20.0 g; $FeSO_4 \cdot 7H_2O$, 65.0 g; biotin, 0.2 g, H_2SO_4, 5 mL.

All cultures were cultivated in basal salt medium (BSM). Unlimited batch culture: the original BSM was adapted (BSM_{adapt}, Table 4.3.1-1) to have all concentrations of the analysed components in the technical range of the on-line device (Table 4.3.1-2). Therefore, the H_3PO_4 content was reduced and subsequently, less concentrated ammonia solution was needed for initial pH adjustment.

Table 4.3.1-1 Media composition for all experiments carried out with different limitations

adapted BSM_{adapt} medium	unlimited culture per litre	P-limited culture per litre	N-limited culture per litre
Glucose monohydrat	33 g	44 g	33 g
H_3PO_4, 85 %	6.85 ml	0 ml	6.85 ml
$CaSO_4 \cdot 2H_2O$	1.17 g	1.17 g	1.17 g
K_2SO_4	18.2 g	18.2 g	18.2 g
$MgSO_4 \cdot 7H_2O$	14.9 g	14.9 g	14.9 g
KOH	4.13 g	0 g	4.13 g
$(NH_4)_2SO_4$	0 g	3.3 g	0 g
Antifoam Structol	300 µl	300 µl	300 µl
initial pH adjustment	25 % NH_4OH	-	25 % NH_4OH
pH adjustment during cultivation	3.4 M NH_4OH	3.4 M NH_4OH	3 M NaOH
PTM 1	4.35 ml	4.35 ml	4.35 ml

Phosphate limited batch culture: BSM_{adapt} was further adapted to have less amount of PO_4^{3-}. Therefore no H_3PO_4 was added and consequently the pH of the medium was already at around 5.4. As no initial pH adjustment was necessary, no NH_4OH was added to the medium. To avoid initially upcoming nitrogen limitation, $(NH_4)_2SO_4$ was added. During the cultivation, NH_4OH was used to maintain the pH and also supplied nitrogen for cell growth.

Nitrogen limited batch culture: BSM_{adapt} was used and pH was kept constant with NaOH [3 M] instead of NH_4OH during the cultivation.

Batch cultivation

Batch cultivations were carried out in 7.5 l volume glass bioreactors (Infors, Switzerland) with a working volume of 4 l. Initial batch medium was sterilized in the bioreactor and pH was adjusted by addition of concentrated ammonium solution. Glucose was sterilized separately and transferred into the bioreactor aseptically. The sterile filtered PTM1 as well as the sterilized antifoam solution were added with a disposable syringe via a septum. Dissolved oxygen (dO_2) was measured with a sterilizable dissolved oxygen electrode (Hamilton, Switzerland). The pH was measured with a sterilizable electrode (Hamilton, Switzerland) and maintained constant at pH 5 with a PID controller by addition of base (Table 4.3.1-1). Base consumption was logged gravimetrically. Cultivation temperature was set to 28°C and agitation was fixed to 1200 rpm. The culture was aerated with 1 vvm of dried air via a sterile filter and off-gas (O_2 and CO_2) was measured on-line with an off-gas monitoring system GA4 (DASGIP, Germany). All data were logged in a process information management system (PIMS; Lucullus, Biospectra, Switzerland).

After the initial batch had finished a repeated batch was carried out. Therefore, the culture volume was reduced to 0.75 l (via the sampling tube) and fresh medium was refilled through a sterile filter up to a volume of 4 l. Hence, a start biomass concentration of 2 $g \cdot l^{-1}$ was obtained after refill. Additionally, the media compositions (Table 4.3.1-1) had to consider residual component concentrations caused by the media refill. With this procedure, several experiments were carried out with the described repeated batch strategy.

Construction of a flow-through cuvette for the on-line sampling device

A special flow-through cuvette was constructed using a commercially available half-micro cuvette, which was drilled at both sides in certain heights to generate a working volume of 1 ml. The lower drilling was connected to a Luer-Lok connector for ingoing flow, the upper drilling was connected to a Luer-Lok T-piece as a drain off.

To characterize the liquid exchange in the flow-through cuvette, a Coomassie solution (210579, Sigma-Aldrich, USA) was pumped through the cuvette, filled with water, at a flow rate of 1 $ml \cdot min^{-1}$. The absorbance at 600 nm was measured in a photometer (U-1100 Hitachi, Japan) in regular intervals. The acceptance criterion for a complete volume exchange in the cuvette was defined with 97% of response. To reduce the volume in the cuvette and thus the required pumping time, 0.5 g of glass beads (G8772, Sigma-Aldrich, USA) were filled into the cuvette. The cuvette was inserted in the on-line port of a photometric robot (CuBiAn XC; OptoCell , Germany) and connected with PTFE tubes (0.8 x1.6 mm; Bartelt, Austria) to a

sterilizable ceramic sampling probe made of Al_2O_3 (pore size o 0.2 µm, filter area 17,8 cm^2, membrane thickness 1.6 mm; iba, Heiligenstadt, Germany). A peristaltic pump (ISM847E; Ismatec, Switzerland) was used to transport the sample from the probe to the cuvette with a flow rate of 1 ml·min^{-1} and the full sampling system (probe, tubings, cuvette) was characterized.

Off-line sampling and biomass dry weight determination

To validate on-line data, frequent off-line samples were taken to analyse the biomass dry weight (BDW) and to measure the concentration of different components in the supernatant. BDW was determined by centrifugation of 5 ml culture broth (5000 rpm, 4°C, 10 min) in a laboratory centrifuge (Sigma 4K15, rotor 11156), washing the pellet with 5 ml deionised water and subsequent drying at 105°C to a constant weight in an oven. The off-line determined biomass concentrations were entered into the PIMS and a continuous BDW signal was generated by linear interpolation for the subsequent calculation of specific rates.

On-line component analysis

Measurements of carbon sources, metabolites, NH_4^+ (hereafter called NH4) and PO_4^{3-} (hereafter called PO4) were performed on-line and off-line with an automatic photometric robot (CuBiAn XC; Innovatis). All photometric assays were carried out at 37°C and are listed in Table 4.3.1-2.

Table 4.3.1-2 Different commercial assays for the on-line determination of carbon sources, metabolites, NH4 and PO4

assay	supplier	assay range	dilution	technical range
glucose (gluc)	Randox	0.05 to 3 g·l^{-1}	1:20	1.0 to 60.0 g·l^{-1}
ethanol (eth)	Enzytec fluid line	0 to 0.25 g·l^{-1}	1:10	0.1 to 2.5 g·l^{-1}
acetate (ace)	Enzytec fluid line	0 to 1.2 g·l^{-1}	1:5	0.05 to 6 g·l^{-1}
glycerol (gly)	Enzytec fluid line	0 to 0.25 g·l^{-1}	1:10	0.1 to 2.5 g·l^{-1}
ammonium (NH4)	Enzytec fluid line	0.1 to 3.75 mM	1:30	3 to 112.5 mM
anorg. phosphate (PO4)	Randox	0 to 10 mM	1:10	0.1 to 100 mM

All listed assays are commercial available assays (Randox, OptoCell GmbH & CO. KG, Germany; Enzytec fluid line, R-Biopharm, Germany) and calibration was carried out by using four standards points in the respective assay range. Due to higher concentrations of the components under culture conditions an automatic dilution (Table 4.3.1-2) was conducted by the system. A recalibration of the methods for ethanol, acetate and ammonium had to be

carried out every two days, whereas the reagents for glucose, glycerol and PO4 showed a stability of up to 4 weeks without recalibration.

Rate and yield calculation

In order to use the on-line determined data for bioprocess quantification, incremental volumetric rates were calculated from on-line measured concentrations according to Equation 4.3.1.1,

$$r_{ci}\left(\frac{t_i - t_{i-1}}{2}\right) = \frac{c_i(t_i) - c_i(t_{i-1})}{(t_i - t_{i-1})} \qquad \text{(Eq. 4.3.1.1)}$$

where t_i and t_{i-1} describe two consecutive measurement time points.

In addition, volumetric rates where used to calculate specific rates with the respective biomass concentration according to Equation 4.3.1.2,

$$q_{Ci} = \frac{r_{ci}(t_i)}{c_x(t_i)} \qquad \text{(Eq. 4.3.1.2)}$$

For all components, rates were calculated with the respective algebraic sign indicating the flux out or into the cell (*i.e.* + = release; - = uptake). All other calculations for rates and yields as well as for elemental balances were carried out according to our previous study [18].

Results and Discussion

Set up of an on-line connected automatic photometric robot

The fully equipped bioreactor system with several on-line sensors was connected to an automatic photometric robot, as shown in Figure 4.3.1-1. The sampling system consisted of a ceramic filtration probe (pore size 0.2 µm), a transport line (length 1.5 m), a controlled pump with a constant flow of 1 ml·min^{-1} and a self-constructed cuvette in the on-line port of the photometric robot.

The advantage of this novel on-line sampling system over more traditional on-line devices is the possibility to determine multiple components in just one sample concomitantly in the photometric robot (*i.e.* 6 different components in this study). Basically any photometric assay can be used with this system demonstrating its broad applicability, for example to determine different substrates and metabolites as well as catalytic activities of different enzymes. This system can either be used with commercially available enzymatic and photometric assay kits, but also allows the development of new off-the-shelf methods and assays, which can be adjusted to the specific requirements of the user, enabling the operator to be more independent from commercially available assay kits

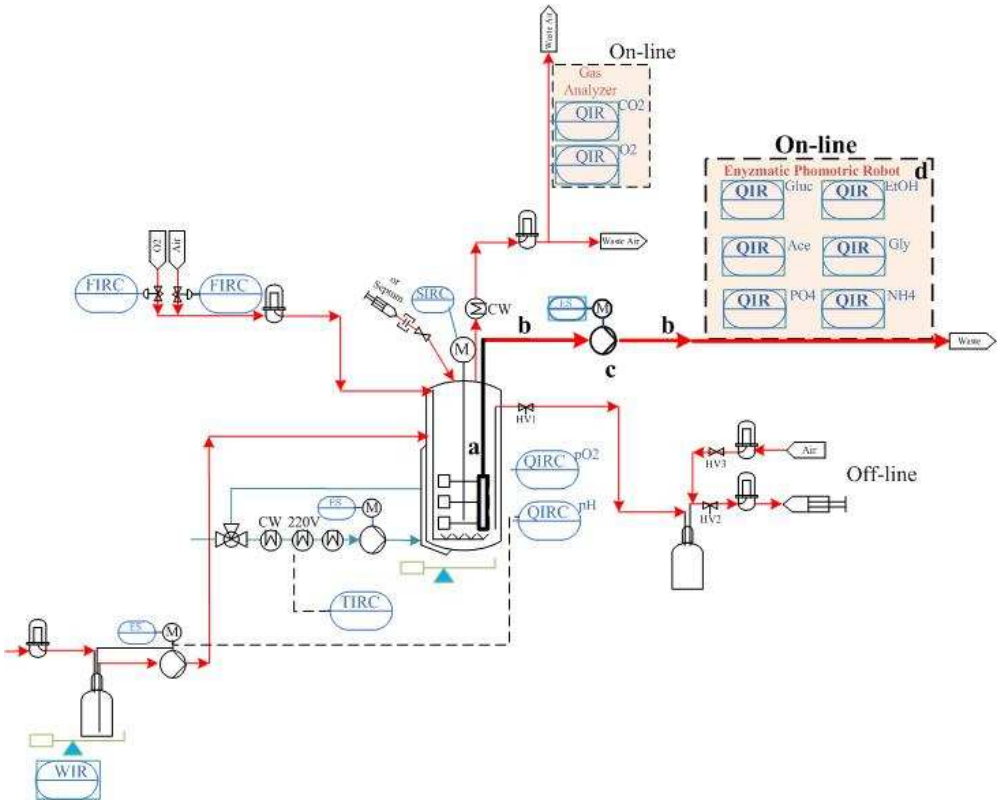


Figure 4.3.1-1 Process and Instrumentation Diagram of the experimental set-up for on-line measurements in batch fermentations of *P. pastoris*. The on-line sampling system consisted of a) a ceramic filtration probe, b) a transport line, c) a pump, d) the self-constructed cuvette and the automatic photometric robot. Nomenclature is according to DIN EN ISO 10628.

Characterisation of a self-constructed flow-through cuvette

A specified sample amount for each analysis was automatically withdrawn from a cuvette installed in the on-line port of the photometric robot. As the device was not provided with an appropriate on-line cuvette, a suitable cuvette had to be constructed and characterized (Figure 4.3.1-2).

For characterization, a Coomassie solution of a certain absorption value was pumped into the water-filled cuvette at a defined rate of 1 $ml \cdot min^{-1}$ and the OD_{600} was measured. Due to a laminar flow profile in the cuvette (the calculated Reynolds number was Re = 1.9), a complete exchange of the solution, defined by an acceptance criterion of 97% of full response, took about 7 minutes (Figure 4.3.1-2C). To cause a faster exchange of the solution and thus shorten the required pumping time, the volume of the cuvette was reduced by adding glass beads (Figure 4.3.1-2A). Consequently, the pumping time was reduced to only 3 minutes to meet the defined acceptance criterion (Figure 4.3.1-2C).

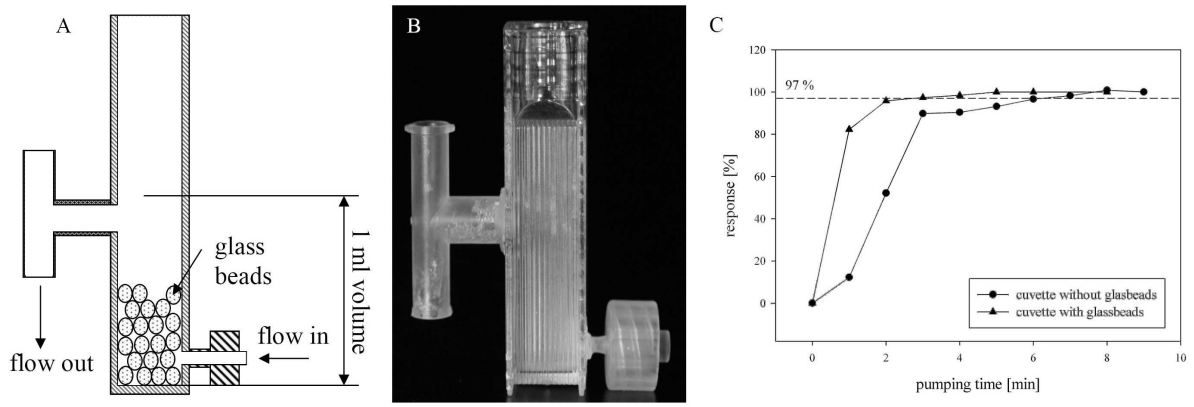


Figure 4.3.1-2 Construction and characterization of a flow-through cuvette; A) schematic illustration; B) picture of the cuvette; C) characterization of liquid exchange in the self-constructed cuvette by pumping a Coomassie solution with a certain OD600 at a flow rate of 1 ml·min^{-1}.

After characterization and improvement of the cuvette, the whole sampling system, comprising the cuvette, the transport line and the ceramic sampling probe, was characterized. In combination, the required total pumping time was determined with 7 minutes to guarantee a representative sample in the cuvette (data not shown).

Bioprocess quantification at unlimited growth of *Pichia pastoris*

On-line vs. off-line data for bioprocess monitoring

To show the feasibility of the described on-line device, a batch cultivation of *P. pastoris* was conducted on the substrate glucose. The medium composition for this batch was defined in a way to supply unlimited growth until glucose depletion. During the experiment several physiological relevant compounds, namely glucose, ethanol, acetate, ammonium and phosphate were measured on-line at the maximum frequency of every ~ 0.6 h by the automatic controlled on-line device. In parallel, off-line samples were taken to check the validity of the on-line measured values and to determine the biomass dry weight (Figure 4.3.1-3).

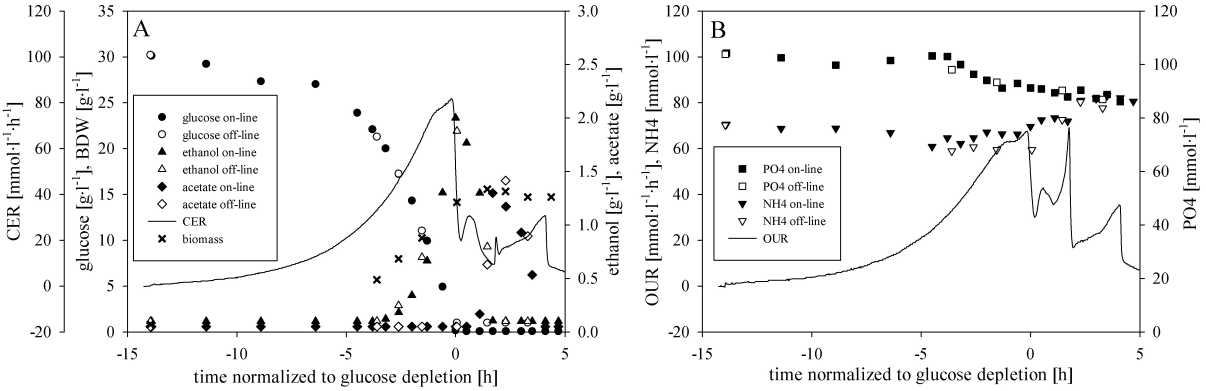


Figure 4.3.1-3 Batch cultivation of *P. pastoris* until carbon depletion referenced at time point 0 h; A) Carbon dioxide evolution rate (CER), on-line/off-line glucose concentration, on-line/off-line ethanol concentration, on-line/off-line acetate concentration; off-line biomass concentration (BDW); B) oxygen uptake rate (OUR), on-line/off-line PO4 concentration, on-line/off-line NH4 concentration.

As shown in Figure 4.3.1-3, on-line and off-line data basically described the same values. Variations in the measured data were ≤ 5 %, which highlights the potential of the described system for the precise determination of relevant process parameters in real-time.

The high frequent on-line determination of carbon sources and substrates was used to identify the metabolic activity of the cells in more detail. As shown in Figure 4.3.1-3A, glucose was taken up exponentially until it was depleted (referenced at time point 0 h). In the latter phase of glucose consumption (-3.5 to 0 h), ethanol was produced up to a concentration of 2 $g \cdot l^{-1}$. After glucose depletion, ethanol was metabolized and converted to acetate, which reached a maximum concentration of 1.4 $g \cdot l^{-1}$ in the bioreactor. These changes in metabolism were also depicted by the carbon dioxide evolution rate (CER, Figure 4.3.1-3) and the oxygen uptake rate (OUR, Figure 4.3.1-3B). Both rates showed an exponential increase until a certain time point (-2 h), after which the rates depicted a more attenuated slope until glucose depletion. Subsequent growth on ethanol and acetate (Figure 4.3.1-3A and B; 0 to 4 h) also caused a characteristic off-gas pattern.

Besides the determination of carbon sources and metabolites, also NH4 and PO4 were determined on-line and off-line. The PO4 concentration showed a slight decrease during the cultivation indicating an uptake by the cells (Figure 4.3.1-3B). In contrast, the NH4 concentration stayed constant, even in the exponential phase of the culture, due to the use of NH_4OH as base for pH titration. However, in the latter phase of the cultivation a slight increase of the NH4 concentration was detected, which was caused by media acidification during acetate production and enhanced pH titration with NH_4OH.

On-line data quality – rate and yield calculation

In order to judge on the quality of the on-line data generated by the on-line device the question arises, whether a simple overlay of on-line and respective off-line data is sufficient? We want to stress, that data are only useful if information can be reliable extracted as we have shown elsewhere [18]. Hence, to further analyse the quality of the on-line measured concentrations and to extract more on-line available information, incremental rates and yields were calculated from on-line sample to on-line sample. In Figure 4.3.1-4A and B the calculated volumetric rates for all on-line measured components are shown. Decreasing values (logical sense for uptake) for the volumetric rate of glucose (r_{gluc}) indicate the increase of biological activity caused by cell growth over time. Interestingly, from the time point where the volumetric rate of ethanol (r_{eth}) increased, the signals for r_{gluc} described a different slope as no further exponential growth was possible. After glucose depletion, the produced ethanol was taken up and converted to acetate, as shown in the volumetric rates for ethanol (r_{eth}) and acetate (r_{ace}). Finally, acetate was consumed and the culture showed no further metabolic activity (Figure 4.3.1-4A).

Besides the volumetric rates for glucose, ethanol and acetate, also the volumetric rates for NH4 and PO4 were determined. The volumetric rate for NH4 (r_{NH4}, Figure 4.3.1-4B) was calculated as a sum of the on-line measured data and the gravimetrically logged consumption of NH_4OH during the cultivation. Both rates (r_{NH4} and r_{PO4}) exhibit a scattered trend (Figure 4.3.1-4B), which was caused only by small variations in the on-line determined values between samples at high absolute values (Figure 4.3.1-4B). Therefore, the calculation of rates arises to be questionable in this case since the absolute difference between on-line measured values was too small. This outcome underlines our previous findings regarding error propagation [18], where small measurement errors of 1% and a sampling frequency of 0.5 h led to huge variations for yield calculations, whereas larger sampling frequencies resulted in a much better quantification of the bioprocess. In analogy to our previous study, a filter was introduced to guarantee that only useful data points of the calculated volumetric rates of the glucose uptake were used for subsequent yield calculations. As shown in Figure 4.3.1-4A, only values where the biological activity was higher than 50 $mmol \cdot g^{-1} \cdot h^{-1}$ for the glucose uptake showed significant differences between the different sampling time points. Thus, the time point for filtering was set to -6 h (see dashed lines in Figure 4.3.1-4A and B) and yield calculations were carried out only from this time point onwards (Figure 4.3.1-4C and D).

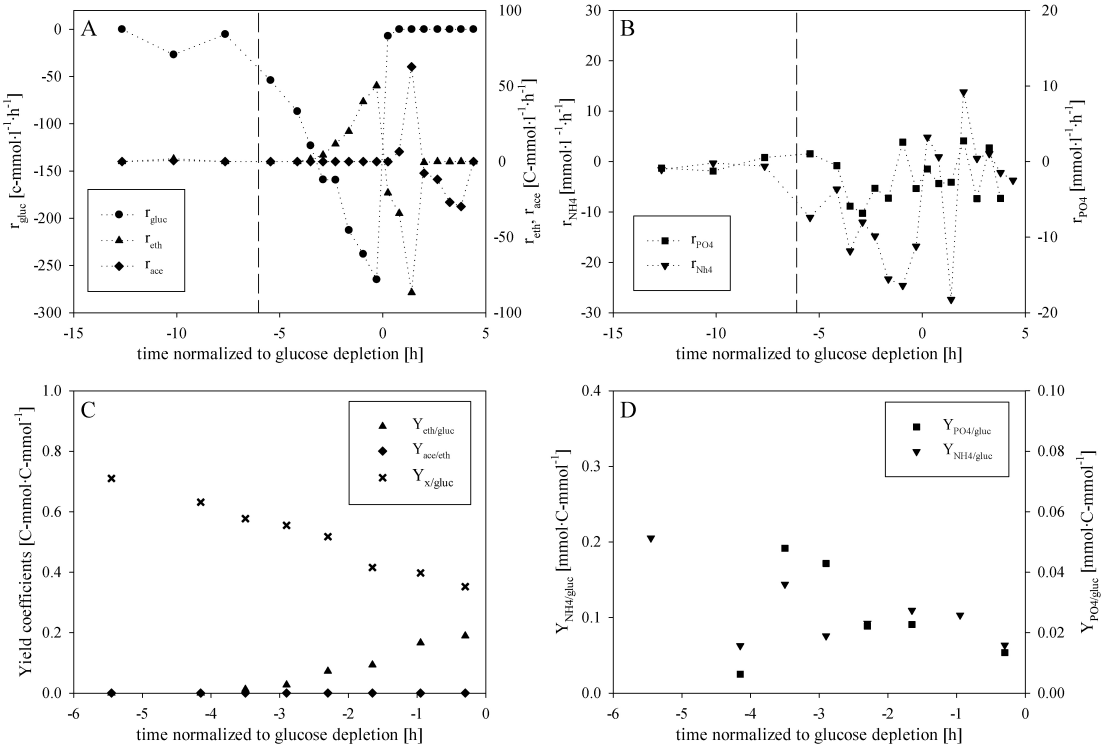


Figure 4.3.1-4 Calculated incremental volumetric rates and yields from on-line measured data; A) volumetric rates for substrate and metabolites (used filter for calculations is indicated with a vertical dashed line); B) volumetric rates for NH4 and PO4 from on-line data (used filter is indicated with a dashed line); C) yield coefficients calculated from volumetric rates in A; D) yield coefficients calculated from volumetric rates in A and B.

Yield calculations from on-line data revealed an inclining yield for ethanol ($Y_{eth/gluc}$) from -3.5 h on, whereas no acetate was produced from glucose (Figure 4.3.1-4C). This indicates a shift of the glucose conversion from biomass to the metabolite ethanol. The off-line determined biomass dry weight was used to check for lower biomass yields in this phase. As shown in Figure 4.3.1-4C, the off-line calculated biomass yield decreased concomitantly with the increasing ethanol yield.

The yields for NH4 and PO4 over glucose before glucose depletion did not show a constant signal but were highly scattered (Figure 4.3.1-4D). This was due to the scattered values of the different volumetric rates (Figure 4.3.1-4B). As shown in Figure 4.3.1-3, the absolute values between two on-line measurements of NH4 and PO4 did not change significantly before glucose depletion and thus the signal-to-noise ratio was quite low. As we have discussed recently [18], a high ratio of the absolute concentration change to the error for the respective measurement represents a prerequisite to ensure needed data quality for a reliable extraction of information from bioprocesses in real-time. To obtain more consistent data for the NH4

and PO4 consumption and more stable signals for the yields, larger absolute changes in the respective concentrations are needed.

Quantification of physiological effects

In order to use the on-line data to further analyse the physiological state of the culture, the off-line biomass dry weight concentrations were used to calculate specific rates (Fig. 3.3.1.5A). The specific ethanol production (q_{eth}) showed increasing values until glucose depletion, whereas the specific glucose uptake (q_{gluc}) adapted to a maximum value at -3 h and did not further decrease (negative sign due to uptake). As the same trend was observed also for the specific oxygen uptake rate (q_{O2}), the production of ethanol was likely caused by a limitation of the oxidative capacity of the cells. Although *P. pastoris* is classified as a Crabtree negative yeast [19], our results are in coincidence with other studies in literature [20-21], where small amounts of ethanol were produced under aerobic conditions and glucose excess in cultivations of *P. pastoris*. Heyland *et al.* concluded on a Crabtree like effect [21], whereas Kern *et al.* detected a potential gene encoding the alternative oxydase (AOD), which controls an alternative respiration pathway (CRR, Cyanid-resistence respiration). The disruption of the described gene led to a decreased ethanol production in *P. pastoris* [20].

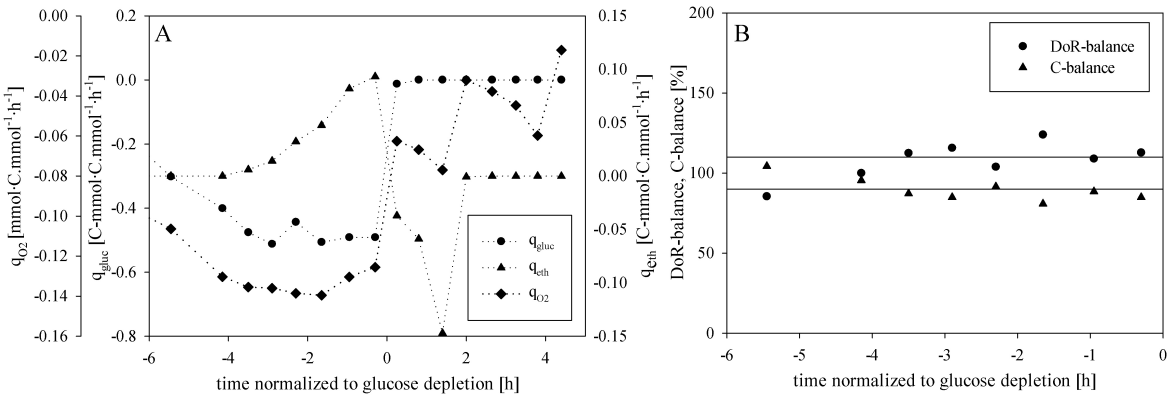


Figure 4.3.1-5 Specific rates and elemental balances for the filtered period (-6 h onwards); A) specific rates of glucose, ethanol and oxygen; B) carbon balance (C-balance) and degree of reduction balance (DoR-balance), horizontal lines indicate 10 % variation from 100 % closing balances according to [18].

To further underline the reliability of the on-line determined data, a consistency check by calculating two elemental balances (carbon-balance and degree of reduction balance) was done using the on-line determined data for the media components in combination with on-line off-gas measurements as well as off-line determined biomass concentrations. As the balances

were close to the acceptance criterion of ± 10 % to closing balances (Figure 4.3.1-5B), the on-line data for rate and yield calculation could be stated as valid.

In summary, the experimental set up comprising the on-line sampling device and the automatic photometric robot delivered reliable on-line data which allowed the real-time quantification of bioprocesses. By calculating rates and yields, conclusions on the physiological regulation of the cells could be drawn.

Bioprocess quantification of cell growth under phosphate limitation

In order to further check the potential of the on-line quantification of physiological regulations in microbial cultures, the *P. pastoris* strain was cultivated in an adapted basal salt medium to consciously drive the culture into a phosphate limitation.

Basically, the P-limited culture showed the same off-gas profile (Figure 4.3.1-6A and B) as the unlimited culture (Figure 4.3.1-3A and B). Phosphate limitation occurred at -2.3 h (Figure 4.3.1-6B), which could be detected in the CER and the OUR signal as a small shoulder (indicated in Figure 4.3.1-6A and B with a vertical straight line). However, glucose was further consumed and again ethanol was produced. In contrast to the unlimited culture, less acetate was formed out of ethanol after glucose depletion. In order to quantify this experiment in the same way as described before, a respective filter was set at -7 h (indicated as a vertical dashed line in Figure 4.3.1-6A and B). Since total PO4 concentrations changed significantly during the cultivation (Figure 4.3.1-6B) the volumetric rates for PO4 could be calculated reliably. Also the volumetric rate for NH4 was less scattered in this experiment (data not shown), which is why a better yield coefficient calculation for NH4 and PO4 (Figure 4.3.1-6C) compared to the unlimited culture (Figure 4.3.1-4C) was possible. The amount of NH4 was correlated to the glucose uptake and a rather constant value of 0.13 mmol·C-mmol^{-1} of glucose was determined. The yield of PO4 showed constant values of 0.02 mmol·C-mmol^{-1} of glucose and dropped to 0 at phosphate limitation.

To check whether the on-line calculated yields for NH4 ($Y_{NH4/gluc}$) and PO4 ($Y_{PO4/gluc}$) before PO4-limitation (-7 h to -2.5 h) relate to their respective consumption by the biomass, a composition analysis of the biomass was done. The results were in a good agreement and showed values of 0.15 mmol·C-mmol^{-1} biomass for nitrogen and 0.02 mmol·C-mmol^{-1} biomass for phosphate.

Surprisingly, the culture was not significantly affected by PO4 limitation as the specific rates for glucose and oxygen remained rather constant until glucose depletion (Figure 4.3.1-6D). Obviously, the culture used alternative phosphate sources, like organic compounds, as

described before [22]. Probably, the time frame in this experiment where the culture was PO4-limited until complete depletion of substrates and metabolites was too short to see physiological regulations of the cells upon PO4 limitation.

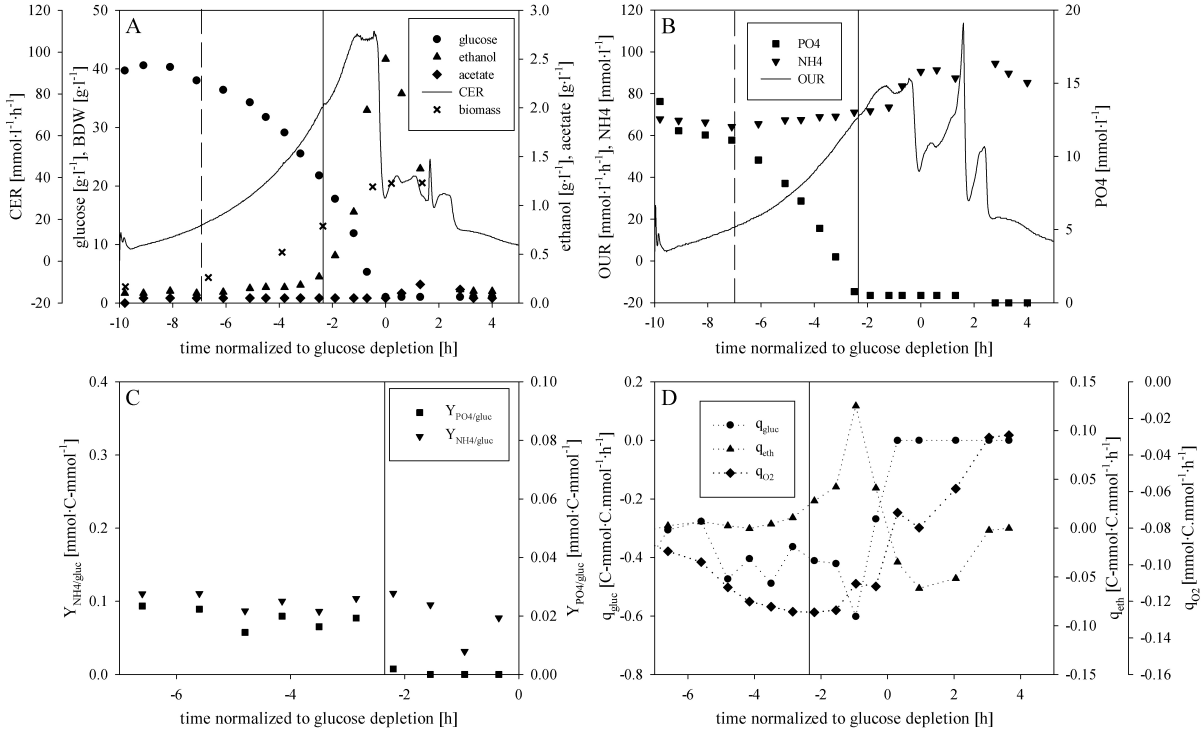


Figure 4.3.1-6 Batch cultivation of *P. pastoris* under phosphate limitation (indicated by vertical straight lines) normalized to glucose depletion at time point 0 h; A) on-line measured data for substrates, metabolites as well as the CER and the off-line biomass dry weight (used filter for calculations is indicated with a vertical dashed line) ; B) on-line measured data for NH4 and PO4 as well as the OUR (used filter for calculations is indicated with a vertical dashed line); C) yield coefficients for NH4 and PO4 on glucose; D) specific rates of glucose (q_{gluc}), ethanol (q_{eth}) and oxygen uptake (q_{O2}).

Bioprocess quantification of cell growth under nitrogen limitation

The same experimental set up was used to analyze the physiological performance of a *P. pastoris* culture under nitrogen limited conditions. Therefore, the basal salt medium was adapted as described in Table 1. The overall culture performance changed significantly when nitrogen limitation started at approximately -2.9 h (indicated as vertical straight lines in Figure 4.3.1-7).

As shown in Figure 4.3.1-7A, the glucose uptake changed upon NH4-limitation from an exponential to a linear consumption, but glucose was still consumed completely. However, in contrast to unlimited and PO4-limited cultures (Figure 4.3.1-3 and Figure 4.3.1-6), less ethanol (0.37 $g \cdot l^{-1}$ compared to 2.0 and 2.5 $g \cdot l^{-1}$, respectively) and no acetate was produced.

Interestingly, both off-gas signals (CER and OUR) became unstable shortly before NH4 depletion at -3 h (Figure 4.3.1-7A and B), indicating that the culture was already limited at a certain low ammonium concentration. In addition to glucose, ethanol and acetate also glycerol was measured during this cultivation, but could not be detected (quantification limit was 0.1 $g \cdot l^{-1}$). This was motivated by the results obtained in another study, where glycerol formation was observed in a *Saccharomyces cerevisiae* culture under nitrogen limitation (EU-project on "Engineering Yeast Starvation" BIO4980562).

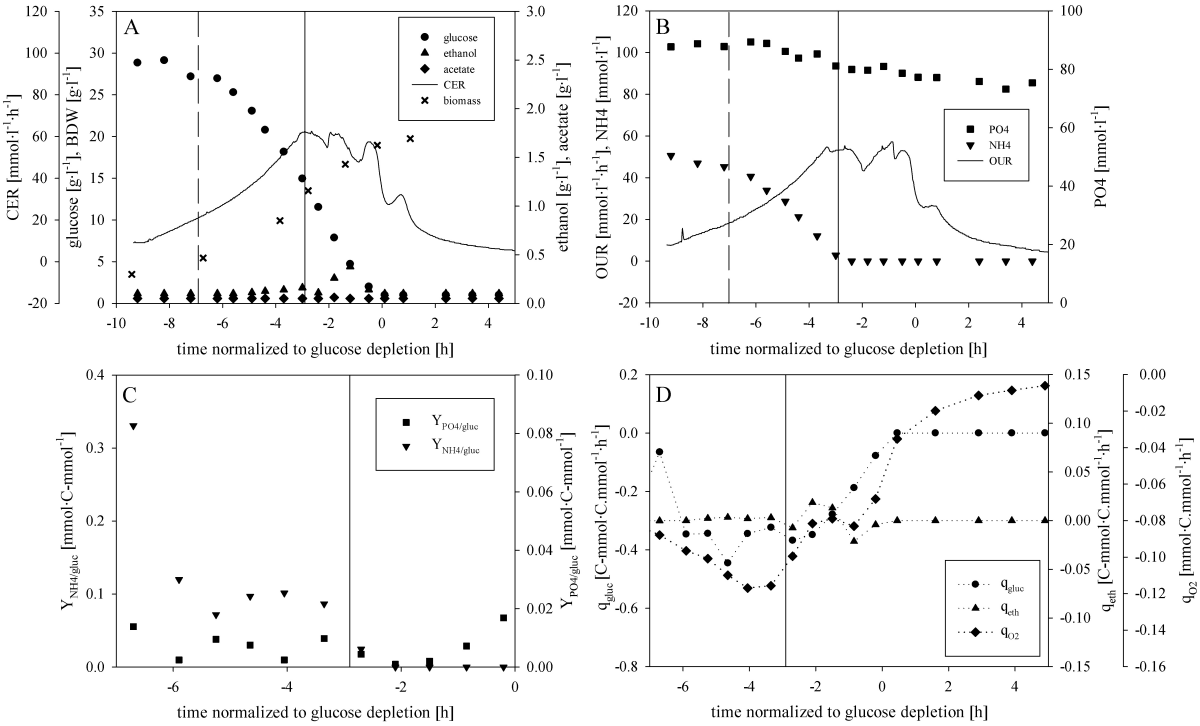


Figure 4.3.1-7 Batch cultivation of *P. pastoris* under nitrogen limitation (indicated by vertical straight lines) referenced on glucose depletion at time point 0 h; A) on-line measured data for substrates, metabolites as well as the CER and the off-line biomass dry weight (used filter for calculations is indicated with a vertical dashed line); B) on-line measured data for NH4 and PO4 as well as the OUR (used filter for calculations is indicated with a vertical dashed line); C) yield coefficients for NH4 and PO4 on glucose; D) specific rates of glucose ($q_{glu}c$) and ethanol (q_{eth}) as well as the specific oxygen uptake rate (q_{O2}).

As shown in Figure 4.3.1-7B, the concentration of NH4 showed an exponential decrease until depletion, whereas the concentration of PO4 decreased just slightly during the whole cultivation time, as also shown in the unlimited culture (Figure 4.3.1-3).

In general, the yields of NH4 and PO4 on consumed glucose before NH4-limitation showed lower and also scattered values compared to the culture under PO4-limitation (Figure 4.3.1-6C), with a very high outlier for $Y_{NH4/gluc}$ at -7 h, which is a very good example for bespoken error propagation from raw measurements (Figure 4.3.1-7A and B). If there are no

significant changes between measured concentrations, and thus the signal-to-noise ratio is low, calculated rates and yields show scattered patterns. In contrast to the NH4-limited batch, in the PO4-limited batch the absolute PO4 concentrations changed to a higher extent between two on-line measurements resulting in more stable values for calculated rates and yields (Figure 4.3.1-6C). However, after nitrogen was depleted in the NH4-limited batch also the calculated yield coefficient $Y_{NH4/gluc}$ dropped to 0 (Figure 4.3.1-7C).

To get more insight into the physiological regulation of the cells in limited conditions, we again used the off-line determined BDW to calculate specific rates (Figure 4.3.1-7D). Before NH4-limitation, the specific rates for glucose (q_{gluc}) and oxygen uptake (q_{O2}) were detected in the same range as for the other experiments described in this study. Shortly before complete nitrogen depletion, q_{O2} inclined and showed a linear increase. With a short delay q_{gluc} followed the same trend until glucose depletion. As only a small amount of ethanol was released into the medium, the previously described limitation and regulation events (Crabtree or alternative respiration) could not be observed to this extent in the NH4-limited culture.

Summarizing, we could show the usefulness of a novel on-line sampling and analysis device comprising an automatic photometric robot for precise on-line measurements of six different components during different batch cultivations of *P. pastoris*. The quality of the on-line data was underlined by rate and yield calculations, where also the importance of the signal-to-noise ratio of measured data points was highlighted and discussed. By considering the off-line determined biomass dry weight specific rates were calculated, which allowed deeper insights in the physiological status of the cells and enabled the discussion on physiological responses on media limitations during highly dynamic process conditions. A reliable on-line device to determine the biomass concentration in the bioreactor, *e.g.* by capacity measurements or different soft sensor devices, would further allow the operator to obtain this information on-line and thus in real-time.

Conclusion

In the present study we introduce a novel on-line analysis device with an automatic photometric robot for fast bioprocess monitoring of microbial cultures. A full characterization of the sampling line comprising a ceramic filtration probe and a self-constructed flow-through cuvette was carried out. The on-line sampling system was connected to a fully equipped bioreactor set up (with on-line analysis of pH, dO_2 and off-gases) to measure various components during different *P. pastoris* batch cultivations:

- Six components (*i.e.* glucose, glycerol, ethanol, acetate, phosphate and ammonium) could be concomitantly analysed with the novel on-line system. No time consuming re-calibration and maintenance work was needed.
- A good correlation between on-line and off-line determined concentrations was found for all components and the data quality was underlined by reliable incremental rate and yield calculations out of the high frequent determined on-line values.
- Based on the on-line data, an early identification of upcoming limitations is possible and allows the operator to control the process in real-time.
- Combining the on-line measured data with the off-line determined biomass dry weight, specific rates were calculated and allowed a deeper insight in the physiological response of the cells to different cultivation conditions.
- We strongly suggest analysing the quality of the data not only by direct comparison to the respective off-line values but rather to check for the ability of extraction of information, such as rates, yields and specific rates. This approach also enables the efficient discussion of physiological effects, independent of scale and initial conditions.

The used photometric robot describes a novel on-line sampling and analysis device, which can be adapted to concomitantly measure numerous components, as in principle any photometric assay can be implemented. Different applications, like optimization of recombinant enzyme expression, strain characterization in early bioprocess development as well as media development and optimization can be supported by the system in either academic or industrial environments. The described tool increases bioprocess understanding and represents a valuable on-line device for Process Analytical Technology (PAT).

Acknowledgements

The authors are very grateful to Prof. Anton Glieder and his workgroup for providing the *P. pastoris* strain used in this study. The study was financially supported by the Austrian Science Fund (FWF Project P24154-N17). Additionally, the authors express their gratitude to Christian Hutterer, who supported the experimental work in the lab.

References

1. FDA: **Guidance for Industry PAT-A framework for Innovative Pharmaceutical Development, Manufacturing, and Quality Assurance.** *www.fda.gov* 2003.
2. ICH: **Q8, Pharmaceutical Development (R1).** *www.ich.org* 2008.
3. Schugerl K: **Progress in monitoring, modeling and control of bioprocesses during the last 20 years.** *Journal of Biotechnology* 2001, **85:**149-173.
4. Vojinovic V, Cabral JMS, Fonseca LP: **Ex situ bioprocess monitoring techniques.** *Chemical Industry & Chemical Engineering Quarterly* 2007, **13:**103-116.
5. Rehbock C, Beutel S, Brueckerhoff T, Hitzmann B, Riechers D, Rudolph G, Stahl F, Scheper T, Friehs K: **Bioprocess analysis.** *Chemie Ingenieur Technik* 2008, **80:**267-286.
6. Callis JB, Illmann DL, Kowalski BR: **Process Analytical Chemistry.** *Analytical Chemistry* 1987, **59:**624-637.
7. Vojinović V, Cabral JMS, Fonseca LP: **Real-time bioprocess monitoring: Part I: In situ sensors.** *Sensors and Actuators B: Chemical* 2006, **114:**1083-1091.
8. Christensen LH, Nielsen J, Villadsen J: **Delay and dispersion in an in situ membrane probe for bioreactors.** *Chemical Engineering Science* 1991, **46:**3304-3307.
9. Filippini C, Moser JU, Sonnleitner B, Fiechter A: **On-line capillary gas chromatography with automated liquid sampling, a powerful tool in biotechnology.** *Analytica Chimica Acta* 1991, **255:**91-96.
10. Herwig C, von Stockar U: **Quantitative analysis of the oxidative metabolism in HXK2- and REG1-deletion mutants of Saccharomyces cerevisiae.** *Enzyme and Microbial Technology* 2002, **31:**698-710.
11. Kaiser C, Pototzki T, Ellert A, Luttmann R: **Applications of PAT-process analytical technology in recombinant protein processes with Escherichia coli.** *Engineering in Life Sciences* 2008, **8:**132-138.
12. Turner C, Gregory ME, Thornhill NF: **Closed-loop control of fed-batch cultures of recombinant Escherichia coli using on-line HPLC.** *Biotechnology and Bioengineering* 1994, **44:**819-829.
13. Herwig C, Doerries C, Marison I, Von Stockar U: **Quantitative analysis of the regulation scheme of invertase expression in Saccharomyces cerevisiae.** *Biotechnology and Bioengineering* 2001, **76:**247-258.
14. Herwig C, Marison I, Von Stockar U: **On-line stoichiometry and identification of metabolic state under dynamic process conditions.** *Biotechnology and Bioengineering* 2001, **75:**345-354.
15. Rocha I, Ferreira EC: **An integrated system for advanced monitoring and control of fed-batch fermentations of recombinant E. coli.** *Comput Appl Biotechnol 2001, Proc Vol IFAC Int Conf, 8th* 2002**:**349-354.
16. Rocha I, Ferreira EC: **On-line simultaneous monitoring of glucose and acetate with FIA during high cell density fermentation of recombinant E. coli.** *Anal Chim Acta* 2002, **462:**293-304.
17. Derfus Gayle E, Abramzon D, Tung M, Chang D, Kiss R, Amanullah A: **Cell culture monitoring via an auto-sampler and an integrated multi-functional off-line analyzer.** *Biotechnol Prog* 2010, **26:**284-292.
18. Wechselberger P, Herwig C: **Model-based analysis on the relationship of signal quality to real-time extraction of information in bioprocesses.** *Biotechnology Progress* 2011, **accepted**.
19. Porro D, Sauer M, Branduardi P, Mattanovich D: **Recombinant protein production in yeasts.** *Molecular Biotechnology* 2005, **31:**245-260.

20. Kern A, Hartner FS, Freigassner M, Spielhofer J, Rumpf C, Leitner L, Froehlich K-U, Glieder A: **Pichia pastoris just in time alternative respiration.** *Microbiology (Reading, United Kingdom)* 2007, **153:**1250-1260.
21. Heyland J, Fu J, Blank LM, Schmid A: **Carbon metabolism limits recombinant protein production in Pichia pastoris.** *Biotechnology and bioengineering* 2011, **108:**1942-1953.
22. Hohmann S: *Stress Response in Yeast.* 1997.

4.3.2. Evaluation and application strategy for online sampling probes

Oliver Spadiut[1], Christian Dietzsch[1], Andreas Posch, Christoph Herwig[*]

[1] these authors contributed equally to this work

Vienna University of Technology, Institute of Chemical Engineering, Research Area Biochemical Engineering, Vienna, Austria

[*]Corresponding Author: Christoph Herwig, Vienna University of Technology, Institute of Chemical Engineering, Research Area Biochemical Engineering, Gumpendorfer Strasse 1a, A-1060 Vienna, Austria. tel.: +43 1 85501 166400, fax.: +43 1 85501 166980, email: christoph.herwig@tuwien.ac.at

Mansucript submitted as a “Technical Report” in revised form to Journal Engineering in Life Sciences

Abstract

A polypropylene and a ceramic sampling probe were tested for the online measurement of substrate and protein concentrations in fed batch cultivations of a recombinant *Pichia pastoris* strain overexpressing the enzyme horseradish peroxidase. Whereas small substrate molecules could be determined precisely under process conditions, online and offline data for enzyme activity and protein content showed offsets for both sampling probes. An easy-to-do multivariate Design of Experiments screening approach revealed the limitations for both sampling probes. Online and offline determined data for enzymatic activity and protein content were fitted to a physiologically meaningful equation. A direct correlation of these equations showed a linear relation between online and offline data for the polypropylene probe and a quadratic relation for the ceramic probe. Using the resulting formulas, observed offsets could be compensated for by mathematical transformation allowing the use of online calculator tools to determine the enzymatic activity and the protein content in quasi real-time. This study demonstrates the usefulness of a Design of Experiments approach to evaluate online sampling probes in a short time and introduces a strategy to obtain data with two different online sampling probes also for large target molecules despite observed offsets.

Practical applications note

Pichia pastoris has evolved into one of the most important host organisms for the heterologous expression of proteins in white biotechnology. Reliable online monitoring strategies for protein production during cultivation processes are very useful to control and optimize these production processes. In the present Technical Report we used two different commercially available sampling probes for the determination of produced proteins in bioreactors. We uncovered that these sampling probes did not meet our specific requirements, since offsets between online and offline determined data were observed. A Design of Experiments screening approach revealed the limitations of each sampling probe in a very short time and thus proved to be a useful tool for characterization and optimization processes in the lab. We proposed a strategy based on mathematical transformation to compensate for the observed offsets. Thus, the tested online sampling probes could still be used and gave good quasi-online data despite observed offsets.

Introduction

Rapid development in genetic engineering allows an enhanced employment of recombinant microorganisms for the industrial production of proteins. A fast and precise determination of substrates and products in these processes is one of the most important requirements to allow better process control and to speed up process development and understanding [1]. Real-time monitoring of process variables enables the operator to take action and interfere with the process, while the process is still being carried out, resulting in higher productivity and enhanced quality control. Therefore, several studies dealt with the development of robust online monitoring devices for bioprocesses in the past decade [2-4].

In general, different aspects of online sampling probes have to be considered: any contamination of the bioprocess by the sampling process must be avoided, a representative sample has to be delivered to the measuring device and the sample composition must not change during the sampling process. Therefore, online sampling probes have to be thoroughly characterized to guarantee reliable results [5].

One goal of our research is the implementation and evaluation of robust online monitoring devices to quantify the formation of extracellularly produced proteins, available substrate molecules and metabolites in bioreactors. In the present study, two different sampling probes were tested for their applicability to determine the concentration of the substrate molecules glucose and glycerol as well as the catalytic activity and the total protein content of an extracellularly produced enzyme in a bioreactor. According to our previous studies, we

conducted fed batch fermentations with a recombinant *P. pastoris* strain overexpressing the enzyme horseradish peroxidase (HRP) [6-7], and used a polypropylene and a ceramic sampling probe for online measurements of bespoken variables.

In this contribution we are demonstrating the applicability of a multivariate Design of Experiments (DoE) screening approach for a fast and easy-to-do evaluation of online sampling probes. We propose a strategy using mathematical transformation to compensate for observed offsets between online and offline determined data allowing the extraction of accurate data from online measurements despite observed offsets.

Material and Methods

Fed batch fermentations of a recombinant P. pastoris strain

We conducted fed batch fermentations of a recombinant *P. pastoris* strain overexpressing the enzyme horseradish peroxidase, according to our previous studies [6-7]. Fed batch cultivations were carried out in a 7.5 l (5 l working volume) glass bioreactor (Infors, Switzerland) equipped with three 6-bladed flatblade Rushton stirrers. The cylindrical probes were installed in distance to the baffles in the bioreactor to reduce any influence caused by changing flow profiles. We conducted these fed batch fermentations in order to develop and test a novel feeding strategy based on the specific substrate uptake rate (q_s) and concomitantly tested the sampling probes for their applicability of online measurements. The detailed fermentation conditions can be found in our previous studies [6-7].

Substrate and product analysis

All measurements of substrate molecules and product concentration were performed online and offline using an automatic photometric robot (CuBiAn XC, Innovatis, Germany). A special flow-through cuvette was constructed using a commercially available half-micro cuvette, inserted in the on-line port of the photometric robot and connected with PTFE tubes (0.8 x1.6 mm; Bartelt, Austria) to either of the sampling probes. A peristaltic pump (ISM847E; Ismatec, Switzerland) was used to transport the sample from the probe to the cuvette with a flow rate of 1 ml/min. Concentrations of glucose and glycerol were determined automatically in cell free samples with commercially available enzymatic assay kits in the photometric robot [6-7]. The total protein content was determined using a Bradford assay and HRP activity was measured with an ABTS assay, as described before [6-7]. All measurements and data acquisition were controlled by a process information management system (Lucullus, Biospectra, Switzerland).

Online sampling probes

The two sampling probes tested were a ceramic sampling probe made of Al_2O_3 (pore size 0.2 µm, filter area 17,8 cm^2, membrane thickness 1.6 mm; iba, Heiligenstadt, Germany) and a polypropylene probe (pore size 0.2 µm, filter area 42,7 cm^2, membrane thickness 1.5 mm; ABC GmbH, Puchheim, Germany). Fully automated sampling was carried out every 3 hours during the whole induction phase. Both probes had cylindrical membranes and were installed in the bioreactor in the direction of flow and were not impeded by baffles. After cleaning procedures, according to the manufacturer's instructions, the membranes were reused.

Biomass determination

Dry cell weight was determined by centrifugation of 5 ml culture broth (5000 rpm, 4 °C, 10 min) in a laboratory centrifuge (Sigma 4K15, rotor 11156), washing the pellet with 5 ml deionized water and subsequent drying at 105°C to constant weight.

Factor screening using a Design of Experiments (DoE) strategy

A 2-level cubic full factorial screening approach with 3 centre points and 1 replicate for all points was set up with the program MODDE (Umetrics AG, Umea, Sweden) to explore the influence of the factors "agitation" (0 - 1200 rpm), "aeration" (0 - 1 vvm) and "biomass concentration" (0 - 77 g/l) as well as their linear interactions on the response parameters "relative HRP activity" and "relative protein content". Twenty-two experiments were necessary to determine the possible influence of the different factors and their linear interactions on each response parameter.

Experimental set-up for the DoE screening approach

A fermentation broth of *P. pastoris* expressing HRP with a biomass dry cell weight of ~77 g/l, an enzymatic activity of 19 U/ml and a protein content of 0.4 mg/ml was used as starting material. To meet the requirements of the DoE design, the culture broth was either diluted to a biomass dry cell weight of ~38.5 g/l or centrifuged to prepare a cell free supernatant (5000 rpm, 4°C, 10 min; Sorval RCBB, rotor H60000A/HBB6). At each biomass concentration, off-line samples were taken, centrifuged (5000 rpm, 4°C, 10 min; Sigma 4K15, rotor 11156) and the determined values for the catalytic activity, the protein content and the substrate concentration were subsequently used as reference values to evaluate online measured data.

Data analysis

Online and offline determined activities for HRP were fitted to an exponential function according to equation (4.3.2.1) by estimating the activity of HRP at induction time zero $a(HRP)_0$, the exponential coefficient β and the deceleration factor α.

$$a(HRP) = a(HRP)_0 \cdot \exp((\beta + \alpha \cdot t) \cdot t) \qquad (4.3.2.1)$$

While the exponential coefficient β was chosen to describe the exponential feeding profile of the limiting substrate in the fed batch cultivations, the deceleration factor α was set in order to compensate for the hydraulic dilution of the volumetric activity in the reaction vessel due to constant feeding. Parameter fitting was performed in Matlab (The Mathworks, USA). Model coefficients were obtained by applying the Levenberg-Marquardt method [8] of minimizing the squared prediction error, fulfilling the constraint of equal exponential factors β for models of online and offline measurements.

Results and Discussion

Online measurement of substrate and product concentrations

A polypropylene and a ceramic online sampling probe were tested for their applicability to measure the concentration of small substrate molecules as well as the enzymatic activity and the protein content in fed batch cultivations of a recombinant *P. pastoris* strain. All measurements of the small substrate molecules glucose and glycerol gave responses of 100 ± 5 % between online and offline determined values, proving that precise measurements of these molecules were possible with both online sampling probes at the tested process conditions. We could further measure exact concentrations of N- and P-sources as well as small metabolites in the bioreactor (*i.e.* ethanol and acetate), which allowed the online calculation of rates and yields and consequently to draw conclusions about the physiological state of the culture (full manuscript submitted). However, offsets between online and offline determined values for the enzymatic activity were observed for both sampling probes (Figure 4.3.2-1). The same offsets were also observed for the total extracellular protein content (data not shown). Interestingly, these offsets were more pronounced for the ceramic sampling probe than for the polypropylene probe (Figure 4.3.2-1).

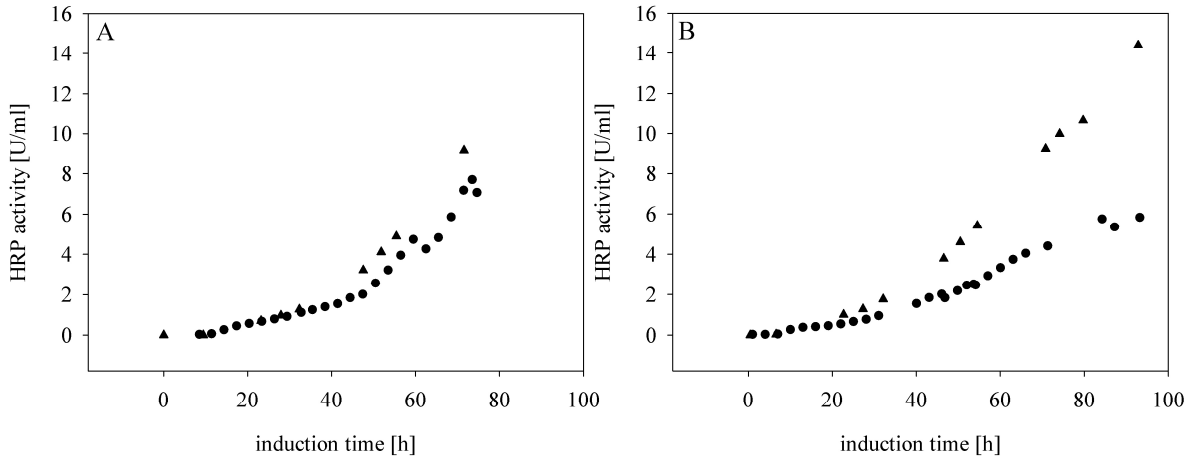


Figure 4.3.2-1 Online and offline measurement of HRP activity in fed batch cultures of P. pastoris. A, polypropylene probe; B, ceramic probe; (circle), online measured data; (triangle), offline measured data.

Multivariate analysis of online sampling probes by a DoE screening approach

To screen for reasons for the observed offsets and to identify the limitations of the online sampling probes under process conditions, a multivariate 2-level cubic full factorial DoE screening approach was set up to analyse the effects of the factors agitation, aeration and biomass concentration (as well as their linear interactions) on the response parameters relative HRP activity and relative protein content.

The analysis of the performed DoE screening approaches revealed that the response parameters did not follow a normal distribution and indicated that not only linear but also quadratic interactions between the factors existed, which caused a low “model validity”. However, the other 3 basic model parameters “model fit”, “estimation of future prediction” and “reproducibility” gave satisfactory results for both sampling probes (*i.e.* $R^2 = 0.689$, $Q^2 = 0.565$, repr. = 0.917 for the polypropylene probe and $R^2 = 0.869$, $Q^2 = 0.769$, repr. = 0.976 for the ceramic probe, respectively), which is why the models could be evaluated and analyzed.

In general, the same effects were observed for both response parameters “relative HRP activity” and “relative protein content” for both sampling probes.

For the polypropylene probe, the quality of the response parameter relative HRP activity decreased significantly with increasing agitation and aeration. At an agitation of 1200 rpm and an aeration of 1 vvm, conditions which are very common for bioreactor cultivations of *P. pastoris*, only less than 65 % of the HRP activity could be measured. However, the biomass concentration was determined as a non-significant factor for the polypropylene probe.

For the ceramic sampling probe, even all three factors (*i.e.* agitation, aeration and biomass concentration) significantly affected online measurements of the HRP activity (Figure 4.3.2-2). At an agitation of 1200 rpm, a biomass concentration of 70 g/l, which can be easily reached in fed batch cultivations of *P. pastoris*, and an aeration of 1 vvm, less than 45 % of the HRP activity could be measured.

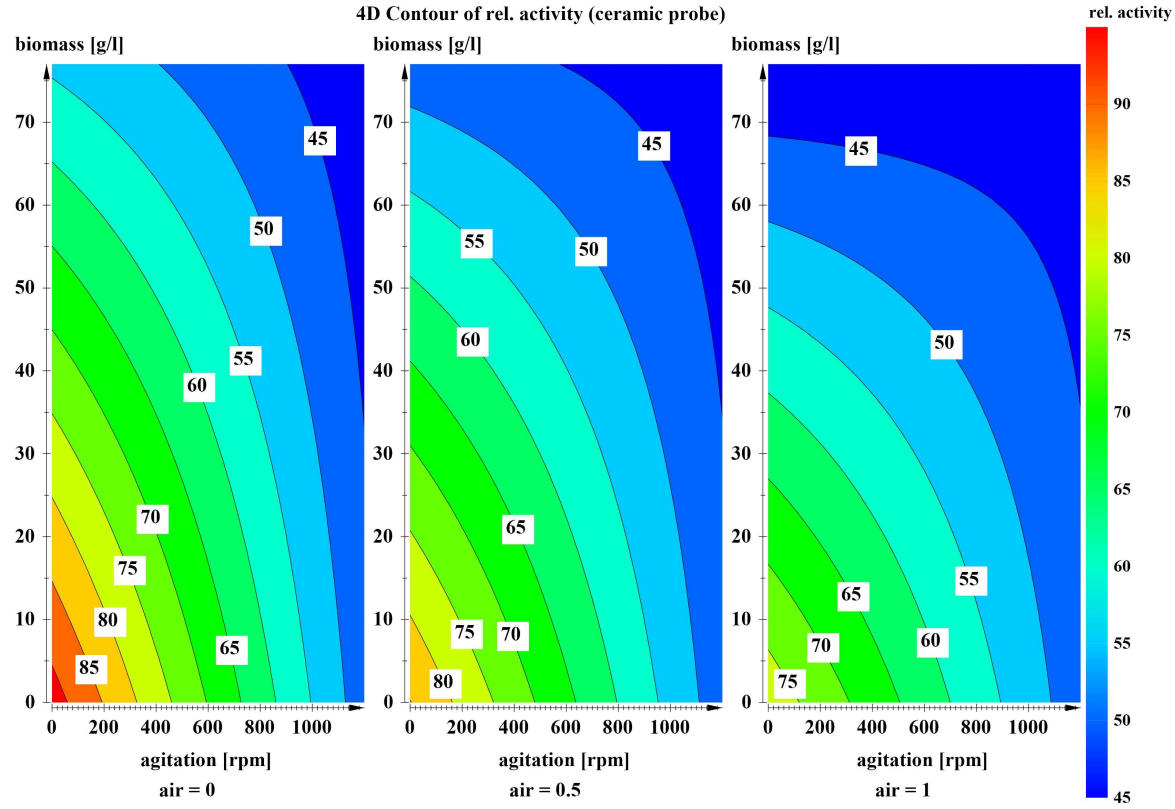


Figure 4.3.2-2 Response surface plot of the factors agitation, aeration and biomass concentration in response to the relative HRP activity for the ceramic sampling probe.

Since the same effects were observed for both response parameters, HRP activity and protein content, for both sampling probes, we concluded that the offsets between online and offline data did not result from a potential reduction of the enzymatic activity of HRP, but that the enzyme was somehow hindered in penetrating the sampling probes. We also ruled out a significant effect of a potential deposition of the enzyme in the membrane, as it has been described before (e.g. [9]), since the randomly performed experiments in the context of the conducted DoE approach clearly showed that the different process parameters described the significant factors causing the observed offsets.

Interpretation of DoE results

For both sampling probes, the factor "agitation" was determined as having the main effect on either of the response parameters. However, since offsets between online and offline data were just observed for the large molecule HRP and not for small substrate molecules, apparently size dependent effects were responsible for this outcome. In mammalian cell culture a similar effect is observed when spin-filters are used to retain mammalian cells in order to operate in perfusion mode. Particles are retained due to steric effects of the rotating system and not due to the filter pore size *per se*, which is why filters with absolute pore sizes larger than the average cell size can be used in these systems [10-12]. Analogically, our results suggest that a kind of inverted spin-filter effect, where not the filter but the liquid rotates, causes difficulties for HRP (44 000 Da) to penetrate the sampling probe, whereas glucose (180 Da) and glycerol (90 Da) can still pass the membranes unimpeded. Increasing aeration in the bioreactor apparently causes similar effects (Figure 4.3.2-2).

The biomass concentration only affected the ceramic sampling probe (Figure 4.3.2-2), which is an asymmetric membrane with varying pore sizes. Probably a biomass layer was formed on top of the ceramic probe during sampling, causing additional filter effects and a size-dependent retention, a phenomenon which has been described as membrane fouling before [13]. Since we were limited to a maximum flowrate of 1 ml/min according to the manufacturer's specifications for the ceramic sampling probe, we were not able to test a higher pumping speed and consequently a higher transmembrane pressure on the quality of the response parameters.

Summarizing, the performed DoE screening approach clearly showed that the two tested sampling probes could not be used for a precise online quantification of HRP at high stirring speed and aeration and/or high cell densities, conditions which are common in fed batch cultivations of *P. pastoris*, due to emerging offsets caused by different process parameters.

Mathematical transformation of online measured data

To be able to extract data from online measurements with the two sampling probes despite the observed offsets, we fitted the data points to different mathematical equations and evaluated the best fit with the highest R^2 values. Since the stepwise feeding profile applied in the conducted fed batches [7] was most accurately described by an exponential function of the type $f(HRP) = f(HRP)_0 \cdot e^{((\beta+\alpha \cdot t) \cdot t)}$, we also tried to describe and fit the offline as well as the online determined data to this equation. As shown in Figure 4.3.2-3, both the online and the

offline determined values described this exponential function for both sampling probes with a very high accuracy (R^2 values of > 0.98).

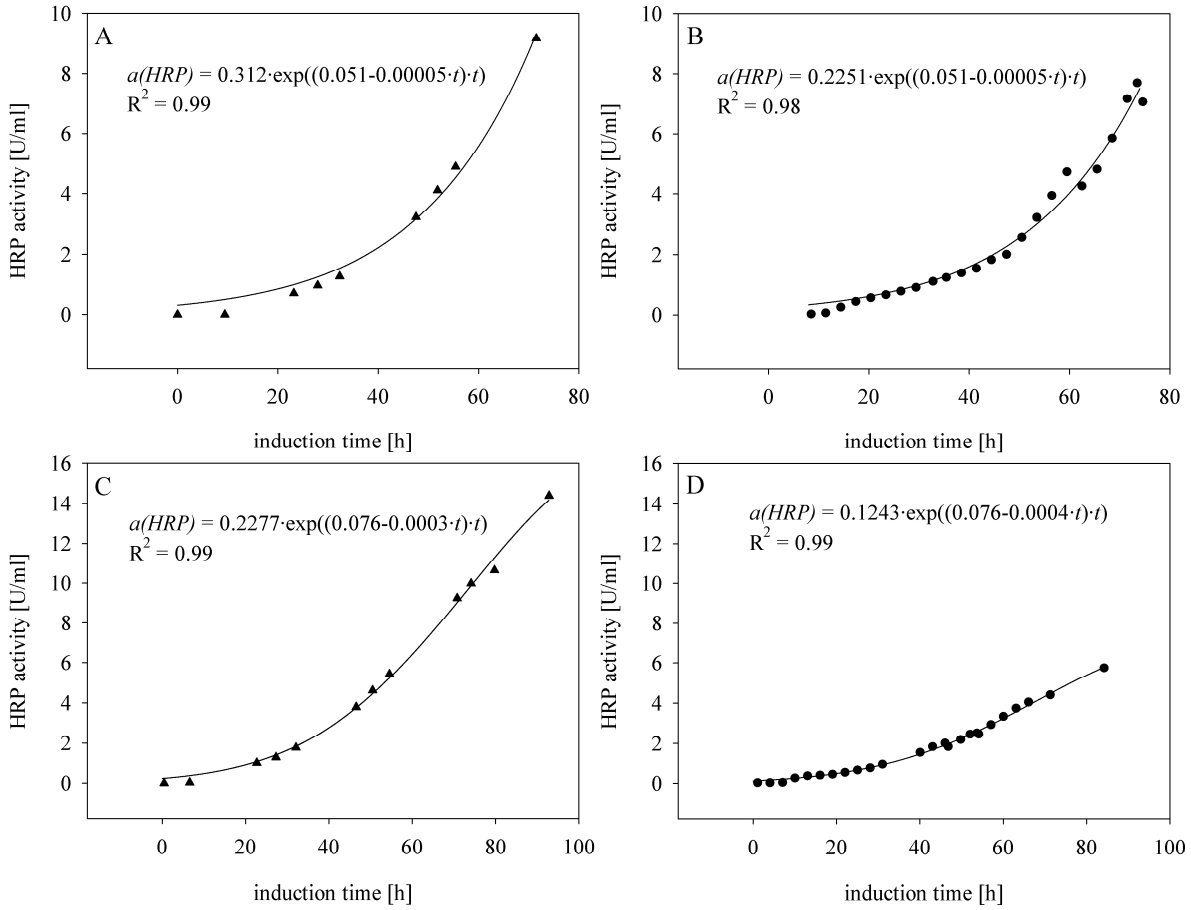


Figure 4.3.2-3 Online and offline determined data of HRP activity for the polypropylene and the ceramic sampling probe. Data points were fitted to the equation a(HRP) = a(HRP)0·e((β+α·t)·t) and resulting equations are shown in the Figure for each sampling probe, respectively. A, offline data polypropylene probe; B, online data polypropylene probe; C, offline data ceramic probe; D, online data ceramic probe; (triangle), offline determined values; (circle), online measured values.

Results from the DoE screening approach identified aeration and agitation to cause an offset between online and offline measurements from the very beginning of the process for both sampling probes, while biomass concentration could be shown to only affect measurements using the ceramic sampling probe (Figure 4.3.2-2). Consequently, the offset caused by aeration and agitation is reflected in a reduced initial $a(HRP)_o$ for all online transformation functions (Figure 4.3.2-3). The biomass accumulation was expected to cause further deviations over process duration for the ceramic probe (Figure 4.3.2-2), which was confirmed in different deceleration factors α for online and offline measurements (Figure 4.3.2-3). While the deceleration factor α was identical for online and offline determined activities for the

polypropylene probe (Figure 4.3.2-3A and Figure 4.3.2-3B), it showed a significant difference for the ceramic probe (Figure 4.3.2-3C and Figure 4.3.2-3D). Analogically to the results from the DoE screening approach, we observed that accumulating biomass caused an accelerated shift between online and offline determined data for the ceramic probe, while the increasing biomass concentration over time did not affect online measurements with the polypropylene probe. Observed differences in the exponential coefficient β (*i.e.* 0.051 for the polypropylene probe and 0.076 for the ceramic probe, respectively; Figure 4.3.2-3) were due to slightly different feeding regimes in the conducted fed batch cultivations of the recombinant *P. pastoris* strain, when either the polypropylene or the ceramic sampling probe were used.

In order to get equations compensating for the observed offsets, we correlated the two decelerated exponential functions obtained for online and offline data for the respective sampling probe.

Consequently, online measured activities could be converted to offline determined data by a simple linear transformation according to equation (4.3.2.2) for the polypropylene probe.

$$a(HRP)_{offline} = a(HRP)_{pp,online} \cdot 1.4 + 0.0025 \qquad (4.3.2.2)$$

For the ceramic probe the conversion of online measured activities to offline determined values could only be accomplished by transformation of higher order according to equation (4.3.2.3), as not only agitation and aeration, but also the biomass concentration significantly affected online measurements.

$$a(HRP)_{offline} = 0.0576 \cdot a(HRP)^2_{ceramics,online} + 1.7984 \cdot a(HRP)_{ceramics,online} \qquad (4.3.2.3)$$

Summarizing, it was possible to successfully correct online measured values by mathematical transformation and thus to compensate for the observed offsets. By using equations (4.3.2.2) and (4.3.2.3) for the polypropylene and the ceramic probe, respectively, online measured data could be corrected to offline determined data with an accuracy of ± 10 %. This mathematical transformation can be done using an online calculation tool in the respective process information management system allowing the quasi real-time monitoring of enzyme activity and protein content in the bioreactor. However, these transformations are dependent on the applied process conditions and have to be determined for changing process variables (*i.e.* used microorganism, stirring speed, optical density, etc.) each time.

In general, the two sampling probes might still give precise online data without any difficulties and without the need of data transformation for systems where less agitation and aeration is needed and where the culture does not grow to high cell densities (*e.g.* in mammalian cell culture). Besides, a precise and reliable online measurement of the small molecules glucose and glycerol is possible with both online sampling probes at process conditions. Consequently, the two studied sampling probes can also be purposefully used for online monitoring of the concentration of substrate molecules as well as other media components and small metabolites during cultivations, allowing a quasi real-time identification of critical process steps, like the starting-point of the fed batch phase or the moment of induction (full manuscript submitted).

Concluding remarks

Here we show the application of two online sampling probes for the determination of substrate concentration, enzymatic activity and protein content in fed batch cultivations of a recombinant *P. pastoris* strain. We highlight the usefulness of using a fast and easy-to-do DoE screening approach for the evaluation of these sampling probes and propose a strategy to compensate for observed offsets between online and offline determined data. Our findings can be summarized as follows:

- both the ceramic and the polypropylene sampling probe can be used to reliably determine the concentration of the small substrate molecules glucose and glycerol at process conditions.
- for both sampling probes offsets between online and offline determined data for the enzymatic activity of HRP and the protein content were observed.
- an easy-to-do multivariate DoE screening approach uncovered the reasons for the observed offsets in a very short time.
- the measured data points for both online and offline determined values for both sampling probes were fitted to the mathematical equation $f(HRP) = f(HRP)_0 \cdot e^{((\beta+\alpha \cdot t) \cdot t)}$, which also described the applied feeding strategy, giving high R^2 values of > 0.98 each time.
- by putting the equations for each sampling probe in direct correlation, the resulting formulas could be used to transform the online measured values to offline determined data and thus to compensate for the observed offsets. This mathematical transformation can be done by online calculator tools allowing a quasi online measurement of the enzymatic activity and the protein content despite observed offsets.

Acknowledgements

We want to thank Cosima Koch for fruitful discussions and Denes Zalai for assistance in the lab.

List of Abbreviations:

ABTS, 2.2' azino bis 3-ethylbenzthiazoline-6-sulphonic acid; DoE, design of experiment; HRP, horseradish peroxidase; vvm, volume air per volume liquid per minute; rpm, rotations per minute

Conflict of interest

The authors have declared no conflict of interest.

References

1. ICH8: **Pharmaceutical Development (R1).** 2008.
2. Schugerl K: **Progress in monitoring, modeling and control of bioprocesses during the last 20 years.** *Journal of Biotechnology* 2001, **85:**149-173.
3. Vojinovic V, Cabral JMS, Fonseca LP: **Ex situ bioprocess monitoring techniques.** *Chemical Industry & Chemical Engineering Quarterly* 2007, **13:**103-116.
4. Rehbock C, Beutel S, Brueckerhoff T, Hitzmann B, Riechers D, Rudolph G, Stahl F, Scheper T, Friehs K: **Bioprocess analysis.** *Chemie Ingenieur Technik* 2008, **80:**267-286.
5. Christensen LH, Nielsen J, Villadsen J: **Delay and dispersion in an in situ membrane probe for bioreactors.** *Chemical Engineering Science* 1991, **46:**3304-3307.
6. Dietzsch C, Spadiut O, Herwig C: **A fast approach to determine a fed batch feeding profile for recombinant Pichia pastoris strains.** *Microbial Cell Factories*, **10:**85.
7. Dietzsch C, Spadiut O, Herwig C: **A dynamic method based on the specific substrate uptake rate to set up a feeding strategy for Pichia pastoris.** *Microbial Cell Factories* 2011, **10**.
8. Marquardt DW: **An Algorithm for Least-Squares Estimation of Nonlinear Parameters.** *Journal of the Society for Industrial and Applied Mathematics* 1963, **11:**431-441.
9. Su TJ, Lu JR, Cui ZF, Thomas RK: **Fouling of ceramic membranes by albumins under dynamic filtration conditions.** *Journal of Membrane Science* 2000, **173:**167-178.
10. Voisard D, Meuwly F, Ruffieux PA, Baer G, Kadouri A: **Potential of cell retention techniques for large-scale high-density perfusion culture of suspended mammalian cells.** *Biotechnology and Bioengineering* 2003, **82:**751-765.
11. Yabannavar VM, Singh V, Connelly NV: **Mammalian cell retention in a spinfilter perfusion bioreactor.** *Biotechnol Bioeng* 1992, **40:**925-933.
12. Yabannavar VM, Singh V, Connelly NV: **Scaleup of spinfilter perfusion bioreactor for mammalian cell retention.** *Biotechnol Bioeng* 1994, **43:**159-164.
13. van de Merbel NC, Lingeman H, Brinkman UAT: **Sampling and analytical strategies in online bioprocess monitoring and control.** *Journal of Chromatography, A* 1996, **725:**13-27.

5. Conclusions and Outlook

The methylotrophic yeast *P. pastoris* has evolved to a widely used expression host for the production of recombinant proteins in the past few years. However, strategies in process development for increased process understanding and process optimization are still mostly based on empiricism. The present study aimed to establish a novel, physiologically based process development strategy for the fast and scalable determination of key parameter sets for a fed batch production process with recombinant *P. pastoris* strains.

- *A fast and easy to do method for parameter transfer from batch to fed batch was successfully developed and applied.*

A fast and easy to do strain performance quantification method was developed to determine strain specific key parameters of recombinant *P. pastoris* strains.

- In contrast to commonly used long lasting continuous cultures, batch pulse experiments were used for the reliable quantification of certain strain specific parameters ($\Delta time_{adapt}$, $q_{s\ adapt}$, $q_{s\ max}$), which were successfully transferred into a fed batch feeding regime, within a short time (Chapter 4.1.1).
- The general applicability of the strategy was confirmed by the execution on different *P. pastoris* phenotypes (Mut^S, Mut^+), which also expressed different recombinant products, like HRP and GalOX (Chapter4.1.2).

- *The key parameter specific substrate uptake rate q_s will play a central role in future process development because it describes a physiological and scalable process parameter.*

The specific substrate uptake rate q_s was successfully applied as a key parameter for process control and process optimization in single as well as in mixed substrate systems.

- In single substrate systems, a dynamic, stepwise increase of the control parameter q_s to $q_{s\ max}$ was identified to be superior to a constant controlled parameter set and a time-dependent effect of the specific productivity was observed (Chapter 4.1.1).
- In mixed feed systems, individual q_s adjustment for both substrates was applied. Different substrate uptake rates for the co-substrate glycerol were detected, where repression effects for protein production or methanol metabolism occurred (Chapter 4.2.1).

Hence, the methodology based on q_s could not only be used for "process development", but was also successfully applied to increase "process understanding".

- *Increased process understanding for correlation of q_s to product generation in* P. pastoris *was achieved.*

The novel strain quantification strategy was used to evaluate and compare different *P. pastoris* clones with an altered methanol utilization pathway.

- The overexpression of the enzyme formaldehyde dehydrogenase (FLD1) turned out to be a promising strategy to increase the efficiency of recombinant *P. pastoris* strains in terms of substrate to product conversion (Chapter 4.2.2).
- Product specific (HRP or CalB) interactions with different MUT pathway enzymes (DAS1, TKL1, FLD1) were identified which highlighted the necessity of a reliable and fast quantification method for key parameter identification.

The methodology allows not only the quantification of the final product titre, but also comprises the analysis of specific production kinetics of different *P. pastoris* strains.

- *An on-line device for fast process monitoring in process development was successfully introduced.*

The presented, novel on-line monitoring device supported "process development" as it can be used for media development, detection of potential limitations events and for product determination.

- An automatic photometric robot was introduced to detect physiological relevant compounds, like substrates and metabolites. On-line measurements speed up data generation in a reliable way and were consequently used for fast physiological response estimation in several *P. pastoris* cultivations (Chapter 4.3.1).
- The novel on-line monitoring system was used for the quantification and qualification of the target product HRP in on-line mode. Compared to the off-line data, the product was determined with offsets in on-line mode. These offsets were caused by changing bioprocess conditions, which cannot be kept constant during protein expression (Chapter 4.3.2).

- *Promising perspective for further extrapolation of achievements.*
  - The fast and reliable quantification of key parameters in batch mode and the possible data transfer into a fed batch production environment describes the major novelty of the present study. Based on precise data exploitation, the batch quantification

principle was identified as a valid tool in process development. In principle, the strategy can be extrapolated to other recombinant protein production systems, where the inducing substrate is assimilated during protein expression. Such systems are e.g. the *P. pastoris* FLD1 promoter system, the *E.coli* L-rhamnose *rha*P_{BAD} promoter or the *E.coli* L-arabinose P_{BAD} system.

- A mechanistic model of product release in relation to the specific substrate uptake rate supplemented with a time dependent variable would allow the optimization of feeding strategies. A remaining question is, whether the boost in specific productivity was caused by the stepwise q_s challenge of the cells, or whether a simple feed forward feeding regime would result in equally high productivities. Such a feeding regime would represent an accelerated exponential profile and variations in the acceleration factor can be used to further optimize recombinant protein production easily.
- The physiological key parameter q_s is a promising control parameter for fed batches and induction processes for recombinant protein production. In carbon limited regimes combined with a reliable estimation of biomass contents, q_s depicts a versatile and universal control parameter which is, compared to the specific growth rate μ less noisy and closely related to induction of recombinant protein expression.
- The transfer of the determined specific rates for the modified strains, e.g. Mut^S FLD1 strain, into a fed batch production regime would confirm higher volumetric productivities, which were shown in batch cultivations. Hence, the industrial applicability of such genetically modified host strains in production processes can be proven.
- The novel on-line device has shown its power to support process development and can be applied to other systems for media development and product quantification in real time. Further investigations are ongoing to overcome observed offsets for large molecules. Modified filtration units comprising larger pore sizes are currently under investigation for the HRP on-line determination.

6. Appendix

Dietzsch C, Spadiut O, Herwig C: **A dynamic method based on the specific substrate uptake rate to set up a feeding strategy for Pichia pastoris.** *Microbial Cell Factories* 2011, **10:**14.

Dietzsch, C.; Spadiut, O.; Herwig, C., **A fast approach to determine a fed batch feeding profile for recombinant Pichia pastoris strains.** *Microb. Cell Fact.* 2011, 10, (1), 85.

Dietzsch et al. Microbial Cell Factories 2011, 10:14
http://www.microbialcellfactories.com/content/10/1/14

MICROBIAL CELL FACTORIES

RESEARCH Open Access

A dynamic method based on the specific substrate uptake rate to set up a feeding strategy for *Pichia pastoris*

Christian Dietzsch, Oliver Spadiut, Christoph Herwig*

Abstract

Background: *Pichia pastoris* is one of the most important host organisms for the recombinant production of proteins in industrial biotechnology. To date, strain specific parameters, which are needed to set up feeding profiles for fed batch cultivations, are determined by time-consuming continuous cultures or consecutive fed batch cultivations, operated at different parameter sets.

Results: Here, we developed a novel approach based on fast and easy to do batch cultivations with methanol pulses enabling a more rapid determination of the strain specific parameters specific substrate uptake rate q_s, specific productivity q_p and the adaption time ($\Delta time_{adapt}$) of the culture to methanol. Based on q_s, an innovative feeding strategy to increase the productivity of a recombinant *Pichia pastoris* strain was developed. Higher specific substrate uptake rates resulted in increased specific productivity, which also showed a time dependent trajectory. A dynamic feeding strategy, where the setpoints for q_s were increased stepwise until a $q_{s\ max}$ of 2.0 mmol·g^{-1}·h^{-1} resulted in the highest specific productivity of 11 U·g^{-1}·h^{-1}.

Conclusions: Our strategy describes a novel and fast approach to determine strain specific parameters of a recombinant *Pichia pastoris* strain to set up feeding profiles solely based on the specific substrate uptake rate. This approach is generic and will allow application to other products and other hosts.

Background

Recombinant protein expression with biological hosts is one of the most examined key processes in the pharmaceutical industry. Numerous products like organic acids, antibiotics, enzymes and amino acids are produced heterologously by recombinant microorganisms. The methylotrophic yeast *Pichia pastoris* is one of the most important host organisms for this purpose.

Several of the published fermentation strategies for *P. pastoris* to date are based on the Invitrogen protocol http://tools.invitrogen.com. This protocol suggests constant feeding profiles for fed batch cultivations, but does not aim to improve production efficiency regarding time and yield or substrate consumption. Based on this protocol, different process strategies were developed to optimize recombinant protein production with *P. pastoris* in the past few years. A commonly used feeding strategy describes a feed forward regime based on a constant specific growth rate μ [1-5]. This strategy results in an exponential feeding profile and does not require complex instrumentation, but μ is also not controlled, and since the cells capacity may change over time, the feeding profiles consider a large safety margin. Another feeding strategy is based on a controlled μ and requires laborious continuous culture investigations and an effective computer controlled operation, based on established growth models and a feedback algorithm requiring expensive online measurement sensors for methanol [2,6-8]. Employing these strategies, the outcome regarding specific productivity and specific growth rate was diverse; some studies showed that the maximal specific productivity did not relate to the maximal specific growth rate [1,3,6,7], whereas another study showed a more or less growth associated productivity [9].

Due to these controversial findings, other parameters were analyzed for their possible correlation with the productivity in the past few years. Khatri and Hoffmann

* Correspondence: christoph.herwig@tuwien.ac.at
Vienna University of Technology, Institute of Chemical Engineering, Research Area Biochemical Engineering, Vienna, Austria

analyzed the specific substrate uptake rate (q_s) and its association with the specific productivity (q_p) in fed-batch cultivations of *P. pastoris* and showed that lower q_s resulted in higher q_p [10,11]. In another study, Cunha *et al*. used a constant feeding rate of methanol during several fed-batch cultivations with different initial biomass contents to monitor q_s over time [12]. Interestingly, Cunha *et al*. also showed increased q_p at lower q_s and clearly stated that q_s was the most important induction parameter. However, despite the obvious effect of q_s on q_p, feeding profiles based on q_s have not been tested yet.

Regardless of which control parameter is chosen to set up feeding profiles for fed batch cultivations, strain specific parameters have to be determined. This can either be done by continuous cultures [13] or by several, consecutive fed batch cultivations, which are operated at different parameter sets [14,15]. These methods have the disadvantage of being very time-consuming and labor-intensive. A robust method to determine these strain specific data and to develop a suitable feeding strategy in a shorter time period is of high interest in biotechnology to speed up process development and to quantitatively screen industrial relevant strains.

In this study, we developed a new and fast method to determine the strain specific data q_s and q_p, as well as the adaptation time of the culture to the substrate methanol, based on easy to do batch cultivations with methanol pulses. The host *P. pastoris* and the product horseradish peroxidase (HRP) were used as a model system, for which we subsequently developed a novel feeding strategy solely based on the determined q_s (Figure 1). Various fed batch cultivations, employing different q_s feeding profiles, were carried out to characterize the strain, hence, to determine yields ($Y_{X/S}$, $Y_{CO2/S}$) and the specific productivity (q_p) to evaluate and improve the feeding strategy. To our knowledge this is the first time that different dynamic feeding profiles only based on q_s were tested to develop a feeding strategy for *P. pastoris* aiming at increased productivity.

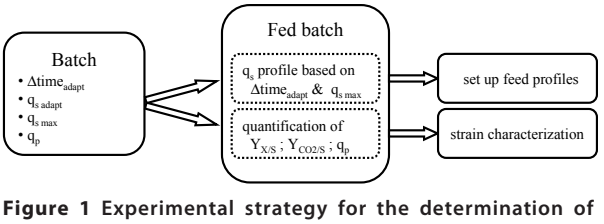


Figure 1 Experimental strategy for the determination of relevant strain specific parameters in a batch cultivation with methanol pulses. These strain specific data ($\Delta time_{adapt}$ - adaptation time; $q_{s\ adapt}$ - specific substrate uptake rate during the adaptation period; $q_{s\ max}$ - maximum specific substrate uptake rate) are then used to set up a feeding profile for *P. pastoris* in fed batch cultivations. The calculated yields ($Y_{X/Y}$, $Y_{CO2/S}$) and the specific productivity (q_p) can consequently be used for strain characterization.

Material and methods

Microorganism and recombinant protein

The *Pichia pastoris* strain KM71 H (*arg4 aox1:arg4*) was transformed with a plasmid containing the gene for the horseradish peroxidase isoenzyme C1A (HRP) and was gratefully provided by Prof. Anton Glieder (Graz University of Technology, Austria). The phenotype of the strain corresponded to an AOX1-deficient clone which is characterized as Mut^S (methanol utilization slow) and HRP was secreted into the fermentation broth.

Horseradish peroxidase is a member of the plant peroxidase super family [16] and catalyzes the oxidation of many substrates using hydrogen peroxide, resulting in oxidized products and in the formation of two molecules of water per molecule of hydrogen peroxide [17-19]. It is a heme-containing glycoprotein with a molecular weight of approximately 44 kDa that has been studied for more than 100 years [19]. Horseradish peroxidase gains more and more industrial relevance in the past few years; it is used in waste water treatment [20,21], analytical diagnostics [22] and for the elimination of H_2O_2 from food and industrial wastewater [23].

Stability of the enzyme horseradish peroxidase in the presence of methanol

To check whether certain concentrations of methanol were affecting the stability of the enzyme horseradish peroxidase, HRP was diluted either in water or in BSM medium to a concentration of 1 U/mL and incubated in a waterbath at 28°C in the presence of up to 20 mg/mL methanol (which corresponds to 2.5% v/v) overnight. At several time points samples were taken and analyzed for catalytic activity and protein content.

Culture Media

Preculture: Yeast nitrogen base media (YNBM), per liter: potassium phosphate buffer (pH 6.0), 0.1 M; YNB w/o Amino acids and Ammonia Sulfate (Difco™), 3.4 g; $(NH_4)_2SO_4$, 10 g; biotin, 400 mg; glucose, 20 g.

Batch/fed batch: Basal salt media (BSM) [24], per liter: 85% phosphoric acid, 26.7 mL; $CaSO_4{\cdot}2H_20$, 1.17 g; K_2SO_4, 18.2 g; $MgSO_4{\cdot}7H_2O$, 14.9 g; KOH, 4.13 g; $C_6H_{12}O_6{\cdot}H_2O$, 44 g, Antifoam Struktol J650, 0.2 mL; PTM1, 4.35 mL; NH_4OH as N-source (see experimental procedure). Trace element solution (PTM1), per litre: $CuSO_4{\cdot}5H_2O$, 6.0 g; NaI 0.08 g; $MnSO_4{\cdot}H_2O$, 3.0 g; $Na_2MoO_4{\cdot}2H_2O$, 0.2 g; H_3BO_3, 0.02 g; $CoCl_2$, 0.5 g; $ZnCl_2$, 20.0 g; $FeSO_4{\cdot}7H_2O$, 65.0 g; biotin, 0.2 g, H_2SO_4, 5 mL.

Feed glucose, per liter: glucose, 250 g; PTM1, 12 mL, Struktol J650, 0.3 mL.

Feed methanol, per liter: methanol, 300 g; PTM1, 4 mL; Struktol J650, 0.3 mL, induction period was carried out in presence of δ-Aminolevulinic acid (δ-ALA), 1 mM.

Base: NH_4OH, concentration was determined by titration with 0.25 M potassium hydrogen phthalate (KHP).

Experimental Procedure

Preculture

Frozen stocks (-80°C) were pre-cultivated in 100 mL of YNBM in 1000 mL shake flasks at 28°C and 200 rpm for max. 24 hours. Then, the preculture was transferred aseptically to the respective culture vessel. The inoculation volume was approximately 10% of the final starting volume.

Batch cultivation and determination of q_s

Batch cultivations were carried out in a 1 L working volume glass bioreactor (Applikon, Netherlands). Basal salt media was sterilized in the bioreactor and pH was adjusted to pH 5.0 by using concentrated ammonia solution after autoclaving. Sterile filtered trace elements were transferred to the reactor aseptically. Dissolved oxygen (dO_2) was measured with a sterilizable polarographic dissolved oxygen electrode (Mettler Toledo, Switzerland). The pH was measured with a sterilizable electrode (Mettler Toledo, Switzerland) and maintained constant with a step controller using ammonia solution (1 to 2 M). Base consumption was determined gravimetrically. Cultivation temperature was set to 28°C and agitation was fixed to 1200 rpm. The culture was aerated with 1.25 vvm dried air and off-gas of the culture was measured by using an infrared cell for CO_2 and a paramagnetic cell for O_2 concentration (Servomax, Switzerland). Temperature, pH, dO_2, agitation as well as CO_2 and O_2 in the off-gas were measured online and logged in a process information management system (PIMS; Lucullus, Biospectra, Switzerland).

After the complete consumption of the substrate glucose, which was indicated by an increase of dissolved oxygen and a drop in off-gas activity, the first methanol pulse of a final concentration of 0.5% (v/v) was conducted with pure methanol (supplemented with PTM1, 12 mL/L of methanol). Following pulses were performed with 1% (v/v), before a last pulse with 2% (v/v) final concentration of methanol was carried out. To investigate the metabolic activity during methanol excess and also the dynamic behavior of the cell metabolism after methanol limitation for several hours, the pulse experiments were performed like this: after methanol was depleted after the "first" pulse (followed by off-gas analysis), an immediate "second" methanol pulse with the same concentration was conducted per day. After methanol depletion, methanol starvation was carried out for several hours before another so-called "first" pulse was applied (Figure 2).

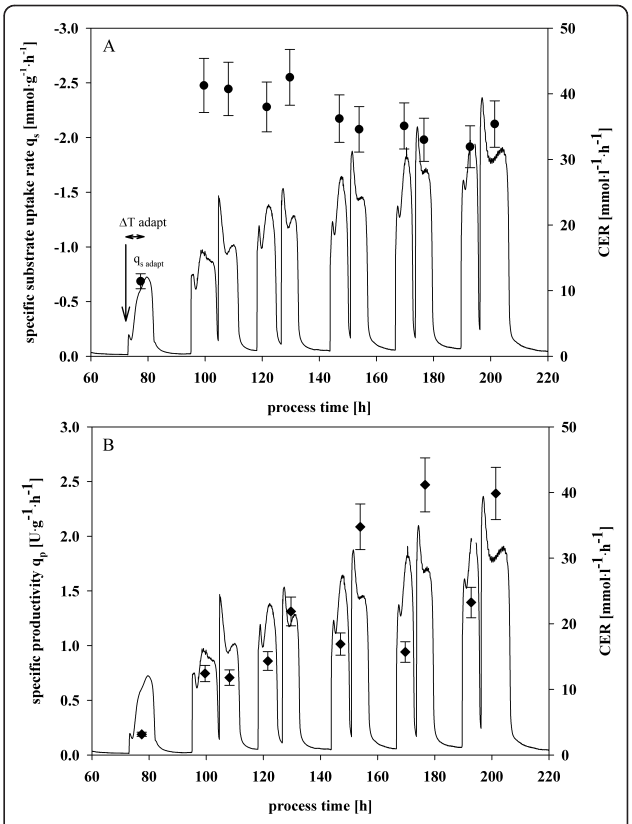


Figure 2 Batch cultivation with methanol pulses of 0.5, 1 and 2% (v/v). A, calculated specific substrate uptake rate q_s (circle) and carbon dioxide evolution rate (CER); B, specific production rate q_p (diamond) and carbon dioxide evolution rate (CER).

For each pulse, at least two samples were taken to determine the concentrations of substrate and product as well as dry cell weight and OD_{600} to calculate the specific substrate uptake rate q_s.

Fed batch cultivations

Fed batch cultures were carried out in a 7.5 L (5 L working volume) glass bioreactor (Infors, Switzerland). Concentrated BSM medium (2-fold concentrated to supply necessary salts for high cell densities) was sterilized in the bioreactor and pH was adjusted to pH 5.0 by using concentrated ammonia solution after autoclaving. The initial volume was set to 1.5 L. Trace elements were filter sterilized and transferred to the reactor aseptically. Dissolved oxygen was measured with a sterilizable polarographic dissolved oxygen electrode (Hamilton, Switzerland). The pH was measured with a sterilizable electrode (Hamilton, Switzerland) and maintained constant using ammonia solution (3 to 5 M). Agitation was set to 1500 rpm. The culture was aerated with at least 1 vvm to avoid oxygen limitation. The dissolved oxygen signal was used to adjust air-in flow manually to keep levels >30% dO_2 at all time points. In case air flow was limited, pure oxygen was added. CO_2

and O_2 were measured as described above. Base consumption and reactor weight were measured gravimetrically. The fed batch feed was measured and controlled using a gravimetrically based PID flow controller.

At several time points during fed batch cultivations, samples were taken and analyzed for accumulated methanol, biomass concentration (dry cell weight and optical density OD_{600}), protein content and enzymatic activity. Based on the total biomass content, feed rates were adjusted manually corresponding to the defined q_s setpoint.

Three different fed batch strategies were tested: fed batch A, where methanol was adjusted to a constant flow during the whole induction phase; fed batch B, where after the adaption time with a $q_{s\ adapt}$, a shift to a high substrate uptake rate of ~90% of $q_{s\ max}$ was done and adjustments to the very same q_s set point during cultivation were performed repeatedly; and fed batches C1 and C2, where after the adaptation period with $q_{s\ adapt}$, the methanol flow was stepwise increased up to $q_{s\ max}$. Values for $q_{s\ adapt}$ and $q_{s\ max}$ had been determined in batch experiments (*vide supra*). An overview of the fed batch cultivations and the corresponding settings is given in Table 1.

Analysis of growth- and expression-parameters

Dry cell weight (DCW) was determined by centrifugation of 5 mL culture broth (5000 rpm, 4°C, 10 min) in a laboratory centrifuge (Sigma 4K15, rotor 11156), washing the pellet with 5 mL deionized water and subsequent drying at 105°C to a constant weight in an oven. Optical density of the culture broth was measured using a spectrophotometer (U-1100 Hitachi, Japan) at a wavelength of 600 nm (OD_{600}). Correlation between dry cell weight measurement and OD_{600} showed a coefficient of regression of R^2 = 0.997 over the full concentration range (data not shown) and could therefore be used for q_s adaptation.

The activity of HRP was determined using a CuBiAn XC enzymatic robot (Innovatis, Germany). Cell free samples (10 μl) were added to 140 μl of 1 mM ABTS (2.2' azino bis 3-ethylbenzthiazoline-6-sulphonic acid) prepared in 50 mM NaOAc buffer (pH 4.5). The reaction mixture was incubated at 37°C and was started by the addition of 20 μl of 0.075% H_2O_2. Changes of absorbance at 415 nm were measured for 80 seconds and rates were calculated. Calibration was done using commercially available horseradish peroxidase (Type VI-A, Sigma-Aldrich, P6782, Lot# 118K76703) as standard at six different concentrations (0.02; 0.05; 0.1; 0.25; 0.5 and 1.0 U/mL). Samples with high enzymatic activity were automatically diluted by the system. Protein concentrations were determined at 595 nm by the Bradford assay [25] using the BioRad Protein Assay Kit with BSA as standard.

Substrate concentrations

Concentrations of methanol were determined in cell free samples by HPLC (Agilent Technologies, USA) equipped with a Supelcoguard column, a Supelcogel C-610 H ion-exchange column (Sigma-Aldrich, USA) and a refractive index detector (Agilent Technologies, USA). The mobile phase was 0.1% H_3PO_4 with a constant flow rate of 0.5 mL/min and the system was run isocratic. Calibration was done by measuring standard points in the range of 0.1 to 10 g/L methanol.

Concentrations of glucose were determined in cell free samples by a commercial enzymatic assay kit using the CuBiAN XC enzymatic robot (Innovatis, Germany). Calibration was done with 4 standard points in the range from 0 to 3 g/L glucose. Samples with higher glucose concentration were diluted automatically by the system.

Data analysis

Measurements of biomass concentration, product concentration and substrate concentration were executed in duplicates: along the observed standard deviation for the single measurement, the error was propagated to the specific rates q_s and q_p as well as to the yield coefficients. The error of determination of the specific rates and the yields was therefore set to 10% and 5%, respectively.

Electrophoresis

To check the purity of the excreted HRP, electrophoresis was done with aliquots of supernatants obtained at different time points during the cultivation of *P. pastoris*

Table 1 Description of feeding strategies for fed batch cultivations based on the specific substrate uptake rate q_s

Fed batch name	Symbol	Description of the strategy
Fed batch A	▲	conventional feeding strategy: long adaptation time and initially adjusted, constant flow rate
Fed batch B	◆	short adaptation time (out of batch exp.) and adjustment to high uptake rate (90% of $q_{s\ max}$) with repeated readjustments
Fed batch C1	▼	short adaptation time (out of batch exp.) and stepwise adjustment of q_s until $q_{s\ max}$ with repeated readjustments
Fed batch C2	●	

expressing the *hrp* gene extracellularly as described by Laemmli *et al.* [26]. SDS-PAGE was performed using a 5% stacking gel and a 10% separating gel in 1× Tris-glycine buffer. Gels were run in the vertical electrophoresis Mini-PROTEAN Tetra Cell apparatus (Biorad; Vienna, Austria) at 150 V for about 2 h. Gels were stained with Coomassie blue. The protein mass standard used was the PageRuler Prestained Ladder (Fermentas; Vienna, Austria).

Results and Discussion

Stability of the enzyme horseradish peroxidase in the presence of methanol

To check whether the enzyme horseradish peroxidase denatures at certain concentrations of methanol, HRP was incubated in the presence of methanol concentrations of up to 2.5% (v/v) at 28°C overnight. No loss in catalytic activity and protein content was detected after this incubation, which is why methanol pulses in batch experiments with concentrations of up to 2.5% (v/v) were theoretically possible without any denaturing effects on HRP.

Determination of strain specific parameters by easy to do batch cultivations with methanol pulses

After depletion of glucose in batch cultivations, a first methanol adaption pulse with a final concentration of 0.5% (v/v) was applied. The adaptation time to the new substrate methanol ($\Delta time_{adapt}$) was determined with 7 hours and was defined by the detection of a maximum in off-gas activity (Figure 2A).

The calculated carbon dioxide evolution rate (CER), signifying metabolic activity, allowed to distinguish different states within the methanol pulses with local minima and maxima (Figure 2). This metabolic behavior of the cells results from inhibition and regulation events in transient conditions, caused by intracellular components like e.g. produced H_2O_2, and transport actions, and has been described for various systems in literature before [27-29].

The high frequent determination of biomass, methanol and product concentrations allowed specific rate calculations for methanol uptake q_s and productivity q_p during the methanol pulses. Specific substrate uptake rates were calculated with 0.8 ± 0.08 $mmol \cdot g^{-1} \cdot h^{-1}$ in the adaptation period ($q_{s\ adapt}$) and with around 2 ± 0.20 $mmol \cdot g^{-1} \cdot h^{-1}$ as a maximum during pulses ($q_{s\ max}$). No difference in the calculated q_s between the "first" and the subsequent "second" pulse on a respective day was observed (Figure 2A). In contrast, the calculated values for q_p (Figure 2B) were very different between the first and the subsequent second pulse. During the first pulse specific productivities of maximum 1.3 ± 0.13 $U \cdot g^{-1} \cdot h^{-1}$ were observed, whereas during the second pulse an increased specific productivity of up to 2.5 ± 0.25 $U \cdot g^{-1} \cdot h^{-1}$ was measured. This increased productivity during the second methanol pulse may be due to the fact that no adaption of the cells to methanol was necessary because all metabolic key functions for methanol assimilation remained in an active state. Thus, recombinant protein expression could start directly and energy was used more efficiently for product formation. This result shows clearly that *Pichia* cultures should be kept induced at all time to obtain maximal productivity. Interestingly, q_p also increased over time for first and second pulses, respectively (Figure 2B). Methanol pulses, where the same final concentration of methanol was used, but which were conducted in the later phase of the batch cultivation, showed higher specific productivities compared to pulses before. Obviously, the culture exhibited a "memory effect" and thus a time-dependence of q_p could be observed in the batch experiment.

As we show here, batch cultivations with methanol pulses allowed a fast identification of strain specific parameters, which are crucial for subsequent fed batch cultivations. The determined maximum specific substrate uptake rate $q_{s\ max}$, represents the upper end of the feed profile respective to q_s. This novel method has the advantage of being less time-consuming and labor-intensive compared to the traditional methods, like continuous cultivations, and additionally allows a free choice of substrate, like e.g. the 2 to 3-fold cheaper glucose instead of glycerol.

Fed batch feeding strategy based on q_s

Based on the batch results, we performed several fed batch cultivations with different feeding profiles based on the specific substrate uptake rate q_s, to find a feeding strategy for a recombinant *P. pastoris* strain.

Feeding profiles

After a batch phase on glucose as substrate (volume 1.5 L), an exponential fed batch cultivation with glucose yielded in biomass concentrations of up to 70 g/L in a volume of 2.5 L. At the end of this fed batch phase, a sample was taken to determine the current biomass concentration by measuring the OD_{600} and the DCW.

Based on the calculated specific methanol uptake rate during adaptation ($q_{s\ adapt}$) from the batch experiment, which was around 0.8 ± 0.08 $mmol \cdot g^{-1} \cdot h^{-1}$, we used a little bit lower $q_{s\ adapt}$ of 0.5 $mmol \cdot g^{-1} \cdot h^{-1}$ methanol for the fed batch cultivations during the adaption time. After $\Delta time_{adapt}$, three different feeding strategies (fed batches A, B and C1/C2) were tested and compared (Figure 3 Table 1).

For fed batch A, which described a conventional feeding strategy as in the Invitrogen protocol, the methanol feed was adjusted to a substrate uptake rate of 1.0 $mmol \cdot g^{-1} \cdot h^{-1}$. The adjustment just happened at the

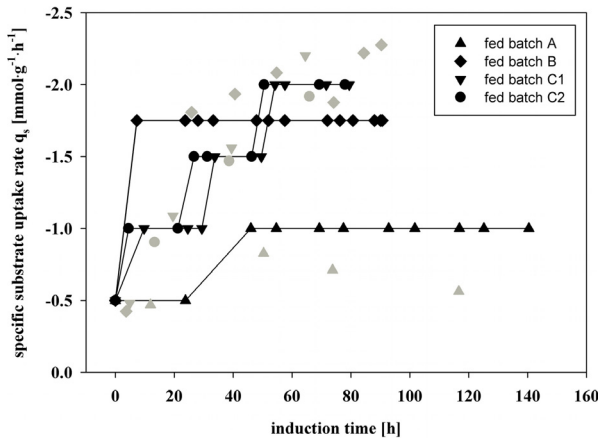


Figure 3 Specific substrate uptake rate profiles which were used for different fed batch cultivations. Theoretical q_s fed batch A (black triangle up), offline determined q_s fed batch A (grey triangle up); theoretical q_s fed batch B (black diamond), offline determined q_s fed batch B (grey diamond); theoretical q_s fed batch C1 (black triangle down), offline determined q_s fed batch C1 (grey triangle down); theoretical q_s fed batch C2 (black circle), offline determined q_s fed batch C2 (grey circle). The error of determination for offline q_s (10%) is not shown for better readability.

beginning and no further readjustments of the feeding rate were performed during the cultivation. Consequently, as biomass still increased, a drop down of q_s over time was observed (Figure 3).

In the other fed batch experiments B, C1 and C2 adjustments of q_s were done after each sampling, based on OD_{600} measurements. As shown in Figure 3 the effective q_s, determined by off-line sampling, and the designated q_s in these fed batch cultivations were very similar. Thus, a frequent determination of the actual biomass concentration in the reactor vessel is necessary, to be able to adjust the feeding rate to the chosen q_s. The development of robust measurement devices for the online determination of the biomass concentration, as e. g. FTIR, capacity probes or soft sensors, could allow an online adjustment of the feeding rate to q_s by an automatic control system in the future, and thus the labor-intensive, frequent sampling procedure would be unnecessary.

Maintenance metabolism vs. cell growth

The relationship between q_s and the specific growth rate was tested and found to be linearly correlated (data not shown). Based on this, the maintenance coefficient for this *P. pastoris* strain could be determined with around 0.5 mmol·g^{-1}·h^{-1} (which equals 0.016 g·g^{-1}·h^{-1}). Very similar results were reported by Jahic *et al.* for fed batch cultures using *P. pastoris* expressing a fusion protein [5] with a maintenance coefficient of 0.013 g·g^{-1}·h^{-1}. When we adjusted q_s to values higher than 1.0 mmol·g^{-1}·h^{-1}, we observed a significant increase in $Y_{X/S}$ and a corresponding decrease in $Y_{CO2/S}$ (Figure 4). That means that at q_s of <1.0 mmol·g^{-1}·h^{-1} most of the energy is obviously used for maintenance metabolism of the cells and thus for the production of CO_2 and not for cell growth or protein production. Interestingly, the same effect on biomass yield was observed by d'Anjou *et al.* using a continuous culture system with a mixed feed strategy [13]. However, we were able to extract this information from time-efficient, dynamic experiments equivalently. Consequently, we employed specific substrate uptake rates of >1.0 mmol·g^{-1}·h^{-1} to guarantee a sufficient amount of energy for both, maintenance and cell growth.

Required methanol concentration for induction - a principle question

When producing recombinant proteins with *P. pastoris*, the principle question is how much methanol has to be fed to guarantee a fully induced AOX promoter. A lot of different studies have examined this topic, with different results. D'Anjou *et al.*, for example, reported that a methanol concentration between 1 and 2 g/L should be maintained in the culture to guarantee fully induced heterologous protein production [13]. Another study of the methanol concentration and its effect on *P. pastoris* Mut^S strains was done by Kupcsulik and Sevella, who showed that the specific productivity of a recombinant human serum albumin expression system showed a maximum at 0.45 g/L of methanol [30]. In contrast to those studies, Cregg reported an even stronger induction of the AOX promoter in limited conditions [31], which

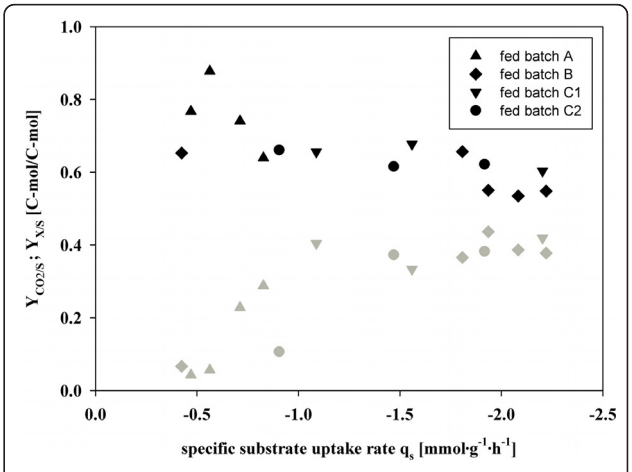


Figure 4 Carbon dioxide yield ($Y_{CO2/S}$) and biomass yield ($Y_{X/S}$) at different specific substrate uptake rates in fed batch cultivations. $Y_{CO2/S}$ fed batch A (black triangle up), $Y_{X/S}$ fed batch A (grey triangle up); $Y_{CO2/S}$ fed batch B (black diamond), $Y_{X/S}$ fed batch B (grey diamond); $Y_{CO2/S}$ fed batch C1 (black triangle down), $Y_{X/S}$ fed batch C1 (grey triangle down); $Y_{CO2/S}$ fed batch C2 (black circle); $Y_{X/S}$ fed batch C2 (grey circle). The error of determined yields (5%) is not shown for better readability.

was the basis for several following studies applying a limited methanol supply for induction [1-3].

For all described fed batch strategies in our study, no significant methanol accumulation was detected (detection limit 0.1 g/L). We clearly show that high residual concentrations of methanol during the production phase are not required for the induction of the *P. pastoris* Mut^S strain, as even higher specific productivities were obtained in limited fed batches with constant substrate uptake rates, compared to the batch cultivations with high methanol concentrations applied in the pulse-experiments (Table 2).

Extracellular protein production and specific productivity q_p

To follow the formation of excreted horseradish peroxidase (HRP) during the induction phase, SDS-PAGE analyses with cell-free supernatants were performed. SDS-PAGE analysis (Coomassie staining, Figure 5) of the cultivation broth taken at different time points did not show a distinct band for HRP, but rather a smear between 60 - 65 kDa. The difference between the reported molecular weight of around 44 kDa for HRP and the observed molecular weight in this study results from the high degree of glycosylation of the recombinant protein expressed in *P. pastoris* with mannose-type oligosaccharides, as described previously for HRP by Morawski *et al.* [32].

The protein content in the cultivation broth increased over time from 0.052 mg/mL after the fed-batch phase with glucose to 0.243 mg/mL at the end of the cultivation, which were around 91 hours of induction. As shown in Figure 5 the increase of the protein content in the cultivation broth is ascribed to an increased amount of HRP, which constituted the majority of secreted proteins in the cultivation broth.

Based on the determined biomass content and the enzymatic activity, the specific productivity q_p during the different fed batch cultivations was calculated. The lowest q_p was obtained in fed batch A, which described a conventional feeding strategy, where also the lowest q_s was used and was not adapted over time (Figure 6). In fed batch B, which considered the actual biomass concentration and where the q_s of 1.75 $mmol \cdot g^{-1} \cdot h^{-1}$ was adapted regularly over time and thus the cells were kept in a certain physiological state, a 2-fold increase in q_p was observed. Clearly, a higher q_s resulted in a higher q_p, which disagrees with the results obtained by Khatri and Hoffmann and Cunha *et al.* [10,11], who stated that lower q_s resulted in higher protein production.

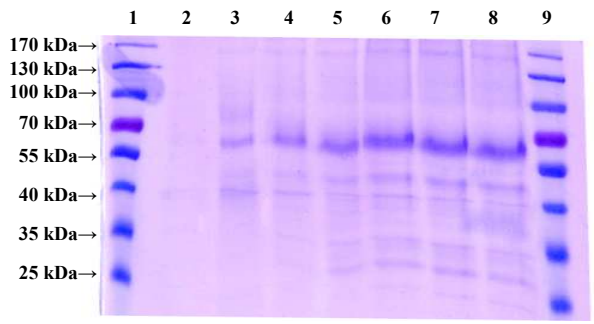


Figure 5 SDS-PAGE analysis of recombinantly produced HRP in fed batch B. Aliquots of the supernatant obtained at different time points during the cultivation of *P. pastoris* expressing the *hrp* gene extracellularly at 28°C were loaded onto the gel. lanes 1 and 9, molecular mass standard; lane 3, not induced (after fed-batch phase with glucose); lane 3-8, 7 h; 33 h; 58 h; 76 h; 88 h; 91 h of induction.

However, a dynamic, stepwise feeding strategy resulted in an even higher productivity compared to the other strategies tested (Figure 6). This dynamic feeding strategy considered $q_{s\ max}$, which had been determined in the batch experiment, as the highest possible substrate uptake rate of the cells, as well as the yield coefficients and the maintenance coefficient as the lower end of efficient energy usage. The feed profile was set up in a way to head off as quickly as possible from the maintenance state of the cells (hence q_s was set to values ≥1.0 $mmol \cdot g^{-1} \cdot h^{-1}$), before q_s was dynamically adapted in steps to $q_{s\ max}$. In fed batch cultivations C1 and C2, where this dynamic feeding strategy was applied, a q_p of around 11 ± 0.11 $U \cdot g^{-1} \cdot h^{-1}$ was determined, which represents a 5.5-fold increase compared to fed batch A.

Time adaptation

The fact that there was still a more than 2-fold difference in q_p between fed batches C1/C2, where q_s was increased stepwise, and fed batch B, where immediately after the adaption period a high q_s of 1.75 $mmol \cdot g^{-1} \cdot h^{-1}$ was applied, indicated another factor being crucial for

Table 2 Comparison of the specific substrate uptake rates q_s and determined maximum specific productivities q_p

Experiment[a]	Methanol concentration	Specific substrate uptake rate q_s [$mmol \cdot g^{-1} \cdot h^{-1}$]	Specific productivity q_p [$U \cdot g^{-1} \cdot h^{-1}$]
Batch with methanol pulses	from 0 to 16 g/L	~ 2	2.5 ± 0.25
Fed batch A ▲	limited	< 1	2.0 ± 0.20
Fed batch B ◆	limited	1.75	5.0 ± 0.50
Fed batch C1 ▼	limited	stepwise up to 2	10 ± 0.10
Fed batch C2 ●	limited	stepwise up to 2	11 ± 0.11

[a]all experiments were performed in the presence of different concentrations of the inducer methanol.

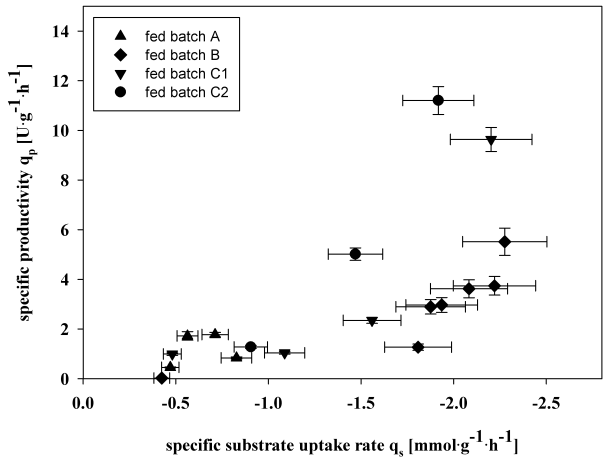


Figure 6 Specific productivity (q_p) plotted against the specific substrate uptake rate (q_s). Fed batch A (triangle up); fed batch B (diamond); fed batch C1 (triangle down); fed batch C2 (circle).

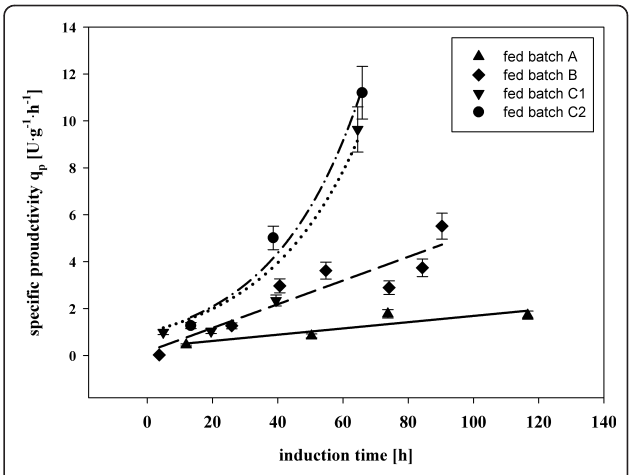


Figure 7 Specific productivity (q_p) in fed batch cultures over the induction time. Fed batch A (triangle up); fed batch B (diamond); fed batch C1 (triangle down); fed batch C2 (circle). Regression lines: linear regression for fed batch A (continuous line); linear regression for fed batch B (discontinuous line); exponential regression for fed batch C1 (dotted line); exponential regression for fed batch C2 (dashed-dotted line).

the specific productivity besides q_s. Therefore, the specific productivity q_p was plotted against the induction time, to analyze possible time-dependent effects (Figure 7). Fed batch A and B were characterized by a linear increase of q_p over time (fed batch B was characterized by a significant steeper slope), whereas fed batches C1 and C2 even showed an exponential increase of the specific productivity. It became obvious that the productivity was directly correlated to the induction time for all experiments conducted in this study, even in the batch experiments (Figure 2). Plantz *et al*. have recently reported an influence of the induction time on q_p as well. They showed increasing product yields for the recombinant production of an interferon with *P. pastoris* in the first period of induction, but a subsequent decrease in product formation and a shift of the energy transfer towards biomass growth during the later phase of the cultivation [8]. In contrast to the study of Plantz *et al*., we clearly showed an increase of q_p over the whole induction time for all experiments in this study (Figure 7).

Furthermore, fed batches C1 and C2 were characterized by an exponential increase of q_p over time, in contrast to a linear increase for fed batches A and B (Figure 7). Obviously, a feeding strategy starting with a lower q_s at the beginning of the induction phase, followed by a subsequent increase with a stepwise, dynamic feeding profile until $q_{s\ max}$ (fed batch C1 and C2), was superior to a feeding strategy, where immediately after the $\Delta time_{adapt}$ a high q_s was applied and kept constant (fed batch B). When the cells had the time to undergo a dynamic, physiological adaptation to the current culture condition before q_s was increased, a boost in q_p was observed, which probably cannot be triggered by constant or exponential feeding regimes (Table 2).

Summarizing, we showed a clear time dependent trajectory for specific product formation, as q_p increased over induction time for all experiments in this study. A tool for the early identification of this effect is of utmost importance in order to consistently compare different feeding profiles.

Conclusions

In this study, we developed a fast and easy to do method based on batch cultivations with methanol pulses to determine strain specific parameters of a *P. pastoris* Mut^S strain expressing the enzyme horseradish peroxidase. A subsequently developed dynamic feeding strategy solely based on q_s, where the cells on the one hand had time to adapt to culture conditions, but were then challenged again repeatedly by a stepwise increase of q_s up to $q_{s\ max}$, resulted in the highest q_p compared to the other strategies tested. Hence, dynamic feeding profiles turned out to be a valuable method to boost the specific productivity. This calls for increased use of dynamic process conditions even for industrial feed profiles. We strongly believe that the strategy presented here can be successfully applied on other microbial expression systems, which is why we are currently testing the applicability of our novel, dynamic approach on other expression systems, like *E. coli*, and other products.

Abbreviations

$\Delta time_{adapt}$: time for adaptation of the culture to the new substrate (methanol) [h]; μ specific growth rate [h^{-1}]; CER: carbon dioxide evolution

rate [mmol·L^{-1}·h^{-1}]; HRP: horseradish peroxidase; Mut^{S}: methanol utilization slow phenotype; PID: proportional-integrative-derivative controller; q_p: specific productivity of horseradish peroxidase [U·g^{-1}·h^{-1}]; q_s: specific substrate uptake rate [mmol·g^{-1}·h^{-1}]; $q_{s\ adapt}$: specific substrate uptake rate during adaptation [mmol·g^{-1}·h^{-1}]; $q_{s\ max}$: maximum specific substrate uptake rate [mmol·g^{-1}·h^{-1}]; rpm: rounds per minute; vvm: volume gas flow per volume liquid per minute; $Y_{CO2/S}$: yield coefficient of carbon dioxide respective to methanol [C-mol·C-mol^{-1}]; $Y_{X/S}$: yield coefficient of biomass respective to methanol [C-mol·C-mol^{-1}].

Acknowledgements
The authors are very grateful to Prof. Anton Glieder and his workgroup for providing the *P. pastoris* Mut^{S} strain expressing the enzyme horseradish peroxidase. Additionally, the authors express their gratitude to D. V. Rial (Consejo Nacional de Investigaciones Científicas y Técnicas CONICET, Argentina), who supported the experimental work in the lab.

Authors' contributions
CD designed and performed the experiments and analyzed data. OS performed some experiments. CD and OS wrote the paper, CH conceived the study and supervised research. All authors read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

Received: 21 December 2010 Accepted: 3 March 2011
Published: 3 March 2011

References

1. Potgieter TI, Kersey SD, Mallem MR, Nylen AC, d'Anjou M: **Antibody expression kinetics in glycoengineered Pichia pastoris.** *Biotechnology and Bioengineering* 2010, **106**:918-927.
2. Trinh LB, Phue JN, Shiloach J: **Effect of methanol feeding strategies on production and yield of recombinant mouse endostatin from Pichia pastoris.** *Biotechnology and Bioengineering* 2003, **82**:438-444.
3. Kobayashi K, Kuwae S, Ohya T, Ohda T, Ohyama M, Tomomitsu K: **High level secretion of recombinant human serum albumin by fed-batch fermentation of the methylotrophic yeast, Pichia pastoris, based on optimal methanol feeding strategy.** *Journal of Bioscience and Bioengineering* 2000, **90**:280-288.
4. Jacobs P, Inan M, Festjens N, Haustraete J, Van Hecke A, Meagher M, Contreras R, Callewaert N: **Fed-batch fermentation of GM-CSF-producing glycoengineered Pichia pastoris under controlled specific growth rate.** *Microbial Cell Factories* 2010, **9**:93.
5. Jahic M, Rotticci-Mulder JC, Martinelle M, Hult K, Enfors SO: **Modeling of growth and energy metabolism of Pichia pastoris producing a fusion protein.** *Bioprocess and Biosystems Engineering* 2002, **24**:385-393.
6. Zhang W, Sinha J, Smith LA, Inan M, Meagher MM: **Maximization of Production of Secreted Recombinant Proteins in Pichia pastoris Fed-Batch Fermentation.** *Biotechnology Progress* 2005, **21**:386-393.
7. Sinha J, Plantz BA, Zhang W, Gouthro M, Schlegel V, Liu C-P, Meagher MM: **Improved production of recombinant ovine interferon-t by Mut+ strain of Pichia pastoris using an optimized methanol feed profile.** *Biotechnology Progress* 2003, **19**:794-802.
8. Plantz BA, Sinha J, Villarete L, Nickerson KW, Schlegel VL: **Pichia pastoris fermentation optimization: energy state and testing a growth-associated model.** *Applied Microbiology and Biotechnology* 2006, **72**:297-305.
9. Ohya T, Ohyama M, Kobayashi K: **Optimization of human serum albumin production in methylotrophic yeast Pichia pastoris by repeated fed-batch fermentation.** *Biotechnology and Bioengineering* 2005, **90**:876-887.
10. Khatri NK, Hoffmann F: **Oxygen-limited control of methanol uptake for improved production of a single-chain antibody fragment with recombinant Pichia pastoris.** *Applied Microbiology and Biotechnology* 2006, **72**:492-498.
11. Khatri NK, Hoffmann F: **Impact of methanol concentration on secreted protein production in oxygen-limited cultures of recombinant Pichia pastoris.** *Biotechnology and Bioengineering* 2006, **93**:871-879.
12. Cunha AE, Clemente JJ, Gomes R, Pinto F, Thomaz M, Miranda S, Pinto R, Moosmayer D, Donner P, Carrondo MJT: **Methanol induction optimization for scFv antibody fragment production in Pichia pastoris.** *Biotechnology and bioengineering* 2004, **86**:458-467.
13. d'Anjou MC, Daugulis AJ: **A rational approach to improving productivity in recombinant Pichia pastoris fermentation.** *Biotechnology and bioengineering* 2001, **72**:1-11.
14. Ren H, Yuan J: **Model-based specific growth rate control for Pichia pastoris to improve recombinant protein production.** *Journal of Chemical Technology and Biotechnology* 2005, **80**:1268-1272.
15. Ren HT, Yuan JQ, Bellgardt KH: **Macrokinetic model for methylotrophic Pichia pastoris based on stoichiometric balance.** *Journal of Biotechnology* 2003, **106**:53-68.
16. Welinder KG: **Amino acid sequence studies of horseradish peroxidase. 4. Amino and carboxyl termini, cyanogen bromide and tryptic fragments, the complete sequence, and some structural characteristics of horseradish peroxidase C.** *European Journal of Biochemistry* 1979, **96**:483-502.
17. Gajhede M: **Horseradish peroxidase.** *Handbook of Metalloproteins* 2001, **1**:195-210.
18. Rodriguez-Lopez JN, Lowe DJ, Hernandez-Ruiz J, Hiner ANP, Garcia-Canovas F, Thorneley RNF: **Mechanism of reaction of hydrogen peroxide with horseradish peroxidase: Identification of intermediates in the catalytic cycle.** *Journal of the American Chemical Society* 2001, **123**:11838-11847.
19. Veitch NC: **Horseradish peroxidase: a modern view of a classic enzyme.** *Phytochemistry (Elsevier)* 2004, **65**:249-259.
20. Lai Y-C, Lin S-C: **Application of immobilized horseradish peroxidase for the removal of p-chlorophenol from aqueous solution.** *Process Biochemistry (Oxford, United Kingdom)* 2004, **40**:1167-1174.
21. Dalal S, Gupta MN: **Treatment of phenolic wastewater by horseradish peroxidase immobilized by bioaffinity layering.** *Chemosphere* 2007, **67**:741-747.
22. Azevedo AM, Martins VC, Prazeres DMF, Vojinovic V, Cabral JMS, Fonseca LP: **Horseradish peroxidase: a valuable tool in biotechnology.** *Biotechnology Annual Review* 2003, **9**:199-247.
23. Hamid M, Khalil ur R: **Potential applications of peroxidases.** *Food Chemistry* 2009, **115**:1177-1186.
24. Cregg JM, Editor: *Pichia Protocols, Second Edition. [In: Methods Mol. Biol. (Totowa, NJ, U. S.)], 2007; 389]* 2007.
25. Bradford MM: **A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding.** *Analytical Biochemistry* 1976, **72**:248-254.
26. Laemmli UK: **Cleavage of structural proteins during the assembly of the head of bacteriophage T4.** *Nature (London, United Kingdom)* 1970, **227**:680-685.
27. Herwig C, von Stockar U: **A small metabolic flux model to identify transient metabolic regulations in Saccharomyces cerevisiae.** *Bioprocess and Biosystems Engineering* 2002, **24**:395-403.
28. Diderich JA, Schepper M, van Hoek P, Luttik MA, van Dijken JP, Pronk JT, Klaassen P, Boelens HF, de Mattos MJ, van Dam K, Kruckeberg AL: **Glucose uptake kinetics and transcription of HXT genes in chemostat cultures of Saccharomyces cerevisiae.** *The Journal of biological chemistry* 1999, **274**:15350-15359.
29. Rizzi M, Theobald U, Querfurth E, Rohrhirsch T, Baltes M, Reuss M: **In vivo investigations of glucose transport in Saccharomyces cerevisiae.** *Biotechnology and Bioengineering* 1996, **49**:316-327.
30. Kupcsulik B, Sevella B: **Effects of methanol concentration on the recombinant Pichia pastoris MutS fermentation.** *Periodica Polytechnica, Chemical Engineering* 2004, **48**:73-87.
31. Cregg JM: **Expression in the methylotrophic yeast Pichia pastoris.** *Gene Expression Systems* 1999, 157-191.
32. Morawski B, Lin ZL, Cirino PC, Joo H, Bandara G, Arnold FH: **Functional expression of horseradish peroxidase in Saccharomyces cerevisiae and Pichia pastoris.** *Protein Engineering* 2000, **13**:377-384.

doi:10.1186/1475-2859-10-14
Cite this article as: Dietzsch *et al.*: A dynamic method based on the specific substrate uptake rate to set up a feeding strategy for *Pichia pastoris*. *Microbial Cell Factories* 2011 **10**:14.

MICROBIAL CELL FACTORIES

RESEARCH Open Access

A fast approach to determine a fed batch feeding profile for recombinant *Pichia pastoris* strains

Christian Dietzsch, Oliver Spadiut and Christoph Herwig*

Abstract

Background: The microorganism *Pichia pastoris* is a commonly used microbial host for the expression of recombinant proteins in biotechnology and biopharmaceutical industry. To speed up process development, a fast methodology to determine strain characteristic parameters, which are needed to subsequently set up fed batch feeding profiles, is required.

Results: Here, we show the general applicability of a novel approach to quantify a certain minimal set of bioprocess-relevant parameters, *i.e.* the adaptation time of the culture to methanol, the specific substrate uptake rate during the adaptation phase and the maximum specific substrate uptake rate, based on fast and easy-to-do batch cultivations with repeated methanol pulses in a batch culture. A detailed analysis of the adaptation of different *P. pastoris* strains to methanol was conducted and revealed that each strain showed very different characteristics during adaptation, illustrating the need of individual screenings for an optimal parameter definition during this phase. Based on the results obtained in batch cultivations, dynamic feeding profiles based on the specific substrate uptake rate were employed for different *P. pastoris* strains. In these experiments the maximum specific substrate uptake rate, which had been defined in batch experiments, also represented the upper limit of methanol uptake, underlining the validity of the determined process-relevant parameters and the overall experimental strategy.

Conclusion: In this study, we show that a fast approach to determine a minimal set of strain characteristic parameters based on easy-to-do batch cultivations with methanol pulses is generally applicable for different *P. pastoris* strains and that dynamic fed batch strategies can be designed on the specific substrate uptake rate without running the risk of methanol accumulation.

Keywords: *Pichia pastoris*, strain characterization, specific substrate uptake rate, batch cultivation, methanol pulse, dynamic feeding profile

Background

Advances in molecular biology, cloning techniques and strain improvement allowed an increasing use of recombinant organisms for the industrial production of a variety of substances like organic acids, antibiotics, enzymes and amino acids. In this context the methylotrophic yeast *Pichia pastoris* is one of the most important host organisms for the expression of recombinant proteins. To meet industrial demands, a fast and easy-to-do characterization of recombinant *Pichia* strains to extract bioprocess-relevant strain characteristic parameters for the subsequent set-up of production processes is essential to speed up process development. Normally, this strain characterization procedure is done by time-consuming experiments, which require complex and costly equipment, like continuous cultures [1,2] or several, consecutive fed batch cultivations, operated at different conditions [3,4]. Parameters, which have to be extracted out of these experiments, describe the best operating conditions for each strain as well as the optimal condition for the adaptation of the culture from the growth substrate (e.g. glucose or glycerol) to the inducer methanol. To date, different strategies are employed regarding the adaptation of *Pichia* to methanol; two prominent examples are: 1) after a fed batch on glycerol or glucose a certain low concentration or flow of methanol is

* Correspondence: christoph.herwig@tuwien.ac.at
Vienna University of Technology, Institute of Chemical Engineering, Research Area Biochemical Engineering, Vienna, Austria

applied to the culture which is then increased to a predefined maximum and constantly maintained throughout the whole cultivation time [5,6], and 2) the glycerol flow in the reactor is decreased following a linear function during a concomitant addition of methanol, a period which is called transition phase, to slowly adapt the culture to methanol [7-10]. These methods are often based on specific experiences with a certain strain, but are nevertheless often used as a general approach for different *Pichia* strains in following studies, without taking into account the specific requirements of the single strains during adaptation.

After adaptation of the culture to methanol, different feeding strategies can be employed for recombinant protein production with *P. pastoris*. Besides the two common strategies of either using a feed forward regime or a controlled specific growth rate (μ) [6,7,9-14], a few studies have also described the importance of the specific substrate uptake rate (q_s) on recombinant protein production [15,16]. A direct correlation between q_s and the specific productivity (q_p) was shown [15,16], and it was clearly stated, that q_s was the most important induction parameter in these experiments [17]. Based on those findings and motivated by problems which occur, when more traditional feeding strategies are applied (e.g. possible accumulation of methanol caused by changing cell capacities during cultivation or the need of expensive monitoring equipment to allow μ-controlled feeding [9,14]), we have focused our research on the specific substrate uptake rate (q_s) and have recently shown optimization potential using dynamic feeding profiles based on this parameter [18].

In our previous study we also developed a fast approach based on batch experiments with methanol pulses to extract a minimal set of strain characteristic parameters (*i.e.* $\Delta time_{adapt}$ - time for adaptation, $q_{s\ adapt}$ - specific substrate uptake rate during adaptation, $q_{s\ max}$ - maximum specific substrate uptake rate), which are required to set up a subsequent feeding regime based on q_s. However, our previous study dealt with the development and the application of this approach for only one recombinant *P. pastoris* Mut^S strain [18]. In the present work, we characterized various *P. pastoris* strains with different phenotypes (Mut^s and Mut^+) expressing different target enzymes using the above mentioned strategy. We analyzed the required time for adaptation to methanol of each strain in detail and could reliably derive certain strain characteristic parameters from pulse experiments to fed batch cultivations. With the variety of used strains in this study, we demonstrate that this approach is generally applicable for different *P. pastoris* strains and is thus a valuable tool for fast process development, which is especially interesting in an industrial environment.

Materials and methods

The experiments conducted in the present study were performed according to our previous study [18], and are thus only described briefly here.

Microorganisms and recombinant proteins

Different *P. pastoris* strains with different phenotypes expressing different target enzymes were used in this study to prove the general applicability of our strategy. A list of the various strains is given in Table 1.

All recombinant genes used in this study were under the control of the AOX1 promoter. The expressed HRP gene coded for the isoenzyme HRP C1A. The strain KM71H PDI HRP concomitantly expressed HRP and the chaperone protein disulfide isomerase (PDI), which was under the control of a modified AOX1 promoter [19]. The strains KM71H, KM71H HRP, KM71H PDI HRP and CBS7435 HRP were gratefully provided by Prof. Anton Glieder (Graz University of Technology, Austria). The strain SMD1168H GalOX was constructed by Spadiut *et al.*, as described elsewhere [20].

Culture Media

Precultures were performed in complex yeast nitrogen base media (YNBM), whereas batch and fed batch cultivations were done in defined basal salt media (BSM; [21]). The glucose feed was prepared with glucose (250 $g \cdot l^{-1}$), trace element solution PTM1 (12 $ml \cdot l^{-1}$) and antifoam Struktol J650 (0.3 ml/l). The methanol feed was composed of methanol (300 $g \cdot l^{-1}$), PTM1 (4 $ml \cdot l^{-1}$) and Struktol J650 (0.3 $ml \cdot l^{-1}$). The induction period for HRP expression was carried out in the presence of δ-Aminolevulinic acid (δ-ALA) in a final concentration of 1 mM. The concentration of the base NH_4OH was determined by titration with 0.25 M potassium hydrogen phthalate (KHP).

Experimental Procedure

Preculture

Frozen stocks (-80°C) were precultivated in 100 ml of YNBM in 1000 ml shake flasks at 28°C and 230 rpm for max. 24 hours.

Table 1 Different *P. pastoris* strains used in this study

Strain	phenotype	expressed enzyme	in this study designated as
KM71H	Mut^S	-	KM71H
KM71H	Mut^S	HRP	KM71H HRP
KM71H	Mut^S	PDI and HRP	KM71H PDI HRP
CBS7435	Mut^S	HRP	CBS7435 HRP
SMD1168H	Mut^+	GalOX	SMD1168H GalOX

Batch cultivation with methanol pulses

Batch cultivations were carried out in a 5 l working volume glass bioreactor (Infors, Switzerland) at 28°C and a fixed agitation speed of 1200 rpm. The culture was aerated with 1 vvm dried air and off-gas was measured by using an infrared cell for CO_2 and a paramagnetic cell for O_2 concentration (Servomex, Switzerland). Process parameters were recorded and logged in a process information management system (PIMS; Lucullus, Biospectra, Switzerland). After the complete consumption of glucose, which was indicated by an increase of dissolved oxygen and a drop in off-gas activity, the first methanol pulse (adaptation pulse) with a final concentration of 0.5% (v/v) was conducted with pure methanol (supplemented with PTM1, 12 $ml \cdot l^{-1}$ of methanol). Following pulses were performed with 1% (v/v) concentration of methanol. For all strains, several pulses were conducted after the adaptation pulse to generate consistent data for each strain. For each methanol-pulse, at least two samples were taken to determine the concentrations of substrate and product as well as dry cell weight and OD_{600} to calculate the specific substrate uptake rate q_s.

Fed batch cultivations

Fed batch cultivations were carried out in a 5 l working volume glass bioreactor (Infors, Switzerland) in 2-fold concentrated BSM medium at 28°C and 1500 rpm. The culture was aerated with at least 1 vvm to keep dissolved oxygen levels > 30%. In case, air flow was limited, pure oxygen was added. The fed batch feed was measured and controlled using a gravimetrically based PID flow controller. At several time points during fed batch cultivations, samples were taken and analyzed for accumulated methanol, biomass concentration (dry cell weight and optical density OD_{600}) and, if applicable, enzymatic activity. Based on the total biomass content, feeding rates were adjusted manually corresponding to the defined q_s set point. All fed batches described in this study were conducted in the same way: after an adaptation period at q_s of 0.5 $mmol \cdot g^{-1} \cdot h^{-1}$, a stepwise increase of q_s up to $q_{s\ max}$ of the respective strain was carried out with step times of 24 hours.

Analysis of growth- and expression-parameters

Dry cell weight (DCW), OD_{600}, substrate concentrations as well as the catalytic activity of HRP were determined as described before [18]. However, in this study the ABTS solution for HRP activity measurements was prepared in 50 mM KH_2PO_4-buffer at pH 6.5 and the calibration range was expanded to 2.0 $U \cdot ml^{-1}$. Also GalOX activity was measured with an ABTS assay; *i.e.* a sample of diluted enzyme (10 µl) was added to 990 µl of assay buffer containing horseradish peroxidase (222 U) (Type VI-A, Sigma-Aldrich, P6782), ABTS (17.7 mg), KH_2PO_4-buffer (50 mM, pH 6.5) and D-galactose (300 mM). The absorbance change at 420 nm (ε_{420} = 42.3 $mM^{-1} \cdot cm^{-1}$) was recorded at 30°C for 180 seconds. One Unit of GalOX activity was defined as the amount of enzyme necessary for the oxidation of 2 µmol of ABTS per min, corresponding to the consumption of 1 µmol of O_2 per min. An additional post-translational activation of GalOX by adding $CuSO_4$ to the samples in the presence of oxygen before activity measurements, as described elsewhere [20], was not executed.

Specific rate calculations

Batch cultivations

To obtain the specific rates for substrate uptake and productivity, samples were taken at certain time points during the methanol pulses (*i.e.* beginning of pulse, maximum off-gas of pulse, end of pulse) and analyzed offline for biomass content, methanol concentration and, if applicable, enzymatic activity. Determined values at the beginning and the end of the respective pulse were used to calculate an average rate of the specific substrate uptake, which was corrected for stripping using Antoine's equation, and the specific productivity. Errors for specific rates were set to 10%, according to our previous study [18]. Online calculated carbon dioxide evolution rate (CER) was divided by actual biomass concentrations to obtain the specific carbon dioxide production rate (qCO_2). In addition, a time derivative of the qCO_2 signal (*i.e.* qCO_2') was calculated using a time window of 30 minutes (15 minutes before and 15 minutes after the actual time point).

Fed batch cultures

During different cultivation periods, representing defined q_s set points, several samples were taken and OD_{600} measurements were used to calculate the actual total biomass content, which allowed adjustments of the methanol feed flow to the actual q_s set point. Specific rates were calculated using DCW and the amount of consumed methanol, which was determined gravimetrically. Presented results correspond to an average value over the respective q_s set point period. Again, errors for specific rates were set to 10%.

Results and Discussion

A fast approach to derive a minimal set of strain characteristic parameters relevant for bioprocess development

Each *P. pastoris* strain was cultivated in an easy-to-do batch system with methanol pulses to obtain certain strain characteristic parameters during the adaptation period ($\Delta time_{adapt}$ - time for adaptation of the culture to methanol, $q_{s\ adapt}$ - specific substrate uptake rate during the adaptation pulse) and the maximum specific substrate uptake rate ($q_{s\ max}$). These parameters were

extracted and consecutively transformed into a feeding profile for fed batch operations based on q_s.

Adaptation of the culture to methanol

After depletion of glucose in batch cultivation, a methanol adaptation pulse with a final concentration of 0.5% (v/v) was applied. The time required to develop a maximum in off-gas activity was used to define $\Delta time_{adapt}$, according to our previous study [18], and is shown here as the specific carbon dioxide production rate (qCO_2; Figure 1).

The used strains showed very different metabolic characteristics during the adaptation to methanol. The shortest $\Delta time_{adapt}$ of 3.5 h was detected for the KM71H strain, which was a Mut^S strain, not carrying a recombinant gene for heterologous protein expression. The other Mut^S strains KM71H HRP, KM71H PDI HRP and CBS7435 HRP (Figure 1B, C and 1D, respectively) showed 2-fold longer adaptation times compared to the KM71H strain (see also Table 2). These results clearly show that recombinant *Pichia* strains, which heterologously produce proteins upon the presence of the inducer methanol, carry an additional biological burden which significantly changes their metabolism and slows down their adaptation to methanol.

Surprisingly, the *P. pastoris* Mut^+ strain SMD1168H GalOX showed the longest adaptation time of 14 h (Figure 1E). However, this maximum was just detected as a result of methanol depletion and thus Monod kinetics, rather than representing the real time point of full methanol adaptation. As shown in Figure 1, the Mut^+ strain SMD1168H GalOX showed a very different metabolic behaviour with a flat slope for qCO_2 compared to the qCO_2 curves of the Mut^S strains, which might be due to intracellular regulation and inhibition events, caused by produced H_2O_2 and the absence of sufficient catalases in the cells. Regulation events like this have been described in various systems before [22-24]. Thus, the determined $\Delta time_{adapt}$ of 14 h for the Mut^+ strain is questionable and the cells had probably been adapted to methanol before.

Consequently, the usefulness of the strain characteristic parameter $\Delta time_{adapt}$, especially with regard to the Mut^+ strain SMD1168H GalOX, was checked by introducing a time derivative of the qCO_2 signal (qCO_2'). Since qCO_2 and the specific growth rate μ are linearly related, the maximum of qCO_2' represents the time point of adaptation of the culture to the new substrate methanol. At the maximum qCO_2' the cells should be already fit for further assimilation to methanol, and thus this point represents a good starting point for consecutive fed batch cultivations without running the risk of methanol accumulation. A good example for this is shown in Figure 1A for the strain KM71H, where a rising slope is shown with a single qCO_2' maximum after 2.1 h. However, the other Mut^S strains tested (KM71H HRP, KM71H PDI HRP and CBS7435 HRP) were characterized by a more bumpy qCO_2 curve, resulting in a qCO_2' signal with several shoulders (Figure 1B, C and 1D, respectively). Apparently, the adaptation of these strains to methanol did not happen as straight-forward as for the strain KM71H, but with local minima and maxima of the metabolic capacity probably caused by regulatory events upon an excess of methanol. For the Mut^+ strain SMD1168H GalOX the specific carbon dioxide production was rather low compared to the Mut^S strains. The maximum in qCO_2' was determined already after 2.7 h, which was by far sooner than the observed $\Delta time_{adapt}$ of 14 h. The quite constant qCO_2' signal over time (Figure 1E), further supports the fact that the cell capacity was already adapted to its maximum after this short time of 2.7 h and that the cells had been fully adapted to methanol much sooner than the $\Delta time_{adapt}$ of 14 h.

In general, the more detailed analysis of the different strains in their adaptation to methanol revealed three different patterns in the qCO_2' signal: 1) with a single maximum, 2) with several shoulders and 3) a quite constant signal over time. This underlines the necessity for individual analyses of different strains in order to quantitatively characterize them during the adaptation phase in the presence of methanol excess. Of course, the observed maximum in off-gas activity is dependent on the affinity of the cells to the substrate methanol. The maximum in qCO_2 could also be reached in terms of substrate limitation and a consequent drop in the qCO_2 signal due to Monod kinetics rather than by the maximum metabolic adaptation to methanol, which in this study can clearly be seen for the Mut^+ strain SMD1186H GalOX (Figure 1E). However, for all strains tested in this study, maximum values of qCO_2' were reached before the maximum off-gas activity (Figure 1 and Table 2), demonstrating that the applied concentration of methanol in the adaptation pulse was high enough to guarantee that the maximum qCO_2' was reached independent of Monod kinetic effects. The validity of qCO_2' as a reliable signal to detect the adaptation of the culture to methanol is further underlined when analyzing the respiratory quotient (RQ) during the adaptation pulse, which is exemplarily shown for the strain KM71H in Figure 2. During the adaptation pulse, RQ fluctuates with local minima and maxima until the signal becomes rather constant indicating the adaptation of the culture to methanol, which actually coincides with the maximum of qCO_2'. Bespoken fluctuations of RQ at the beginning of the adaptation pulse represent the differences in catabolic and anabolic activity of the adapting cells. Similar effects have been observed for *Saccharomyces cerevisiae* [22,25,26]. During the

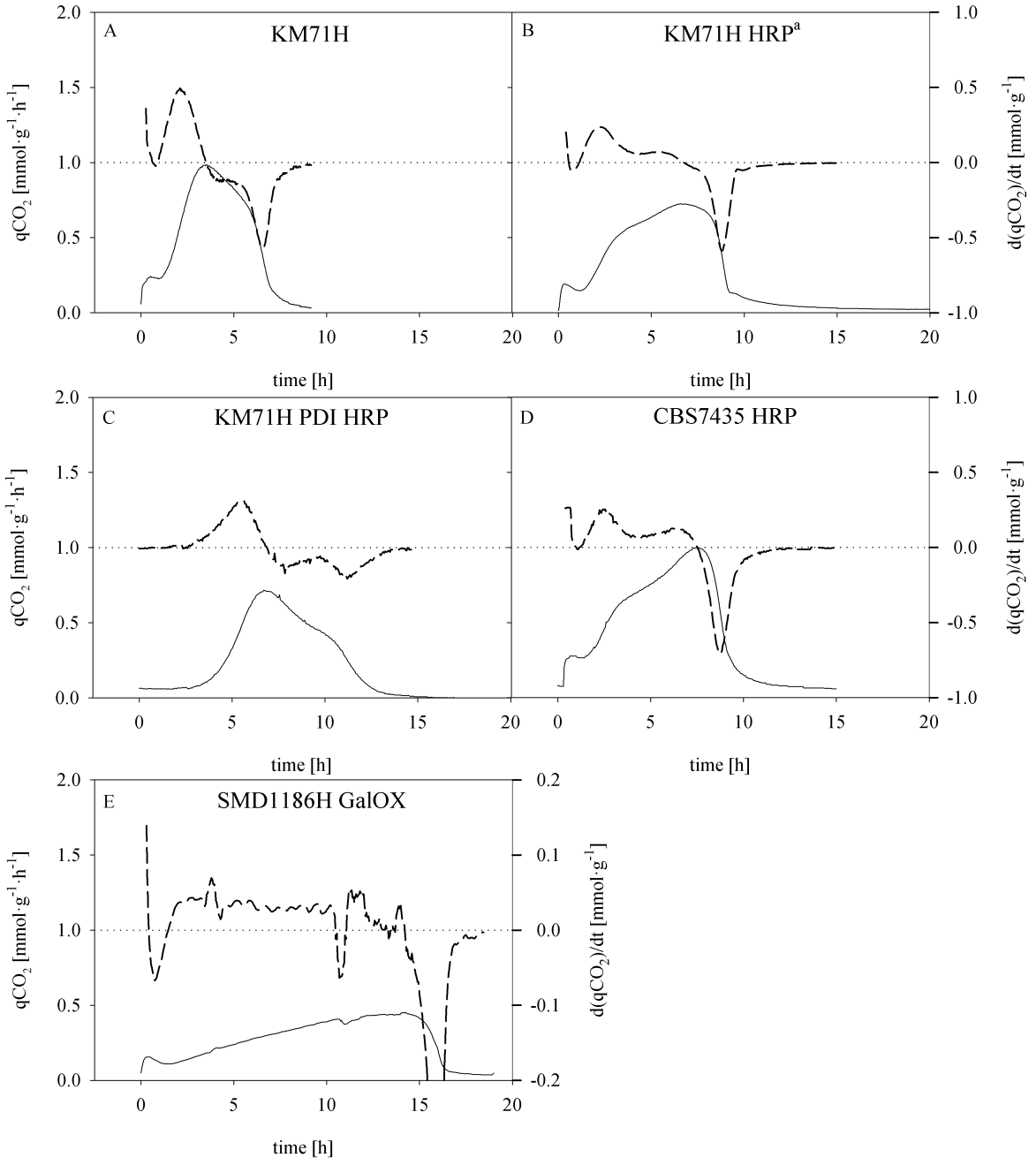


Figure 1 Adaptation pulses with 0.5% (v/v) methanol after glucose depletion for different *P. pastoris* strains. Straight line, specific carbon dioxide production rate qCO_2; dashed line, time-derivative of the specific carbon dioxide production rate qCO_2' ($d(qCO_2)/dt$); A, KM71H; B, KM71H HRP[a]; C, KM71H PDI HRP; D, CBS7435 HRP; E, SMD1168H GalOX. [a]data taken from [18]

following pulses, RQ shows a rather constant signal indicating that the cells had already been adapted to the new substrate. These findings validate the parameter qCO_2' as a reliable indicator for methanol adaptation.

However, despite the advantage of describing the time point of adaptation of the culture to methanol more accurately, the use of qCO_2' as a parameter to determine the starting point of the following fed batch could be risky because of the described fluctuations in qCO_2 (Figure 1). On the other hand, the maximum off-gas activity ($\Delta time_{adapt}$) is a parameter which safely describes methanol adaptation. Another advantage of using $\Delta time_{adapt}$ instead of qCO_2' is the fact that due to no significant biomass increase during the adaptation

Table 2 Batch experiments with methanol pulses to determine strain specific parameters of different *P. pastoris* strains.

	KM71H	KM71H HRP[a]	KM71H PDI HRP	CBS7435 HRP	SMD1168H GalOX
$\Delta time_{adapt\ Batch}$ [h]	3.5	7	7	7.5	14
max. qCO_2' [h]	2.1	2.3	5.7	2.5	2.7
$q_{s\ adapt}$ [mmol·g^{-1}·h^{-1}]	0.96 ± 0.10	0.80 ± 0.08	0.56 ± 0.06	0.77 ± 0.08	0.48 ± 0.05
$q_{s\ max}$[b] [mmol·g^{-1}·h^{-1}]	1.94 ± 0.19	2.00 ± 0.20	1.08 ± 0.10	1.54 ± 0.15	2.62 ± 0.26
$q_{p\ max}$[b] [U·g^{-1}·h^{-1}]	-	2.5 ± 0.25	6.3 ± 0.63	4.25 ± 0.43	200.8 ± 20.1

[a] data taken from [18]

[b] representing the maximum value determined out of all pulses

pulses, the carbon dioxide evolution rate (CER), which can easily be derived in online mode, can be used to determine $\Delta time_{adapt}$, as also shown in our previous study [18], and thus describes a valuable online tool for process monitoring and control. Consequently, we stuck to $\Delta time_{adapt}$ as a minimum and safe parameter for complete methanol adaptation, as we have done previously [18], while the maximum of qCO_2' should be regarded as a possible minimum prerequisite to start the fed batch feed.

Determination of the specific substrate uptake rates ($q_{s\ adapt}$ and $q_{s\ max}$) using batch cultivations with repeated methanol pulses

The frequent determination of biomass and methanol concentrations allowed specific rate calculations for methanol uptake (q_s) during the methanol pulses. Adaptation pulses of 0.5% (v/v) methanol were used to determine the parameter $q_{s\ adapt}$ (specific substrate uptake rate during the adaptation pulse) for each strain (Table 2). The knowledge of $q_{s\ adapt}$ allows the operator to

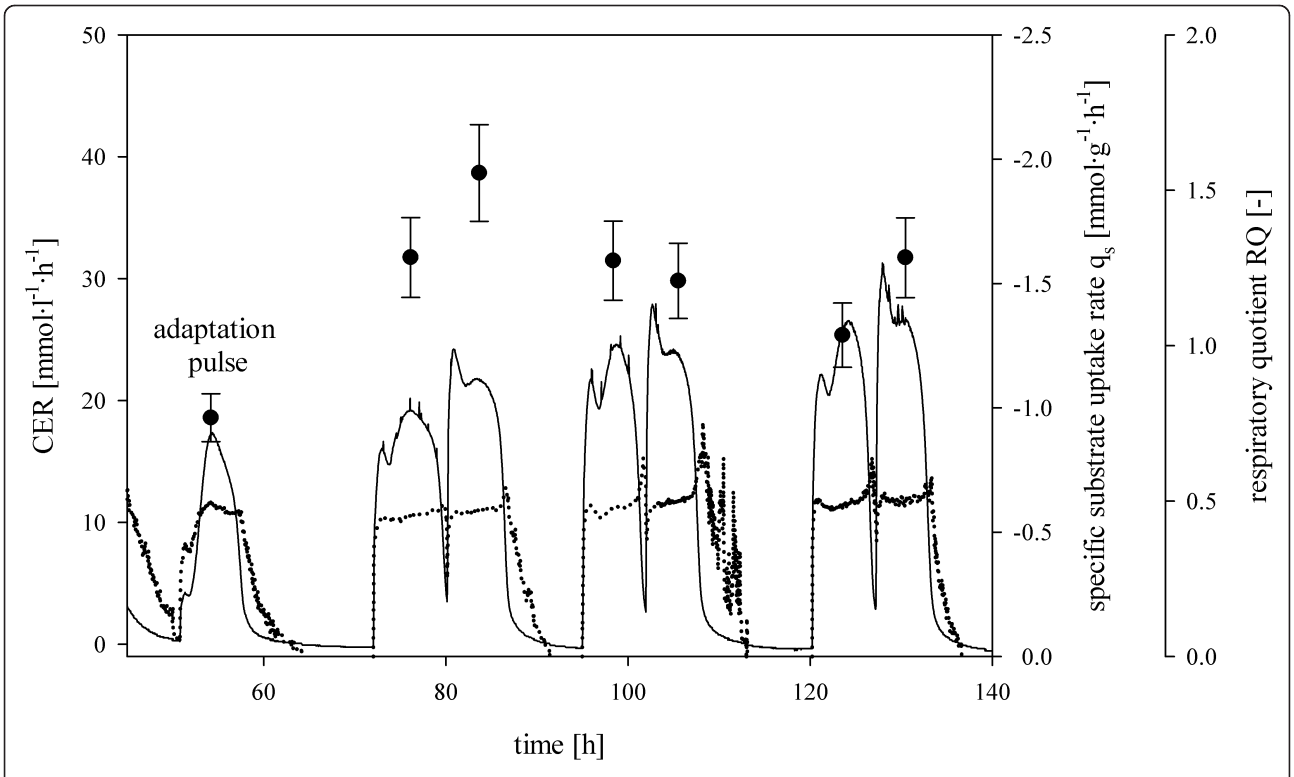


Figure 2 Batch cultivation of the strain KM71H with a 0.5% (v/v) methanol pulse for adaptation and 1% (v/v) methanol pulses for $q_{s\ max}$ determination. Straight line, calculated carbon dioxide evolution rate (CER); dotted line, respiratory quotient RQ (CER/OUR); black dot, specific substrate uptake rate (q_s) for each pulse.

adjust a specific, optimal flow of methanol during the adaptation for each single strain, thus preventing methanol accumulation.

After the methanol of the adaptation pulse was depleted, several pulses with 1% (v/v) methanol were conducted to determine the maximum specific substrate uptake rate ($q_{s\ max}$) for each *P. pastoris* strain. In Figure 2, this strategy is exemplarily shown for the Mut^S strain KM71H, for which the specific substrate uptake rate was calculated with 0.96 ± 0.09 $mmol \cdot g^{-1} \cdot h^{-1}$ in the adaptation period ($q_{s\ adapt}$) and with 1.94 ± 0.19 $mmol \cdot g^{-1} \cdot h^{-1}$ as a maximum during pulses (Figure 2 and Table 2). All *P. pastoris* strains were characterized with the above mentioned strategy and results are summarized in Table 2. Very different values for the single strain characteristic parameters of the different *P. pastoris* strains were determined and it becomes evident, that *P. pastoris* strains require specific conditions for an optimal adaptation to methanol and that the maximum levels of methanol uptake differ significantly between the single strains.

Summing up, our results underline the importance of analyzing different *P. pastoris* strains in more detail during methanol adaptation and regarding the maximum substrate uptake rate. By a more detailed analysis of each strain, which can be done in a fast way by the strategy described here, the bioprocess-relevant strain characteristic parameters $\Delta time_{adapt}$, $q_{s\ adapt}$ and $q_{s\ max}$ can be easily extracted and used for setting up a consecutive fed batch experiment based on q_s. Additionally, the more detailed individual analysis of each strain during the adaptation phase delivers important information for an early process development.

Fed batch design based on batch cultivations with methanol pulses

After characterization experiments in batch cultivations, fed batch experiments using q_s based feeding profiles were carried out, according to our previous study [18]. Besides proving the general applicability of this feeding strategy on different *P. pastoris* strains, we wanted to check for parameter consistency, *i.e.* whether $q_{s\ max}$, which had been determined in batch experiments before, could be reached in fed batch cultivations without observable methanol accumulation.

After a batch phase on glucose as substrate (volume 1.5 l), an exponential fed batch cultivation with glucose yielded in biomass concentrations of up to 70 g/l in a final volume of 2.5 l. As soon as glucose was depleted, a sample was taken to determine the current biomass concentration by measuring the OD_{600} and the DCW. Afterwards, all cultures were induced with a flow corresponding to a q_s below $q_{s\ adapt}$ to guarantee a certain safety margin. The observed adaptation times during the fed batch cultivations for all strains are summarized in Table 3. All the Mut^S strains were characterized by slightly longer adaptation times in fed-batches compared to the batch cultivations, which might be due to the mode of providing methanol, *i.e.* in batch experiments methanol was pulsed into the reactor, resulting in a temporal excess of methanol, whereas in fed batch cultivations methanol was slowly fed into the bioreactor according to the actual biomass content, which is why it took the cells longer to fully adapt to the new substrate methanol.

However, the Mut^+ strain SMD1168H GalOX showed a completely different behaviour: the adaptation time in the fed batch was much shorter than the one observed in the batch experiment, which might be due to the fact that the sudden excess of methanol in the batch-pulse experiment resulted in inhibition events caused by produced H_2O_2 and thus $\Delta time_{adapt}$, which had been determined in the batch experiment, did not really describe the time point of adaptation of the culture to methanol, but rather qCO_2' (*vide supra*).

However, as soon as the maximum in off-gas activity was reached in the fed batch cultivations, the feed was increased to 1.0 $mmol \cdot g^{-1} \cdot h^{-1}$ and then stepwise (0.5 $mmol \cdot g^{-1} \cdot h^{-1}$/step) every 24 hours up to $q_{s\ max}$. Since $q_{s\ max}$ for the strain KM71H HRP PDI had been determined with just 1.08 $mmol \cdot g^{-1} \cdot h^{-1}$, smaller increases of 0.25 $mmol \cdot g^{-1} \cdot h^{-1}$ in the q_s steps were performed. This strategy, describing a stepwise increase of q_s to $q_{s\ max}$, was chosen to allow the detection of possible

Table 3 Results of dynamic fed batch cultivations of different *P. pastoris* strains based on q_s.

	KM71H	KM71H HRP[a]	KM71H PDI HRP	CBS7435 HRP	SMD1168H GalOx
$\Delta time_{adapt\ Fedbatch}$ [h]	5.5	9.7	10.4	9.2	5.3
$q_{s\ max}$ determined in batch experiments [$mmol \cdot g^{-1} \cdot h^{-1}$]	1.94 ± 0.19	2.00 ± 0.21	1.08 ± 0.10	1.54 ± 0.15	2.62 ± 0.26
q_s reached without methanol accumulation [$mmol \cdot g^{-1} \cdot h^{-1}$]	1.79 ± 0.18	1.92 ± 0.19	1.22 ± 0.12	1.64 ± 0.16	2.44 ± 0.24
$q_{p\ max}$[b] [$U \cdot g^{-1} \cdot h^{-1}$]	-	11.0 ± 1.10	7.09 ± 0.10	6.48 ± 0.65	139 ± 13.9

[a] data taken from [18]

[b] representing the maximum value determined out of all q_s periods

dependencies between q_s and q_p. At several time points during each step, samples were taken and, based on the apparent biomass content (estimated by OD_{600} measurements) feeding rates were adjusted manually corresponding to the defined q_s set point. Since these regular adjustments of q_s to the actual biomass content were performed, the feeding profile actually represented an accelerated exponential feeding profile, which has proven to result in higher specific productivities compared to other feeding profiles tested [18].

In Figure 3, this fed batch strategy, which was applied for all *P. pastoris* strains in this study, is exemplarily shown for the Mut^s strain KM71H and the Mut$^+$ strain SMD1186H GalOX. The q_s set points were increased stepwise to a value of 2.00 mmol·g^{-1}·h^{-1} for the KM71H strain, and, as shown in Figure 3A, methanol accumulation was only observed when the feeding rate exceeded values above the respective $q_{s\ max}$ of 1.94 ± 0.19 mmol·g^{-1}·h^{-1}. When we stopped feeding after ~ 70 h, the accumulated methanol was consumed immediately.

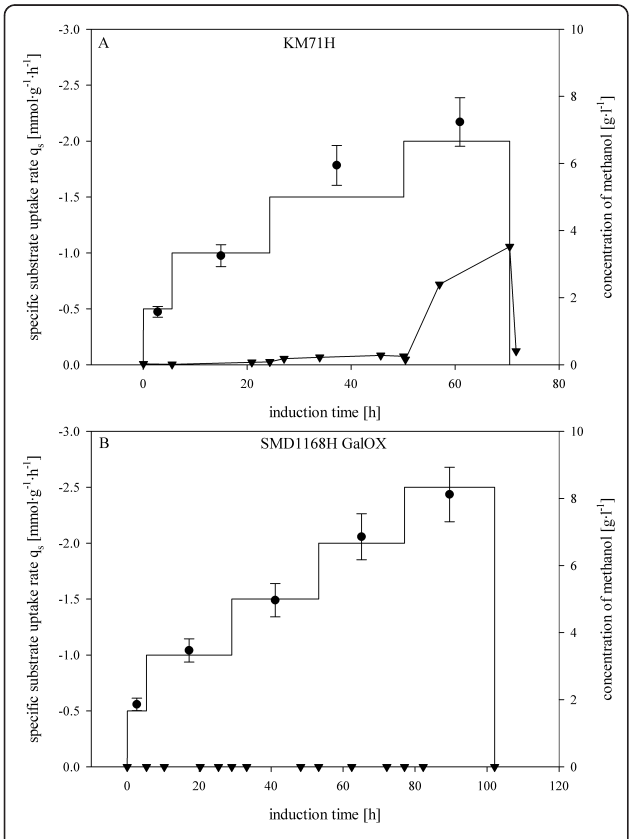


Figure 3 Fed batch cultivations of different *P. pastoris* strains on methanol with a stepwise increase of q_s to $q_{s\ max}$. A, *P. pastoris* Mut^S strain KM71H; B, *P. pastoris* Mut$^+$ strain SMD1186H GalOX; straight line, set point for q_s; black dot, calculated q_s values; black triangle, methanol concentration in the supernatant.

For the strain SMD1168H GalOX, the q_s set point was stepwise increased to 2.5 mmol·g^{-1}·h^{-1} ($q_{s\ max}$ = 2.62 mmol·g^{-1}·h^{-1}) and no methanol accumulation was detected when these q_s steps were conducted (Figure 3B). The same dynamic feeding strategy was applied to the other *P. pastoris* strains and all essential results are summarized in Table 3.

As shown in Table 3, values for $q_{s\ max}$, which had been determined in batch pulsing experiments before, were reached in fed batch experiments without methanol accumulation. However, when q_s set points were further increased, methanol accumulation was observed. This proves that the values for $q_{s\ max}$ from batch experiments can be found again in consecutive fed batch experiments for different *P. pastoris* strains and thus shows the great potential of this approach: by determining $q_{s\ max}$ in fast and easy-to-do batch experiments, the operator does not run the risk of overfeeding methanol in consecutive fed batch cultivations. However, we recommend a maximum feed flow below $q_{s\ max}$ to guarantee a certain safety margin.

Regarding the productivity, for all the strains, except for SMD1168H GalOX, same or even higher specific productivities were obtained in fed batch cultures compared to the batch experiments, signifying that volumetric productivities were higher in fed batch cultivations compared to batch experiments (Table 2 and Table 3). This shows that our approach employing dynamic feeding profiles [18] can be successfully applied on different *P. pastoris* strains. The lower $q_{p\ max}$ of the strain SMD1168H GalOX in fed batch cultivation probably resulted from copper-limitation [20], but was not investigated any further, since it was not goal of this study to optimize the production of recombinant enzymes of the single *P. pastoris* strains.

Summing up, in this study we show the general applicability of a fast approach to determine certain strain characteristic parameters, which were extracted out of batch experiments and were verified in subsequent fed batch cultures of different *P. pastoris* strains, making this approach a valuable tool for fast bioprocess development.

Conclusions

In the present study we prove that the fast approach to determine bioprocess-relevant strain characteristic parameters and the novel dynamic feeding strategy based on q_s, which we have described recently for one recombinant *P. pastoris* strain [18], are applicable for a variety of *P. pastoris* strains with different phenotypes producing different recombinant proteins. This underlines the great potential of this strategy as a fast and simple tool to quantify a minimal set of parameters needed to set

up consecutive fed batch regimes, which is particularly important for industry, where a fast process development is essential.

Our strategy describes:

1. a batch experiment with
• a 0.5% (v/v) methanol adaptation pulse to determine $\Delta time_{adapt}$ and $q_{s\ adapt}$
• at least 4 consecutive 1.0% (v/v) methanol pulses to determine $q_{s\ max}$
2. a dynamic fed batch feeding strategy based on q_s, where after the adaptation of the culture to methanol (described by a maximum in off-gas activity), q_s set points can be increased to $q_{s\ max}$ without observable methanol accumulation

We further show that a detailed analysis of the adaptation to methanol reveals a variability of adaptation characteristics of the different strains, highlighting that an individual analysis of potentially new strains in this respect is required to allow quantitative strain characterization and to derive parameters necessary for a consecutive fed batch set-up. The parameter $\Delta time_{adapt}$ safely describes the transition condition during methanol adaptation. Since also the carbon dioxide evolution rate (CER), as well as the oxygen uptake rate (OUR), can be used to determine $\Delta time_{adapt}$, it further describes an online available data source allowing real-time monitoring and controlling of bioprocesses, which is essential under the aspect of Process Analytical Technology (PAT).

In this study we show that easy-to-do batch experiments with methanol pulses delivered valid and safe strain characteristic parameters which were consistent and precise enough to set up fed batch feeding profiles based on the specific substrate uptake rate. Our strategy is faster than the usually used continuous cultures or consecutive fed batch cultivations, and therefore allows faster process development. Besides, the strategy described here can be carried out using standard equipment without the need of cost-intensive tools; only a standard bioreactor connected to an off-gas analysis system and an HPLC or GC to determine methanol concentrations are required to carry out the experiments.

List of Abbreviations

$\Delta time_{adapt}$: mtime for adaptation of the culture to the new substrate (methanol) [h]; μ: specific growth rate [h^{-1}]; ABTS: 2, 2' azino bis 3-ethylbenzthiazoline-6-sulphonic acid; CER- carbon dioxide evolution rate [$mmol \cdot l^{-1} \cdot h^{-1}$]; $d(qCO_2)/dt$: derivative of qCO_2; DCW: dry cell weight; HPLC: high performance liquid chromatography; HRP: horseradish peroxidise; GalOX: galactose 6-oxidase; Mut^+: methanol utilization phenotype; wildtype; Mut^S: methanol utilization slow phenotype; OD_{600}: optical density at 600 nm [AU]; PID: proportional-integrative-derivative controller; qCO_2: specific carbon dioxide production rate [$mmol \cdot g^{-1} \cdot h^{-1}$]; qCO_2': derivative of qCO_2; q_p: specific productivity [$U \cdot g^{-1} \cdot h^{-1}$]; $q_{s\ adapt}$: specific substrate uptake rate during adaptation [$mmol \cdot g^{-1} \cdot h^{-1}$]; $q_{s\ max}$: maximum specific substrate uptake rate [$mmol \cdot g^{-1} \cdot h^{-1}$]; q_s: specific substrate uptake rate [$mmol \cdot g^{-1} \cdot h^{-1}$]; rpm: rounds per minute; RQ: respiratory quotient; Vvm: volume gas flow per volume liquid per minute

Acknowledgements

The authors are very grateful to Prof. Anton Glieder and his workgroup for providing all *P. pastoris* Mut^S strains expressing the enzyme horseradish peroxidase. Additionally, the authors express their gratitude to Josef Schulze, who supported the experimental work in the lab.

Authors' contributions

CD designed and performed the majority of the experiments, analyzed the data and drafted the manuscript. OS designed and performed some experiments and finalized the manuscript. CH conceived the study and supervised research. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

Received: 9 August 2011 Accepted: 27 October 2011
Published: 27 October 2011

References

1. d'Anjou MC, Daugulis AJ: **A rational approach to improving productivity in recombinant Pichia pastoris fermentation.** *Biotechnology and bioengineering* 2001, **72**:1-11.
2. Curvers S, Linnemann J, Klauser T, Wandrey C, Takors R: **Recombinant protein production with Pichia pastoris in continuous fermentation - kinetic analysis of growth and product formation.** *Engineering in Life Sciences* 2002, **2**:229-235.
3. Ren H, Yuan J: **Model-based specific growth rate control for Pichia pastoris to improve recombinant protein production.** *Journal of Chemical Technology and Biotechnology* 2005, **80**:1268-1272.
4. Ren HT, Yuan JQ, Bellgardt KH: **Macrokinetic model for methylotrophic Pichia pastoris based on stoichiometric balance.** *Journal of Biotechnology* 2003, **106**:53-68.
5. Invitrogen: **Pichia Fermentation Process Guidelines.** 2002.
6. Jahic M, Rotticci-Mulder JC, Martinelle M, Hult K, Enfors SO: **Modeling of growth and energy metabolism of Pichia pastoris producing a fusion protein.** *Bioprocess and Biosystems Engineering* 2002, **24**:385-393.
7. Trinh LB, Phue JN, Shiloach J: **Effect of methanol feeding strategies on production and yield of recombinant mouse endostatin from Pichia pastoris.** *Biotechnology and Bioengineering* 2003, **82**:438-444.
8. Zhang W, Bevins MA, Plantz BA, Smith LA, Meagher MM: **Modeling Pichia pastoris growth on methanol and optimizing the production of a recombinant protein, the heavy-chain fragment C of botulinum neurotoxin, serotype A.** *Biotechnology and Bioengineering* 2000, **70**:1-8.
9. Zhang W, Sinha J, Smith LA, Inan M, Meagher MM: **Maximization of Production of Secreted Recombinant Proteins in Pichia pastoris Fed-Batch Fermentation.** *Biotechnology Progress* 2005, **21**:386-393.
10. Plantz BA, Sinha J, Villarete L, Nickerson KW, Schlegel VL: **Pichia pastoris fermentation optimization: energy state and testing a growth-associated model.** *Applied Microbiology and Biotechnology* 2006, **72**:297-305.
11. Potgieter TI, Kersey SD, Mallem MR, Nylen AC, d'Anjou M: **Antibody expression kinetics in glycoengineered Pichia pastoris.** *Biotechnology and Bioengineering* 2010, **106**:918-927.
12. Kobayashi K, Kuwae S, Ohya T, Ohda T, Ohyama M, Tomomitsu K: **High level secretion of recombinant human serum albumin by fed-batch fermentation of the methylotrophic yeast, Pichia pastoris, based on optimal methanol feeding strategy.** *Journal of Bioscience and Bioengineering* 2000, **90**:280-288.
13. Jacobs P, Inan M, Festjens N, Haustraete J, Van Hecke A, Meagher M, Contreras R, Callewaert N: **Fed-batch fermentation of GM-CSF-producing glycoengineered Pichia pastoris under controlled specific growth rate.** *Microbial Cell Factories* 2010, **9**:93.
14. Sinha J, Plantz BA, Zhang W, Gouthro M, Schlegel V, Liu C-P, Meagher MM: **Improved production of recombinant ovine interferon-t by Mut+ strain of Pichia pastoris using an optimized methanol feed profile.** *Biotechnology Progress* 2003, **19**:794-802.
15. Khatri NK, Hoffmann F: **Oxygen-limited control of methanol uptake for improved production of a single-chain antibody fragment with**

recombinant Pichia pastoris. *Applied Microbiology and Biotechnology* 2006, **72**:492-498.
16. Khatri NK, Hoffmann F: **Impact of methanol concentration on secreted protein production in oxygen-limited cultures of recombinant Pichia pastoris.** *Biotechnology and Bioengineering* 2006, **93**:871-879.
17. Cunha AE, Clemente JJ, Gomes R, Pinto F, Thomaz M, Miranda S, Pinto R, Moosmayer D, Donner P, Carrondo MJT: **Methanol induction optimization for scFv antibody fragment production in Pichia pastoris.** *Biotechnology and bioengineering* 2004, **86**:458-467.
18. Dietzsch C, Spadiut O, Herwig C: **A dynamic method based on the specific substrate uptake rate to set up a feeding strategy for Pichia pastoris.** *Microbial Cell Factories* 2011, **10**:14.
19. Hartner FS, Ruth C, Langenegger D, Johnson SN, Hyka P, Lin-Cereghino GP, Lin-Cereghino J, Kovar K, Cregg JM, Glieder A: **Promoter library designed for fine-tuned gene expression in Pichia pastoris.** *Nucleic Acids Research* 2008, **36**:e76/71-e76/15.
20. Spadiut O, Olsson L, Brumer H: **A comparative summary of expression systems for the recombinant production of galactose oxidase.** *Microb Cell Fact* 2010, **9**:68.
21. Cregg JM: **Pichia Protocols, Second Edition.** In *Methods Mol Biol.* Edited by: Cregg JM. Totowa, NJ, U. S.; 2007:268.
22. Herwig C, von Stockar U: **A small metabolic flux model to identify transient metabolic regulations in Saccharomyces cerevisiae.** *Bioprocess and Biosystems Engineering* 2002, **24**:395-403.
23. Diderich JA, Schepper M, van Hoek P, Luttik MA, van Dijken JP, Pronk JT, Klaassen P, Boelens HF, de Mattos MJ, van Dam K, Kruckeberg AL: **Glucose uptake kinetics and transcription of HXT genes in chemostat cultures of Saccharomyces cerevisiae.** *The Journal of biological chemistry* 1999, **274**:15350-15359.
24. Rizzi M, Theobald U, Querfurth E, Rohrhirsch T, Baltes M, Reuss M: **In vivo investigations of glucose transport in Saccharomyces cerevisiae.** *Biotechnology and Bioengineering* 1996, **49**:316-327.
25. Duboc P, Von Stockar U, Villadsen J: **Simple generic model for dynamic experiments with Saccharomyces cerevisiae in continuous culture: decoupling between anabolism and catabolism.** *Biotechnology and Bioengineering* 1998, **60**:180-189.
26. Duboc P, Cascao-Pereira LG, von Stockar U: **Identification and control of oxidative metabolism in Saccharomyces cerevisiae during transient growth using calorimetric measurements.** *Biotechnology and Bioengineering* 1998, **57**:610-619.

doi:10.1186/1475-2859-10-85
Cite this article as: Dietzsch *et al.*: A fast approach to determine a fed batch feeding profile for recombinant *Pichia pastoris* strains. *Microbial Cell Factories* 2011 **10**:85.