QUANTITATIVE CHARACTERIZATION OF A RECOMBINANT *PICHIA PASTORIS* MUT$^+$ STRAIN SECRETING AVIDIN USING TRANSIENT CONTINUOUS CULTURES

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PREFACE

Ce manuscrit finalise quatre superbes années de ma vie et j'aimerais sincèrement remercier toutes les personnes qui ont contribué à la réussite de ce travail autant sur le plan scientifique que sur le plan humain.

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Carmen Jungo

Lausanne, le 7 mars 2007

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SUMMARY

The objective of this thesis was to characterize the growth stoichiometry, the specific recombinant protein productivity and the regulation of the alcohol oxidase (AOX) enzyme of a *Pichia pastoris* Mut^+ strain, performing conventional chemostat cultures and transient changes of culture parameters in continuous cultures. A *P. pastoris* strain secreting avidin was chosen as case study. The investigation focused on an analysis of the influence of specific growth rate, culture temperature and on use of mixed substrates.

First, a chemically defined medium was designed for the production of recombinant biotin-free avidin in continuous cultures. Indeed, one problem with heterologous expression systems secreting avidin is that biotin, which is an essential vitamin for most microorganisms, binds strongly to the produced recombinant avidin. The addition of low amounts of biotin (20 $\mu g\ L^{-1}$ biotin for a cell density of 8 $g\ L^{-1}$) resulted in stable chemostat cultures on methanol with the concomitant production of biotin-free avidin.

The influence of the specific growth rate on growth stoichiometry, recombinant avidin productivity and specific alcohol oxidase activity was studied with chemostat cultures on glycerol and on methanol. Results showed that the substrate consumption rate for maintenance purposes was low: 0.007 and 0.011 C-mol $C\text{-}mol^{-1}\ h^{-1}$ for growth on glycerol and methanol, respectively. No recombinant avidin was detected in cultures growing on glycerol, but a partial derepression of the synthesis of AOX was observed. During chemostat cultures on methanol, the specific AOX activity was 20 to 100-fold higher than in chemostat cultures on glycerol and the specific avidin production rate was growth-associated, increasing linearly with dilution rate. No relationship between the specific avidin production rate and the specific AOX activity could be established, although avidin was expressed under the control of the AOX1 promoter.

The applicability of a new and dynamic cultivation method, which consists in increasing linearly the temperature during continuous culture, was investigated. Comparison of culture characteristics determined during pseudo-steady state continuous cultures with linear increase of temperature at a rate of $0.1°C\ h^{-1}$ and during chemostat cultures showed that this technique can be used as a fast and accurate tool for the characterization of host cells according to cultivation temperature. Results showed that cultivation at a lower temperature than the optimal growth temperature of 30°C did not influence significantly the recombinant avidin productivity.

The regulation of the AOX enzyme was investigated in continuous cultures on glycerol, methanol or mixed substrate cultures on glycerol and methanol with sudden changes of nutrient supply or pulses of methanol. Results showed that use of mixed feeds of glycerol and methanol allowed faster adaptation of cellular metabolism after growth on glycerol due to faster synthesis of methanol dissimilating enzymes. Pulse experiments showed that during cultures on methanol or during mixed substrate cultures, a sudden increase in the consumption rate of methanol after a transient increase in methanol led to the excretion of toxic intermediates (formaldehyde, formic acid) and to wash-out of the culture. This sudden increase in methanol consumption rate is due to the high amount of AOX synthesized during growth at low residual methanol concentrations.

The use of mixed feeds of glycerol and methanol or sorbitol and methanol during the induction phase in place of methanol as sole carbon source was investigated by performing pseudo-steady state continuous cultures with linear changes in the methanol fraction of the feed medium. This technique was validated with comparisons with results obtained during high cell density fed-batch cultures. The volumetric avidin production rate could be increased by up to 30% with mixed feeds due to higher biomass yields during mixed substrate growth (10% increase with a feed of 60% methanol - 40% C-mol C-mol^{-1} glycerol at 0.06 h^{-1}, 30% increase with a feed of 43% methanol - 57% C-mol C-mol^{-1} sorbitol at 0.03 h^{-1}). Moreover, heat production and oxygen consumption rates could be significantly reduced using mixed substrate growth (reduction of 28% with 60% methanol - 40% C-mol C-mol^{-1} glycerol at 0.06 h^{-1}; reduction of 38% with 43% methanol - 57% C-mol C-mol^{-1} sorbitol at 0.03 h^{-1}), which is very useful for the performance of high cell density cultures. By contrast with glycerol, control of residual sorbitol concentration is not a critical point because sorbitol is a non-repressive carbon source with respect to AOX expression. Indeed, accumulation of sorbitol in the culture medium did not affect the specific avidin production rate.

Finally, based on the results obtained during steady-state and transient continuous cultures, an optimal high cell density fed-batch strategy was proposed at a 2 L scale for the production of recombinant avidin with the studied strain. A performance of 1.06 mg h^{-1} avidin could be achieved with an exponential mixed feed of 43% methanol and 57% C-mol C-mol^{-1} sorbitol at 0.03 h^{-1} during the induction phase.

Keywords: *Pichia pastoris*, avidin, alcohol oxidase, mixed substrates, transient continuous cultures, calorimetry, high cell density.

RÉSUMÉ

L'objectif de cette thèse était de caractériser la stoichiométrie, la productivité spécifique en protéine recombinante et la régulation de l'enzyme alcool oxidase (AOX) d'une souche de *Pichia pastoris* Mut^+, en réalisant des cultures continues chemostat et des cultures continues avec des changements transitoires de paramètres de culture. Une souche *P. pastoris* sécrétant de l'avidine a été choisie comme étude de cas. L'étude était concentrée sur une analyse de l'influence du taux de croissance spécifique, la température et sur l'utilisation de substrats mixtes.

Un milieu défini a d'abord été conçu pour la production d'avidine libre, non-liée à de la biotine, en cultures continues. En effet, un problème avec les systèmes d'expression sécrétant de l'avidine est que la biotine, une vitamine essentielle pour la plupart des microorganismes, se lie fortement à l'avidine produite. L'addition de faibles quantités de biotine (20 $\mu g\ L^{-1}$ pour une densité cellulaire de 8 $g\ L^{-1}$) a permis d'effectuer des cultures chemostat sur méthanol stables avec production concomitante d'avidine libre.

L'influence du taux de croissance spécifique sur la stoichiométrie, la productivité d'avidine et l'activité spécifique d'AOX a été étudiée avec des cultures chemostat sur glycérol et sur méthanol. Les résultats ont montré que la vitesse de consommation du substrat pour la maintenance était faible: 0.007 et 0.011 C-mol C-mol^{-1} h^{-1} sur glycérol et méthanol, respectivement. De l'avidine recombinante n'a pas été détectée lors de cultures sur glycérol, mais une dérépression partielle de la synthèse d'AOX a été observée. Lors de chemostats sur méthanol, l'activité spécifique d'AOX était 20-100 fois plus élevée que lors de chemostats sur glycérol et la productivité spécifique d'avidine était liée à la croissance, augmentant linéairement avec le taux de dilution. Aucune relation entre la productivité spécifique d'avidine et l'activité spécifique d'AOX n'a pu être établie, bien que l'avidine aie été exprimée sous le contrôle du promoteur AOX1.

L'application d'une nouvelle méthode de culture dynamique, consistant à augmenter linéairement la température en culture continue, a été examinée. Des comparaisons des caractéristiques de culture déterminées lors d'augmentations de température à une vitesse de 0.1°C h^{-1} avec les résultats lors de chemostats ont montré que cette technique en mode pseudo-stationnaire pouvait être utilisée pour la caractérisation rapide et précise de cellules hôtes en fonction de la température. Les résultats ont montré qu'une diminution de la température en dessous de celle optimale pour la croissance de 30°C n'influençait pas significativement la productivité d'avidine.

La régulation de l'AOX a été étudiée en cultures continues sur glycérol, méthanol ou substrats mixtes de glycérol et méthanol avec des changements soudains d'apport nutritif ou des pulses de méthanol. Les résultats ont montré que l'utilisation de substrats mixtes permettait une adaptation plus rapide du métabolisme après croissance sur glycérol grâce à une synthèse plus rapide des enzymes de dissimilation du méthanol. Des pulses de méthanol ont montré que lors de cultures sur méthanol ou substrats mixtes, une augmentation soudaine de la consommation de méthanol après un pulse conduit à l'excrétion d'intermédiaires toxiques (formaldéhyde, acide formique) et à un *wash-out* de la culture. Cette augmentation soudaine de la consommation de méthanol est due à la synthèse élevée d'AOX lors de faibles concentrations résiduelles de méthanol.

L'utilisation de substrats mixtes de glycérol et méthanol ou de sorbitol et méthanol pendant la phase d'induction à la place de méthanol a été examinée avec des cultures continues pseudo-stationnaires avec des changements linéaires de la fraction de méthanol dans le milieu d'alimentation. Cette technique a été validée avec des comparaisons avec des cultures fed-batch à haute densité cellulaire. La productivité volumétrique d'avidine a pu être augmentée de 30% grâce à des rendements en biomasse plus élevés sur substrats mixtes (augmentation de 10% avec 60% méthanol et 40% C-mol C-mol^{-1} glycérol à 0.06 h^{-1}; augmentation de 30% avec 43% méthanol et 57% C-mol C-mol^{-1} sorbitol à 0.03 h^{-1}). De plus, la production de chaleur et la consommation d'oxygène ont été diminuées significativement sur substrats mixtes (réduction de 28% avec 60% méthanol-40% C-mol C-mol^{-1} glycérol à 0.06 h^{-1}; réduction de 38% avec 43% méthanol-57% C-mol C-mol^{-1} sorbitol à 0.03 h^{-1}), ce qui est très utile à haute densité cellulaire. Contrairement au glycérol, le contrôle de la concentration résiduelle de sorbitol n'est pas critique car le sorbitol n'est pas une source de carbon répressive par rapport à l'expression d'AOX. En effet, une accumulation de sorbitol dans le milieu de culture n'a pas affectée la productivité spécifique d'avidine.

Finalement, basé sur les résultats obtenus en cultures continues, stationnaires ou transitoires, une stratégie optimale fed-batch à haute densité cellulaire à l'échelle de 2L a été proposée pour la production d'avidine avec la souche étudiée. Une performance de 1.06 mg h^{-1} d'avidine a été atteinte avec une alimentation exponentielle de 43% methanol et 57% C-mol C-mol^{-1} sorbitol à 0.03 h^{-1} pendant la phase d'induction.

Mots-clés: *Pichia pastoris*, avidine, alcool oxidase, substrats mixtes, cultures continues transitoires, calorimétrie, haute densité cellulaire.

CHAPTER 1

INTRODUCTION

1. A BRIEF HISTORY OF *PICHIA PASTORIS* FROM THE 60'S TO TODAY

About forty years ago, Koichi Ogata first described the ability of certain yeast species to utilize methanol as sole source of carbon and energy (Ogata et al. (1969)). During the last three decades, interest in the study of methylotrophic yeasts has increased dramatically.

By definition, methylotrophic yeasts are able to utilize methanol as the sole carbon and energy source. All strains identified to date belong to only four genera: *Hansenula, Pichia, Candida* and *Torulopsis* (Faber et al. (1995)).

Methylotrophic yeasts were first isolated from soil, rotten fruits and the gut of insects (Gellissen (2000)). During the 1970's, these yeasts were first evaluated for the production of single-cell protein to be used primarily as high protein animal feed.

The species *Pichia pastoris* was initially developed by the Phillips Petroleum Company (USA) for the production of single-cell protein for feed stock. Cultivation techniques were developed for maintaining *P. pastoris* in large-volume continuous cultures and at cell densities higher than 100 g L^{-1} dry cell weight. However, because of the oil crisis of the 1970's, the production of single-cell protein from methanol was not financially interesting. In the 1980's, Phillips Petroleum contracted with the Salk Institute Biotechnology/Industrial Associates, Inc. (SIBIA, La Jolla, CA, USA) to develop *P. pastoris* as an organism for heterologous protein expression. Researchers at SIBIA isolated the AOX1 gene, coding for alcohol oxidase 1, and its promoter and developed vectors, strains, and methods for molecular genetic manipulation of *P. pastoris*. Currently, the *P. pastoris* expression system is available as a kit from Invitrogen Corporation (Carlsbad, CA, USA). This expression system is extensively used for the expression of heterologous proteins, for which many examples can be found in the literature (Cregg et al. (1993); Hollenberg and Gellissen (1997); Cereghino and Cregg (2000); Gellissen (2000); Cereghino et al. (2002); Daly and Hearn (2005)). More recently, *P. pastoris* has been used to express therapeutic proteins that have entered clinical trials (Gerngross

(2004)). Further development in the field of therapeutic glycoprotein production with *P. pastoris* strains is expected due to recent advances in genetic engineering of human glycosylation pathways into yeasts (Choi et al. (2003); Hamilton et al. (2003); Bobrowicz et al. (2004)). With the ability to replicate certain human glycosylation patterns, yeast-based expression platforms offer an attractive alternative to current mammalian cell culture processes due to a variety of advantages including faster growth rates (and hence shorter culture times), cheaper operating costs, simple chemically defined media, no viral contamination and shorter development times from gene to protein.

In order to develop optimal culture strategies for the achievement of maximal levels of recombinant proteins with *P. pastoris* strains, it is important to have a good understanding of the influence of culture conditions on the physiology and the regulation of this expression system. According to the literature, factors such as temperature (Whittaker and Whittaker (2000); Li et al. (2001); Jahic et al. (2003b); Li et al. (2003); Shi et al. (2003)), pH (Sreekrishna et al. (1997); Inan et al. (1999)), composition of the feed medium (Boze et al. (2001); Xie et al. (2005); Jungo et al. (2007)) and specific growth rate (D'Anjou and Daugulis (2001)) have to be optimized in order to achieve high productivities of recombinant protein with *P. pastoris* strains.

2. OBJECTIVES AND STRATEGY OF THE THESIS

2. 1. Objectives

The goal of this thesis was to characterize the growth stoichiometry, the specific heterologous protein productivity and the regulation of the alcohol oxidase enzyme of a recombinant *Pichia pastoris* Mut^+ strain.

A recombinant *Pichia pastoris* Mut^+ strain expressing and secreting avidin was chosen as case study. The investigation focused on an analysis of the influence of specific growth rate, culture temperature and nutritional regime. In particular, the use of mixed substrates (methanol and glycerol, methanol and sorbitol) during the induction phase, which is conventionally performed on methanol as sole carbon source, was evaluated.

2. 2. Strategy

In order to have a better insight into the physiology and the regulation of recombinant *P. pastoris* Mut^+ strains, cultures in continuous mode were essentially performed because they allow for a good control of cultivation conditions. Conventional chemostat

cultures and continuous cultures with transient changes of culture parameters were performed. Indeed, part of the work of this thesis was based on transient experiments in continuous cultures at pseudo-steady state in order to do strain characterization with fewer experiments and therefore to speed up bioprocess development. Moreover, in order to gain insights into the regulation of the metabolism of *Pichia pastoris* Mut^+ strains, transient experiments in continuous cultures with sudden changes in cultures parameters were also performed.

This quantitative study of the physiology and metabolic regulation in continuous cultures enabled the design of an optimal high cell density fed-batch strategy for the production of recombinant avidin with a *P. pastoris* Mut^+ strain.

3. METABOLISM AND EXPRESSION SYSTEM OF *PICHIA PASTORIS*

3. 1. Glycerol utilisation pathways

In standard cultures with recombinant *P. pastoris* strains for the production of foreign proteins with induction by methanol, *P. pastoris* is usually grown first on glycerol as carbon source, since biomass yield and maximum specific growth rate are higher for growth on glycerol than for growth on methanol. Moreover, during growth on glycerol, recombinant protein expression is repressed, which avoids the selection of non-expressing mutants during biomass generation.

Use of glucose as carbon source is usually avoided because higher amounts of the by-product ethanol is observed than during growth on glycerol (Macauley-Patrick et al. (2005)). Residual ethanol concentrations repress the alcohol oxidase promoter, even at levels of around 10-50 mg L^{-1} (Inan and Meagher (2001b)).

The simplified metabolic pathways on glycerol are schematically shown in figure 1 (Ren et al. (2003)).

The glycerol catabolic pathway includes phosphorylation 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 (Nevoigt and Stahl (1997)). The dihydroxyacetone phosphate formed enters the glycolytic pathway. A few yeast species have an alternative pathway for dissimilating glycerol which involves a NAD-dependent glycerol

dehydrogenase and a dihydroxyacetone kinase. However this pathway seems to be of less importance (Nevoigt and Stahl (1997)).

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 many 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. The pentose phosphate pathway, which serves to generate NADPH and enables the synthesis of pentose sugars, and gluconeogenesis, which regulates the provision of buiding blocks for biosynthetic reactions, were not represented in figure 1 for simplification but are of course also involved in the metabolism of *P. pastoris*.

The alcoholic fermentation starting from pyruvate may be triggered by a limitation in the respiratory capacity (Sonnleitner and Käppeli (1986)) and/or by a limitation in the pyruvate dehydrogenase bypass (conversion of acetaldehyde to acetate) (Lei et al. (2001)). In this fermentative bypass, pyruvate is converted to acetaldehyde by pyruvate decarboxylase and further reduced to ethanol by alcohol dehydrogenase. Ethanol may also be used as a substrate, if the limitations mentioned above are removed (Inan and Meagher (2001b); Lei et al. (2001)). In this case, ethanol will be oxidized to acetaldehyde, then to acetate, and finally converted to acetyl-CoA by acetyl-CoA synthetase (Pronk et al. (1996)). However, according to Ren et al. (2003), the residual ethanol concentration measured during growth on glycerol is negligible. Hence, for simplification, ethanol formation can be neglected when oxygen is not limiting. In contrast to *Saccharomyces cerevisiae*, which is a Crabtree-positive yeast, *P. pastoris* is Crabtree-negative.

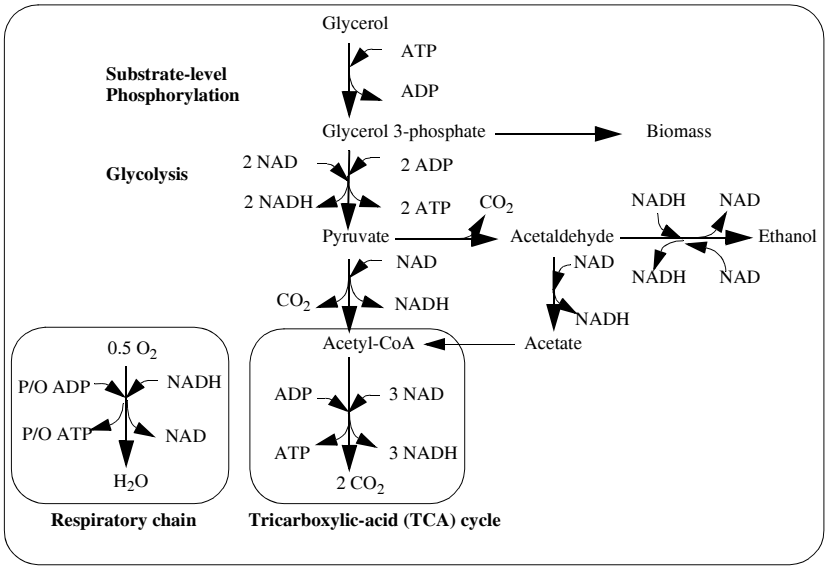

Figure 1: Simplified metabolism of *Pichia pastoris* growing on glycerol

3. 2. Methanol utilisation pathways

The facultative methylotrophic yeast species that belong to the four genera *Hansenula, Pichia, Candida* and *Torulopsis* share a specific methanol utilization pathway (Sibirny et al. (1988); Sahm (1977); Gleeson and Sudbery (1988); Gellissen (2000); Zhang et al. (2000)). The main reactions involved in the methanol utilization pathways are schematically represented in figure 2.

Adaptation to growth on methanol is associated with induction of alcohol oxidase (AOX), dihydroxyacetone synthase (DAS) and several other enzymes involved in methanol metabolism (Hartner and Glieder (2006)). In cells grown on methanol, AOX can account for up to 35% of total cell protein, but is virtually absent in cells grown on glucose, glycerol or ethanol. The enzyme alcohol oxidase catalyses the first step in the dissimilation of methanol, which consists in the oxidation of methanol to formaldehyde and hydrogen peroxide using molecular oxygen. This reaction takes place within specialized organelles called peroxisomes (Veenhuis et al. (1983)) in order to avoid the

toxic effect of hydrogen peroxide concomitantly produced during this reaction (Sreekrishna and Kropp (1996)). Peroxisomal catalase ensures the degradation of hydrogen peroxide to water and molecular oxygen. In cells grown on methanol, peroxisomes can account for over 80% of the cell volume.

Formaldehyde enters the cytosol to some extent, where it forms a complex with reduced glutathione and is oxidized to carbon dioxide by two subsequent dehydrogenase reactions. Glutathione-dependent formaldehyde dehydrogenase catalyzes the production of formate, from which carbon dioxide is produced by the action of formate dehydrogenase. 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 (Lee et al. (2002)).

Formaldehyde can be reduced to methanol by the NADH-dependent formaldehyde reductase enzyme (Sibirny et al. (1988)). Formaldehyde reductase and alcohol oxidase form a futile cycle which regulates the cellular content of formaldehyde and NADH.

The remaining formaldehyde reacts in a transketolase reaction with xylulose-5-phosphate (Xu_5P) to yield dihydroxyacetone (DHA) and glyceraldehyde-3-phosphate (GAP). This reaction is catalysed by the peroxisomal dihydroxyacetone synthase. The formed C_3 compounds are further assimilated within the cytosol and will serve as building blocks for biomass synthesis. Dihydroxyacetone is phosphorylated by a dihydroxyacetone kinase (Lüers et al. (1998)), and subsequently, in an aldolase reaction with glyceraldehyde-3-phosphate, it forms fructose-1,6-bisphosphate (FBP), which is then converted to fructose-6-phosphate (F_6P) by a phosphatase. Fructose-6-phosphate enters the pentose phosphate pathway to regenerate xylulose-5-phosphate. For every three cycles, one net molecule of glyceraldehyde-3-phosphate is produced and utilized in order to form biomass by standard reactions of gluconeogenesis.

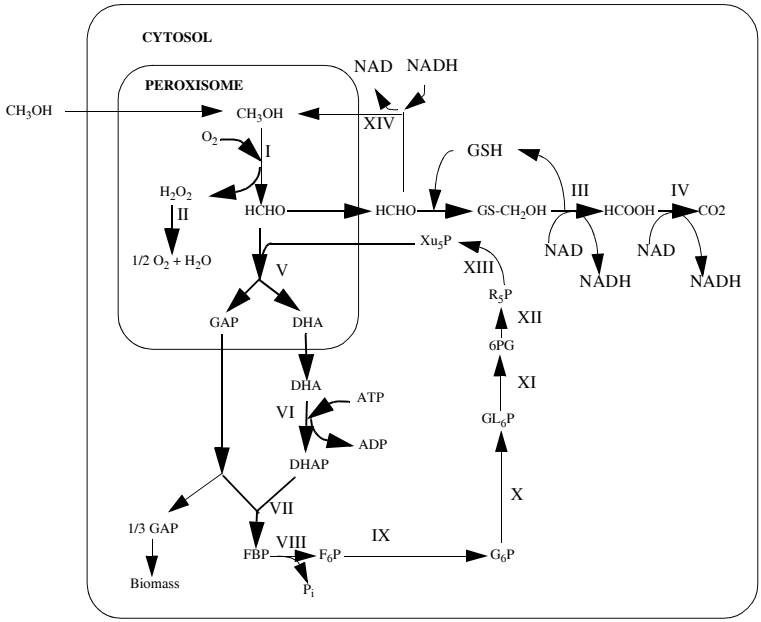

Figure 2: The methanol utilization pathway and its compartmentalization in methylotrophs (Egli et al. (1982b); Gleeson and Sudbery (1988); Sibirny et al. (1988); Gellissen (2000); Zhang et al. (2000)) I, alcohol oxidase; II, catalase; III, glutathione-dependent formaldehyde dehydrogenase; IV, formate dehydrogenase; V, dihydroxyacetone synthase; VI, dihydroxyacetone kinase; VII, fructose 1,6-bisphosphate aldolase; VIII, fructose 1,6-bisphosphatase; IX, glucose-6-phosphate isomerase; X, glucose-6-phosphate dehydrogenase; XI, gluconolactonase; XII, 6-phosphogluconate dehydrogenase; XIII, ribulose-5-phophate-3-epimerase; XIV, formaldehyde reductase; GAP, glyceraldehyde 3-phosphate; DHA, dihydroxyacetone; DHAP, dihydroxyacetone phosphate; FBP, fructose 1,6-bisphosphate; F_6P, fructose 6-phosphate; G_6P, glucose 6-phosphate; GL_6P, gluconolactone-6-phosphate; 6PG, 6-phosphogluconate; R_5P, ribulose-5-phosphate; Xu_5P, xylulose 5-phosphate; GSH, reduced glutathione (L-gamma-Glutamyl-L-Cysteinylglycine); GS-CH_2OH, S-hydroxymethylglutathione.

The simplified metabolic pathways of the central metabolism in methylotrophic yeasts, in the presence of methanol as carbon source, is schematically shown in figure 3 (Ren et al. (2003)).

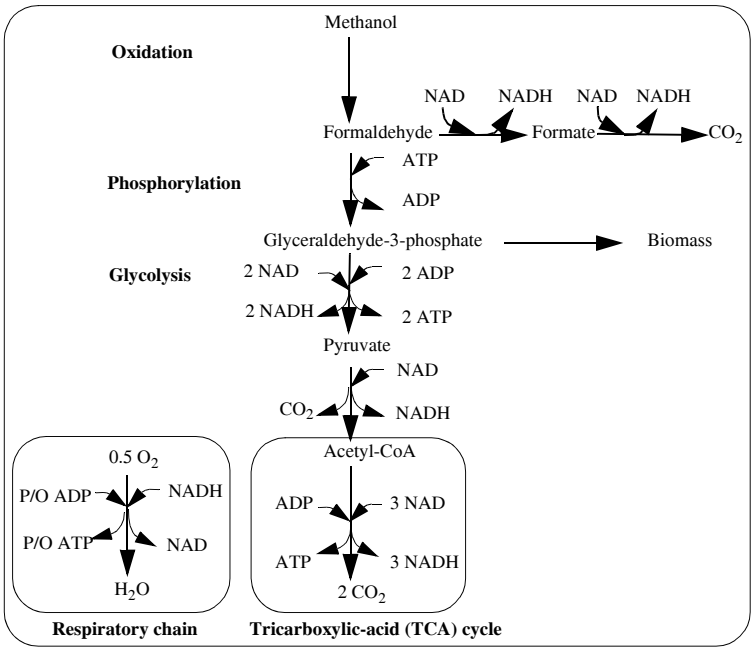

Figure 3: Simplified metabolism of *Pichia pastoris* growing on methanol.

The TCA cycle is essential in supplying energy for the methylotrophic growth of yeasts. Most energy comes from the assimilatory pathway, while the primary role of the enzymes formaldehyde dehydrogenase and formate dehydrogenase is to protect the cell from toxic levels of formaldehyde caused by a high residual methanol concentration in the medium (Sibirny et al. (1990)).

The main function of the respiratory chain is of course to yield energy in the form of ATP.

3. 3. Expression with the AOX1 promoter

Heterologous proteins are usually expressed in *P. pastoris* strains under the control of the strong, tightly regulated, and methanol-induced alcohol oxidase promoter AOX1.

This promoter drives the expression of the enzyme alcohol oxidase 1 in the wild-type strain.

Ellis et al. (1985) first isolated the AOX1 gene. The genome of *P. pastoris* contains two alcohol oxidase genes, AOX1 and AOX2 (Koutz et al. (1989)). AOX1 is responsible for the majority of alcohol oxidase activity in the cell (Cregg et al. (1989)). Large amounts of this enzyme are synthesized because alcohol oxidase has a very low affinity for oxygen (Couderc and Baratti (1980), Veenhuis et al. (1983)). Indeed, in cultures on methanol as carbon source, AOX can account for over 30% of the total cell protein (Couderc and Baratti (1980)). This strong AOX1 promoter can therefore be used to drive the expression of recombinant proteins to high levels, up to 12 g L^{-1} of recombinant protein (Cregg et al. (1993)).

Expression of AOX1 is tightly regulated at the level of transcription (Ellis et al. (1985)) and is controlled by both repression/derepression and induction mechanisms (Tschopp et al. (1987a); Ozimek et al. (2005)) similar to the regulation of alcohol oxidase in the methylotrophic yeast *H. polymorpha* (Egli et al. (1980)). However, unlike for *H. polymorpha*, the presence of methanol is essential to induce high levels of transcription (Tschopp et al. (1987a)).

With regard to methanol-utilizing ability, three phenotypes of expression strains are available (Stratton et al. (1998)):

- Methanol utilization positive (Mut^+): presence of functional AOX1 and AOX2 genes; growth on methanol at the wild-type rate.
- Methanol utilization slow (Mut^S): the AOX1 gene is disrupted, only the AOX2 gene is functional; methanol metabolism is dependent on the transcriptionally weaker AOX2 gene.
- Mehanol utilization negative (Mut^-): both AOX1 and AOX2 genes are disrupted; methanol cannot be metabolized at all, but methanol is necessary for induction of recombinant protein expression.

The maximum specific growth rate on methanol is 0.14 h^{-1}, 0.04 h^{-1} and 0.0 h^{-1} for Mut^+, Mut^S and Mut^- *P. pastoris* strains, respectively (Stratton et al. (1998)).

The standard protocol for the expression of recombinant proteins under the control of the AOX1 promoter is usually performed in three-stage high cell density fed-batch cultures (Stratton et al. (1998)). First, cells are grown in batch culture on glycerol in order to achieve high cell densities rapidly while repressing foreign gene expression since

the AOX1 promoter is repressed by unlimited growth on glycerol (Tschopp et al. (1987a)). Secondly, glycerol is fed at limiting concentrations in order to increase biomass concentration further and to derepress the methanol metabolic machinery. This transition phase allows to derepress the enzymes necessary for the dissimilation of methanol gradually and reduces the time necessary for the cells to adapt to growth on methanol (Chiruvolu et al. (1997)). Finally, recombinant protein production is induced by the addition of methanol in fed-batch mode.

3. 4. Expression with alternative promoters to the AOX1 promoter

The *P. pastoris* glyceraldehyde-3-phosphate dehydrogenase gene (GAP) (Waterham et al. (1997)) and glutathione-dependent formaldehyde dehydrogenase (FLD1) (Shen et al. (1998)) promoters are interesting alternatives to the AOX1 promoter.

Glyceraldehyde-3-phosphate dehydrogenase is involved in glycolysis and gluconeogenesis and is often expressed constitutively and at high levels. Waterham et al. (1997) isolated and employed the GAP promoter for expression of recombinant β-lactamase. They showed that this promoter provides strong constitutive expression on glucose at levels comparable to that seen with the AOX1 promoter. Various carbon sources have been tested for use with GAP promoters, such as glucose, glycerol, oleic acid and methanol, with glucose found to result in the highest expression levels.

Glutathione-dependent formaldehyde dehydrogenase is a key enzyme in methylotrophic yeasts and is required for the dissimilation of methanol as a carbon source and certain alkylated amines such as methylamine as nitrogen source. Shen et al. (1998) isolated and employed the FLD1 promoter for expression of recombinant β-lactamase. They showed that this promoter is strongly and independently induced by either methanol as sole carbon source (with ammonium sulfate as nitrogen source) or methylamine as sole nitrogen source (with glucose as carbon source). The levels of β-lactamase produced under the control of the FLD1 promoter were comparable to those obtained with the commonly used AOX1 promoter.

The GAP and the FLD1 promoters for expression of recombinant proteins with *P. pastoris* represent interesting alternatives to the AOX1 promoter because use of methanol as carbon source can be avoided with these promoters. Development of fermentation protocols for the optimal expression of recombinant proteins under the control of the GAP or the FLD1 promoter is necessary. Resina et al. (2005) developed a high cell density fed-batch cultivation strategy for recombinant *Rhizopus oryzae* lipase produc-

tion in *P. pastoris* using the FLD1 promoter, with methylamine and sorbitol as nitrogen and carbon sources, respectively, during the induction phase. Results showed that the productivity in recombinant protein could be increased by up to 1.9-fold compared to expression with the AOX1 promoter.

Recently the 3-phosphoglycerate kinase (PGK1) promoter has been examined as an alternative to constitutive heterologous expression in *P. pastoris* (de Almeida et al. (2005)). The α-amylase gene from *Bacillus subtilis* was placed under the control of the PGK1 promoter in *P. pastoris* as case study. Comparisons were done with the AOX1 promoter for growth on various carbon sources: glucose, glycerol and methanol. Similary to the GAP promoter, the PGK1 promoter showed high efficiency of expression on these three carbon sources, with the highest expression level on glucose. Expression level on methanol as carbon source was higher than with the AOX1 promoter.

3. 5. Influence of temperature on growth and recombinant protein production in *P. pastoris* cultures

Temperature is an important environmental parameter for cell growth. Microorganisms do not have the ability to regulate their internal temperature. Hence, cell temperature is always equal to environmental temperature and all cellular biochemical reactions taking place depend directly on the external temperature. Moreover, not only the reaction rates, but also metabolic regulation, nutritional requirements and biomass composition will be affected by temperature.

According to their temperature optima, organisms can be classified in three groups: 1) psychrophiles (T_{opt} < 20°C), 2) mesophiles (T_{opt} = 20 to 50°C), and 3) thermophiles (T_{opt} > 50°C), Shuler and Kargi (2002a).

Most processes with *P. pastoris* are run at an optimum temperature for growth of 30°C (Wegner (1983)) since few studies have been carried out on the effect of temperature on recombinant protein production in *P. pastoris*. It has been stated that temperatures above 32°C can be detrimental to protein expression and may lead to cell death (Invitrogen (2002)). Inan et al. (1999) observed that temperatures above 30°C were not appropriate for the production of a recombinant peptide. It is well known that elevated temperatures result in cell death, which will lead to cell lysis and higher protease activity in fermentation media.

Lower cultivation temperatures can influence yields of recombinant proteins because the rate of proteolysis is lowered for kinetic reasons, and because proteolysis is reduced due to lower cell death and protease release to the medium. Expression of recombinant proteins at lower temperature also helps to reduce protein misfolding and to produce more properly folded proteins to be secreted into the culture medium (Georgiou and Valax (1996)). It has been reported that a lower cultivation temperature with *P. pastoris* cultures can lead to an increase in the yield of recombinant proteins (Chen et al. (2000); Whittaker and Whittaker (2000); Li et al. (2001); Hong et al. (2002); Sarramegna et al. (2002); Jahic et al. (2003a); Jahic et al. (2003b); Li et al. (2003); Shi et al. (2003)). Table 2 summarizes the values of the lower temperatures used during these processes in order to improve recombinant protein production with *P. pastoris* strains (Table 1). Howerver, it has to be pointed out that temperatures below 30°C do not significantly influence the production of other recombinant proteins expressed by *P. pastoris* strains (Inan et al. (1999); Curvers et al. (2001); Kupesulik and Sevella (2005)).

Table 1: Some examples of use of lower temperatures during *P. pastoris* processes for the improvement of recombinant protein production

Reference	Produced recombinant protein	Temperature during induction phase on methanol
Chen et al. (2000)	Human α-galactosidase A	25°C
Whittaker and Whittaker (2000)	Galactose oxidase	25°C
Li et al. (2001)	Herring Antifreeze protein	23°C
Hong et al. (2002)	Laccase	20°C
Sarramegna et al. (2002)	Human μ-opioid receptor	15, 20, 25°C
Shi et al. (2003)	Single-chain antibody	15°C
Li et al. (2003)	Reg IV	26°C
Jahic et al. (2003a)	Fusion protein composed of a cellulose-binding module and lipase B	22°C
Jahic et al. (2003b)	Fusion protein composed of a cellulose-binding module and lipase B	Temperature limited fed-batch control (T_{min} =12°C)

3. 6. Influence of pH on growth and recombinant protein production in *P. pastoris* cultures

Hydrogen-ion concentration (pH) affects the activity of enzymes and therefore the growth rate. The optimal pH for growth may be different from that for product formation. The pH optimum for growth for many bacteria ranges from 3 to 8; for yeasts, from 3 to 6; for molds, from 3 to 7; for plant cells, from 5 to 6; and for animal cells, from 6.5 to 7.5 (Shuler and Kargi (2002a)). Many microorganisms have mechanisms to maintain intracellular pH at a relatively constant level in the case of fluctuations in environmental pH. However, when the extracellular pH differs from the optimal value, the maintenance-energy requirements increase. Hence, bioprocesses are often performed at constant pH in the optimal pH range for growth.

In high cell density fed-batch cultures with *P. pastoris* strains, the final protein yield is sometimes reduced because of proteolytic degradation (Clare et al. (1991); Scorer et

al. (1993); Kobayashi et al. (2000)). One of the methods of minimizing proteolytic degradation is to set the pH during the methanol induction phase at a value that is not optimal for protease activity. Another possibility is to add amino acid rich supplements, such as peptone or casamino acids, to the culture medium (Clare et al. (1991)). The peptone can act as alternative and competing substrate for one or more proteases and can also repress protease induction caused by nitrogen limitation. However this possibility has the drawback of working with a medium that is no longer well defined, which will complicate downstream processes in the case of secreted recombinant proteins.
P. pastoris is capable of growing over a relatively broad pH range from 3.0 to 7.0, more preferably and usually about 3.5 to 5.5. This range has little effect on the growth rate (Wegner (1983); Inan et al. (1999)), which allows considerable freedom in adjusting the pH to one that is not optimal for a problem protease (Sreekrishna et al. (1997)). Inan et al. (1999) determined the optimum pH, temperature and glycerol feed rate for the production of a recombinant peptide: the pH was the most significant factor affecting yield, specific yield and specific activity of the produced peptide. In the literature, different pH values have been found to be optimal from a recombinant protein's stability point of view. One possibility is to lower proteolytic degradation by a pH drop from 5.0 during the growth phase on glycerol to 3.0 during the induction phase on methanol (Scorer et al. (1993)) since alkalic and neutral proteases are generally responsible for damage to recombinant proteins in the culture broth. However, depending on the nature of the produced recombinant protein, different optimal pH ranges have been reported in the literature. Table 2 provides some examples.

Table 2: Some examples of optimal pH values for the production of recombinant proteins with *P. pastoris* strains

Reference	Produced recombinant protein	Optimal pH for recombinant protein production
Zhu et al. (1995)	Coffee bean α-galactosidase	6.5[a]; 4-5[b]
Inan et al. (1999)	Hookworm anticoagulant peptide	7.0[b]
Chen et al. (2000)	Human α-galactosidase A	6.0[a,b]
Kobayashi et al. (2000)	Human serum albumin	> 5.9[b]
Koganesawa et al. (2002)	Cytokine growth-blocking peptide	3.0
Sarramegna et al. (2002)	Human μ-opioid receptor	10.0[a]
Jahic et al. (2003a)	Fusion protein composed of a cellulose-binding module and lipase B	4.0[b]
Shi et al. (2003)	Single-chain antibody	7.0[a]
Damasceno et al. (2004)	Single-chain Fv antibody fragment	3.0[b]
Kupesulik and Sevella (2005)	Human serum albumin	5.6[b]

a. shake flask cultures
b. high cell density fed-batch cultures in bioreactors

Care has to be taken when setting the pH of the medium to low values, since inefficient utilization of carbon source may occur resulting in low biomass yields (Chiruvolu et al. (1998)). This is due to energy being required to overcome the large difference between intracellular pH (around 6.0) and external pH of the medium (Dombek and Ingram (1987), Imai and Ohno (1995)).

3. 7. Some advantages and drawbacks of *P. pastoris* as an expression system

P. pastoris is widely used as an expression system for heterologous proteins (Cregg et al. (1993); Hollenberg and Gellissen (1997); Cereghino and Cregg (2000); Gellissen

(2000); Cereghino et al. (2002); Gerngross (2004); Daly and Hearn (2005)) because many characteristics of this yeast make it an attractive expression system.

First of all, techniques for genetic modifications are available. They are similar to those described for *S. cerevisiae* and expression vectors are provided by the Invitrogen company and can be integrated directly into the *P. pastoris* genome in order to maximize stability of expression systems.

P. pastoris has a strong preference for respiratory growth, as opposed to fermentative growth. This is a major advantage relative to the well-known yeast *S. cerevisiae* because *P. pastoris* can be cultured to extremely high cell densities (> 100 g L^{-1} cell dry weight), without production of overflow metabolites. This characteristic is especially important for secreted proteins, as the concentration of product in the medium is roughly proportional to the concentration of cells in culture.

P. pastoris efficiently produces recombinant proteins intracellularly, but recombinant proteins can also be secreted into the fermentation broth. Wegner (1990) pointed out that both yeast and mammalian secretion-signal sequences have been found to function with this yeast. This greatly simplifies downstream processing as the cells do not have to be disrupted in order to recover recombinant proteins. Moreover, purification of secreted recombinant proteins is facilitated by the fact that *P. pastoris* itself secretes very few proteins to the cultures medium (Tschopp et al. (1987b); Cereghino and Cregg (2000)).

P. pastoris has also the ability to perform some eukaryotic post-translational modifications, such as processing of signal sequences, folding, disulfide bridge formation, certain types of lipid addition and O- and N-linked glycosylation. This ability is also a major advantage over bacterial expression systems. However, in contrast to mammals, where O-linked oligosaccharides are composed of a variety of sugars, *P. pastoris* is only capable of adding O-oligosaccharides composed of mannose residues. Moreover, Daly and Hearn (2005) pointed out that although *P. pastoris* does not generally hyperglycosylate, there are some recombinant proteins expressed by *P. pastoris* strains which have been found to be hyperglycosylated, in a similar way to *S. cerevisiae*.

In spite of the inability of *P. pastoris* to modify proteins with human glycosylation structures, recent advances in genetic engineering of human glycosylation pathways into yeasts have shown significant promise and are challenging the current dominance of therapeutic protein production based on mammalian cell culture (Choi et al. (2003); Ha-

milton et al. (2003); Bobrowicz et al. (2004)). An interesting point is that «humanized» glycoproteins with exceptional glycan uniformity could be obtained with glycoengineered *P. pastoris* strains (Gerngross (2004)).

Usually recombinant proteins are expressed under the control of the strong, tightly regulated and methanol-induced alcohol oxidase promoter AOX1, which drives the expression of the enzyme alcohol oxidase 1 in the wild-type strain. An advantage with this tight regulation is that if recombinant proteins are toxic to the yeast, growth under repressing carbon sources avoids selection of non-expressing mutants during biomass generation.

Defined and inexpensive media can be used for large-scale production of heterologous proteins. The medium components consist of pure carbon sources (glycerol, methanol), biotin, salts, trace elements, and water. The medium is free of undefined ingredients that can be sources of pyrogens or toxins and is therefore compatible with the production of human pharmaceuticals.

However, the *Pichia pastoris* expression system has also several drawbacks. At high cell densities, high amounts of heat are produced during the induction phase on methanol because of the high enthaply of combustion of methanol (-727 kJ C-mol^{-1}, Weast (1980)). High oxygen consumption rates are also achieved during the induction phase on methanol, so that very high oxygen transfer rates are required. Technical adaptations such as high performance stirrers, high cooling capacities and aeration with pure oxygen are usually necessary for the achievement of high cell densities with *P. pastoris* cultures grown on methanol, even in laboratory scale reactors. In large-scale *P. pastoris* cultures, the amount of methanol required by the cells can be potentially dangerous due to the flammability of methanol. Because of safety and economic aspects, the use of pure oxygen also represents a drawback with this expression system.

Another drawback is that the *P. pastoris* expression system has been patented. Research Corporation Technologies (Tucson, AZ, USA) are the current holders of the patent for the *P. pastoris* expression system, which is available as a kit from Invitrogen Corporation (Carlsbad, CA, USA). This implies that royalties have to be paid by companies who want to use this expression system for recombinant protein production.

4. SINGLE AND MULTIPLE NUTRIENT LIMITED GROWTH

The study of nutrient limitations in biological processes is important since nutrient limitation is one of the most powerful tools to force cultures to perform in a predetermined way. The metabolism of a culture depends on the limitation under which the culture is grown. Hence, the application of appropriate nutrient limitations can be used as a means to force the metabolism into the optimal state for synthesis of a desired product.

4. 1. Single nutrient limitations in chemostat cultures: Kinetic and stoichiometric considerations

A simple unstructured mathematical model was developed by von Stockar and Auberson (1992) for the quantitative prediction for the behaviour of chemostat cultures of yeasts. According to von Stockar and Auberson (1992), growth kinetics in chemostats is governed by the uptake rate of the limiting substate and not vice versa. Growth is a consequence of substrate uptake and not the cause.

With chemoheterotrophs, it is usually the carbon and energy substrate that limits growth in chemostat culture, hence the kinetics of the whole process are governed by the uptake kinetics of this substrate. Usually carbon sources (for instance glucose) are taken up by facilitated diffusion or active transport, hence the uptake kinetics can be described by writing an equation analogous to Michaelis-Menten kinetics (Shuler and Kargi (2002b)):

$$q_S = q_S^{max} \cdot \frac{C_S}{C_S + K_S} \qquad (1)$$

where q_S is the specific substrate consumption rate, q_S^{max} the maximal specific substrate consumption rate, C_S the concentration of the limiting nutrient and K_S the Monod substrate saturation constant.

The specific growth rate is obtained by multiplying equation 1 by the respective stoichiometric yield.

$$\mu = Y_{X/S} \cdot q_S^{max} \cdot \frac{C_S}{C_S + K_S} \qquad (2)$$

where $Y_{X/S}$ is the biomass yield. The last equation corresponds to the Monod equation.

At low dilution rates, maintenance effects are often observed, with the biomass concentration decreasing at low dilution rates because part of the carbon source is not used for growth but for maintenance purposes. In order to extend the model for substrate consumption due to maintenance, the stoichiometry of the growth equation ($Y_{X/S}$, $Y_{N/S}$ yields for instance) is assumed to stay unchanged, but an additional consumption of carbon and energy substrate proceeds in parallel (equation 3).

$$q_S = q_S^{growth} + m \tag{3}$$

where q_S^{growth} is the specific substrate consumption rate for growth and m is the specific substrate consumption rate for maintenance purposes. q_S^{growth} is stoichiometrically related to the specific growth rate μ as follows:

$$\mu = Y_{X/S} \cdot q_S^{growth} \tag{4}$$

By substituting q_S^{growth} in equation 3 by means of equation 4 and dividing by μ, an expression for the apparent growth yield ($Y_{X/S}^{app}$) resulting from both growth and maintenance is obtained (equation 5), which is known as the linear Herbert-Pirt equation.

$$\frac{q_S}{\mu} = \frac{1}{Y_{X/S}^{app}} = \frac{1}{Y_{X/S}} + \frac{m}{\mu} \tag{5}$$

By combining equations 1 and 5, one obtains a relationship between μ and C_S (equation 6), which replaces Monod's equation (equation 2).

$$\mu = Y_{X/S} \cdot \left(q_S^{max} \cdot \frac{C_S}{C_S + K_S} - m \right) \tag{6}$$

Since growth kinetics in chemostat cultures are determined by the uptake rate of the limiting substrate, the substate uptake kinetics also determine the uptake kinetics of all the non-limiting substrates present in the culture broth. The non-limiting substrates cannot therefore be consumed according to their intrinsic, biological uptake kinetics.

Let us consider nitrogen as non-limiting substrate and carbon as limiting substrate. A mass balance for the nitrogen source over the whole bioreactor in continuous mode leads to the following balance (it is supposed that the nitrogen source is in liquid state):

$$D \cdot (C_{N,f} - C_N) = q_N \cdot X + \frac{dC_N}{dt} \quad (7)$$

where $C_{N,f}$ and C_N are the nitrogen source concentrations in the feed medium and in the culture medium, q_N is the specific nitrogen source consumption rate, D the dilution rate and X the dry cell weight concentration.
The accumulation term dC_N/dt is zero under steady-state growth conditions, hence one obtains the following balance for steady-state growth conditions:

$$D \cdot (C_{N,f} - C_N) = q_N \cdot X \quad (8)$$

Figure 4 represents schematically the intrinsic kinetic model for nitrogen source uptake (bold line). The straight line represents the terms of the left part of equation 8, which corresponds to the difference between nitrogen source influx and outflux from the chemostat culture.

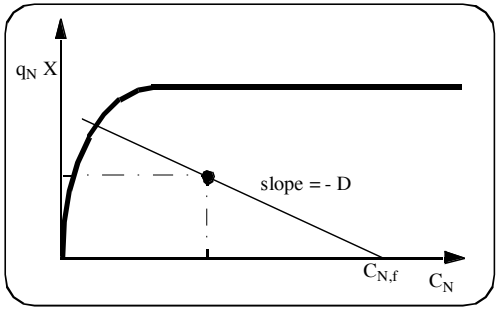

Figure 4: Amount of nitrogen source consumption per unit time as a function of the residual concentration of nitrogen source in the culture medium during single carbon source limited growth. Bold line: Intrinsic kinetic model for nitrogen source uptake, straight line: Difference between the nitrogen source influx and outflux during chemostat culture.

If the nitrogen source would be the limiting substrate, the culture would operate at the intersection of the kinetic curve (bold line) and the straight operating balance line.

However, because the carbon source is limiting growth, the nitrogen uptake rate will be lower and given for instance by the horizontal dashed line (figure 4).
von Stockar and Auberson (1992) defined therefore a *limiting substrate* in the following manner: «A limiting substrate is one which is taken up according to the intrinsic biological kinetics and for which the culture operates on the respective kinetic curve (bold line in figure 4). A non-limiting substrate will be taken up at a lower rate dictated by stoichiometric considerations».
For nutrients taken up by free diffusion, the relationship between the residual concentration of the limiting nutrient and its specific uptake rate is somewhat different from equation 1. For instance, small lipid-soluble molecules such as glycerol, methanol and ethanol are transported into yeasts to an appreciable extent by free diffusion (Walker (1998)). The driving force will depend on both the intracellular concentration and the extracellular concentration of limiting substrate. In fact, molecules will move down a concentration gradient (from high to low concentration) that is thermodynamically favorable. Hence,

$$J_S = K_P \cdot (C_{S,E} - C_{S,I}) \tag{9}$$

where J_S is the flux of nutrient S across the membrane (C-mol dm^{-2} s^{-1}), K_p is the permeability (dm s^{-1}), $C_{S,E}$ and $C_{S,I}$ are respectively the extracellular and intracellular concentrations of nutrient S (C-mol L^{-1}), Shuler and Kargi (2002b). The specific uptake rate of nutrients taken up by free diffusion can be expressed by multiplying equation 9 by the specific area of cells, α (expressed in dm^2 C-mol^{-1}), Duboc and von Stockar (1998). Therefore,

$$q_S = K_P \cdot \alpha \cdot (C_{S,E} - C_{S,I}) \tag{10}$$

Hence, in this case, equation 6 will become

$$\mu = Y_{X/S} \cdot (K_P \cdot \alpha \cdot (C_{S,E} - C_{S,I}) - m) \tag{11}$$

The diffusion rate of the limiting nutrient must equal the intracellular conversion rate which can be described by a Michaelis-Menten saturation kinetic expression with respect to the intracellular limiting nutrient concentration:

$$q_s = q_S^{max} \cdot \frac{C_{S,I}}{C_{S,I} + K_S} \qquad (12)$$

With equations 10 and 12, one can calculate the specific limiting nutrient conversion rate q_S as a function of the extracellular concentration of S ($C_{S,E}$).

At high extracellular residual concentrations of nutrient S, growth is always limited by the enzymatic conversion of nutrient S because the enzymatic machinery reaches a saturation limit. However, at low residual concentrations of nutrient S, the relative importance of the reaction rate, as compared to the transfer rate can be evaluated by comparing the initial slopes of the two curves: for equation 12, q_S^{max}/K_S and for equation 10, $K_P \alpha$ respectively. If $q_S^{max}/K_S << K_P\alpha$, the uptake rate of nutrient S is mainly limited by its intracellular enzymatic conversion, and if $q_S^{max}/K_S >> K_P\alpha$, the uptake rate of nutrient S is mainly limited by the transfer through the cell membrane (Duboc and von Stockar (1998)).

4. 2. Stoichiometric relationships between limiting and non-limiting substrates

If cells are growing in continuous culture with no other limitation than for instance the carbon source, they do so according to a defined stoichiometry. Hence, the specific uptake (or production) rates of all other substrates (or products) should be tightly coupled to the carbon source uptake rate. For example, in the case where the carbon source is the limiting substrate and the nitrogen source is in excess, if the uptake rates of these substrates (q_C and q_N respectively) are measured as a function of dilution rate and plotted against one another a straight line should result (figure 5a). The slope of this line corresponds to the stoichiometric yield of nitrogen source with respect to carbon source consumption rates ($Y_{N/C}$).

In a chemostat culture limited by the carbon source, we can consider that «all» of this single limiting substrate fed to the culture is consumed. Hence the specific substrate consumption rate q_C on the x axis of figure 5a will be equal to the specific substrate supply rate.

At low supply of carbon source (point A in figure 5a), the uptake rate of the nitrogen source, which is determined by the straight line of slope $Y_{N/C}$, will be smaller than the nitrogen source supply rate (horizontal line in figure 5a). Therefore a certain amount of the nitrogen source is present in excess and will remain unused in the culture broth.

As the supply rate of the carbon source is increased, the consumption rates of the carbon source and of the nitrogen source will increase until the latter reaches the rate at which the nitrogen source is supplied to the culture (point B in figure 5a). At this point, the nitrogen source becomes also limiting and the culture can be considered as dually limited in carbon and nitrogen source. At higher supply rates of carbon source, two different limiting behaviours can result.

If the metabolism remains strictly the same, i.e. the ratio of nitrogen to carbon source uptake rates ($Y_{N/C}$) stays constant, the culture will be single limited in nitrogen source. Part of the carbon source supplied to the culture can no longer be taken up (point C in figure 5a). The data can also be plotted as a function of the ratio of carbon to nitrogen supply rates $C_{C,f}/C_{N,f}$ (figure 5b).

Another possibility is that the culture can change its metabolism in such a way that the excess carbon source is also completely consumed. In this situation, the excess carbon source supply is accumulated for instance as intracellular carbohydrates (polyhydroxyalkanolates in the case of *Pseudomonas oleovorans,* Durner (1998), Zinn (1998), or glycogen in the case of yeasts, Duboc (1997)), or overflow metabolites are produced (for instance ethanol in the case of Crabtree positive yeasts, Duboc (1997)). The yield of nitrogen source with respect to carbon source consumption rates ($Y_{N/C}$) will be lower because the culture cannot take up more nitrogen than the amount indicated by the horizontal line (figure 6, supply C), but degrades an amount of carbon source which is higher than before.

If the supply rate of carbon source is further increased (figure 6, supply D), single nitrogen limitation can be observed. Part of the carbon source supplied to the culture can no longer be taken up by the cells.

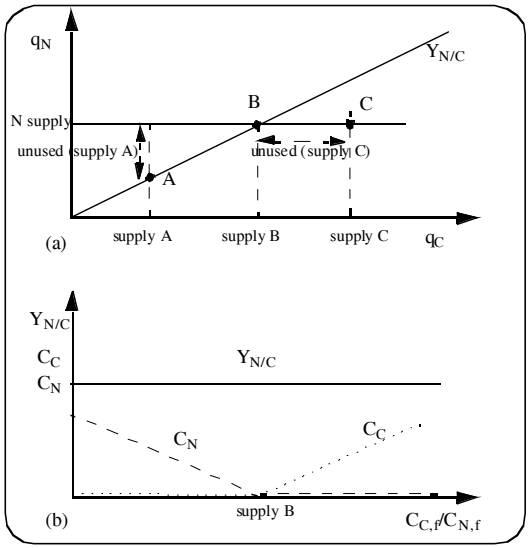

Figure 5: Consumption kinetics of the carbon and the nitrogen source for a hypothetical culture with a strictly constant metabolism. (a) nitrogen consumption rate as a function of carbon source consumption rate for low (A), medium (B), and high (C) carbon source supply rates. (b) ratio of nitrogen to carbon consumption rates ($Y_{N/C}$) and residual carbon source (C_C) and nitrogen source (C_N) concentrations as a function of carbon to nitrogen feed ratio ($C_{C,f}/C_{N,f}$). Adapted from von Stockar et al. (1995).

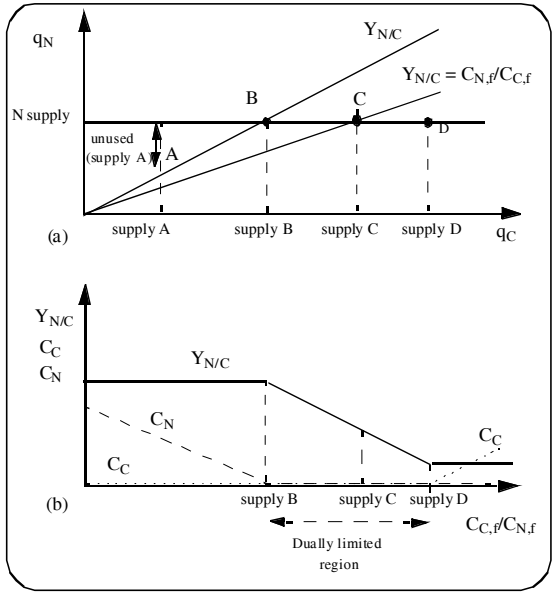

Figure 6: Consumption kinetics of the carbon and nitrogen source for a culture exhibiting dual carbon and nitrogen limitations : (a) and (b) see legend in figure 5. Adapted from von Stockar et al. (1995).

4. 3. Classification of dual limiting nutrients

In general, nutrients can be grouped according to their physiological function. Four cases of dual nutrient limitation can be recognized (Baltzis and Fredrickson (1988); Egli (1995); Zinn (1998)).

Homologous nutrients are nutrients that fulfill the same physiological function during growth (Harder and Dijkhuizen (1976)). For example various pairs of sugars often fulfill the same physiological function in heterotrophic organisms. Other authors used the synonyms *entirely* or *perfectly substitutable substrates* (Baltzis and Fredrickson (1988); Léon and Tumpson (1975); Ramakrishna et al. (1997)) or *mixed substrates* (Egli (1995); Harder and Dijkhuizen (1976); Harder and Dijkhuizen (1982); Narang et al. (1997)).

Heterologous nutrients satisfy different physiological requirements (Harder and Dijkhuizen (1982)). One nutrient cannot be replaced by the other. Ammonium ions and

glucose in synthetic media, for organisms like *Escherichia coli,* are examples: ammonium ions supply elemental nitrogen and NH_2 moieties, whereas glucose supplies elemental carbon and available energy. Synonyms for such substrates include *non-homologous* (Harder and Dijkhuizen (1976)), *non-interactive* (Bader (1978)), *complementary* (Baltzis and Fredrickson (1988); Straight and Ramkrishna (1994); Ramakrishna et al. (1997)) or *essential nutrients* (Tilman (1980)).

Two additional combinations of nutrients can be distinguished when a nutrient can fulfill two functions at the same time. For instance, one substrate can be *partially homologous* to a second substrate, whereas the second substrate is *entirely homologous* to the first substrate: for example an amino acid and glucose in medium used to cultivate a strain of bacteria. The amino acid is homologous and therefore substitutable for glucose because it can supply the cells' requirement for carbon and available energy as well as for nitrogen and amino groups whereas glucose can only supply the requirements for carbon and available energy. In Baltzis and Fredrickson (1988), this case is called *partially substitutable and entirely substitutable substrates*.

Finally combinations of substrates can be *partially homologous and partially heterologous*. A corresponding synonym for this case is *partially substitutable and partially complementary* (Baltzis and Fredrickson (1988)). Glutamine and glucose in media used for animal cell cultures can be incorporated in this class. They are homologous because both are used to supply available energy, and they are heterologous because glutamine supplies the cells' need for this essential amino acid, whereas glucose supplies their need for ribose moieties.

4. 4. Prediction of dual heterologous nutrient limited growth for chemostat culture

Egli and Quayle (1986) proposed a stoichiometric approach to predict dual nutrient limited growth during chemostat cultures simultaneously limited by heterologous nutrients. The borders of the limited growth regime at a particular growth rate can be predicted from the substrate biomass yields using equation 14, given below for the case of carbon and nitrogen as limiting nutrients. The concentration of biomass in the culture (X) can be calculated from equation 13 where $C_{C,f}$ and $C_{N,f}$ are the concentrations of carbon and nitrogen sources in the feed, C_C and C_N are the residual concentrations of carbon and nitrogen sources in the culture, and $Y_{X/C}$ and $Y_{X/N}$ are the

biomass yields for carbon and nitrogen sources, respectively, measured under either carbon-limited or nitrogen-limited growth conditions (at the same dilution rate). Under double substrate limitation, both C_C and C_N are negligible, equation 13 can therefore be rearranged into equation 14 to yield the $C_{C,f}/C_{N,f}$ ratio of the inflowing medium.

$$X = (C_{C,f}-C_C) * Y_{X/C} = (C_{N,f}-C_N) * Y_{X/N} \quad (13)$$

$$\frac{C_{C,f}}{C_{N,f}} = \frac{Y_{X/N}}{Y_{X/C}} = \frac{1}{Y_{N/C}} \quad (14)$$

The two borders ot the limited growth regime at a given growth rate can be calculated from the substrate biomass yields determined during either carbon- or nitrogen- limited growth.

Chemostat experiments showed that the predicted and the measured dual limited growth regimes were identical (Egli and Quayle (1986); Gräzer-Lampart et al. (1986); Durner et al. (2001); Durner et al. (2000)).

Dual limited growth regimes are also a function of the dilution rate (D) and not only of the composition of the feed medium $C_{C,f}/C_{N,f}$. Egli (1991) called this growth regime the *dual nutrient limited zone*: this growth regime can be represented graphically in a D versus $C_{C,f}/C_{N,f}$ diagram (figure 7). The zone has a *banana shape*. The dual nutrient limited zone is broader at lower growth rates, becomes narrower at higher growth rates and is not detectable at the maximum growth rate.

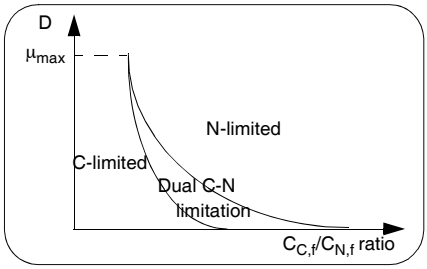

Figure 7: Dual carbon-nitrogen limitation is dependent on the medium composition (feed ratio $C_{C,f}/C_{N,f}$) and the dilution rate D of the chemostat.

According to equation 13, in order to obtain double-substrate-limited growth with substrates S_1 and S_2, the growth yields of either $Y_{X/S1}$ or $Y_{X/S2}$ (or both) have to be different

for growth conditions solely limited by S_1 as compared to those solely limited by S_2. Hence, the wider the range within which a cell component affected by the substrate(s) studied can vary, the more extended the double-substrate-limited growth regime will be. The transition growth regime becomes narrower with increasing dilution rates because differences in cellular composition and growth yield are markedly more pronounced in slow growing cells (Egli and Quayle (1986); Egli (1991)). Moreover, the zone of dual limitation is moved towards higher $C_{S1,f}/C_{S2,f}$ ratios at low dilution rates because of higher substrate consumption for maintenance purposes at lower dilution rates.

The shape and position of the banana are determined by the kind of substrate limitation and by the substrate themselves. For instance, for the dual C/N limitation, for highly oxidized carbon sources (giving lower growth yield coefficients) the dual limited growth regime will be shifted towards higher C/N ratios (Egli (1991)). Unfortunately, little data on *dual nutrient limited zones* is available for other combinations of nutrients than carbon and nitrogen. For instance, the influence of the carbon to essential trace metals ratios in the feed medium and the dilution rate on regimes of growth limitation have not been investigated. Only the calculation of boundaries for glucose/magnesium has been done for *K. pneumonia* (Egli (1991)).

4. 5. Mixed substrate growth by dual carbon source limitations in cultures with methylotrophic yeasts

Methanol and glucose

In the 1980's, Egli and coworkers investigated the use of mixed feeds of methanol and glucose in carbon-limited continuous cultures with the methylotrophic yeasts *Hansenula polymorpha* and *Kloeckera* sp. 2201, a strain of *Candida boidinii* (Egli et al. (1980); Egli et al. (1982b); Egli et al. (1982a); Egli et al. (1983); Egli et al. (1986)). Both the influence of dilution rate and the influence of the methanol-glucose ratio in the feed medium were analyzed on the growth stoichiometry and on the specific activity of several key enzymes involved in the dissimilation of methanol, in particular alcohol oxidase, catalase, formaldehyde dehydrogenase and formate dehydrogenase.

Derepression of methanol dissimilating enzymes was observed during glucose-limited continuous cultures (Egli et al. (1980)). Although the extent to which derepression occured was different for each enzyme, higher specific enzyme activities in the cells were

observed at low dilution rates for all enzymes. It was pointed out that since the residual substrate concentation decreases with decreasing dilution rates in chemostat cultures, it is most likely that the extent to which derepression is observed relates to the residual concentration of glucose. Except for alcohol oxidase, the extent to which derepression of methanol dissimilating enzymes occured was similar for both methylotrophic yeasts. With *H. polymorpha,* the specific AOX activity in cells grown at low dilution rates was 10-20% of that of methanol-limited cells grown at the same dilution rate and derepression was observed up to dilution rate of about 0.4 h^{-1}. However, with *Kloeckera* sp. 2201, the repression of AOX was only relieved at dilution rates below 0.1 h^{-1} and to a smaller extent than in *H. polymorpha*.

During mixed substrate chemostat cultures on methanol and glucose, both carbon sources were consumed simultaneously to completion by *H. polymorpha* and *Kloeckera* sp. 2201 at a dilution rate of 0.15 and 0.14 h^{-1} respectively, independent of the methanol-glucose ratio in the feed medium (Egli et al. (1982b)). The biomass produced was found to be about the same as the sum ot the individual biomasses theoretically possible from each substrate. The specific activities of alcohol oxidase, catalase, formaldehyde dehydrogenase and formate dehydrogenase increased as the proportion of methanol in the feed medium increased from 0 to 40% and reached the levels found in cells grown on methanol as sole carbon source at the same dilution rate. Further increase in the methanol fraction in the feed medium did not enhance the specific enzyme activities.

The influence of dilution rate during mixed substrate growth on methanol and glucose was investigated with a mixture of 38.8% glucose and 61.2% w w^{-1}methanol (Egli et al. (1982a)) such that all methanol dissimilating enzymes were fully induced at low dilution rates. It was shown that during mixed substrate growth, methanol was utilized at dilution rates significantly higher than the maximum specific growth rate achieved on methanol as sole carbon source. Hence, higher productivities can be achieved with mixed substrates than with methanol as sole carbon source.

In order to have a better understanding of mixed substrate growth of *H. polymorpha* on methanol and glucose, the utilization of mixtures of methanol and glucose of various composition were investigated at different dilution rates (Egli et al. (1986)). The specific growth rate at which the transition from mixed substrate growth to growth on glucose

occured was related to the methanol-glucose ratio in the feed medium: it increased with decreasing proportions of methanol in the feed medium.

Methanol and glycerol

The use of mixed feeds of glycerol and methanol during the induction phase of high cell density fed-batch cultures for the production of recombinant proteins with *P. pastoris* strains has been examined by several authors. A number of authors showed that the presence of glycerol during the induction phase in fed-batch processes can lead to improved productivities (Cregg et al. (1993); Loewen et al. (1997); McGrew et al. (1997); Katakura et al. (1998); Zhang et al. (2003)). According to the literature, as long as the residual glycerol concentration in the culture medium does not reach levels sufficient to repress the AOX1 promoter or cause the excretion of inhibitory metabolites such as ethanol or acetate (Mardon (1995)), the specific rate of methanol consumption and the recombinant protein productivity should not be diminished.

However, some authors claim that the optimal level of protein expression is not achievable with mixtures of glycerol and methanol due to a partial repression of the AOX1 promoter by glycerol (Sreekrishna et al. (1997); Hellwig et al. (2001))

Although there are contradictory points of view in the literature about the benefits of performing mixed substrate cultures on glycerol and methanol, mixed feeds on glycerol and methanol take advantage of the higher cell densities and feeding rates possible with growth on glycerol, providing that glycerol is present at limiting concentration in order to prevent repression of heterologous protein production and that methanol does not accumulate to toxic levels.

One interesting point with mixed feeds of glycerol and methanol is that for a given growth rate and for the same amount of total carbon source, the heat production rate and the oxygen consumption rate on mixed-substrate cultures will in all probability be lower than in cultures on methanol as sole carbon source. Heat production and oxygen consumption rates are closely correlated: for each mole of oxygen consumed during aerobic growth, 460 kJ heat is generated (Roels (1983)). Oxygen consumption rate is diminished with mixed feeds because less oxygen is necessary for the oxidation of glycerol than of methanol. The enthalpies of combustion of methanol and glycerol are -727 kJ C-mol^{-1} (Weast (1980)) and -549.5 kJ C-mol^{-1}(von Stockar et al. (1993)), respectively. Since the enthalpy of combustion of glycerol is lower than that of methanol,

less heat will be released in cultures with mixed feeds of glycerol and methanol than with feeds of methanol.

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 scale. As a matter of fact, oxygen transfer and reactor cooling frequently represent the major technical limitations for high cell density operations with *P. pastoris* strains (Hensing et al. (1995); Mardon (1995); Schilling et al. (2001); Jenzsch et al. (2004)). Therefore, any method which reduces the oxygen consumption rate and the heat production rate without affecting recombinant protein productivity is welcomed.

Methanol and sorbitol

Some authors pointed out that carbon sources other than glycerol might further improve the fed-batch strategy with recombinant *P. pastoris* strains.

For instance, Inan and Meagher (2001a) studied growth and levels of recombinant protein expression with a *P. pastoris* Mut$^-$ strain expressing β-galactosidase on mixtures of 0.5% methanol and 1% of either glycerol, glucose, ethanol, acetate, alanine, sorbitol, mannitol or trehalose as carbon sources in shaken flasks. Results showed that alanine, sorbitol, mannitol and trehalose did not repress foreign gene expression and could be used as carbon source for Mut$^-$ *P. pastoris* strains with methanol required as an inducer. Glucose, glycerol, ethanol and acetate supported growth but repressed the expression of β-galactosidase. Hence, instead of feeding methanol as sole carbon source, mixtures of methanol and either alanine, mannitol, sorbitol or trehalose could be fed during the induction phase for Mut$^+$ and Mut^S *P. pastoris* strains employing the AOX1 promoter driven heterologous protein expression. Indeed, if the feeding of these substrates does not affect the specific recombinant protein productivity and if biomass yields are higher than for growth on methanol, the volumetric recombinant protein productivity can be increased.

According to the results of Inan and Meagher (2001a), with trehalose or alanine, lower maximal specific growth rates and lower biomass yields are achieved than on sorbitol or mannitol, which makes the latter two substrates more interesting candidates. The best recombinant protein expression level was achieved with a mixture of sorbitol and methanol.

The benefits of using mixtures of sorbitol and methanol during the induction phase of high cell density *P. pastoris* cultures has been reported by other authors (Sreekrishna

et al. (1997); Thorpe et al. (1999); Boze et al. (2001); Xie et al. (2005)). Boze et al. (2001) reported for instance that compared with a high cell density fed-batch culture on methanol as sole carbon source during the induction phase, the specific recombinant protein productivity was higher when using mixed feeds of sorbitol and methanol but was lower when using mixed feeds of glycerol and methanol. This is in all probability because, by contrast with glycerol, sorbitol is a non-repressing carbon source with respect to the AOX1 promoter (Thorpe et al. (1999)).

Use of mixed feeds of sorbitol and methanol also presents other advantages, including the lower heat production rate and the lower oxygen consumption rate for growth on sorbitol than for growth on glycerol or methanol. The enthalpy of combustion of dissolved sorbitol was not found in the literature but can be assumed to be very close to the enthalpy of combustion of mannitol (-507.8 kJ C-mol^{-1}, Weast (1980)), which is an isomer of sorbitol. Hence, the enthalpy of combustion of sorbitol is about 8% lower than that of glycerol and about 30% lower than that of methanol. In all probability, for a given growth rate, less heat will be released in cultures with mixed feeds of sorbitol and methanol than with mixed feeds of glycerol and methanol or with feeds of methanol alone. Since the degree of reduction of sorbitol (4.33) is lower than those of glycerol (4.66) and methanol (6.00), less oxygen will be consumed during mixed substrate growth on sorbitol and methanol than on mixed feeds of glycerol and methanol or on methanol as sole carbon source.

However, to our knowledge, no detailed analysis on the influence of the methanol-sorbitol ratio in the feed medium on growth stoichiometry and recombinant protein productivity in *P. pastoris* cultures has been reported. Studies were performed in batch or fed-batch cultures with simultaneously changing ratios of substrates, changing feed rates and changing specific growth rates (Thorpe et al. (1999); Boze et al. (2001); Inan and Meagher (2001a); Xie et al. (2005)).

In this thesis the influence of the methanol-glycerol and the methanol-sorbitol ratio in the feed medium on growth stoichiometry and recombinant avidin productivity were investigated by performing transient continuous cultures with linearly increasing or decreasing methanol fractions in the feed medium at constant dilution rates. This technique was described previously for the analysis of the influence of the C/N ratio in the feed medium on the production of polyhydroxyalkanoates (Zinn (1998); Maskow and Babel (2000); Maskow and Babel (2002)).

5. PRODUCTION OF RECOMBINANT AVIDIN IN *P. PASTORIS*

Avidin is a glycosylated protein found in avian, reptilian, and amphibian egg white and is widely used in biotechnological and biomedical applications (Wilchek and Bayer (1990)) due to its exceptionally high affinity to (+)-biotin. The active form of hen avidin is a homotetramer composed of four singly glycosylated subunits. Each monomer has a very high affinity for biotin (K_a in the range of 10^{15} M^{-1}, Wilchek and Bayer (1990)), the highest known affinity between any ligand and a protein. The biotin-avidin technology has found numerous applications in various fields of biotechnology, including ELISA, immunolabelling, affinity targeting, drug delivery, and recently in enantioselective catalysis (Skander et al. (2004)).

The primary source for the commercial production of avidin is the large-scale extraction and purification from chicken egg white. This large-scale purification of chicken avidin requires a large number of eggs because chicken avidin is a minor constituent of egg white (approximately 0.05% of egg white proteins (Koser (1968))). Hence, much effort has been invested in producing avidin in heterologous expression systems. Production of biologically active recombinant avidin isoforms has been reported in *Escherichia coli* (Airenne et al. (1994), Hytönen et al. (2004)), baculovirus-infected insect cells (Airenne et al. (1997)), maize (Hood et al. (1997)) and in *Pichia pastoris* (Zocchi et al. (2003)).

Zocchi et al. (2003) designed a clone of *P. pastoris* expressing and secreting a recombinant glycosylated avidin with an acidic isoelectric point (pI = 5.4) because the high isoelectric point of avidin (pI = 10.5) can limit its field of application. Recombinant avidin was secreted at approximately 330 mg L^{-1} of culture supernatant in high cell density fed-batch cultures (up to 160 g L^{-1} of dry cell weight). The ability to grow at high cell densities and the secretion of the recombinant active avidin in the culture medium provided an attractive alternative to other expression systems.

This thesis discusses and proposes an optimal high cell density fed-batch strategy for the production of recombinant glycosylated avidin, expressed and secreted by the strain designed by Zocchi et al. (2003). Steady-state and transient continuous cultures were essentially performed in order to analyse quantitatively the physiology of this recombinant host secreting avidin.

6. PHYSIOLOGICAL STUDIES USING TRANSIENT EXPERIMENTS IN CONTINUOUS CULUTRES

Nowadays the possibility of modifying the genetic make-up of a cell to produce virtually any protein, to introduce new functionalities or to adapt them to process needs brings new challenges to the bioprocess development field. An enormous amount of recombinant mutants are made available, which have to be tested under process conditions and for which optimal culture processes have to be developed.

As a matter of fact, host cells used in bioprocesses need to be cultured in an environment that provides optimal conditions for maximal protein expression. Factors such as temperature, pH and nutrient concentrations play an important role and it is necessary to find the optimal combinations of these factors.

Often massive parallel experimentations are performed in batch cultures using miniaturized reaction systems. However, albeit the batch culture is convenient and looks easy, from a physiological point of view, it is highly dynamic and difficult to control. Cultures in continuous mode are more suited for physiological studies because they allow to grow cells under well defined growth conditions. Usually, continuous cultures are performed in chemostat mode. Steady-state growth is achieved in chemostat cultures after three to five liquid residence times and it indeed allows growth under well defined conditions. However, the performance of several chemostat cultures is usally very time-consuming, especially when a large number of steady-state data points are required. This is in all probability the main reason why the characterization of host cells is seldom performed in continuous cultivation.

In order to speed up strain characterization in continuous cultivation, transient continuous cultures under pseudo-steady state growth conditions can be performed instead of performing several chemostat cultures. After having achieved steady-state growth during chemostat cultivation, one culture parameter is changed at a sufficiently low rate in order to achieve pseudo-steady state growth conditions. This technique is called the A-stat technique and is defined as a continuous culture with smooth controlled change (acceleration rate) of a cultivation parameter. The use of the A-stat technique for the study of the influence of dilution rate on growth characteristics of various microorganisms has been described in detail by several authors (Paalme and Vilu (1992); Paalme et al. (1995); Paalme et al. (1997a); Paalme et al. (1997b); van der Sluis et al. (2001); Barbosa et al. (2003)). Changes of nutrient concentrations in the feed medium during

continuous cultivation at pseudo-steady state have also been reported, in particular for the analysis of the carbon to nitrogen ratio in the feed medium (Zinn (1998); Maskow and Babel (2000); Maskow and Babel (2002)).

On the other hand, transient experiments in continuous cultures with sudden changes of one culture parameter provide quantitative insights into the physiology and the process relevant regulation kinetics of host cells. Such experiments are usually done in the following manner: the culture is first grown at steady-state in continuous cultivation, and the metabolism is suddenly challenged by forcing the culture through a transient, either by applying a pulse to the culture, by a shift-up or shift-down of the dilution rate or a substrate feeding rate (van Urk et al. (1988); Sonnleitner and Hahnemann (1994); van Kleeff et al. (1996); Duboc et al. (1998a); Flikweert et al. (1999); Herwig et al. (2001)). Such transient experiments are a highly powerful way to investigate the metabolism of a culture. Indeed, it is possible to obtain a better insight into physiology and metabolism, while obtaining quantitative data on the kinetics of regulation. When the metabolism of a culture is challenged by applying pulses or a shift-up, it tries to increase the metabolic fluxes through the various parts of metabolism as fast as possible in order to adapt to the new conditions and to meet the demands imposed by it. Since all parts of the metabolism cannot adapt to the new conditions at the same rate, a metabolic imbalance results, intermediate products accumulate in the cells and are then secreted into the fermentation medium. Monitoring the appearance and consumption of these metabolites quantitatively during transients enables identification of the adaptation kinetics of the various parts of metabolism and thus insights into process relevant regulation phenomena (Duboc et al. (1998b); Herwig and von Stockar (2002b); Herwig and von Stockar (2002a); von Stockar et al. (2003)). Such experiments also permit rapid and effective strain and mutant characterization and yield quantitative data for mathematical models, for rapid and rational bioprocess development and control (Duboc et al. (1998a); Jobé et al. (2003)).

The metabolic imbalances observed after a shift-up and pulses have been studied in more detail by subjecting continuous steady-state cultures to nutrient limitations. Different metabolic responses have been observed in response to nutrient-limited growth conditions:

- Variation of the basic cellular composition (proteins, RNA, carbohydrate), (Cooney et al. (1976); Cooney et al. (1996); Larsson et al. (1993)).

- Accumulation of storage compounds such as polyhydroxyalkanoates (Durner (1998); Durner et al. (2000); Durner et al. (2001); Zinn (1998); Maskow and Babel (2002)), exopolysaccharides (Reeslev et al. (1996)), glycogen.
- Change of the physiological efficiency: for example biomass/ATP consumed and biomass/CO_2 produced. This phenomenon is often called metabolic uncoupling (Tsai and Lee (1990)**;** Liu et al. (1999); von Stockar et al. (1995); Cooney et al. (1996); Larsson et al. (1993)).
- Excretion of metabolic intermediates into the culture broth, called overflow metabolism (Cooney et al. (1976); von Stockar et al. (1995); Cooney et al. (1996); Larsson et al. (1993)).

Hence, by understanding and controlling nutrient limitations, metabolism can be forced in a desired direction, for example to promote or avoid the formation of a product (Neijssel et al. (1993)).

In this thesis, transient experiments in continuous cultures with sudden changes of nutrient supply, pulses of nutrients or slow linear changes of nutrient concentrations in order to maintain pseudo-steady state growth were evaluated for the quantitative characterization of a *P. pastoris* Mut^+ strain secreting recombinant glycosylated avidin, as a basis for obtaining powerful tools for process development and strain characterization. In particular, the advantages of mixed substrates during the induction phase of recombinant protein were investigated.

Another variable, which is often deliberately changed in the course of recombinant protein production, is temperature. Many processes are run at an optimum temperature for growth because only few studies describe the effect of temperature on recombinant protein production. However, a lower cultivation temperature during the induction phase can influence yields of recombinant proteins favorably because the rate of proteolysis is diminished for kinetic reasons, and because proteolysis is reduced due to reduced cell death and protease release to the medium (Jahic et al. (2003b)). Expression of recombinant proteins at lower temperature also helps to reduce protein misfolding (Georgiou and Valax (1996)).

In this thesis, pseudo-steady state experiments in continuous cultures with linear changes in cultivation temperature were performed and evaluated for the quantitative characterization of a *P. pastoris* Mut^+ strain secreting recombinant glycosylated avidin. It is documented in the literature that lower cultivation temperature with *P. pastoris*

strains can lead to higher yields of recombinant proteins (Chen et al. (2000); Whittaker and Whittaker (2000); Li et al. (2001); Hong et al. (2002); Sarramegna et al. (2002); Jahic et al. (2003a); Jahic et al. (2003b); Li et al. (2003); Shi et al. (2003)). These experiments constitute a basis for evaluating linear temperature changes in pseudo-steady state continuous cultures as a tool for process development and strain characterization.

7. On-line tools used for the monitoring of metabolic activity during transient experiments

A prerequisite for the analysis of transient experiments are on-line monitoring tools which enable to quantitatively follow microbial activity, secretion and consumption rates of all major metabolites as a function of time.

This has been achieved by applying on-line calorimetry and on-line monitoring of carbon dioxide production and oxygen consumption rates.

Calorimetry is an excellent and rapid on-line tool for the monitoring of metabolic activity (von Stockar et al. (1997); Duboc et al. (1998a); Duboc et al. (1999)). It is therefore a very useful on-line method for the detection of shifts in metabolism in response to dynamic growth conditions. This is particularly true since the sum of all ongoing enzymatic reactions (metabolism) in living systems results in a net heat production. Although heat production is non-specific, its measurement can be used to investigate growth, biomass or product formation, since the heat released during growth is linearly correlated with other process variables, such as biomass, carbon dioxide production, or oxygen uptake rate (Birou (1986); Menoud (1996); Duboc (1997); Voisard (2001)). Moreover, the heat signal can be used to carry out an enthalpy balance (von Stockar et al. (1993)), which can be used as an additional constraint in data reconciliation procedures for the verification of the validity of all measurements (Stephanopoulos et al. (1998)).

8. Structure of the thesis

This thesis has been structured in 9 chapters: an introduction, seven chapters dealing with experimental results and a chapter with general conclusions and perspectives. A list of symbols is provided at the end of each chapter.

Chapter 1 gives a general introduction on the methylotrophic yeast *P. pastoris* as an expression system of recombinant proteins. Limited growth of microorganisms and in particular mixed substrate growth of methylotrophic yeasts is introduced. This chapter also gives a brief introduction on the use of transient experiments in continuous cultures for strain characterization. Moreover, the objectives and the stucture of the thesis are presented in this chapter.

Chapter 2 is devoted to the design of a chemically defined medium for the production of biotin-free avidin in continuous cultures with a *P. pastoris* strain secreting recombinant avidin. Biotin concentration in the culture medium was optimized, taking into account the fact that biotin is an essential nutrient for optimal growth of *P. pastoris* and that avidin is a molecule binding biotin with a very high affinity.

Chapter 3 provides a quantitative analysis of the influence of specific growth rate on recombinant avidin productivity and synthesis of the alcohol oxidase enzyme in *P. pastoris*. Results for chemostat cultures on methanol or glycerol at various dilution rates are presented and discussed.

Chapter 4 is devoted to the study of the influence of culture temperature on specific growth rate of *P. pastoris* and on recombinant avidin productivity. Transient experiments in continuous cultures with linear changes of culture temperature under pseudo-steady state growth conditions were evaluated as a tool for strain characterization.

Chapter 5 is devoted to the study of the regulation of the alcohol oxidase enzyme in continuous *P. pastoris* cultures subjected to sudden changes of nutrient supply (transition from growth on glycerol to growth on methanol or to mixed substrate growth on glycerol and methanol) or to sudden increase in residual methanol concentration (pulse of methanol).

Chapter 6 introduces the use of transient nutrient gradients in continuous cultures for the rapid and rational analysis of the use of mixed substrate feeds of glycerol and methanol in recombinant *P. pastoris* cultures. The advantages of mixed feeds of methanol and glycerol during the induction phase of high cell density fed-batch cultures are discussed.

Chapter 7 presents the advantages of using mixed feeds of sorbitol and methanol for the production of recombinant proteins with *P. pastoris*. A quantitative analysis of the influence of the methanol-sorbitol ratio was done with transient nutrient gradients in continuous cultures.

Chapter 8 discusses and proposes an optimal strategy for the production of recombinant avidin in *P. pastoris* high cell density fed-batch cultures.

Chapter 9 contains the general conclusions and perspectives of this work.

9. NOMENCLATURE

AOX	Alcohol oxidase	
ADP	Adenosine diphosphate	
ATP	Adenosine triphosphate	
C_i	Concentration of nutrient i	C-mol L^{-1}
D	Dilution rate	h^{-1}
DAS	Dihydroxyacetone synthase	
DHA	Dihydroxyacetone	
DHAP	Dihydroxyacetone phosphate	
FAD	Flavin adenine dinucleotide	
FBP	Fructose 1,6-bisphosphate	
F_6P	Fructose 6-phosphate	
FLD	Glutathione-dependent formaldehyde dehydrogenase	
GAP	Glyceraldehyde-3-phosphate	
G_6P	Glucose 6-phosphate	
GL_6P	Gluconolactone-6-phosphate	
J_S	Flux of nutrient S	C-mol dm^{-2} s^{-1}
K_a	Affinity constant	
K_S	Monod substrate saturation constant	C-mol L^{-1}
K_P	Permeability	dm s^{-1}
m	Specific substrate consumption rate for maintenance purposes	C-mol C-mol^{-1} h^{-1}
NADH	Nicotinamide adenine dinucleotide	
NADPH	Nicotinamide adenine dinucleotide phosphate	
q_i	Specific consumption rate of i	C-mol C-mol^{-1} h^{-1}
pI	Isoelectric point	
P/O	Effectiveness coefficient of oxidative	

	phosphorylation	
6PG	6-phosphate-gluconate	
R_5P	Ribulose 5-phosphate	
T	Temperature	°C
TCA	Tricarboxylic acid	
X	Biomass concentration	C-mol L^{-1}
Xu_5P	Xylulose-5-phophate	
$Y_{X/i}$	Biomass yield on substrate i	C-mol C-mol^{-1}
α	Specific area of cells	dm^2 C-mol^{-1}
μ	Specific growth rate	h^{-1}

Subscripts

C	Refers to the carbon source
E	Refers to extracellular
f	Refers to the feed medium
I	Refers to intracellular
max	Maximal
min	Minimal
N	Refers to the nitrogen source
opt	Optimal
S	Refers to the limiting nutrient

Superscripts

app	Apparent
growth	Refers to growth purposes

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CHAPTER 2

OPTIMISATION OF CULTURE CONDITIONS WITH RESPECT TO BIOTIN REQUIREMENT FOR THE PRODUCTION OF RECOMBINANT AVIDIN IN *PICHIA PASTORIS*

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1. ABSTRACT

Due to its very high affinity to biotin, avidin is one of the most widely exploited proteins in modern biotechnological and biomedical applications. Since biotin is an essential vitamin for the growth of many microorganisms, we examined the effect of biotin deficiency on growth for a recombinant *P. pastoris* strain expressing and secreting a recombinant glycosylated avidin. The results showed that biotin deficiency lowers growth rate and biomass yield for *P. pastoris*. Substitution of biotin in the medium by the two structurally unrelated compounds, aspartic acid and oleic acid, which do not bind to recombinant avidin was analyzed quantitatively.

These two compounds had a growth promoting effect in biotin-deficient medium, but did not replace biotin completely. Indeed, in chemostat culture, wash-out occured after about six liquid residence times and recombinant avidin productivity was lowered.

However, addition of low amounts of biotin (20 μg L^{-1} of biotin for a cell density of 8 g L^{-1}) resulted in stable chemostat cultures on methanol with the production of recombinant biotin-free avidin. The specific avidin production rate was 22 μg g^{-1} h^{-1} at a dilution rate of 0.06 h^{-1}.

Keywords: Biotin; Avidin; Aspartic acid; Oleic acid; *Pichia pastoris.*

2. INTRODUCTION

Avidin is a glycosylated protein which is widely used in biotechnological and biomedical applications (Wilchek and Bayer (1990)) because of its exceptionally high affinity to (+)-biotin, also called vitamin H. The active form of hen-avidin is a homotetramer composed of four singly glycosylated subunits, each monomer having a very high affinity for biotin (K_a in the range of 10^{15} M^{-1}, Wilchek and Bayer (1990)). As a result of this property, biotin-avidin technology has found numerous applications in various fields of biotechnology, including ELISA, immunolabelling, affinity targeting, drug delivery, and recently in enantioselective catalysis (Skander et al. (2004)).

Avidin is found naturally in avian, reptilian and amphibian egg white, but only in relatively small amounts (approx. 0.05% of egg white proteins (Koser (1968))). Since large-scale purification requires large numbers of eggs, much effort has been invested in the production of recombinant avidin in heterologous expression systems. Indeed, production of biologically active recombinant avidin isoforms has been reported in *Escherichia coli* (Airenne et al. (1994), Hytönen et al. (2004)), baculovirus-infected insect cells (Airenne et al. (1997)) and maize (Hood et al. (1997)). Recently, the methylotrophic yeast *Pichia pastoris* was chosen as a host for the expression of recombinant avidin in order to perform enantioselective hydrogenation with artificial metalloenzymes based on the biotin-avidin technology (Collot et al. (2003), Skander et al. (2004), Zocchi et al. (2003a), Ward et al. (2003)). For this purpose, Zocchi et al. (2003b) designed a clone of *P. pastoris* expressing and secreting a recombinant glycosylated avidin with an acidic isoelectric point. Recombinant avidin was secreted at approximately 330 mg L^{-1} of culture supernatant in high cell density fed-batch cultures (up to 160 g L^{-1} of dry cell weight). The ability to grow at high cell densities and the possibility to obtain active avidin directly in the medium provided an attractive alternative to other expression systems.

However, one problem with heterologous expression systems secreting avidin is the fact that biotin, which is an essential nutrient for most microorganisms, binds strongly to the produced recombinant avidin, which makes it virtually impossible to quantify the amount of recombinant avidin produced.

Therefore, the production of recombinant avidin in heterologous expression systems is usually performed on complex or semi-defined media (Airenne et al. (1994), Airenne et al. (1997), Hytönen et al. (2004)). Zocchi et al. (2003b) produced recombinant avidin in

fed-batch mode using a chemically defined medium with an excess of biotin in the batch phase preceeding the production of avidin but replacing the former by a mixture of vitamins (thiamine, pyridoxine, alanine, riboflavin and inositol) during the induction phase.

In order to perform physiological studies with this recombinant *P. pastoris* strain in continuous cultures, biotin was replaced by the mixture of vitamins used by Zocchi et al. (2003b) during the induction phase. However, continuous cultures with this mixture of vitamins in place of biotin always resulted in wash-out. In order to study the physiology of *P. pastoris* in defined physiological states, it is imperative to perform chemostat cultures attaining a similar performance both with respect to growth and avidin production as obtained by Zocchi et al. (2003b). The goal of this paper was to find a solution to the problem that stable growth of *P. pastoris* in continuous cultures necessitates biotin in the medium, which impairs the production of biotin-free avidin.

The question therefore arose as to what other biochemical compounds could perform the metabolic role normally played by biotin. Although biotin is only present in tiny amounts in cells and tissues, it is an essential nutrient for most organisms. According to Koser (1968) only 1 $\mu g\ L^{-1}$ or less is usually ample to support growth of microorganisms, although biotin is involved in many metabolic processes. For instance, in mitochondria, biotin is a coenzyme of pyruvate carboxylase, which catalyses the carboxylation of pyruvate to oxaloacetate (Wallace et al. (1998)). Biotin also participates in fatty acid synthesis, since it is a co-enzyme of acetyl CoA carboxylase, which catalyzes the carboxylation of acetyl CoA to malonyl CoA (Wakil et al. (1958), Wakil and Gibson (1960), Numa et al. (1965)), the first step in fatty acid synthesis. Biotin acts as a co-enzyme in other carboxylation reactions, for instance, with propionyl CoA carboxylase for the breakdown of isoleucine, threonine and methionine, and with β-methylcrotonyl CoA carboxylase for the catabolism of leucine (Koser (1968)). Biotin plays an important metabolic role in many other biochemical reactions, for example in the synthesis of nucleic acids, some amino acids, proteins and carbohydrates (Koser (1968)).

Relatively few compounds structurally related to biotin can substitute for this vitamin. Among these compounds, it was reported that the methyl ester of biotin has essentially the same activity as free biotin, and oxybiotin, dethiobiotin, biocytin and biotin sulfoxide exhibit some degree of activity (Koser (1968); Firestone and Koser (1960); Axelrod et

al. (1947); Krueger and Peterson (1948)). Although biotin derivatives have lower binding affinity for avidin than biotin (Torreggiani et al. (2000), Kuhn and Kollman (2000)), the substitution of biotin by biotin derivatives was not addressed in this study because the formation of complexes between the produced recombinant avidin protein and biotin derivatives would not be avoided. Green (1975) summarized values of the dissociation constants of avidin complexes with various biotin derivatives and pointed out that biotin derivatives such as biotin sulfone, the methyl ester of biotin and dethiobiotin, still bind very firmly to avidin. Indeed, the imidazolidone ring of biotin is mainly responsible for binding with avidin, and the partial modification of the valeryl side-chain carboxylate group of biotin does not prevent the formation of the complex with avidin, but affects only the association constant of the complex (Torreggiani and Fini (1999), Torreggiani et al. (2000)).

Some authors showed that biotin can be replaced by the two structurally unrelated compounds aspartic and oleic acids. Koser (1968) gives examples of microorganisms where one or both of these compounds were used as substitutes for biotin. Suomalainen and Keränen (1962) reported that a mixture of aspartic and oleic acids permitted the same extent and rate of growth of Baker's yeast as did biotin under aerobic conditions. Unsaturated long-chain fatty acids added to the growth medium with aspartic acid compensate for biotin deficiency in fatty acid synthesis reactions. Suomalainen and Keränen (1962) pointed out that unsaturated fatty acids compensate better for biotin deficiency than saturated ones because they have a lower melting point and penetrate yeast cells more easily. Aspartic acid added to a biotin-deficient growth medium acts in the construction of other amino acids and biochemicals in the citric acid cycle. Among biochemicals that may be synthesized from aspartic acid are asparagine, arginine, lysine, methionine, threonine, isoleucine, and several nucleotides.

However, other authors claimed that aspartic and oleic acids replace biotin only partially and that the growth promoting effect of these two compounds is not equal to that of optimal amounts of biotin. For instance, Adler et al. (1981) used aspartic acid and Tween 80 (polyoxyethylene sorbitan monooleate), which is an excellent source of oleic acid, as substitutes for biotin with cultures of *Saccharomyces cerevisiae* and a biotin-requiring mutant of *Aspergillus nidulans*. The growth promoting effect was not comparable to that of optimal amounts of biotin: lower growth rates and lower biomass yields were achieved with these two substitutes. According to Adler et al. (1981), a very large

inoculum which could have contained significant amounts of biotin was used by Suomalainen and Keränen (1962) and could explain the discrepancy with their results.

The present study aimed at determining whether biotin is an essential nutrient for the efficient growth for *P. pastoris* and, if not, to find alternatives in order to perform continuous cultures with a *P. pastoris* strain secreting recombinant avidin, a molecule binding biotin with a very high affinity. Biotin was replaced by two structurally unrelated compounds, aspartic acid and oleic acid, an attempt to avoid the presence of biotin in the medium and binding to the produced recombinant avidin.
The goal was to examine if biotin could be replaced by these two compounds in order to produce recombinant avidin in continuous mode on a chemically defined medium without biotin or to find another solution to the problem that stable growth of *P. pastoris* in continuous cultures necessitates biotin in the medium, which impairs the production of biotin-free avidin.

3. MATERIALS AND METHODS

3. 1. Microorganism, inoculum preparation and media

A recombinant *Pichia pastoris* Mut^+ strain was kindly provided by Andrea Zocchi from the university of Neuchâtel (Switzerland). Zocchi et al. (2003b) designed a clone of *P. pastoris* expressing and secreting a recombinant glycosylated avidin (recGAvi) with an acidic isoelectric point. Stock cultures were stored at -80°C in a 20 g L^{-1} glycerol and 9 g L^{-1} NaCl solution. Cells were re-activated in a 1 liter baffled shake flask containing 100 mL of complex YPG medium at 30°C for 24 h. Two cultures in baffled shaken flasks of 100 mL each were used to prepare the inoculum. After 24 h at 30°C, the two precultures were centrifuged at 3000 rpm during 10 min at 4°C and pellets were resuspended in 10 mL of sterile water. This cell suspension was immediately utilized to inoculate the bioreactor containing 1L of defined medium.
The complex YPG medium contained 20 g L^{-1} glycerol (Sigma-Aldrich, Steinheim, Germany), 6 g L^{-1} yeast extract (OXOID, Hampshire, England), 5 g L^{-1} Bacto Peptone (Becton, Le Pont de Claix, France). This medium was sterilized by heating at 120°C during 20 min.

The defined medium used for cultures in bioreactor was based on the medium developed by Egli and Fiechter (1981) and contained per liter: 40 g glycerol or 20 g methanol as carbon source, 15.26 g NH_4Cl, 5.62 g KH_2PO_4, 1.18 g $MgSO_4$ $7H_2O$, 0.9 g EDTA $2H_2O$, 110 mg $CaCl_2$ $2H_2O$, 75 mg $FeCl_3$ $6H_2O$, 28 mg $MnSO_4$ H_2O, 44 mg $ZnSO_4$ $7H_2O$, 8 mg $CuSO_4$ $5H_2O$, 8 mg $CoCl_2$ $6H_2O$, 5.2 mg Na_2MoO_4 $2H_2O$, 8 mg H_3BO_3, 1.2 mg KI, and 0.4 mL of antifoam agent (Struktol SB 2121, Schill and Seilacher, Hamburg, Germany). Various vitamins were used in this study: a mixture of vitamins (24 mg L^{-1} thiamine HCl, 24 mg L^{-1} pyridoxine HCl, 24 mg L^{-1} alanine, 12 mg L^{-1} riboflavin and 4.8 mg L^{-1} myo-inositol) based on the medium used by Zocchi et al. (2003b), excess of biotin at a concentration of 2 mg L^{-1}, or small amounts of biotin, namely concentrations of 2 and 20 μg L^{-1}.

All the components of the defined medium were obtained by Fluka, Buchs, Switzerland, except glycerol which was obtained from Sigma-Aldrich, Steinheim, Germany. The defined medium was sterilized by filtration (0.22 μm, Steritop, Millipore Corporation, Billerica, USA).

3. 2. Culture conditions

Growth experiments were undertaken using a bench-scale heat-flux calorimeter RC1 (Mettler Toledo AG, Greifensee, CH) modified for biotechnological applications with a working volume of 1.6 L. Characteristics of this so called Bio-RC1 can be found in Marison et al. (1998). Continuous cultures were performed at 1.050 L of culture volume.

Temperature was maintained at 30°C and pH at 5.0 by the automatic addition of 2M KOH. The heat of neutralization was calculated from the consumption of KOH 2M according to Meier-Schneiders et al. (1995). A polarographic pO_2 probe (InPro 6800, Mettler Toledo, Greifensee, Switzerland) was used to monitor dissolved oxygen, with respect to air saturated medium. Aeration rate was set at 2.0 NL min^{-1} of air (2 vvm) using a thermal mass flow controller (5850E, Rosemount Brooks, Veenendaal, The Netherlands). The air was saturated with water immediately prior to entry in the calorimeter. The stirring rate was fixed at 800 rpm to maintain dissolved oxygen levels at > 80% air saturation.

All liquid inlet flows were quantified gravimetrically. The medium feed rate was controlled by a controller based on the on-line monitoring of the reservoir balance. The temperature of all feeds was adjusted to the reactor temperature prior to entering the calorimeter.

Torque measurements were used to detect baseline shifts of the heat flux (Menoud et al. (1995)). Since the release of CO_2 from the medium is an endothermic process, the heat flow induced by CO_2 stripping was taken into account according to Meier-Schneiders et al. (1995). Heat losses to the environment were calculated by using a heat transfer coefficient for the bioreactor of 0.133 W K^{-1}, which was determined as described in Birou (1986).

3. 3. Continuous cultivation on methanol

Cultures were first grown in batch mode on a mixture of 20 g L^{-1} of glycerol and 20 g L^{-1} of methanol. Methanol was consumed only after all the glycerol had been consumed since high glycerol concentrations are known to have a repressing effect on the expression of the methanol dissimilating enzymes. After all glycerol had been exhausted, methanol was consumed which allowed the induction and synthesis of methanol dissimilating enzymes and prepared the cells for chemostat growth on methanol.
As soon as all the methanol had been consumed (indicated by a sudden increase in the dissolved oxygen level), the supply of the feed medium was initiated. All chemostat cultures were performed at a dilution rate of 0.06 h^{-1}.

3. 4. Substrate and metabolite analysis

Culture samples (10-12 mL) were collected using a purpose-built auto-sampler (Cannizzaro (2002)) and kept at 2°C for up to 12h before handling. Samples for the analysis of avidin quantification were handled immediately after cooling for 10 minutes at 2°C.
The concentration of biomass was determined gravimetrically as dry cell weight. Samples were spun down in a pre-weighed glass-sample tube and the pellet was dried at 100°C to constant weight after two washing steps of the pellet with ultrapure water.
Glycerol and methanol were determined by HPLC analysis (1100 series, Agilent Technologies, Palo Alto, USA). An ion exchange chromatography column (Supelcogel H 300 mm, Supelco, Bellefonte, USA) with a guard column (Superlguard C610H, Supelco, Bellefonte, USA) was used at 60 °C. A 5 mM sulphuric acid solution in ultrapure water was applied at a constant eluent flow rate of 0.6 mL min^{-1}. Metabolites were measured using a refractive index detector. The detection limit was 0.01 g L^{-1} for glycerol and 0.05 g L^{-1} for methanol.
The HPLC device used for aspartic acid determination included different units: an autosampler (HPLC autosampler 465, Kontron Instrument, Watford, UK), a pump (Shi-

madzu LC-10AD, Kyoto, Japan), a resin based column (Aminex HPX-87C column, BIO-RAD, USA), and a refractive index detector (RI Detector ERC-7515A, Erma CR.INC, Tokyo, Japan). A 2 mM calcium nitrate solution in ultrapure water was applied at a constant eluent flow of 0.8 mL min^{-1} at 57 bars and 85 °C for 35 minutes and 20 μm of sample was injected.

The carbon dioxide evolution rate in the bioreactor off-gas was determined using an infrared (PSA 402, Servomex, Crowborough, UK) analyser. The measured values were corrected for water vapour according to Duboc and von Stockar (1998).

3. 5. Recombinant avidin quantification

The recombinant avidin was quantified by biotin-4-fluorescein titration of binding sites (Kada et al. (1999)), assuming a tetrameric form of the recombinant avidin, with four active-binding sites (Zocchi et al. (2003b)). Biotin-4-fluorescein was obtained by Fluka, Buchs, Switzerland.

Since the detection limit of this method is about 10 mg L^{-1} of avidin, it was necessary to concentrate the samples 15 fold by ultrafiltration (Centriplus, 30 KDa, Millipore Corporation, Bedford, USA) before titration with biotin-4-fluorescein, the molecular weight of the active form of avidin being 65.4 kDa (Zocchi et al. (2003b)).

Volumetric and specific avidin productivities were calculated at steady state according to equations (1) and (2) respectively:

$$R_P = C_P \cdot D \tag{1}$$

$$q_P = \frac{C_P \cdot D}{X} \tag{2}$$

where D (h^{-1}), X (g L^{-1}) and C_P (μg L^{-1}) respresent respectively the dilution rate, dry cell weight and avidin concentration.

3. 6. Black box stoichiometry and elemental composition of biomass

The elemental composition of biomass and the black box stoichiometry on a C-molar basis were determined previously in chemostat cultures under aerobic growth conditions (Jungo et al. (2006)). The elemental composition of biomass did not change sig-

nificantly according to dilution rate. The black box stoichiometries for growth on glycerol and methanol were described by the following equations:

$$CH_{8/3}O + Y_{O2/S}\,O_2 + Y_{NH3/S}\,NH_3 \rightarrow Y_{X/S}\,CH_{1.764}O_{0.469}N_{0.182} + Y_{CO2/S}\,CO_2;\ \Delta_r H_S^{*\circ} \quad (3)$$

$$CH_4O + Y_{O2/S}\,O_2 + Y_{NH3/S}\,NH_3 \rightarrow Y_{X/S}\,CH_{1.691}O_{0.502}N_{0.176} + Y_{CO2/S}\,CO_2;\ \Delta_r H_S^{*\circ} \quad (4)$$

S represents the limiting carbon source, $Y_{i/S}$ the stoichiometric coefficients in mole or C-mole per C-mole of substrate, where is can be S (substrate), O_2, CO_2, NH_3 or X (biomass). $\Delta_r H_S^{*\circ}$ is the standard molar enthalpy change caused by reactions described in equations 3 and 4, with CO_2, H_2O and NH_3 as reference state. The first formula on the right-hand side of equations 3 and 4 represents one C-mole of dried biomass.

3. 7. Check of data consistency and reconciliation of yield coefficients in chemostat cultures on methanol

Five yields have to be determined in the black box stoichiometry, namely $Y_{O2/S}$, $Y_{NH3/S}$, $Y_{X/S}$, $Y_{CO2/S}$ and $Y_{Q/S}$. The heat yield $Y_{Q/S}$ represents the molar enthalpy change caused by reactions described respectively in equations 3 and 4 per C-mole of substrate. Measurements of dry cell weight, carbon source concentrations, carbon dioxide in the exhaust gas and heat generation were used in order to calculate three of them, namely biomass, carbon dioxide and heat yields. Together with the constraints provided by the carbon, nitrogen, degree of reduction and enthalpy balances a redundancy of

$$L = -\,(\,5 - (3 + 4)) = 2 \quad (5)$$

resulted.

The redundancy of the system was used in order to estimate the ammonia and oxygen yields and to reconcile the data.

Indeed, the calculated yields were checked for consistency and reconciled based on a χ^2-test as proposed by Wang and Stephanopoulos (1983) and Stephanopoulos et al. (1998). The test value h is given by the sum of the weighted squares of the residuals ε:

$$h = \varepsilon \cdot P^{-1} \cdot \varepsilon \quad (6)$$

where P is the variance-covariance matrix. Assuming measurement errors of 5%, a test value h lower than 5.99 permitted to assume a 95% significance level that no gross errors and no metabolites other than the ones appearing in equations 3 and 4 had affected the balances.

4. RESULTS AND DISCUSSION

4. 1. The effect of biotin deficiency in chemostat cultivation on methanol

Zocchi et al. (2003b) used a mixture of vitamins composed of thiamine HCL, pyridoxine HCl, alanine, riboflavin and myo-inositol during the induction phase on methanol of high cell density fed-batch cultures with the studied *P. pastoris* strain. In order to perform physiological studies in chemostat cultivation on methanol with this recombinant strain secreting avidin, we examined if this mixture of vitamins could support growth in a chemically defined medium to which no biotin was added.

Two carbon-limited chemostat cultures on 20 g L^{-1} of methanol were performed at a dilution rate of 0.06 h^{-1}, one with an excess of biotin and one with the mixture of vitamins proposed by Zocchi et al. (2003b) but no biotin. For the chemostat culture with biotin, a concentration of 2 mg L^{-1} of biotin was chosen in order to have a large excess which allowed to compensate for the binding of the produced recombinant avidin to biotin. In figure 1, the progress of dry cell weight and residual methanol concentrations for these two experiments were compared as a function of the liquid residence time, τ. The culture with an excess of biotin remained stable for more than six liquid residence times and no wash-out was observed. No avidin was detected since the excess of biotin prevents quantification using the applied analytical method. Since the biotin-avidin affinity is so high, biotin-free avidin cannot be recovered in cultures with an excess of biotin. Replacement of biotin by the mixture of vitamins resulted in the biomass concentration falling and the residual methanol concentration increasing after 4.4 liquid residence times leading to wash-out of the culture.

A concentration of 2.6 mg L^{-1} of avidin was measured immediately prior to wash-out for the culture with a mixture of vitamins. Volumetric and specific avidin production rates were 163 $\mu g\ L^{-1}\ h^{-1}$ and 21 $\mu g\ g^{-1}\ h^{-1}$ respectively.

The chemostat culture supplemented with vitamins in place of biotin was repeated at other dilution rates (0.03 and 0.09 h^{-1}) in order to confirm this behaviour. After about four liquid residence times, the culture always resulted in wash-out (data not shown). This clearly showed that the mixture of vitamins was not able to support growth of *P. pastoris* in a biotin deficient medium and could not be used as a substitute for biotin in chemostat cultures. In all experiments there was a carry-over of biotin from the batch phase, which was exhausted after about 4 liquid residence times.

At steady state, the average reconciled yield coefficients for the chemostat culture with 2 mg L^{-1} of biotin were: $Y_{X/S}$ = 0.48 C-mol C-mol^{-1}, $Y_{CO2/S}$ = 0.51 mol C-mol^{-1}, $Y_{O2/S}$ = 0.98 mol C-mol^{-1}, $Y_{NH3/S}$ = 0.08 mol C-mol^{-1} and $Y_{Q/S}$ = - 489 kJ C-mol^{-1}. The statistical test value h was 0.32, which is lower than the threshold value of 5.99 for a redundancy of two and a confidence level of 95%. Before wash-out of the culture, the reconciled yield coefficients for the chemostat culture with the mixture of vitamins were of the same magnitude as those with an excess of biotin, considering an error of 5% on the measured yields.

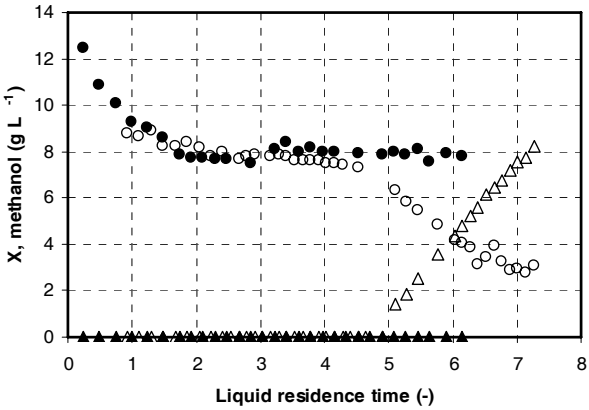

Figure 1: Comparison of chemostat cultures on 20 g L^{-1} of methanol with an excess of biotin (2 mg L^{-1} biotin) and with the mixture of vitamins proposed by Zocchi et al. (2003b). Dry cell weight X (●) and methanol (▲) concentrations with an excess of biotin. Dry cell weight X (❍) and methanol (△) concentrations with the vitamin mixture. Data was represented in terms of liquid residence time, τ.

4. 2. Aspartic and oleic acids as replacements for biotin in batch cultures on glycerol

In order to perform chemostat cultures on methanol with the avidin-secreting *P. pastoris* strain, we examined whether biotin could be replaced by other compounds, structurally unrelated to biotin, yet fulfilling the same physiological functions as this vitamin. Since many authors claimed that aspartic acid and oleic acid had a growth promoting effect in biotin-free media, we examined whether this was the case with the *P. pastoris* strain used in this study.

Batch cultures on 40 g L^{-1} of glycerol were carried out in order to evaluate if these two compounds stimulated growth in the absence of biotin and whether they could replace biotin. Batch cultures are advantageous compared with chemostat cultures for preliminary experiments because the former can be carried out in three days, including down time, whereas chemostat cultures at suitable dilution rates take at least one week. Because of the toxic effect of methanol, cell growth is inhibited in cultures on methanol with high residual substrate concentrations (Katakura et al. (1998)). Hence, batch cultures on methanol as carbon source should be performed with a low (< 1% w/v) initial methanol concentration. Since the residual biotin contained in the inoculum might be sufficient to support growth for batch cultures with low initial methanol concentrations, batch cultures on 40 g L^{-1} of glycerol were performed for the evaluation of the growth promoting effect of aspartic and oleic acids in biotin-free medium.

Three batch cultures on 40 g L^{-1} of glycerol are shown in figures 2, 3, and 4, one with an excess of biotin (2 mg L^{-1} of biotin), one culture to which no biotin was added, and one with 4 g L^{-1} of aspartic acid and 1 g L^{-1} of oleic acid in place of biotin.

According to Suomalainen and Keränen (1962), for yeasts, the biotin-like effect of unsaturated long-chain fatty acids, such as oleic acid, is only manifested if aspartic acid is also present. Similarly it was also pointed out that if aspartic acid is added alone to the culture medium in place of biotin, growth is promoted only to a certain extent but is not as efficient as with biotin in the culture medium. Hence both supplements were added to the culture medium and no culture was performed with only oleic acid or aspartic acid in place of biotin.

The results presented in figures 2 and 3 for growth with an excess of biotin and without biotin, respectively, show clearly that addition of biotin allows optimal growth of *P. pastoris*. In the absence of biotin, the growth rate and the final biomass concentration were

21% and 39% lower, respectively. Moreover, analysis of the continuous, on-line heat signal shows that in the absence of biotin growth was not exponential.

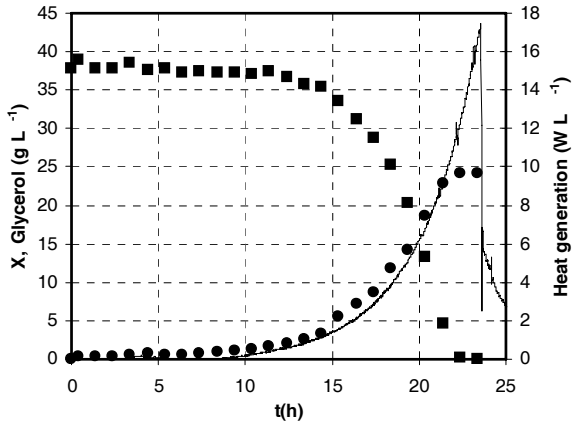

Figure 2: Batch culture on 40 g L^{-1} of glycerol with 2 mg L^{-1} of biotin. Dry cell weight X (●) and glycerol (■) concentrations, heat generation (continuous line).

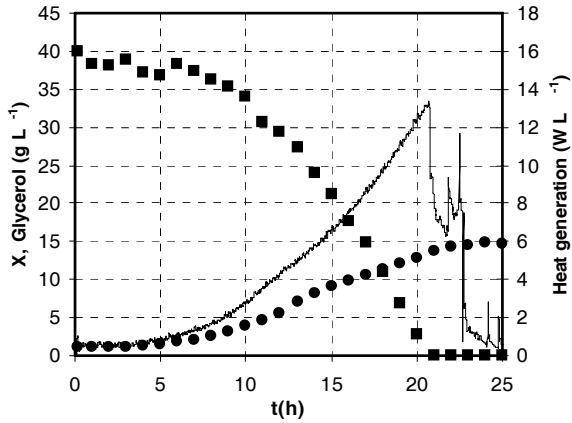

Figure 3: Batch culture on 40 g L^{-1} of glycerol without biotin or any supplement. Dry cell weight X (●) and glycerol (■) concentrations, heat generation (continuous line).

With the addition of aspartic and oleic acids (figure 4), exponential growth was observed until all aspartic acid was consumed, which corresponded to 13 hours after inoculation. The exhaustion of aspartic acid and the associated metabolic transition are clearly reflected by the sudden change of the heat signal. It was also observed that no ammonium was consumed until the complete exhaustion of aspartic acid. This suggests that aspartic acid was preferentially consumed and repressed inorganic ammonium uptake. Thus aspartic acid was used not only as a substitute for biotin, but also as nitrogen source and in all probability as carbon source. As a result the culture became limited by aspartic acid, before complete consumption of glycerol, at a biomass concentration of 8.9 g L^{-1}. After all the aspartic acid had been consumed, linear and non-exponential growth was observed. Due to the low solubility of aspartic acid in water, it was not possible to increase the concentration in the medium. It is therefore not possible to perform batch cultures on glycerol with a biomass concentration higher than approximately 9 g L^{-1} without being first limited by aspartic acid.

The results showed that the use of aspartic and oleic acids as supplements allowed better growth than with a biotin-free medium and that these two compounds actually

stimulate growth of *P. pastoris*. Growth rate and growth yields were however not as high as in presence of biotin.

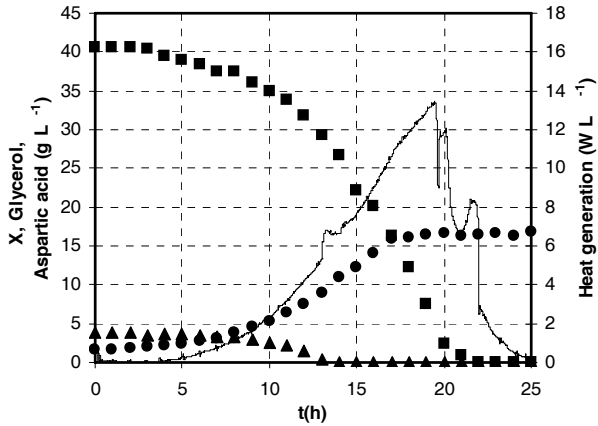

Figure 4: Batch culture on 40 g L^{-1} of glycerol with 4 g L^{-1} of aspartic acid and 1 g L^{-1} of oleic acid as substitutes for biotin in the culture medium. Dry cell weight X (●), glycerol (■) and aspartic acid (▲) concentrations, heat generation (continuous line).

4. 3. Aspartic and oleic acids as replacements for biotin in chemostat cultivation on methanol

The growth promoting effect of aspartic and oleic acids was finally examined in a continuous culture on 10 g L^{-1} of methanol, at a dilution rate of 0.06 h^{-1}. The goal was to perform a long-term stable chemostat culture on methanol with a biotin-free medium in order to avoid the irreversible binding of biotin to the produced recombinant avidin. The feed medium contained 4 g L^{-1} of aspartic acid. Due to the low solubility of aspartic acid in water, its concentration in the feed medium could not be increased. Because of the extremely low solubility of oleic acid in water, oleic acid was not added to the feed medium, but pulses of 0.9 mL of oleic acid were performed twice a day in order to perform the experiment at an average oleic acid concentration of 1 g L^{-1} in the culture.

Figure 5 shows the progress of dry cell weight, methanol and aspartic acid concentrations and carbon dioxide production rate during this chemostat experiment as a function of the liquid residence time. Pulses of oleic acid induced an increase in the carbon

dioxide production rate and in the biomass concentration. Oleic acid had the added benefit of acting as an anti-foam agent, however periodic addition resulted in a lowering of the reactor volume and lead to a transient state for about 3 hours. Hence, a periodic steady state was reached during this continuous culture.

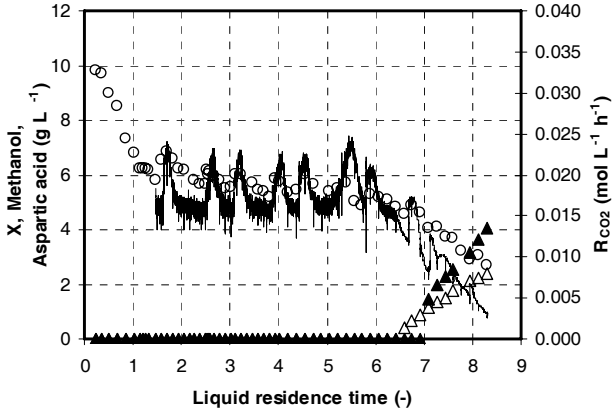

Figure 5: Chemostat cultures on 10 g L^{-1} of methanol with 4 g L^{-1} aspartic acid and 1 g L^{-1} oleic acid as substitutes for biotin. Dry cell weight X (❍), methanol (▲) and aspartic acid (△) concentrations, and carbon dioxide production rate (R_{CO2}, continuous line). Data was represented in terms of liquid residence time, τ.

After approximately six liquid residence times, biomass concentration and carbon dioxide production rate began to decrease and wash-out of the culture was finally observed. Hence, compared with the mixture of vitamins proposed by Zocchi et al. (2003b), aspartic and oleic acids had a slightly better growth promoting effect, since wash-out occured already after four residence times with the mixture of vitamins. However, aspartic and oleic acids did not fulfil all the metabolic functions of biotin since it was not possible to perform a long-term stable chemostat culture on methanol.

Moreover, the use of aspartic acid and oleic acid as supplements in place of biotin did not lead to increased specific avidin productivities. Indeed, the avidin concentration was 1.6 mg L^{-1} after 5.27 liquid residence times, which corresponds to a specific avidin production rate of 17 μg g^{-1} h^{-1}, which is about 20% lower than that determined with

the chemostat experiment with the mixture of vitamins proposed by Zocchi et al. (2003b).

Moreover, the insolubility of oleic acid and the low solubility of aspartic acid in water pose additional problems to the use of these two compounds as supplements for biotin in chemostat cultivation. Aspartic acid has many metabolic functions, including as carbon source, nitrogen source and growth supplement (biotin replacement), however, unlike batch cultures, the presence of ammonium enabled the culture not to be limited by nitrogen source. However, since the culture was carbon-limited, the residual aspartic acid concentration was very low (below the detection limit of the HPLC method). Hence, insufficient aspartic acid was available to act as growth supplement to replace biotin. It would be expected that higher amounts of aspartic acid in the feed medium, for the same methanol concentration, might increase the stability of methanol-limited chemostat cultures. However, the concentration of aspartic acid in the feed medium could not be increased since the solubility limit had been attained.

4. 4. Chemostat cultivation on methanol with low amounts of biotin

According to Koser (1968), only very small amounts of biotin, of the order of 1μg L^{-1}, should be sufficient for optimal growth of microorganisms. Hence, a chemostat culture on 20 g L^{-1} of methanol and 2 μg L^{-1} of biotin was carried out at a dilution rate of 0.06 h^{-1}. However, wash-out was attained after about four liquid residence times, in the same manner as with the mixture of vitamins composed of thiamine, pyridoxine, alanine, riboflavin and inositol. A second chemostat culture on 20 g L^{-1} of methanol with

20 μg L^{-1} of biotin was performed and was stable for 14 liquid residence times (figure 6), after which the culture was stopped.

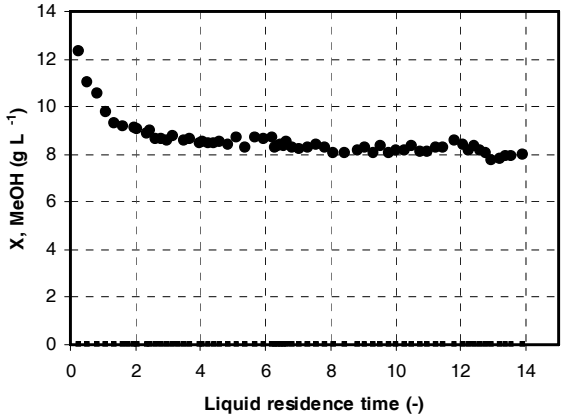

Figure 6: Chemostat culture on 20 g L^{-1} of methanol and 20 μg L^{-1} of biotin. Dry cell weight X (●) and residual methanol (■) concentrations. Data was represented in terms of liquid residence time, τ.

At steady state, the average reconciled yield coefficients were $Y_{X/S}$ = 0.47 C-mol C-mol^{-1}, $Y_{CO2/S}$ = 0.56 mol C-mol^{-1}, $Y_{O2/S}$ = 1.05 mol C-mol^{-1}, $Y_{NH3/S}$ = 0.08 mol C-$mol^{-1}$and $Y_{Q/S}$ = - 522 kJ C-mol^{-1}. The statistical test value h was of 1.30 which is lower than the threshold value of 5.99 for a redundancy of two and a confidence level of 95%. Considering an error of 5% on the calculated yield coefficients, the growth stoichiometry was therefore the same as the one determined with an excess of biotin, which means that 20 μg L^{-1} of biotin are sufficient to support growth of *P. pastoris* to at least 8 g L^{-1} of dry cell weight.

Moreover, similar avidin productivities were obtained with 20 μg L^{-1} of biotin as with the absence of biotin (with the mixture of vitamins), which means that the presence of small amounts of biotin did not hinder the production of biotin-free recombinant avidin. Indeed, with the chemostat culture supplemented with 20 μg L^{-1} biotin (figure 6) the avidin concentration was 2.7 mg L^{-1} at steady state and the volumetric and specific avidin production rates were 166 μg L^{-1} h^{-1} and 22 μg g^{-1} h^{-1}, respectively.

It was quite surprising that the amount of excreted recombinant avidin detected in cultures containing 20 μg L^{-1} biotin or biotin-free, was similar (2.7 and 2.6 mg L^{-1} respectively). This is because 2.7 mg L^{-1} avidin was measured in the culture containing 20 μg L^{-1} biotin, while theoretically up to 1.3 mg L^{-1} would have been expected to bind to the 20 μg L^{-1} biotin added to the medium. As a result 1.4 mg L^{-1} avidin should have been measured.

One explanation for these results might be that the rate of uptake of biotin by *P. pastoris* was faster than the characteristic time of binding of biotin with avidin. According to Wilchek and Bayer (1990), the rate constant for binding of biotin to avidin is $7x10^7$ M^{-1} s^{-1} at pH 5 and 25°C. Under these conditions, the calculated characteristic binding time of biotin to avidin is 0.47 s^{-1}.

Biotin is taken up by active transport in many microorganisms, such as *E. coli* (Piffeteau and Gaudry (1985), Prakash and Eisenberg (1974)) and *S. cerevisiae* (Rogers and Lichstein (1969a)). For biotin concentrations of 20 μg L^{-1}, the uptake rate of biotin is 7.5 pmol mg^{-1} min^{-1} in *S. cerevisiae* resting cells (Rogers and Lichstein (1969a)) and 0.25 pmol mg^{-1} min^{-1} in *S. cerevisiae* cells growing on glucose (Rogers and Lichstein (1969b)). The calculated characteristic time for uptake of biotin is therefore 80 s and 40 min for resting and growing *S. cerevisiae* cells, respectively, which is much higher than the characteristic time for binding of biotin to avidin. This suggests that the uptake rate of biotin by *P. pastoris* cells must be faster than for *S. cerevisiae* cells, since in the chemostat culture with 20 μg L^{-1} of biotin the level of produced recombinant avidin was as high as the culture to which no biotin was added and yield coefficients were as high as in the chemostat culture with an excess of biotin.

Rogers and Lichstein (1969b) pointed out that when avidin was added to cultures with an excess of biotin (concentrations of biotin higher than that necessary for optimal growth), *S. cerevisiae* developed the ability to take up larger amounts of biotin than in cultures with no addition of avidin. They also showed that the uptake rate of biotin depended on the concentration of biotin in the culture medium. Larger amounts of biotin were taken up in cultures when *S. cerevisiae* was grown with low (but sufficient) amounts of biotin (< 9 μg L^{-1}). Moreover, according to the results of Rogers and Lichstein (1969a,b) the nature of the transport system (active transport or free diffusion) seemed to depend on the concentration of biotin in the culture medium. Biotin was taken up by active transport in cultures with low concentrations of biotin and in all proba-

bility by facilitated diffusion in cultures with an excess of biotin. Hence, uptake rate of biotin appears not only to depend on the organism but also on the composition of the culture medium (biotin concentration, presence of avidin in culture medium) and on the experimental growth conditions (temperature, pH). A detailed analysis of the nature of the transport system and the uptake kinetics of biotin in *P. pastoris* would therefore be necessary in order to analyze in-depth the results of this work.

Moreover, additional experiments (data not shown) showed that the results of this study at low cell densities could also be applied for high cell density fed-batch cultures. Indeed, the use of 400 μg biotin during a one-liter high cell density fed-batch culture allowed optimal growth of *P. pastoris* up to 160 g L^{-1} dry cell weight and the concomitant production of recombinant biotin-free avidin at the same specific productivity as in low cell density chemostat cultures.

4. 5. Growth of *P. pastoris* with an excess of avidin in the culture medium

In order to determine if the avidin-biotin complex could be assimilated by *P. pastoris* cells, or if biotin uptake is faster than the binding of biotin to avidin under the studied experimental conditions, a batch culture was performed on 40 g L^{-1} of glycerol with an excess of avidin. The culture medium contained 20 μg L^{-1} biotin and 8.33 mg L^{-1} hen egg white avidin in order to have an excess of avidin. Results are presented in figure 7. Profiles of biomass, glycerol concentration and heat generation were similar to the batch culture without biotin in the culture medium (figure 3). These results show that the complexed biotin cannot be assimilated by *P. pastoris* cells. Hence, the fact that low amounts of biotin (20 μg L^{-1} of biotin for a dry cell weight concentration of 8 g L^{-1}) allow optimal growth of *P. pastoris* cells and the concomittant production of recombi-

nant biotin-free avidin implies that biotin uptake must be faster than binding of avidin and biotin under the studied experimental conditions.

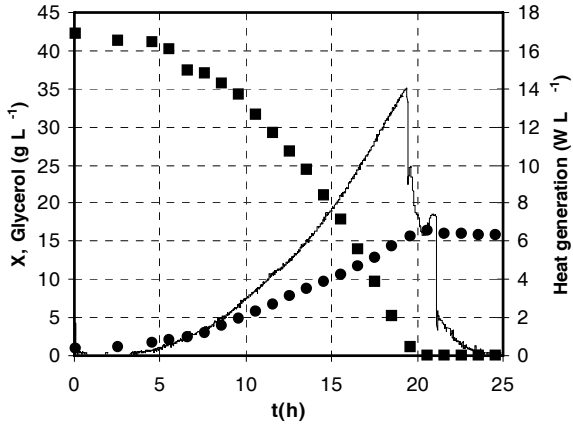

Figure 7: Batch culture on 40 g L^{-1} of glycerol with an excess of avidin (0.020 mg biotin and 8.33 mg avidin per L of medium). Dry cell weight X (●) and glycerol (■) concentrations, heat generation (continuous line).

5. CONCLUSIONS

The results of the batch and continuous cultures on glycerol and methanol showed that biotin is an essential vitamin for the growth of *P. pastoris*. Biotin deficiency results in lower growth rates and lower biomass yields in batch cultures and in wash-out in continuous cultures. However, optimal growth was observed during about 4 liquid residence times in continuous cultures with no biotin in the feed medium, and optimal growth was also observed during the induction phase on methanol of high cell density fed-batch cultures performed by Zocchi et al. (2003b) with no biotin in the feed medium. These observations lead to the conclusion that even if no biotin is supplied, stocks of biotin accumulated in the cells during the batch phase performed with an excess of biotin can support growth during a certain time in a biotin-free environment.

The structurally unrelated compounds, aspartic acid and oleic acid, had a growth promoting effect in biotin-free medium. However, the performance of chemostat cultures on methanol with aspartic and oleic acids as supplements in a biotin-free medium re-

sulted in wash-out after approximately 6 liquid residence times, thus these two compounds could not replace biotin completely.

The results show that cultures to which small amounts of biotin have been added (20 $\mu g\ L^{-1}$ of biotin for a cell density of about 8 $g\ L^{-1}$) attain growth yields as high as those with an excess of biotin and biotin-free avidin production levels as high as those with no addition of biotin.

The experiments performed in this study suggest that the time available for biotin uptake was shorter than the time required for binding of biotin to avidin. More experiments would be necessary to confirm this hypothesis. For instance, a quantitative characterization of the biotin transport system in *P. pastoris* should enable to verify if biotin uptake is faster than the binding of biotin to avidin under the studied experimental conditions.

6. ACKNOWLEDGEMENTS

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7. NOMENCLATURE

AOX	Alcohol oxidase	
C_P	Concentration of avidin	$\mu g\ L^{-1}$
D	Dilution rate	h^{-1}
h	Statistical test value	
$\Delta_r H_S^{*\circ}$	Standard molar enthalpy change of reaction r per C-mol of substrate consumed	$kJ\ C\text{-}mol^{-1}$
L	Redundancy	
P	Variance-covariance matrix	
q_P	Specific avidin production rate	$\mu g\ g^{-1}\ h^{-1}$
R_P	Volumetric avidin production rate	$\mu g\ L^{-1}\ h^{-1}$
X	Cell dry weight	$g\ L^{-1}$
$Y_{j/i}$	Yield coefficient of substance j on substance i	$C\text{-}mol\ C\text{-}mol^{-1}$

ε	residuals	
τ	Liquid residence time	h

Subscripts

i	Refers to compound i
j	Refers to compound j
S	Refers to the limiting nutrient
Q	Heat
P	Refers to the produced recombinant avidin

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CHAPTER 3

QUANTITATIVE CHARACTERIZATION OF THE REGULATION OF THE SYNTHESIS OF ALCOHOL OXIDASE AND OF THE EXPRESSION OF RECOMBINANT AVIDIN IN A *PICHIA PASTORIS* MUT$^+$ STRAIN

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1. ABSTRACT

Pichia pastoris is increasingly used as a host for the expression of heterologous proteins both in academic research and at industrial scale. It is therefore important to have a good understanding of the regulation of its expression system. Usually, heterologous proteins are expressed under the control of the AOX1 promoter, which drives the expression of alcohol oxidase 1 in the wild-type strain.

The regulation of the synthesis of alcohol oxidase and the recombinant protein productivity were investigated in chemostat cultures on glycerol and on methanol as sole carbon sources for a *P. pastoris* Mut$^+$ strain expressing and secreting a recombinant avidin.

Under glycerol limitation, alcohol oxidase was derepressed, and both on glycerol and on methanol, the specific AOX activities increased with decreasing dilution rate. However, specific AOX activities in cultures on glycerol were only 1 to 5% of the activities measured in cultures on methanol, and recombinant avidin could only be detected in cultures on methanol.

The recombinant avidin production rate increased linearly with dilution rate in cultures on methanol. A growth-associated linear model was applied in order to predict the specific recombinant avidin productivity as a function of dilution rate.

The black box stoichiometry was also characterized quantitatively as a function of dilution rate. After validation of the steady-state data with a statistical hypothesis testing

method, analysis revealed that because of the low maintenance demand of *P. pastoris*, the stoichiometry did not change significantly in the range of dilution rate studied.

Keywords: Derepression; Alcohol oxidase; Avidin; *Pichia pastoris.*

2. INTRODUCTION

Interest in the study of methylotrophic yeasts, which are by definition able to utilize methanol as the sole carbon and energy source, has increased dramatically in the last decades. All strains identified to date belong to only four genera: *Hansenula*, *Pichia*, *Candida* and *Torulopsis* (Faber et al. (1995)).

Methylotrophic yeasts were first isolated from soil, rotten fruits and the gut of insects (Gellissen (2000)). Initially, these yeasts were evaluated for the production of single-cell protein to be used primarily as high protein animal feed. However, the production of single-cell protein from methanol was not financially interesting.

Nowadays the species *Pichia pastoris* is extensively used for the expression of heterologous proteins, several examples can be found in Gellissen (2000). Many reviews describe the general features of this yeast expression system (Faber et al. (1995), Cereghino and Cregg (2000), Cregg and Higgins (1995), Daly and Hearn (2005), Romanos et al. (1992), Vedvick (1991)) and examining advances in its development and application (Cregg et al. (1993), Hollenberg and Gellissen (1997), Romanos (1995), Sreekrishna et al. (1997)).

A commonly used approach for heterologous protein production is to express the gene of interest under the control of the AOX1 promoter. In the wild-type *P. pastoris* strain, this promoter controls the expression of alcohol oxidase 1 which is the first enzyme in methanol metabolism (Cregg et al. (1989)). There are actually two copies of the alcohol oxidase (AOX) gene in the genome of *P. pastoris*, called AOX1 and AOX2. The AOX1 promoter regulates 85% of AOX production while the AOX2 promoter is less active (Cregg et al. (1989)). In methanol-grown cells, AOX can account for up to 35% of the total cell protein (Sreekrishna et al. (1997)). This is believed to be due to the fact that AOX has a low affinity for oxygen; the cell therefore compensates by producing the enzyme in large amounts (Koutz et al. (1989)).

The AOX1 promoter is induced by methanol, but repressed in the presence of excess glycerol. Typically, recombinant *P. pastoris* cells are grown in batch culture on glycerol to repress foreign gene expression followed by induction after the addition of methanol

in the fed-batch phase. With regard to methanol-utilizing ability, three phenotypes of expression strains are available (Stratton et al. (1998)): methanol utilization positive (Mut^+), methanol utilization slow (Mut^S) and methanol utilization negative (Mut^-). Mut^+ strains contain both AOX1 and AOX2, Mut^S strains contain only AOX2 and Mut^- strains are defective in both AOX1 and AOX2. The three different phenotypes can be distinguished by their specific growth rates on methanol which are respectively of 0.14 h^{-1}, 0.04 h^{-1} and 0 h^{-1} (Stratton et al. (1998)).

Since there is increasingly interest in using *P. pastoris* as an expression system for heterologous proteins, it is of course important to have a good understanding of the physiology of this species. For instance, little is known about the relationship between central metabolism and recombinant protein productivity. Normally the metabolism is adjusted to produce only the minimum amount of essential metabolites, together possibly with small amounts of non-essential secondary metabolites, or for instance recombinant proteins. This implies that practically all of the carbon source can be considered to be converted into biomass and the end products of energy metabolism (which correspond essentially to CO_2 in the case of *P. pastoris* grown in aerobic conditions). A better comprehension of the relationship between central metabolism and recombinant protein production could improve experimental design and planning with the studied strain.

A few authors have investigated the relationship between growth and heterologous protein production with *P. pastoris* strains. In chemostat cultures, it was reported that heterologous protein productivity is growth-associated and increases linearly with dilution rate. The recombinant protein productivity of a Mut^S *P. pastoris* strain secreting sea raven antifreeze protein was investigated in chemostat cultures on mixed feeds of glycerol and methanol by D'Anjou and Daugulis (2001). The specific recombinant protein productivity increased more or less linearly with dilution rate between 0.01 and 0.09 h^{-1}. Curvers et al. (2001) worked with a Mut^+ *P. pastoris* strain secreting human chymotrypsinogen B and found also a linear correlation between specific recombinant protein productivity and dilution rate. In fed-batch cultures, contradictory relationships between recombinant protein productivity and specific growth rate were reported. For instance, Cunha et al. (2004) and Kobayashi et al. (2000) reported that for low growth rates the specific recombinant protein productivity decreased when the growth rate increased,

and above 0.025 and 0.015 h^{-1} respectively, the productivity did not depend on the growth rate (Cunha et al. (2004), Kobayashi et al. (2000)).

On the other hand, with *P. pastoris* strains, little is known about the regulation of the synthesis of alcohol oxidase as a function of growth rate. A detailed study on the regulation of the synthesis of catabolic enzymes has already been made by Egli et al. (1980) for the two methylotrophic yeasts *Hansenula polymorpha* and *Kloeckera* sp. 2201. It was shown that in chemostat cultures on glucose the synthesis of alcohol oxidase increased with decreasing dilution rates. Although it is known for a long time that AOX is strongly repressed in *P. pastoris* strains by carbon sources such as glycerol and glucose and is induced by carbon starvation (Tschopp et al. (1987)) and methanol, no study has described the regulation of the synthesis of this enzyme as a function of growth rate. Moreover, a relationship between alcohol oxidase level in the cells and recombinant protein productivity could be conceivable with heterologous proteins expressed under the control of the AOX1 promoter, alcohol oxidase being the first key enzyme for methanol metabolism.

This study focuses on the quantitative analysis of the regulation of the synthesis of alcohol oxidase and of the expression of recombinant protein as a function of growth rate in a *P. pastoris* Mut^+ strain expressing and secreting recombinant glycosylated avidin. Cultures were performed in continuous mode since it allows for control of cultivation conditions, and hence of cell physiology.

The aim is to determine if and how recombinant avidin production is related to growth and to which extent the alcohol oxidase expression is repressed in the presence of glycerol as carbon source. To achieve this, single carbon source limited continuous cultures were performed at various dilution rates on either glycerol or methanol.

In addition, the chemostat cultivation mode enabled determining the whole growth stoichiometry, including biomass, carbon dioxide, oxygen, nitrogen source and heat yields, using a reconciliation procedure involving a consistency test of the data and the evaluation of the best estimates of the measured and non-measured yields.

3. MATERIALS AND METHODS

3. 1. Microorganism, inoculum preparation and media

A recombinant *Pichia pastoris* Mut^+ strain was kindly provided by Andrea Zocchi from the university of Neuchâtel (Switzerland). Zocchi et al. (2003) designed a clone of *P. pastoris* expressing and secreting a recombinant glycosylated avidin (recGAvi) with an acidic isoelectric point. Stock cultures were stored at -80°C in a 20 g L^{-1} glycerol and 9 g L^{-1} NaCl solution. Cells were re-activated in a 1 liter baffled shake flask containing 100 mL of complex YPG medium at 30°C for 24 h. Two cultures in baffled shaken flasks of 100 mL each were used to prepare the inoculum. After 24 h at 30°C, the two precultures were centrifuged at 3000 rpm during 10 min at 4°C and the pellets were resuspended in 10 mL of sterile water. This cell suspension was immediately utilized to inoculate the bioreactor containing 1L of defined medium.

The complex YPG medium contained 20 g L^{-1} glycerol (Sigma-Aldrich, Steinheim, Germany), 6 g L^{-1} yeast extract (OXOID, Hampshire, England), 5 g L^{-1} Bacto Peptone (Becton, Le Pont de Claix, France). This medium was sterilized at 120°C during 20 min.

The defined medium used for batch cultures and in the reservoir feed bottle for continuous cultures was based on the medium developed by Egli and Fiechter (1981) and contained per liter: 40 g glycerol or 20 g of methanol as carbon source, 15.26 g NH_4Cl, 5.62 g KH_2PO_4, 1.18 g $MgSO_4$ $7H_2O$, 0.9 g EDTA $2H_2O$, 110 mg $CaCl_2$ $2H_2O$, 75 mg $FeCl_3$ $6H_2O$, 28 mg $MnSO_4$ H_2O, 44 mg $ZnSO_4$ $7H_2O$, 8 mg $CuSO_4$ $5H_2O$, 8 mg $CoCl_2$ $6H_2O$, 5.2 mg Na_2MoO_4 $2H_2O$, 8 mg H_3BO_3, 1.2 mg KI, 1.74 mg biotin, and 0.4 mL of antifoam agent (Struktol SB 2121, Schill and Seilacher, Hamburg, Germany). For the cultures on methanol, only 20 μg L^{-1} of biotin was added to the medium because higher concentrations of biotin would interfere with the quantification of the produced recombinant avidin protein. All components of the defined medium were obtained from Fluka, Buchs, Switzerland, except glycerol which was obtained from Sigma-Aldrich, Steinheim, Germany. The defined medium was sterilized by filtration (0.22 mm, Steritop, Millipore Corporation, Billerica, USA).

3. 2. Culture conditions

Growth experiments were undertaken using a bench-scale heat-flux calorimeter RC1 (Mettler Toledo AG, Greifensee, CH) modified for biotechnological applications with a

working volume of 1.6 L. Characteristics of this so called Bio-RC1 can be found in Marison et al. (1998).

Temperature was maintained at 30°C and pH at 5.0 by the automatic addition of 2M KOH. The heat of neutralization was calculated from the titrimetric consumption of KOH 2M according to Meier-Schneiders et al. (1995). A polarographic pO_2 probe (InPro 6800, Mettler Toledo, Greifensee, Switzerland) was used to monitor dissolved oxygen, with respect to air saturated medium. Aeration rate was set at a constant value of 2.0 NL min^{-1} of air (2 vvm) using a thermal mass flow controller (5850E, Rosemount Brooks, Veenendaal, The Netherlands). The air was satured with water before its entry in the calorimeter. Stirring rate was fixed at a constant value of 800 rpm in order to maintain dissolved oxygen levels above 80% air saturation throughout the experiments.

All liquid inlet flows were quantified gravimetrically. The feed pump rate was controlled by a proportional controller based on the on-line monitoring of the reservoir scale. The temperature of all feeds was adjusted to reactor temperature before they entered the calorimeter.

Torque measurements were used to detect baseline shifts of the heat flux Menoud et al. (1995). As the release of CO_2 from the medium is an endothermic process, the heat flow induced by CO_2 stripping was taken into account according to Meier-Schneiders et al. (1995). The heat losses to the environment were calculated by using a heat transfer coefficient for the bioreactor of 0.133 W K^{-1}, which was determined as described in Birou (1986).

3. 3. Continuous culture

Cultures were grown in batch culture on 40 g L^{-1} of glycerol until the exhaustion of the initial glycerol charge was indicated by a sudden increase in the dissolved oxygen level.

For continuous cultures on glycerol, the supply of the feed-medium was immediately started after the depletion of glycerol at the end of batch growth.

For continuous cultures on methanol, sterile methanol (3 mL) was added to the medium four hours after all glycerol in the batch phase was consumed. One hour after methanol consumption began, the supply of feed-medium was initiated. This transition phase was done in order to derepress the methanol metabolic machinery and to allow the cells to transition smoothly from glycerol to methanol growth.

Samples were withdrawn periodically (the frequency was determined by the dilution rate) for testing substrate levels and measuring cell density. Samples for the determination of specific alcohol oxidase activity and recombinant protein concentration were carried out only at steady state.

The system was considered to have reached steady state after at least three liquid residence times, when the variations in dissolved oxygen, cell density, carbon dioxide concentration in the exhaust gas and heat signal were lower than the experimental errors on these measurements.

The range of dilution rates studied was 0.02 to 0.22 h^{-1} for cultures on glycerol and 0.03 to 0.12 h^{-1} for cultures on methanol, the maximum specific growth rates (determined in batch cultures) being of 0.24 and 0.14 h^{-1} respectively on these two carbon sources.

3. 4. Substrate and metabolite analysis

Culture samples (about 10 mL) were collected using a purpose built auto-sampler (Cannizzaro (2002)) and kept at 2°C for up to 12 hours before handling. Samples for the analysis of alcohol oxidase activity and avidin quantification were handled immediately after cooling for 10 minutes at 2°C.

The concentration of biomass was determined gravimetrically as dry cell weight. A sample of the fermentation broth was spun down in a pre-weighed glass-sample tube and the pellet was dried at 100°C to constant weight.

The C-molar composition of biomass $CH_{xH}O_{xO}N_{xN}$ was determined by elemental analysis as described in Gurakan et al. (1990). Cultures samples were collected at steady state, centrifuged and washed three times with deionized water. After the final washing/centrifugation step, the cell pellets were spread as a thin layer of about 2 mm on Petri dishes and stored at -20 °C. The frozen cell pellets were freeze-dried for 24 hours. Analysis of carbon, nitrogen and hydrogen content was made with a CHN analyzer (Carlo Erba Instruments, Rodano, Italy). Oxygen content was calculated by difference (Gurakan et al. (1990)). The ash content was determined by placing weighed quantities (0.5-1.0 g) of lyophilized biomass samples in ceramic crucibles followed by incubation at 550 °C during 6 hours (Gurakan et al. (1990)). For each carbon source, the biomass composition stayed constant over the investigated dilution rate ranges.

Glycerol and methanol were determined by HPLC analysis (1100 series, Agilent Technologies, Palo Alto, USA). An ion exchange chromatography column (Supelcogel H

300 mm, Supelco, Bellefonte, USA) with a guard column (Superlguard C610H, Supelco, Bellefonte, USA) was used at 60 °C. A 5 mM sulphuric acid solution in ultrapure water was applied at a constant eluent flow rate of 0.6 mL min^{-1}. Metabolites were measured using a refractive index detector. The detection limit was of 0.01 g L^{-1} for glycerol and of 0.1 g L^{-1} for methanol.

The carbon dioxide production rate in the bioreactor off-gas was determined using an infrared (PSA 402, Servomex, Crowborough, UK) analyser. The measured values were corrected for water vapour according to Duboc and von Stockar (1998).

3. 5. Recombinant avidin quantification

Recombinant avidin was quantified by biotin-4-fluorescein titration of binding sites (Kada et al. (1999)), assuming a tetrameric form of the recombinant avidin, with four active-binding sites (Zocchi et al. (2003)). Biotin-4-fluorescein was obtained by Fluka, Buchs, Switzerland.

Since the detection limit of this method is approximately 10 mg L^{-1} avidin, it was necessary to concentrate the samples about 15 times by ultrafiltration (Centriplus, 30 KDa, Millipore Corporation, Bedford, USA) before titration with biotin-4-fluorescein, the molecular weight of the active form of recombinant avidin being of 65.4 kD (Zocchi et al. (2003)).

The volumetric and specific avidin productivities have been calculated at steady state according to equations (1) and (2) respectively:

$$R_P = C_P \cdot D \ (\text{mg L}^{-1}\ \text{h}^{-1}) \tag{1}$$

$$q_P = \frac{C_P \cdot D}{X} \ (\text{mg g}^{-1}\ \text{h}^{-1}) \tag{2}$$

where D (h^{-1}), X (g L^{-1}) and C_P (mg L^{-1}) respresent respectively the dilution rate, dry cell weight and avidin concentration.

3. 6. Preparation of cell-free extracts

For the preparation of cell-free extracts, fermentation broth samples of 2 mL were immediately cooled at 0-2°C and were subsequently centrifuged at 14000 rpm for 1 min and at 4°C. Pellets of biomass were washed once with 100 mM potassium phosphate buffer, pH 7.4 at 4°C. Washed biomass samples were stored at -20°C.

Cell extracts were prepared by placing the washed biomass samples in a vibration mill (MM 300, Retsch, Haan, Germany) with about the same volume of glass beads (0.40-0.45 mm diameter, B Braun Melsungen AG, Melsungen, Germany) as the volume of washed biomass pellet and with 400 μL of breaking buffer. The breaking buffer is a 50 mM potassium phosphate buffer (pH 7.4) containing 50 g L^{-1} glycerol, 1 mM EDTA (Fluka, Buchs, Switzerland) and 1 mM phenylmethanesulfonyl fluoride (Fluka, Buchs, Switzerland). Twelve cycles were run at a vibrational frequency of 30 Hz during 30 s. Between each cycle the samples were placed on ice. Alternating vortexing with cooling keeps the cell extracts cold and reduces denaturation of proteins. Cell debris was removed by centrifugation (6 min at 4500 rpm and at 4°C). Enzyme activities were determined immediately after cell disruption. The Bradford method (Protein Assay, Bio-rad, München, Germany) was used to determine total protein concentration. Bovine serum albumin (Fluka, Buchs, Switzerland) served as standard.

3. 7. Alcohol oxidase activity measurement

Alcohol oxidase (EC 1.1.3.13) was assayed by adapting a method developed by Azevedo et al. (2004a), Azevedo et al. (2004b) and Vojinovic et al. (2004). The activity of alcohol oxidase (AOX) was determined using a bi-enzymatic colorimetric assay comprising horseradish peroxidase (HRP) and the reducing substrates phenol-sulfonic acid (PSA) sodium salt dihydrate and 4-aminoantipyrine (AAP). Methanol oxidation by molecular oxygen catalysed by AOX yields formaldehyde and hydrogen peroxide, which is further reduced by HRP with the concomitant formation of a quinoneimine dye.

The activity of AOX was determined spectrophotometrically by monitoring the increase in absorbance at 500 nm for 8 min (COBAS MIRA, Roche, Basel, Switzerland). All kinetic studies were performed at 32°C using a standard assay reaction mixture, containing 0.4 mM AAP, 25 mM PSA and 2 U mL^{-1} HRP in 100 mM potassium phosphate buffer, pH 7.4. 5 μL of sample was added to 200 μL of the standard assay reaction mixture. The reaction was initiated by the addition of 25 μL of methanol.

A linear calibration curve was established by analysing standard solutions of alcohol oxidase (Sigma-Aldrich, Steinheim, Germany) between 0.3 and 5.5 U mL^{-1}.

PSA and AAP were obtained from Fluka, Buchs, Switzerland, HRP was obtained form Sigma-Aldrich, Steinheim, Germany.

The specific activity of alcohol oxidase was expressed in U mg^{-1} total proteins. One unit (U) of AOX corresponds to the oxidation of 1.0 µmole of methanol to formaldehyde per min at pH 7.5 at 25°C.

3. 8. Black box stoichiometry

According to the black box model, the growth reaction on a C-molar basis under aerobic conditions on glycerol can be written as:

$$CH_{8/3}O+Y_{O2/S}\,O_2+Y_{NH3/S}\,NH_3 \rightarrow Y_{X/S}\,CH_{xH}O_{xO}N_{xN}+Y_{CO2/S}\,CO_2+Y_{Q/S} \quad (3)$$

and that on methanol:

$$CH_4O+Y_{O2/S}\,O_2+Y_{NH3/S}\,NH_3 \rightarrow Y_{X/S}\,CH_{xH}O_{xO}N_{xN}+Y_{CO2/S}\,CO_2+Y_{Q/S} \quad (4)$$

where S represents the limiting carbon source and $Y_{Q/S}$ the heat yield in kJ C-mol^{-1}. The formation of water in the black box reaction equations was eliminated because a reduction balance was used instead. The "generalized degree of reduction" corresponding to the hypothetical combustion to CO_2, H_2O and NH_3 was considered (Duboc et al. (1999)).

The volumetric production or consumption rates R_i (C-mol L^{-1} h^{-1}) were calculated from a steady-state balance over the chemostat:

$$D \cdot (Ci_{in} - Ci_{out}) + R_i = 0 \quad (C-mol \cdot L^{-1} \cdot h^{-1}) \quad (5)$$

where D (h^{-1}) represents the dilution rate and C_i (C-mol L^{-1}) the concentration of the substance i.

The specific consumption and production rates q_i were calculated by dividing the volumetric rate by the average dry cell weight concentration at steady state.

The yield of substance j on substance i was calculated as

$$Y_{j/i} = \frac{R_j}{R_i} \quad (C-mol \cdot C-mol^{-1}) \quad (6)$$

On both substrates, five yields have to be determined. The measurements of dry cell weight, carbon source concentrations, carbon dioxide in the exhaust gas and heat generation were used in order to calculate three of them, namely biomass, carbon dioxide

and heat yields. Together with the constraints provided by the carbon, nitrogen, degree of reduction and enthalpy balance a redundancy (L) of

$$L = -(5 - (3 + 4)) = 2 \quad (7)$$

resulted.

The redundancy of the system was used in order to estimate the ammonia and oxygen yields and to reconcile the data.

3. 9. Check of data consistency and reconciliation of yield coefficients

Carbon (R_C) and enthalpy ($R_{\Delta H}$) recoveries were calculated according to the two following equations respectively obtained by carbon and enthalpy balances:

$$R_C = Y_{X/S} + Y_{CO2/S} \quad (8)$$

$$R_{\Delta H=} (Y_{X/S}\, \Delta_C H_X^{\circ *} + Y_{Q/S})/\Delta_C H_S^{\circ *} \quad (9)$$

where $\Delta_C H_X^{\circ *}$ and $\Delta_C H_S^{\circ *}$ are the modified enthalpies of combustion of biomass and substrate in kJ C-mol^{-1}.

Moreover, the yields were checked for consistency and reconciled based on a χ^2-test as proposed by Wang and Stephanopoulos (1983) and Stephanopoulos et al. (1998). The reconciled yield coefficients are the best yield estimates that minimize the magnitude of the residuals ε and are determined by minimizing the sum of squared errors scaled according to their variance. The test value h is given by the sum of the weighted squares of the residuals ε:

$$h = \varepsilon \cdot P^{-1} \cdot \varepsilon \quad (10)$$

where P is the variance-covariance matrix. Assuming measurement errors of 5%, a test value h lower than 5.99 permitted to assume a 95% significance level that no gross errors and no metabolites other than the ones appearing in equations 3 and 4 had affected the balances.

4. RESULTS

4. 1. Steady-state growth stoichiometry

Elemental composition of biomass

Over the studied dilution rate ranges, no significant changes in the elemental composition of biomass were observed. Hence, an average biomass composition was used for calculations. Table 1 summarizes the average results of biomass composition and ash content for growth on glycerol and on methanol as sole carbon sources in chemostat cultures. The generalized degree of reduction (γ^*_X) and molar mass (M_X) of biomass were calculated as described in Duboc et al. (1999). The modified enthalpy of combustion of biomass $\Delta_C H_X^{0*}$ was obtained by multiplying the average generalized degree of reduction of biomass by the oxycaloric quotient ($Q^*_{O,X}$). According to Duboc et al. (1999), the oxycaloric quotient has an average value of -115.52 kJ e^--mol^{-1} for yeasts.

Table 1: Average values for elemental analysis, ash content (f_{ash}), molar mass (M_X), generalized degree of reduction (γ_X*) and modified enthalpy of combustion ($\Delta_C H_X^{\circ}$*) of biomass in chemostat cultures on glycerol and on methanol. Mass fractions are expressed as g per g biomass. e^- represents the available electrons in biomass. The standard deviation is indicated in parenthesis.

Carbon source	x_H g g^{-1}	x_O g g^{-1}	x_N g g^{-1}	f_{ash} g g^{-1}, %	M_X g C-mol^{-1}	γ^*_X e^-	$\Delta_C H_X^{\circ *}$ kJ C-mol^{-1}
Glycerol	1.764	0.469	0.182	10.43	26.61	4.28	-494.6
	(0.041)	(0.007)	(0.002)	(0.09)	(0.11)	(0.06)	(6.9)
Methanol	1.691	0.502	0.176	6.03	25.77	4.16	-480.4
	(0.023)	(0.025)	(0.006)	(0.08)	(0.16)	(0.03)	(3.9)

Reconciled yield coefficients

The measured variables (dry cell weight, residual carbon source concentrations, carbon dioxide production rate and heat release) as well as the carbon and enthalpy recoveries are shown in figures 1 and 2 for growth on glycerol and methanol respectively, for the various studied steady states. The corresponding calculated reconciled yield coefficients are presented in figures 3 and 4 for growth on glycerol and methanol respectively. For all the analyzed steady states, the statistical test indicated that the data was consistent. Actually the test values h were always lower than the threshold value of 5.99 (figure 5).

For cultures on glycerol, no fermentation by-products, such as ethanol or acetate, were detected, and *P. pastoris* used glycerol entirely to generate biomass and CO_2. This supports the notion that *P. pastoris* grows exclusively in a respiratory manner and is thus an efficient biomass and protein producer. Likewise, for cultivations on methanol, no overflow product, such as formaldehyde or formic acid were detected, and the statistical test was never rejected (figure 5). Therefore *P. pastoris* used methanol entirely to generate biomass and CO_2.

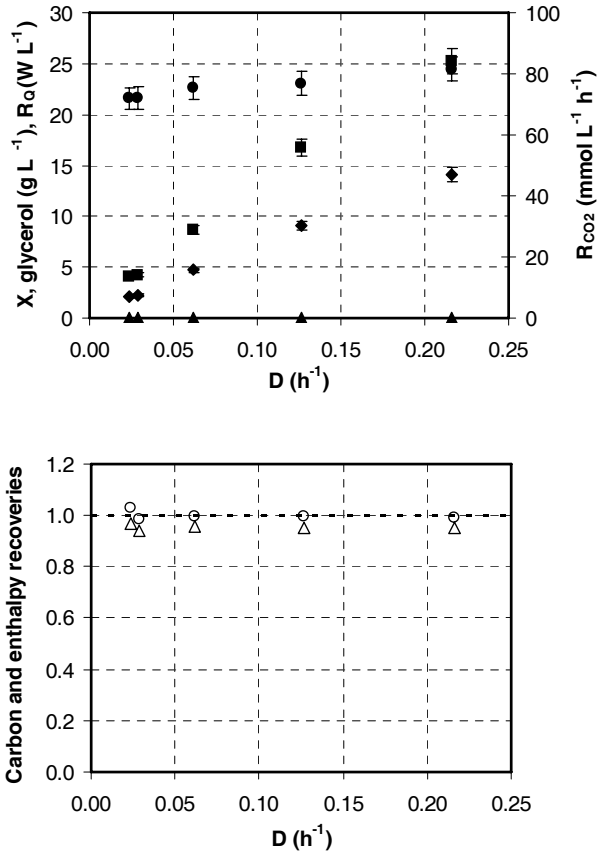

Figure 1: Dry cell weight (X, ●), residual glycerol concentration (▲), power (R_Q, ◆) and carbon dioxide production rate (R_{CO2}, ■) for cultures on glycerol as limiting substrate as a function of the dilution rate, and corresponding carbon (○) and enthalpy (△) recoveries.

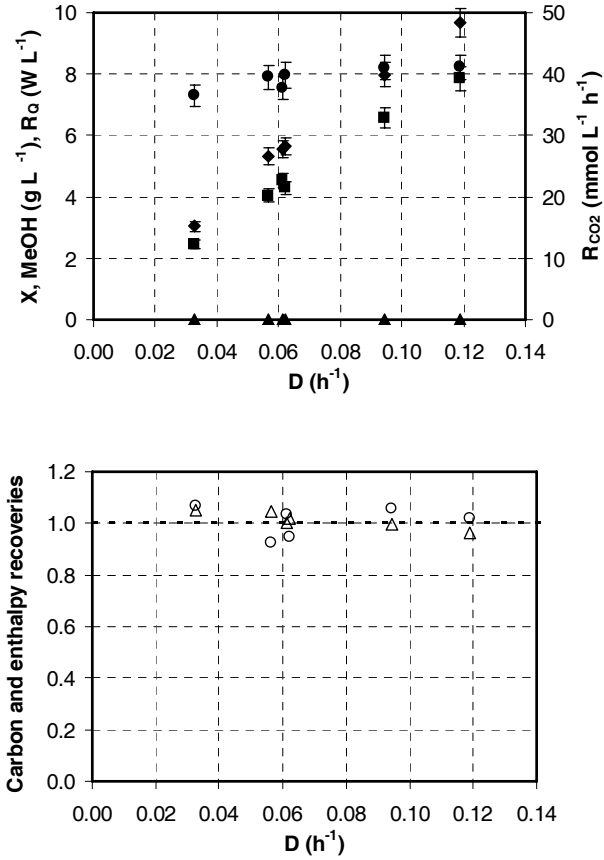

Figure 2: Dry cell weight (X, ●), residual methanol concentration (▲), power (R_Q, ◆) and carbon dioxide production rate (R_{CO2}, ■) for cultures on methanol as limiting substrate as a function of the dilution rate, and corresponding carbon (❍) and enthalpy (△) recoveries.

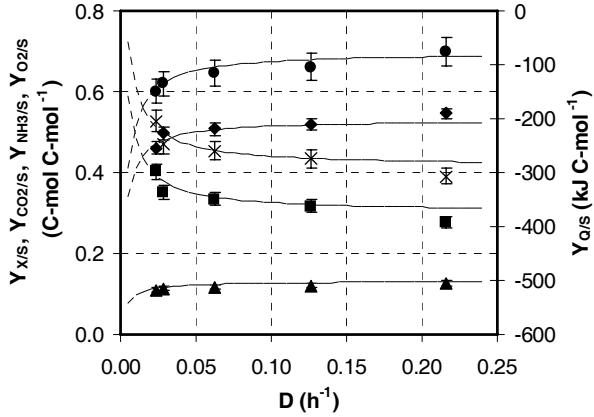

Figure 3: Reconciled biomass (●), carbon dioxide (■), ammonia (▲), oxygen (x) and heat yields (◆) for cultures on glycerol as limiting substrate as a function of the dilution rate. Dashed-lines represent the simulation of yield coefficients as a function of dilution rate with a simple, unstructured mathematical model.

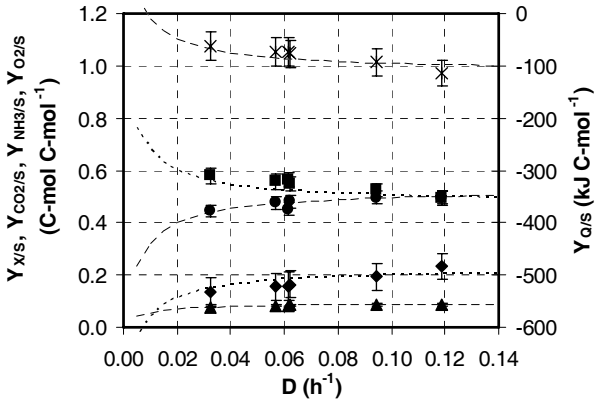

Figure 4: Reconciled biomass (●), carbon dioxide (■), ammonia (▲), oxygen (x) and heat yields (◆) for cultures on methanol as limiting substrate as a function of the dilution rate. Dashed-lines represent the simulation of yield coefficients as a function of dilution rate with a simple, unstructured mathematical model.

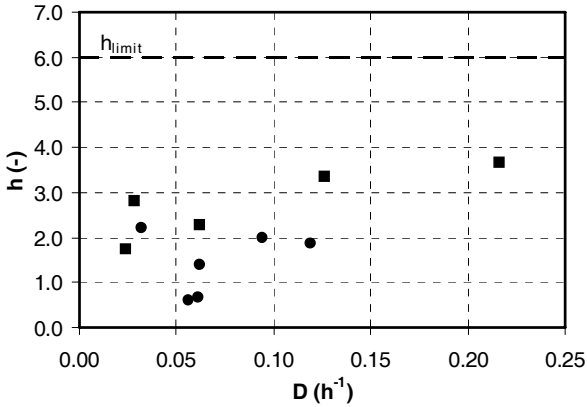

Figure 5: Test value h calculated with reconciliation procedure with a 95% confidence level for cultures on glycerol (■) and on methanol (●). The threshold value to reject hypothesis with a 95% confidence level and a degree of redundancy of 2 is of 5.99.

Maintenance requirements

The overall growth reaction depends on the environmental conditions, for instance on the dilution rate applied. However, for both substrates, the yield coefficients did not vary considerably in the studied dilution rate ranges (figure 3 and 4) indicating a low maintenance demand of *P. pastoris*.

The true growth yield coefficients ($Y_{X/S}^{true}$) and the empirical maintenance coefficients (m) were estimated by plotting the specific glycerol and methanol consumption rates as a function of the dilution rate (figure 6). The maintenance coefficients were of 0.007 and 0.011 C-mol C-mol^{-1} h^{-1} for growth on glycerol and methanol respectively (which correspond to maintenance coefficients of 0.009 and 0.014 g g^{-1} h^{-1} respectively). The true growth yield coefficients related only to energy source consumption for growth purposes (if there were no maintenance) were 0.71 and 0.53 C-mol C-mol^{-1} for growth on glycerol and methanol respectively (which correspond to true growth yield coefficients of 0.61 and 0.42 g g^{-1} respectively).

By comparison, on methanol, *Hansenula polymorpha* has a maintenance coefficient of 0.028 C-mol C-mol^{-1} h^{-1} at 35°C and *Candida boidinii* a maintenance coefficient of 0.031 C-mol C-mol^{-1} h^{-1} at 33°C (Heijnen and Roels (1981)), which is about three times higher than found for *P. pastoris* strains. Jahic et al. (2002) determined a maintenance coefficient of 0.013 g g^{-1} h^{-1} (which corresponds to 0.010 C-mol C-mol^{-1} h^{-1}) for fed-batch cultures with a *P. pastoris* strain on methanol, which is very close to the value obtained in this study.

The true growth yield coefficient of 0.42 g g^{-1} for cultures on methanol is close to the value of 0.41 g g^{-1} determined by Hazeu and Donker (1983) for a *H. polymorpha* strain growing on methanol.

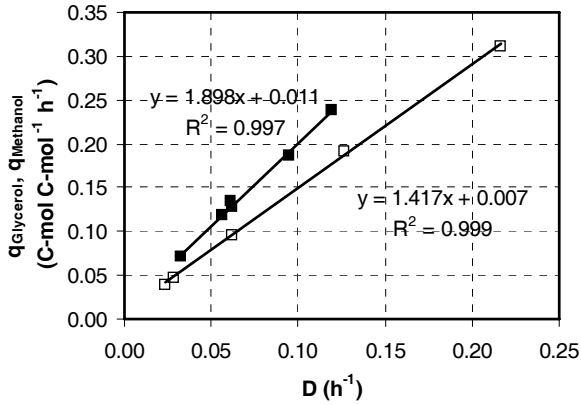

Figure 6: Specific methanol (■) and glycerol (□) consumption rates at steady state as a function of dilution rate.

Simple, unstructured model for black box stoichiometry based on maintenance requirements

In order to predict quantitatively the growth stoichiometry as a function of dilution rate, a simple, unstructured mathematical model describing growth in chemostat cultures limited by carbon source (S) and accounting for maintenance effects was applied.

Assuming that the specific substrate consumption rate required for maintenance is constant, the biomass concentration (X) can be expressed as a function of the dilution rate with the following equation:

$$X = \frac{D \cdot (C_{S,in} - C_S) \cdot Y_{X/S}^{true}}{D + m \cdot Y_{X/S}^{true}} \text{ (C-mol L}^{-1}\text{)} \qquad (11)$$

where $C_{S,in}$ and C_S correspond to the carbon source concentration in the feed medium and to the residual carbon source concentration in the reactor respectively.

The biomass yield was calculated as a function of dilution rate by dividing equation (11) by the amount of substrate consumed at steady state, and by applying the maintenance coefficient and the true biomass yield coefficient experimentally determined in figure 6. Since the degree of freedom of the black box model is of one (5 yields minus 4 balances), all the remaining yield coefficients could be calculated as a function of dilution rate. The predictions of this model were compared to the reconciled yield coefficients in figures 3 and 4. In the studied dilution rate ranges, good agreement was obtained between the reconciled yields and the simulations. Moreover, at low dilution rates, this model provides an estimate of all the yield coefficients and avoids the need to perform very time-consuming steady-state experiments.

4. 2. Regulation of the synthesis of alcohol oxidase

Specific AOX activities have been measured at various dilution rates in chemostat cultures on glycerol as limiting substrate. A partial derepression of the AOX promoter took place, even though glycerol was not dependent on this enzyme for its assimilation. Even if the synthesis of AOX is repressed in the presence of glycerol (Daly and Hearn (2005)), this enzyme is expressed at low concentrations of glycerol, as is the case in glycerol-limited continuous cultures.

The results presented in figure 7 show that the level of AOX in the cells gradually increased with decreasing dilution rate. Catabolite repression was relieved to a higher extent at lower dilution rates. However, cells of chemostat cultures on glycerol showed only 1 to 5% of the AOX activity of that of cultures on methanol at the same dilution rate. Specific AOX activities were also determined in cell-free extracts of *P. pastoris* grown at various dilution rates in chemostat cultures on methanol as limiting substrate (figure 7). The specific AOX activities were 20 to 100 fold higher than in chemostat cultures on

glycerol and increased with decreasing dilution rates, but in a different manner than with cultures on glycerol. A similar trend has been observed with the methylotrophic yeast *Hansenula polymorpha* grown in chemostat cultures on methanol by Dijken et al. (1976).

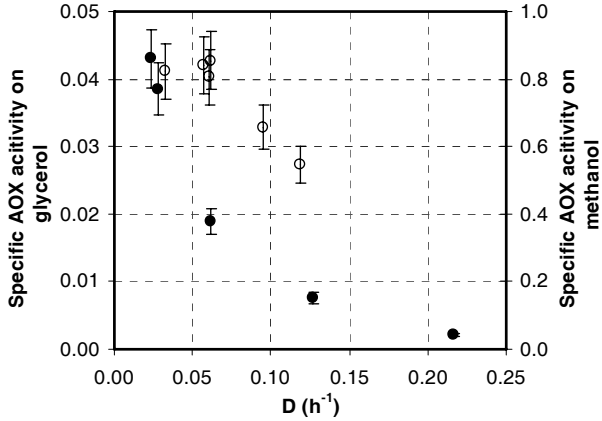

Figure 7: Specific activities of alcohol oxidase (AOX) expressed in U mg^{-1} total intracellular proteins in cell-free extracts of *P. pastoris* grown in chemostat cultures on glycerol (●, left axis) and on methanol (❍, right axis) as limiting substrate, as a function of the dilution rate.

4. 3. Recombinant avidin production

Even if a partial derepression of alcohol oxidase was observed in chemostat cultures on glycerol as limiting substrate, no recombinant avidin could be detected.

However, for each chemostat culture performed on methanol as carbon source, the titration of binding sites by biotin-4-fluorescein allowed to check the functionality and to quantify the secreted recombinant avidin. Knowing the concentration of avidin, equations 1 and 2 were applied in order to calculate the volumetric and the specific avidin production rates as a function of dilution rate which corresponds to the specific growth rate in steady-state continuous cultures. The results are presented in figure 8 and show that avidin production was growth-associated. In fact, the specific productivity of avidin increased linearly with dilution rate between 0.03 and 0.12 h^{-1}.

A linear regression was applied to the calculated specific productivities as a function of dilution rate (figure 8). The specific avidin productivity could be expressed as a function of dilution rate with the following equation (for the studied range of dilution rates):

$$q_P = Y_{P/X} * D + \beta \quad (12)$$

The avidin formation was related to growth by a constant yield coefficient $Y_{P/X}$ (126.4 ± 31.1 μg g^{-1}). The second term β (12.7 ± 2.4 μg g^{-1} h^{-1}) includes the avidin formation proportional to the cell density.

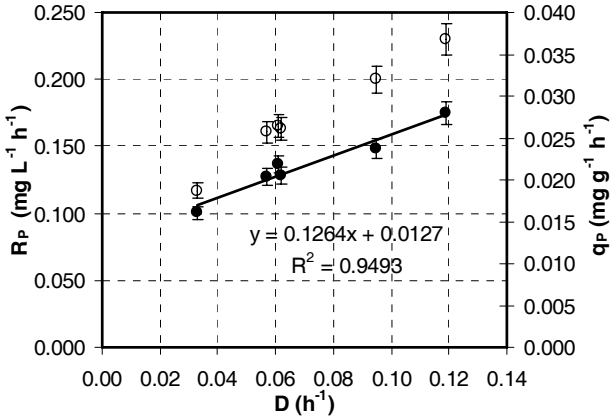

Figure 8: Steady-state volumetric (○, R_P) and specific (●, q_P) productivities of recombinant avidin as a function of dilution rate in chemostat cultures on methanol.

5. DISCUSSION

5. 1. Regulation of the synthesis of alcohol oxidase

For chemostat cultures on glycerol as limiting substrate, the extent of repression of AOX synthesis in *P. pastoris* is most likely a function of the residual glycerol concentration in the reactor. This behavior has already been pointed out by Egli et al. (1980) for the two methylotrophic yeasts *Hansenula polymorpha* and *Kloeckera* sp. 2201 growing in continuous cultures on glucose as limiting substrate. As the dilution rate de-

creases, the residual concentration of glycerol also decreases (Herbert et al. (1956)) and repression by glycerol is relieved to a greater extent.

The AOX profiles for *P. pastoris* showed that the repression of AOX was relieved until dilution rates close to the maximum specific growth rate. In this view *P. pastoris* shows a behavior similar to that of *H. polymorpha* (Egli et al. (1980)). However, concerning the amount of expressed AOX, *P. pastoris* shows a behavior similar to *Kloeckera* sp. 2201 since cells of chemostat cultures on glycerol contained only 1 to 5% of the AOX activity of that of chemostat cultures on methanol at the same dilution rate.

Hence, even if all methylotrophic yeasts show some common features of their expression patterns, the regulation of the synthesis of AOX are not identical and have to be analyzed for each species.

In contrast to *H. polymorpha*, for which significant levels of AOX can be detected on alternative carbon sources such as glycerol, sorbitol or ribose (Gleeson and Sudbery (1988)), with *P. pastoris* high levels of AOX were only expressed in chemostat cultures on methanol. However, as already pointed out, the specific AOX activity increased with decreasing dilution rates with a similar trend than with *H. polymorpha* (Dijken et al. (1976)). The same authors pointed out that if the rate limiting step in methanol metabolism is the oxidation of methanol, a small variation in the rate of methanol oxidation should affect the growth rate. Therefore, at low dilution rates, the residual methanol concentration decreases and the low affinity of AOX for methanol rapidly slows down the rate of methanol oxidation and lower growth rates are expected. However, at low dilution rates, the cells maintain a high rate of substrate oxidation by increasing the amount of the rate limiting enzyme.

5. 2. Recombinant avidin productivity

As reported by Curvers et al. (2001) and by D'Anjou and Daugulis (2001) for two other recombinant *P. pastoris* strains, a linear relationship between specific growth rate and specific production rate of the secreted recombinant protein was determined. Indeed, for chemostat cultures on methanol, the specific recombinant avidin productivity increased linearly with dilution rate between 0.03 and 0.12 h^{-1}.

Growth-associated products are usually those which are essential to the function of the organism. Cell components such as cell walls and essential enzymes are generally coupled to growth. Since the expression of avidin is under the control of the promoter

of alcohol oxidase, which is the first key enzyme for methanol metabolism, it is not surprising that the production of avidin is growth-coupled.

However, no correlation between specific AOX activity and specific recombinant avidin production rate could be established. The specific AOX activity rather seemed to be a function of the residual carbon source concentration, which diminishes as the dilution rate decreases. On the other hand, the specific recombinant avidin production rate varied as a function of metabolic rates. Indeed, as the dilution rate was increased, the specific consumption and production rates of the metabolites of the central metabolism increased proportionally to the dilution rate and influenced directly the specific product formation rate.

6. CONCLUSIONS

The black box stoichiometry of a recombinant *P. pastoris* strain expressing and secreting glycosylated avidin has been characterized quantitatively in chemostat cultures as a function of dilution rate on glycerol and on methanol as sole carbon sources. The yield coefficients remained almost constant over a broad range of dilution rates (down to about 0.03 h^{-1}) because of the low maintenance demand of *P. pastoris*. A systematic method using statistical hypothesis testing was applied in order to validate the measurements and the proposed black box models.

Although the expression of the AOX enzyme was derepressed under glycerol-limited growth conditions, the AOX enzyme was only weakly expressed in cultures on glycerol. It was therefore not possible to detect any recombinant avidin in cultures on glycerol. Both in cultures on glycerol and on methanol, the specific AOX activity increased with decreasing dilution rate as has been pointed out by Egli et al. (1980) for two other methylotrophic yeasts.

For chemostat cultures on methanol, recombinant avidin productivity could be described by a growth-associated linear model. Indeed, volumetric and specific recombinant avidin production rates increased linearly with dilution rate between 0.03 and 0.12 h^{-1}. However, no relationship with specific AOX activities could be established, although its promoter was used for the expression of recombinant avidin. Other factors such as metabolic rates of central metabolism were modified as a function of dilution rate and may have influenced the recombinant avidin production rate.

Further insight will be gained by studying the mixed metabolism of *P. pastoris* growing simultaneously on glycerol and methanol in continuous cultures. Corresponding experiments are already under way in our laboratory.

7. ACKNOWLEDGEMENTS

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8. NOMENCLATURE

AOX	Alcohol oxidase	
C_i	Concentration of substance i	C-mol L^{-1}
D	Dilution rate	h^{-1}
f_{ash}	Ash content	g g^{-1}
h	Statistical test value	
$\Delta_C H_i^{\circ *}$	Standard modified entahlpy of combustion of i	kJ C-mol^{-1}
L	Redundancy	
m	Maintenance coefficient	C-mol C-$mol^{-1}h^{-1}$
M_X	Molar mass of biomass	g C-mol^{-1}
P	Variance-covariance matrix	
q_i	Specific production or consumption rate of i	C-mol C-$mol^{-1}h^{-1}$
$Q^*_{O,X}$	Oxycaloric quotient	kJ C-mol
R_i	Volumetric production or consumption rate of i	C-mol L^{-1} h^{-1}
R_C	Carbon recovery	
$R_{\Delta H}$	Enthalpy recovery	
X	Cell dry weight	C-mol L^{-1}
x	Atomic coefficient for one C-mol of biomass	g g^{-1}
$Y_{j/i}$	Yield coefficient of substance j on substance i	C-mol C-mol^{-1}
$Y_{j/i}^{true}$	True yield coefficient	C-mol C-mol^{-1}
β	Avidin formation proportional to the cell density	μg g^{-1} h^{-1}
ε	residuals	

γ^*	Generalized degree of reduction	
μ	Specific growth rate	h^{-1}

Subscripts

i	Refers to compound i
j	Refers to compound j
max	Maximal
S	Refers to the limiting nutrient
Q	Heat
P	Refers to the produced recombinant avidin

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CHAPTER 4

INFLUENCE OF CULTURE TEMPERATURE ON GROWTH STOICHIOMETRY AND PRODUCTIVITY OF RECOMBINANT AVIDIN EXPRESSED AND SECRETED BY A *PICHIA PASTORIS* MUT$^+$ STRAIN

1. ABSTRACT

Cultivation at temperatures lower than the optimal temperature for growth is often used with *P. pastoris* strains in order to increase the productivity of recombinant protein. Optimisation of culture temperature for maximal recombinant protein expression with *P. pastoris* strains is often performed in high cell density fed-batch cultures at various temperatures or in continuous cultures using chemostat cultivation. In order to have an exhaustive picture of the influence of culture temperature on recombinant protein productivity would require repeated chemostat or fed-batch cultures which are very time-consuming. In the present study, we investigated the applicability of a new and dynamic cultivation method, which consists in increasing the temperature linearly during continuous culture after having achieved steady-state growth conditions.

Comparison of culture characteristics, determined during pseudo-steady state continuous cultures with a linear increase of temperature at a rate of 0.1°C h^{-1}, with results obtained at steady-state during chemostat cultures, showed that this technique can be used as a fast and accurate tool to determine the influence of culture temperature on the expression system of host cells.

A recombinant *P. pastoris* Mut$^+$ strain expressing and secreting avidin was chosen as a model system. The influence of culture temperature and specific growth rate on recombinant avidin production rate were investigated with response surface methodology. Since the maximal specific growth rate depends on the culture temperature, the experimental design was defined prior to continuous cultures by performing batch cultures on methanol at various temperatures.

Cultivation at temperatures lower than the optimal temperature for growth did not significantly affect recombinant avidin productivity. However, recombinant avidin productivity increased linearly with specific growth rate.
Keywords: Temperature; Avidin; *Pichia pastoris*; Transient; A-stat.

2. INTRODUCTION

Most processes involving recombinant *P. pastoris* strains are run at an optimum temperature for growth of 30°C (Wegner (1983)). However, it has been reported that recombinant protein productivity increases with lower cultivation temperatures (Chen et al. (2000); Whittaker and Whittaker (2000); Li et al. (2001); Hong et al. (2002); Sarramegna et al. (2002); Jahic et al. (2003a); Jahic et al. (2003b); Li et al. (2003); Shi et al. (2003)). According to these authors, lower cultivation temperature during the induction phase can improve the protein yield by reducing the rate of proteolysis due to kinetic reasons, combined with reduced proteolysis due to a reduction in cell death and protease release to the medium. Moreover, growth at lower temperatures is a means to facilitate correct protein folding due to a slower rate of protein synthesis and a decrease in the driving force for protein self-association (Georgiou and Valax (1996)).
However, there are also reports that cultivation at lower temperatures either did not lead to higher recombinant protein productivity and could even lead to a reduction with certain recombinant *P. pastoris* strains. For instance, Inan et al. (1999) and Curvers et al. (2001) showed that temperatures between 25 and 30°C were all optimum for the yield of recombinant proteins expressed by *P. pastoris* strains and that temperatures below 25°C led to lower productivities. It has also been reported that temperature had no significant effect on the productivity of recombinant human serum albumin expressed by a *P. pastoris* Mut^S strain (Kupesulik and Sevella (2005)).
Traditionally, physiological strain characterization is performed in continuous cultures at steady state. Such experiments are very time-consuming since many steady states have to be attained to achieve an exhaustive picture of the strain under physiologically adapted conditions. In this study, the possibility to analyse the influence of the culture temperature on recombinant protein production rate was investigated under pseudo-steady-state growth conditions by increasing the culture temperature during continuous cultivation on methanol after having achieved steady-state growth conditions. Such transient experiments in continuous cultures allow to speed up strain character-

ization and process development. More precisely, the culture temperature was increased linearly at a rate of 0.1°C h^{-1} from 24 to 32°C, and from 30 to 40°C at a dilution rate of 0.06 h^{-1} in order to investigate quantitatively the influence of the culture temperature on the growth stoichiometry and the recombinant protein productivity for a *P. pastoris* Mut^+ strain expressing and secreting a recombinant glycosylated avidin. Similar transient experiments for the study of the influence of dilution rate on growth stoichiometry have been reported (Paalme and Vilu (1992); Paalme et al. (1995); Paalme et al. (1997a); Paalme et al. (1997b); van der Sluis et al. (2001); Barbosa et al. (2003)). This technique has been termed the A-stat technique and is defined as a continuous culture with smooth controlled change (acceleration rate) of a cultivation parameter.

Results obtained during A-stat cultures with a linear change in temperature were compared with results obtained during chemostat cultures. Moreover, in order to analyse both the influence of culture temperature and specific growth rate on recombinant avidin production rate, additional chemostat cultures were performed in the experimental design. Since the maximal specific growth rate depends on culture temperature, the experimental design was determined prior to continuous cultures by performing batch cultures on methanol at various temperatures.

Response surface methodology, using a linear model with first order interactions, was applied in order to describe and analyse the influence of culture temperature and dilution rate on specific avidin production rate.

The influence of culture temperature on the specific activity of alcohol oxidase (AOX) was also investigated since it is a key enzyme catalyzing the first oxidation step in the dissimilation of methanol. Moreover, recombinant avidin was expressed under the control of the AOX1 promoter.

3. MATERIALS AND METHODS

3. 1. Microorganism, inoculum preparation and media

A recombinant *Pichia pastoris* Mut^+ strain was kindly provided by Andrea Zocchi from the University of Neuchâtel (Switzerland). Zocchi et al. (2003) designed a clone of *P. pastoris* expressing and secreting a recombinant glycosylated avidin (recGAvi) with an acidic isoelectric point. Precultures were prepared in complex YPG medium at 30°C for 24h as described previously (Jungo et al. (2006)).

The complex YPG medium contained 20 g L^{-1} glycerol (Sigma-Aldrich, Steinheim, Germany), 6 g L^{-1} yeast extract (OXOID, Hampshire, England), 5 g L^{-1} Bacto Peptone (Becton, Le Pont de Claix, France). This medium was sterilized by heating at 120°C during 20 min.

The defined medium used for batch and continuous cultures was developed by Egli and Fiechter (1981) and was modified as described in a previous study (Jungo et al. (2007)) for optimal expression of recombinant avidin. It contained per liter: 15.26 g NH_4Cl, 5.62 g KH_2PO_4, 1.18 g $MgSO_4$ $7H_2O$, 0.9 g EDTA $2H_2O$, 110 mg $CaCl_2$ $2H_2O$, 75 mg $FeCl_3$ $6H_2O$, 28 mg $MnSO_4$ H_2O, 44 mg $ZnSO_4$ $7H_2O$, 8 mg $CuSO_4$ $5H_2O$, 8 mg $CoCl_2$ $6H_2O$, 5.2 mg Na_2MoO_4 $2H_2O$, 8 mg H_3BO_3, 1.2 mg KI, 20 μg biotin, and 0.4 mL of antifoam agent (Struktol SB 2121, Schill and Seilacher, Hamburg, Germany). Batch cultures were performed on 10 g L^{-1} methanol. Prior to initiating continuous cultures on 20 g L^{-1} methanol, the cultures were first grown in batch mode on 20 g L^{-1} glycerol and 20 g L^{-1} methanol. The pH was adjusted to 5.0 with KOH 4M.

All components of the defined media were obtained from Fluka, Buchs, Switzerland, except glycerol which was obtained from Sigma-Aldrich, Steinheim, Germany. The defined media were sterilized by filtration (0.22 μm, Steritop, Millipore Corporation, Billerica, USA).

3. 2. Culture conditions

Growth experiments were undertaken using a bench-scale reaction calorimeter RC1 (Mettler Toledo AG, Greifensee, CH) modified for biological reactions with a working volume of 1.6 L. Characteristics of this so called Bio-RC1 which can be operated like standard bench-scale bioreactors can be found in Marison et al. (1998). The steady-state culture volume was 1.050 L.

Batch cultures on methanol were performed at either 15, 18, 21, 25, 30, 35 or 37°C. Temperature was maintained at 30°C during batch growth prior to chemostat cultures performed at either 24, 30, 32, 34 or 36°C. pH was always maintained at 5.0 by the automatic addition of 2M KOH. A polarographic pO_2 probe (InPro 6800, Mettler Toledo, Greifensee, Switzerland) was used to monitor dissolved oxygen, with respect to air-saturated medium. Aeration rate was set a constant value of 2 L min^{-1} air (2 vvm) using a thermal mass flow controller (5850E, Rosemount Brooks, Veenendaal, The Netherlands). The air was saturated with water prior to entry in the bioreactor. Stirring rate was

set at a constant value of 800 rpm in order to maintain dissolved oxygen levels above 80% air saturation throughout the experiments.
All liquid inlet flows were quantified gravimetrically. The feed pump rate was regulated by a controller based on the on-line monitoring of the reservoir scale.

3. 3. Continuous cultivation on methanol

The culture was first grown in batch phase at 30°C on a mixture of 20 g L^{-1} glycerol and 20 g L^{-1} methanol. Methanol was consumed only after all the glycerol had been depleted since high glycerol concentations are known to have a repressing effect on the expression of the enzymes necessary for the dissimilation of methanol. The onset of methanol consumption was accompanied by the induction and synthesis of methanol dissimilating enzymes and prepared the cells for chemostat growth on methanol.
As soon as all the methanol had been consumed (indicated by a sudden increase in the dissolved oxygen level), the supply of feed-medium containing 20 g L^{-1} methanol as carbon source was initiated.

3. 4. A-stat experiments

Two transient continuous cultures were performed at a dilution rate of 0.06 h^{-1}. Linear increases in reactor temperature, from 24 to 32°C, and from 30 to 40°C, were begun after approximately five liquid residence times. Initiation of the temperature gradients was dependent on having attained steady state conditions, at the starting temperatures of 24 and 30 °C, as determined by on-line exhaust CO_2 and off-line cell dry weight measurements. The reactor temperature was linearly increased at a rate of 0.1°C h^{-1}.

3. 5. Substrate and metabolite analysis

Culture samples (about 10 mL) were collected using a purpose built auto-sampler (Cannizzaro (2002)) and kept at 2°C for up to 12h before handling.
Methanol and dry cell weight concentrations were determined as described previously (Jungo et al. (2007)).
The carbon dioxide production rate in the bioreactor exhaust gas was determined using an infrared analyzer (series 2500, Servomex, Crowborough, UK). The measured values of carbon dioxide were corrected for water vapour according to Duboc and von Stockar (1998).

3. 6. Preparation of cell-free extracts and alcohol oxidase activity measurement

The procedures for the preparation of cell-free extracts and the measurement of specific alcohol oxidase activity have been described previously (Jungo et al. (2006)).
The specific activity of alcohol oxidase was expressed in U mg^{-1} total protein. One unit (U) of AOX corresponds to the oxidation of 1.0 µmole of methanol to formaldehyde per min at pH 7.5 at 25°C.

3. 7. Recombinant avidin quantification

Recombinant avidin was quantified by biotin-4-fluorescein titration of binding sites (Kada et al. (1999)), assuming a tetrameric form of recombinant avidin, with four active-binding sites (Zocchi et al. (2003)).
Since the detection limit of this method is about 10 mg L^{-1} avidin, it was necessary to pre-concentrate samples approximately 15-fold by ultrafiltration (Centriplus, 30 kDa, Millipore Corporation, Bedford, USA) before titration with biotin-4-fluorescein.

3. 8. Black box stoichiometry and elemental composition of biomass

On a C-molar basis, the black box stoichiometry was described by the following equation for growth on methanol as carbon source.

$$CH_4O + Y_{O2/S}\, O_2 + Y_{NH3/S}\, NH_3 \rightarrow Y_{X/S}\, CH_{xH}O_{xO}N_{xN} + Y_{CO2/S}\, CO_2 \qquad (1)$$

$Y_{i/S}$ represents the stoichiometric coefficients in mole or C-mole per C-mole of substrate, where i can be O_2, CO_2, NH_3 or X (biomass). x_H, x_O and x_N represent respectively the atomic coefficients of hydrogen, oxygen and nitrogen of one C-mol of biomass. The generalized degree of reduction with CO_2, H_2O and NH_3 as reference state was used.
The C-molar composition of biomass for growth on methanol was determined by elemental analysis (Gurakan et al. (1990)). Cultures samples were collected at steady state during the chemostat cultures performed at various dilution rates and temperatures. Samples were centrifuged and washed three times with deionized water. After the final washing/centrifugation step, the cell pellets were spread as a thin layer of about 2 mm on Petri dishes and stored at -20 °C. The frozen cell pellets were freeze-

dried for 24 hours. Analysis of carbon, nitrogen and hydrogen content was made with a CHN analyzer (Carlo Erba Instruments, Rodano, Italy). Oxygen content was calculated by difference (Gurakan et al. (1990)). The C-molar composition of biomass for growth on methanol at 30°C was determined previously (Jungo et al. (2006)).

3. 9. Check of data consistency and reconciliation of yield coefficients

The yield of substance j on substance i was calculated from

$$Y_{j/i} = \frac{R_j}{R_i} \quad (C-mol/C-mol) \tag{2}$$

where R_i and R_j are the volumetric production or consumption rates of substances i and j, respectively.

During continuous cultures at steady state or during transient A-stat cultures, the measurements of dry cell weight, carbon source concentrations and carbon dioxide in the exhaust gas were used in order to calculate two of the four yields of the black box stoichiometry (equation 1), namely biomass and carbon dioxide yields.

Together with the constraints provided by the carbon, nitrogen and degree of reduction balances, a redundancy of 1 resulted. The redundancy of the system was used in order to estimate the unknown yields and to reconcile the data.

The yields were checked for consistency and reconciled based on a χ^2-test (Wang and Stephanopoulos (1983); Stephanopoulos et al. (1998)). The obtained reconciled yield coefficients are the best yield estimates that minimize the magnitude of the residuals ε and are determined by minimizing the sum of squared errors scaled according to their variance. The test value h is given by the sum of the weighted squares of the residuals ε:

$$h = \varepsilon \cdot P^{-1} \cdot \varepsilon \tag{3}$$

where P is the variance-covariance matrix. Assuming measurement errors of 5% on yield coefficients, a test value h lower than 3.84 for a redundancy of 1 permitted to assume a 95% significance level that no gross errors and no metabolites other than the ones appearing in equations 1 had affected the balances.

4. RESULTS AND DISCUSSION

4. 1. Influence of temperature on maximal specific growth rate in batch cultures

The maximal specific growth rates during growth on methanol was determined in batch cultures at various temperatures (figure 1). Many models have been developed in order to describe the maximal specific growth rate as a function of temperature, for instance the models proposed by Hinshelwood (1946), Ratkowsky et al. (1982), Ratkowsky et al. (1983), Zwietering et al. (1991) and Rosso et al. (1993). Using the minimal residual sum of squares criterion, the empirical model of Rosso et al. (1993) (equation 4) was considered to be the most suitable.

$$\mu_{max} = \frac{\mu_{opt} \cdot (T - T_{max}) \cdot (T - T_{min})^2}{(T_{opt} - T_{min}) \cdot ((T_{opt} - T_{min}) \cdot (T - T_{opt}) - (T_{opt} - T_{max}) \cdot (T_{opt} + T_{min} - 2 \cdot T))} \quad (4)$$

Where T_{min} (°C) is the temperature below which growth is no longer observed, T_{max} (°C) is the temperature above which no growth occurs, T_{opt} (°C) is the temperature at which the maximum specific growth rate equals its optimal value μ_{opt} (h^{-1}).

One advantage with the model proposed by Rosso et al. (1993) is that its four parameters have a biological interpretation, which facilitates the setting of a simple parameter starting value. According to measurement values, the following starting values were used: T_{min} = 15°C, T_{max} = 38°C, T_{opt} = 30°C and μ_{opt} = 0.14 h^{-1}. Integration of equation 4 was carried out numerically using the program Matlab and a least-squares optimization algorithm. The following parameters were obtained: T_{min} = 13.5°C, T_{max} = 38.0°C, T_{opt} = 28.2°C and μ_{opt} = 0.13 h^{-1}. The simulation of the maximal specific growth rate as a function of the temperature with the model proposed by Rosso et al. (1993) is shown in figure 1 (continuous line).

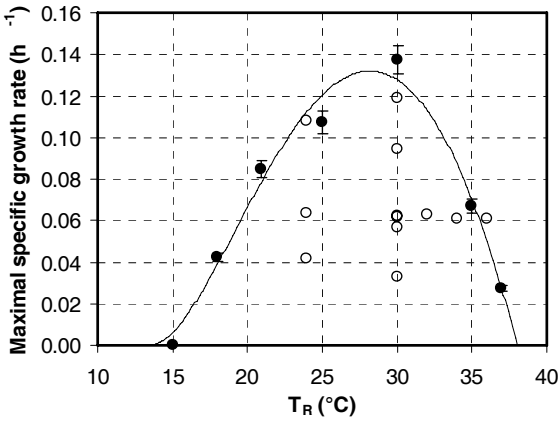

Figure 1: Measured maximal specific growth rates (●) as a function of the culture temperature in batch cultures on methanol. The continuous line represents the simulation of the maximal specific growth rate as a function of temperature according to the model developed by Rosso et al. (1993). The experimental design of all the chemostat cultures included in this study is also respresented on this figure (○).

4. 2. Influence of temperature on growth stoichiometry and elemental composition of biomass in chemostat cultures

After the determination of the experimental design with batch cultures and application of Rosso's model (figure 1), two transient continuous cultures with linear increase of temperature at a rate of 0.1°C h^{-1} were performed at a dilution rate of 0.06 h^{-1} (between 24 and 32°C and between 30 and 40°C). The goal was to investigate the influence of the culture temperature on growth stoichiometry and recombinant avidin productivity and to evaluate the performance of transient continuous cultures with linear increase in temperature as a tool for strain characterization. Various chemostat cultures were performed in order to validate the results obtained during the two transient continuous cultures and to complete the experimental design. A summary of the chemostat cultures is shown in figure 1. The chemostat cultures performed previously at 30°C (Jungo et al. (2006)) have been included in the present analysis.

The profiles of dry cell weight concentration and carbon dioxide production rate are shown in figures 2A and 2B for the transient continuous cultures performed between 24 and 32°C and between 30 and 40°C, respectively. In order to evaluate if the decrease in dry cell weight measurements between 24 and 32°C (t = 0 - 81 h, figure 2A) and between 30 and 36.6°C (t = 0 - 66 h, figure 2B) were significant, the correlation coefficients for linear regressions of these measurements according to time were calculated. The correlation coefficients were 0.87 and 0.69 respectively. The decreases in dry cell weight concentrations were therefore significant with a confidence level higher than 99.9% since the calculated correlation coefficients exceed tabulated correlation coefficient r values for a probability level of 0.001 (Frontier et al. (2001)).

The choice of the acceleration rate of the culture temperature was cross-checked because too fast a change in culture temperature could result in deviation from pseudo steady-state growth conditions. The specific growth rate was derived from the dry cell weight measurements according to Barbosa et al. (2003). Figures 3A and 3B show the specific growth rates calculated from a transient biomass balance and the specific growth rate as a percentage of the dilution rate during the two transient continuous cultures with linear increase in temperature. In both transient experiments, the specific growth rate dropped not lower than 80% of the dilution rate, but during most of the cultivation time it was higher than 90% of the dilution rate, except during wash-out of the culture. Indeed, during the transient continuous culture with increase in temperature from 30 to 40°C, wash-out was observed at about 36.9°C (t = 69 h, figure 2B), which led to a fast decrease in dry cell weight concentration (figure 2B) and in specific growth rate (figure 3B). According to Barbosa et al. (2003), A-stat cultures can be considered to be in pseudo-steady state when the growth rate is higher than 80% of the dilution rate.

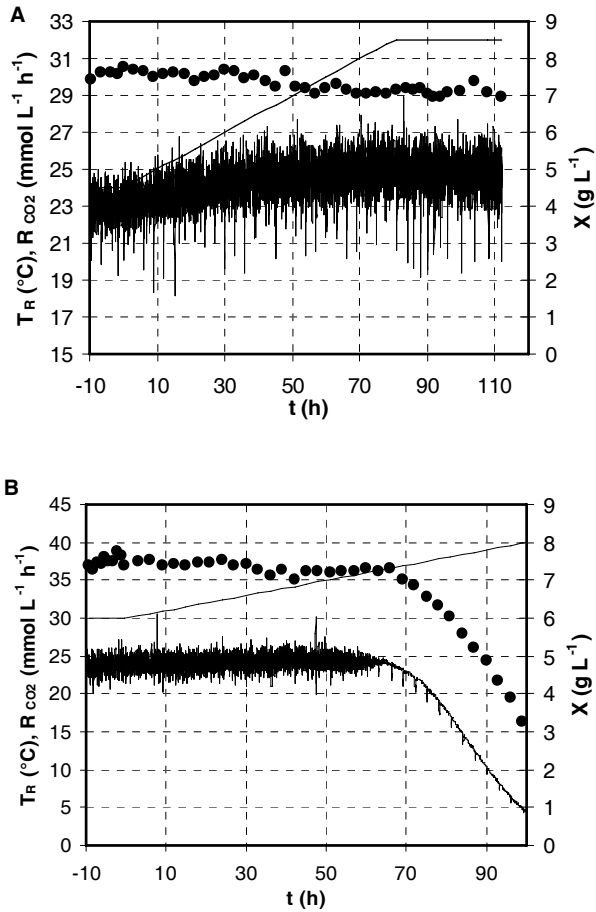

Figure 2: Dry cell weight concentration X (●) and carbon dioxide production rate (continuous line) as a function of time during the two transient continuous cultures with linear increase in culture temperature T_R (straight line) at 0.06 h^{-1}. Figure A: culture with linear increase of temperature from 24 to 32°C. Figure B: culture with linear increase of temperature form 30 to 40°C. Time zero corresponds to the beginning of the linear increase in temperature for both experiments.

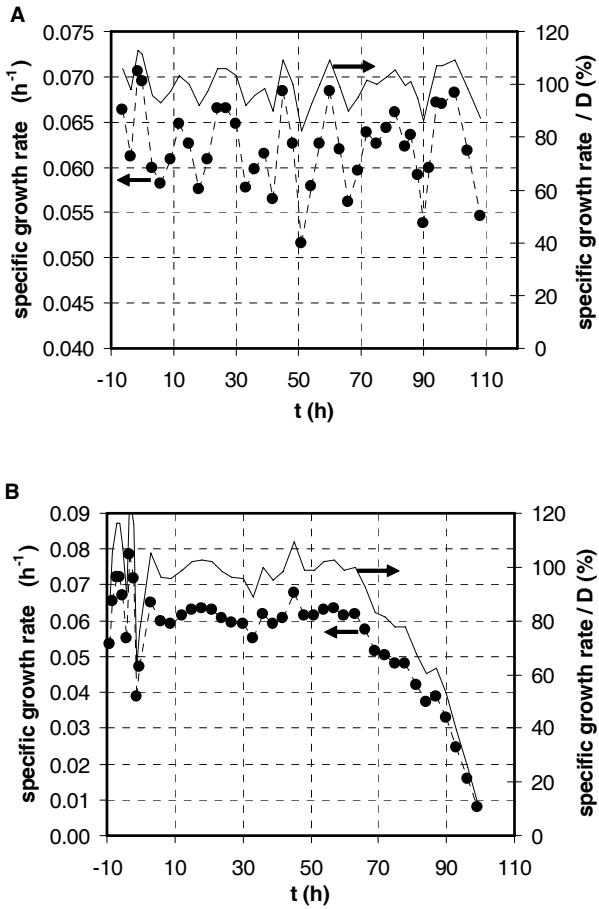

Figure 3: Specific growth rates (●) calculated from dry cell weight concentration and ratio of specific growth rate versus dilution rate according to time. Figure A: culture with linear increase of temperature from 24 to 32°C. Figure B: culture with linear increase of temperature form 30 to 40°C. Time zero corresponds to the beginning of the linear increase in temperature for both experiments.

The reconciled yield coefficients measured during the two transient continuous cultures with linear increase in culture temperature are presented in figures 4A and 5A. The results obtained during chemostat cultures are also represented in these figures for com-

parison. Very good agreement was obtained between the yield coefficients determined during chemostat cultures and during transient cultures with a linear increase in culture temperature. The yield coefficients only varied by about 8% between 24 and 32°C and by about 4% between 30 and 36.6°C. These changes in yield coefficients were however significant. Indeed, the calculated correlation coefficients for linear regressions of yield coefficients according to temperature exceed tabulated correlation coefficients r values for a probability level of 0.001 (Frontier et al. (2001)). Although maintenance requirements increase with increasing temperatures (Heijnen and Roels (1981); Roels (1983)), the yield coefficents were only affected slightly by temperatures between 24 and 36.6°C because maintenance requirements are low for *P. pastoris* strains (Jungo et al. (2006)). At cultivation temperatures higher than 36.6°C, the decrease in yield coefficient was due to wash-out of the culture (figure 5A) because the dilution rate exceeded the maximal specific growth rate (see figure 1). An attempt was made to perform a chemostat culture at 37°C but steady-state growth conditions could not be achieved at a dilution rate of 0.06 h^{-1}.

In figure 4B and 5B, it can be observed that the statistical test value h was always below the threshold value of 3.84 (h_{limit}) for a confidence level of 95% and a redundancy of 1, except at temperatures above 38°C (figure 5B) because of wash-out of the culture.

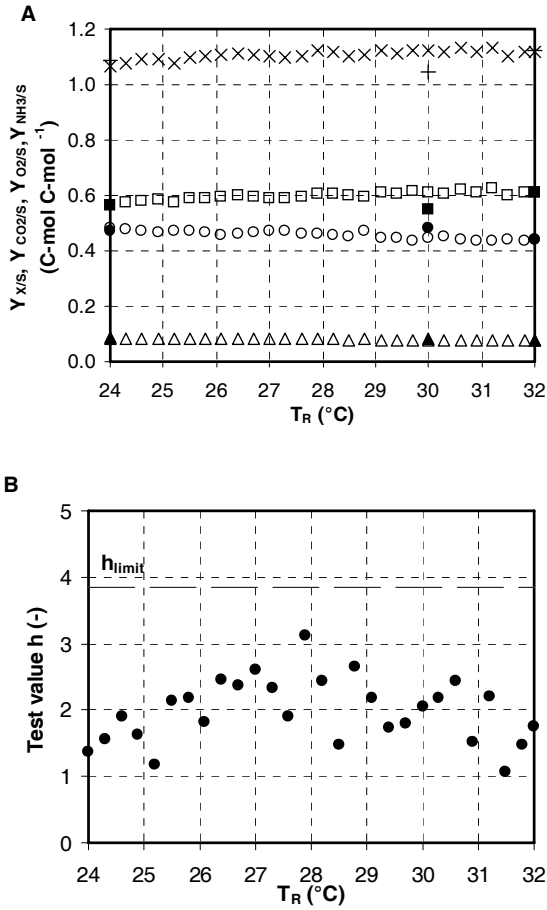

Figure 4: A: Reconciled biomass (○), carbon dioxide (□), oxygen (x) and ammonia (△) yield coefficients according to culture temperature during A-stat culture at 0.06 h^{-1} with linear increase in temperature from 24 to 32 °C. Steady-state reconciled biomass (●), carbon dioxide (■), oxygen (+) and ammonia (▲) yield coefficients according to culture temperature during chemostat cultures at 0.06 h^{-1}. B: Corresponding calculated statistical test values h calculated with reconciliation procedures with a 95% confidence level during A-stat culture. The threshold value (discontinuous line) to reject hypothesis with a 95% confidence level and a degree of redundancy of 1 is of 3.84 (h_{limit}).

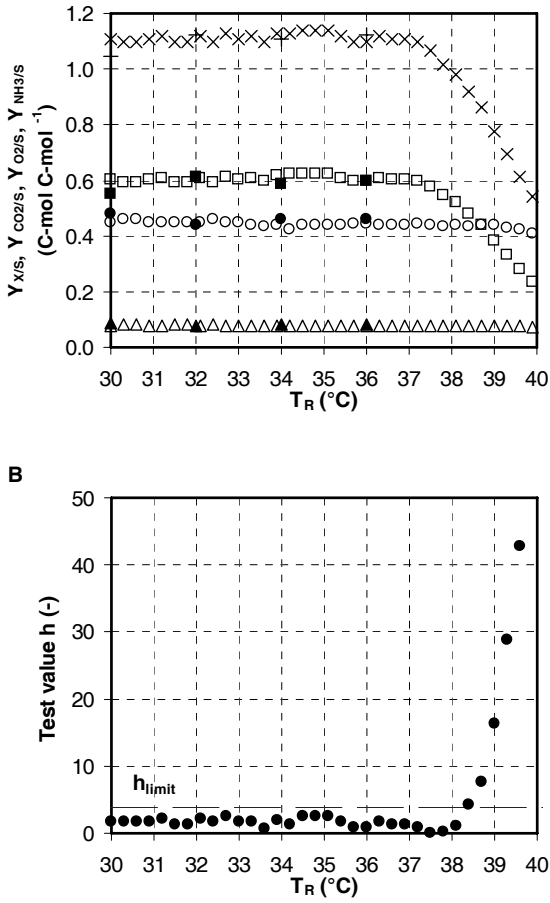

Figure 5: A: Reconciled biomass (○), carbon dioxide (□), oxygen (x) and ammonia (△) yield coefficients according to culture temperature during A-stat culture at 0.06 h^{-1} with linear increase in temperature from 30 to 40 °C. Steady-state reconciled biomass (●), carbon dioxide (■), oxygen (+) and ammonia (▲) yield coefficients according to culture temperature during chemostat cultures at 0.06 h^{-1}. B: Corresponding calculated statistical test values h calculated with reconciliation procedures with a 95% confidence level during A-stat culture. The threshold value (discontinuous line) to reject hypothesis with a 95% confidence level and a degree of redundancy of 1 is of 3.84 (h_{limit}).

The reconciled yield coefficients determined during the three chemostat cultures at 24°C are presented in figure 6 according to the dilution rate. The maintenance coefficient (m) and the true biomass yield ($Y_{X/S}^{true}$) were determined at 24°C by plotting the specific methanol consumption rate at steady-state during these three chemostat cultures as a function of the dilution rate. The maintenance coefficient was of 0.009 ± 0.002 C-mol C-mol^{-1} h^{-1} and the true yield was 0.55 ± 0.01 C-mol C-mol^{-1}, which is very close to the values determined at 30°C. Indeed, previous chemostat cultures at 30°C showed that the maintenance coefficient was 0.011 ± 0.004 C-mol C-mol^{-1} h^{-1} and the true yield was 0.53 ± 0.02 C-mol C-mol^{-1} (Jungo et al. (2006)). Hence, maintenance requirements at 24°C and at 30°C are comparable.

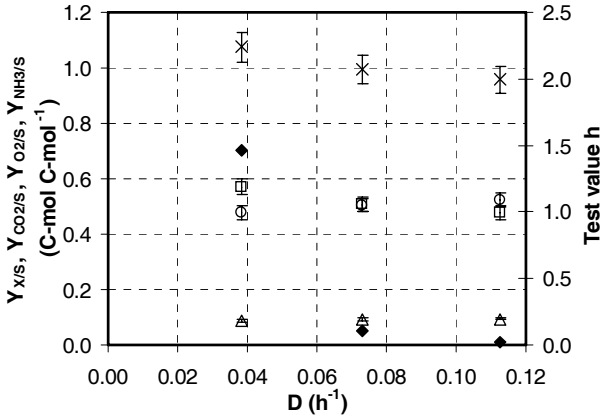

Figure 6: Reconciled biomass (❍), carbon dioxide (□), oxygen (x) and ammonia (△) yield coefficients according to dilution rate during chemostat cultures at 24°C. Statistical test values h (◆) calculated with reconciliation procedures with a 95% confidence level during A-stat culture. The threshold value to reject hypothesis with a 95% confidence level and a degree of redundancy of 1 is of 3.84.

Elemental composition of biomass

For all chemostat cultures, the elemental composition of biomass was determined after having achieved steady-state growth conditions. Figure 7A shows the elemental composition of biomass at various temperatures for chemostat cultures performed at a dilution rate of 0.06 h^{-1} and figure 7B presents the elemental composition of biomass at 24°C for various dilution rates. These results show that in the studied range of temper-

atures and dilution rates, no significant changes in the elemental composition of biomass was observed.

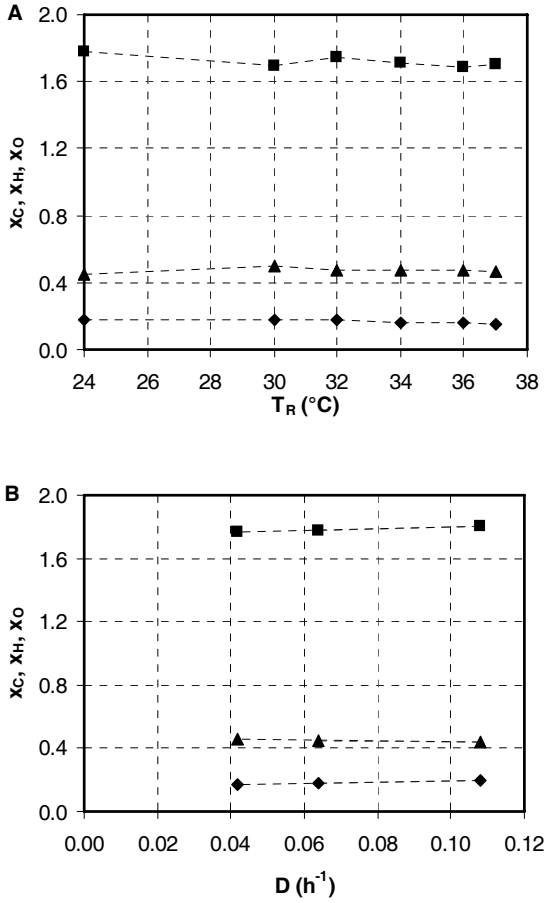

Figure 7: Elemental compositon for one C-mol of biomass. Atomic coefficients of hydrogen (x_H, ■), oxygen (x_O, ▲) and nitrogen (x_N, ◆). Figure A: results for chemostat cultures at a dilution rate of 0.06 h^{-1} as a function of temperature. Figure B: results for chemostat cultures at 24°C as a function of dilution rate.

4. 3. Influence of temperature on specific avidin production rate

Figures 8A and 8B present the influence of culture temperature on the specific recombinant avidin productivity. Results obtained at steady state during chemostat cultures and during the two transient continuous cultures with linear increase in temperature are represented for comparison. Very good agreement was obtained between the measurements at steady state and during the transient experiments. Taking into account the errors on the measurements of the specific avidin production rate, it can be noted that cultivation temperatures lower than the optimal growth temperature of 30°C do not lead to enhanced avidin productivity. Indeed, between 24 and 32°C the temperature did not influence significantly recombinant avidin production rate. Figure 8B shows that recombinant avidin productivity is diminished during cultivation at temperatures above 32°C. Similar results showing no enhanced recombinant protein production rate at lower temperatures than the optimal growth temperature of 30°C for *P. pastoris* strains have been reported by other authors (Inan et al. (1999); Curvers et al. (2001); Kupesulik and Sevella (2005)). The decrease in recombinant avidin productivity at temperatures higher than 32°C is in all probability due to increased cell death, which leads to cell lysis and higher protease activity in fermentation media.

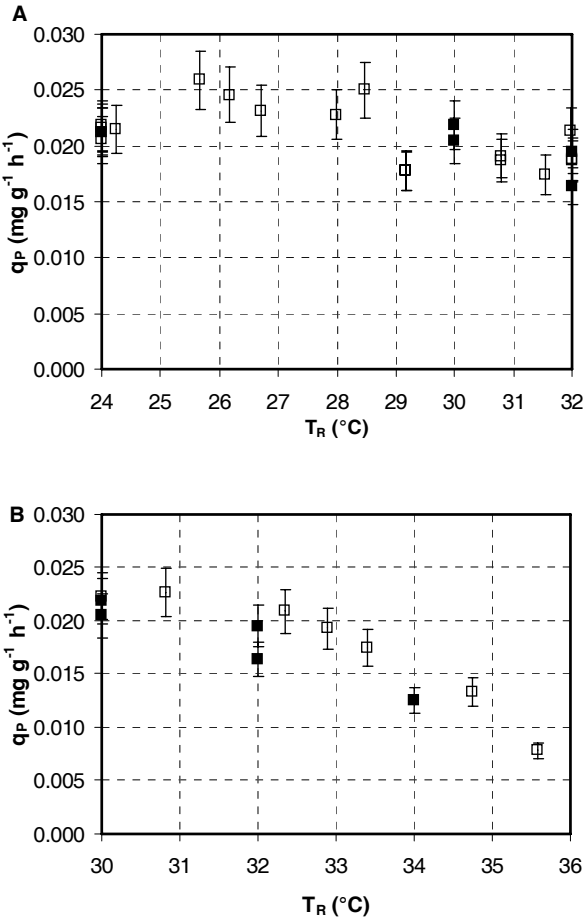

Figure 8: Specific avidin production rates (q_P, □) according to culture temperature during two A-stat cultures at a dilution rate of 0.06 h^{-1}. Figure A: linear increase of temperature from 24 to 32 °C. Figure B: linear increase of temperature from 30 to 40 °C. The specific avidin production rates (q_P, ■) during chemostat cultures were also represented according to culture temperature.

Figure 9 shows the influence of dilution rate on volumetric and specific recombinant avidin productivity for three chemostat cultures performed at 24°C. The specific productivity of avidin increased linearly with dilution rate. Hence, avidin production was

growth-associated. A linear regression was applied to the calculated specific avidin productivities as a function of dilution rate in order to express the specific avidin productivity according to the following equation:

$$q_P = Y_{P/X} * D + \beta \quad (5)$$

where $Y_{P/X}$ is the yield coefficient of avidin per amount of dry cell weight (127.9 ± 24.2 $\mu g\ g^{-1}$) and β corresponds to the specific avidin production rate proportional to the cell density (13.0 ± 1.8 $\mu g\ g^{-1}\ h^{-1}$).

Previous results for chemostat cultures at 30°C (Jungo et al. (2006)) are in very good agreement with the values found at 24°C which confirms that temperature does not significantly affect recombinant avidin productivity between 24 and 30°C.

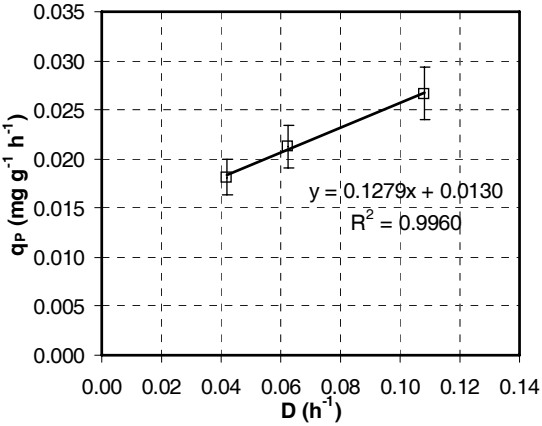

Figure 9: Specific avidin production rates (q_P, □) according to dilution rate at 24°C during chemostat cultures .

Response surface methodology

A first order model (equation 6) was used to regress the specific avidin production rate as a function of two factors: temperature and specific growth rate. First order interactions between the two analyzed factors were taken into account in this model. Data determined during chemostat cultures in this study and during chemostat cultures

performed previously at 30°C (Jungo et al. (2006)) were used for this analysis. The experimental design is summarized in figure 1.

Since the current experimental design does not match an orthogonal design of experiments, it contains correlations that decrease its robustness. Variance inflation factors (VIF) were calculated in order to predict the reliability of the actual design. A full description of the methods used in order to verify the reliability of a model is provided by Goupy (1999) and Myers and Montgomery (2002). The variance inflation factors are measures of multi-collinearity between factors. The larger the variance inflation factors, the more severe the multi-collinearity. The effect of multi-collinearity is to introduce a near-linear dependence in the columns of the model matrix and implies that the regression coefficients of the model are very poorly estimated.

Some authors have suggested that if any variance inflation factors exceed 10, then multi-collinearity is a problem and the experimental design should be changed. Other authors consider this value too liberal and suggest that the variance inflation factors should not exceed 4 or 5 (Myers and Montgomery (2002)).

The calculated inflation factors for the first order model with interactions are displayed in figure 10. The inflation factors were low, which means that the four coefficients of the model were only poorly affected by correlations. The planned experimental design was therefore satisfying in order to construct a first order model with interactions. This analysis validated a priori the set of steady-state experiments chosen for the study of dilution rate and temperature on the specific recombinant avidin production rate.

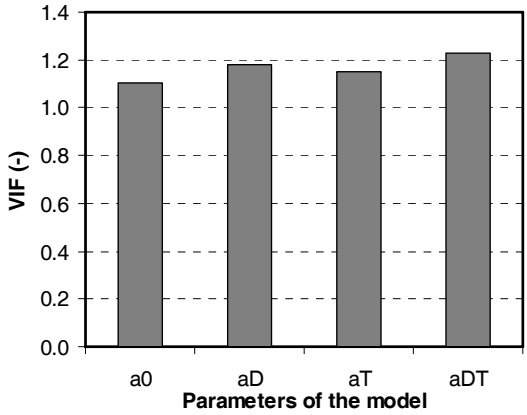

Figure 10: Variance inflation factors (VIF) for first order model with interactions for the analysis of influence of dilution rate and temperature; a_0, a_D, a_T and a_{DT} are respectively the four parameters of the linear model with interactions presented in equation 6.

The results of the analysis of variance (ANOVA) are presented in table 1 and figure 11 shows the response surface plot of the specific avidin production rate based on equation 6. The analysis of variance (ANOVA, table 1) shows that the p-value, which represents the probability that our F value would be obtained by chance (random error) alone, is very small (1.17×10^{-8}) and therefore validates the description of the specific avidin production rate with the linear model described by equation 6 on the basis of our measurements.

$$q_P = 2.1405 \cdot 10^{-2} + 6.2634 \cdot 10^{-3} \cdot D - 2.1175 \cdot 10^{-3} \cdot T_R + 1.1290 \cdot 10^{-3} \cdot D \cdot T_R \qquad (6)$$

Table 1: ANOVA for significance of regression of specific avidin productivity q_P with linear model described by equation 6.

Source of variance	Sum of squares	Degrees of freedom	Mean square	F	p-value
Regression	5.17E-03	3	1.72E-03	318.2	1.17E-08
Residuals	4.33E-05	8	5.41E-06		
Total	5.21E-03	11	4.74E-04		

Figure 11 shows that the specific recombinant avidin production rate is mainly a function of the dilution rate, which corresponds to the specific growth rate, and that temperature does not significantly affect specific recombinant avidin productivity.

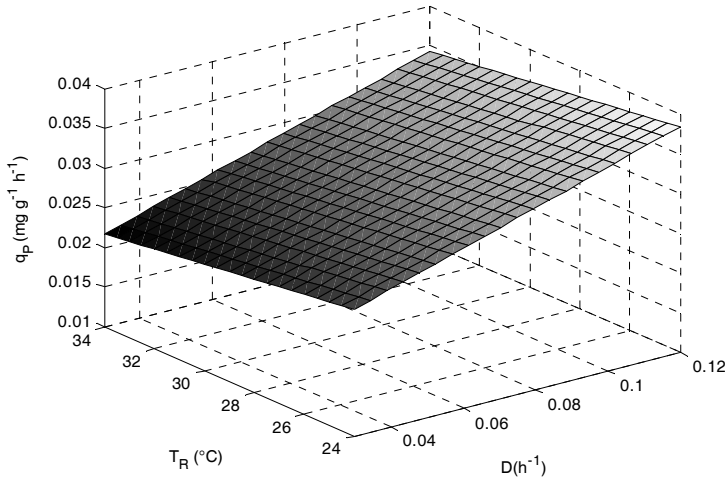

Figure 11: Response surface of specific avidin productivity (q_P) to the dilution rate (D) and the culture temperature (T_R) based on equation 6.

4. 4. Influence of temperature on specific AOX activity

Recombinant avidin was expressed under the control of the strong, tightly regulated, and methanol-induced alcohol oxidase promoter AOX1. This promoter drives the expression of the enzyme alcohol oxidase 1 in the wild-type strain. This enzyme catalyses

the first step in the dissimilation of methanol which consists in the oxidation to formaldehyde and hydrogen peroxide using molecular oxygen (Veenhuis et al. (1983)).
Figures 12A and 12B show the influence of culture temperature on specific alcohol oxidase activity, determined during chemostat and A-stat continuous cultures at a dilution rate of 0.06 h^{-1}. Good agreement was obtained between values determined at steady state during chemostat cultures and during pseudo steady-state A-stat continuous cultures.
In figure 12A, it can be noted that the specific AOX activity was higher at temperatures lower than the optimal growth temperature of 30°C (about 1.2-fold higher specific AOX activity at 24°C compared to 30°C). The decrease in specific AOX activity as the temperature was increased from 24 to 32°C was significant with a confidence level of more than 99.9%. Indeed, the correlation coefficient for linear regression of specific AOX activity measurements according to culture temperature was 0.80 and exceeded tabulated correlation coefficient r values for a probability level of 0.001 (Frontier et al. (2001)).
An increase in AOX activity upon lowering the culture temperature was also observed by Jahic et al. (2003b). A sharp decrease in specific AOX activity can be observed in figure 12B for temperatures above 36°C because of wash-out of the culture during the A-stat experiment performed between 30 and 40°C.

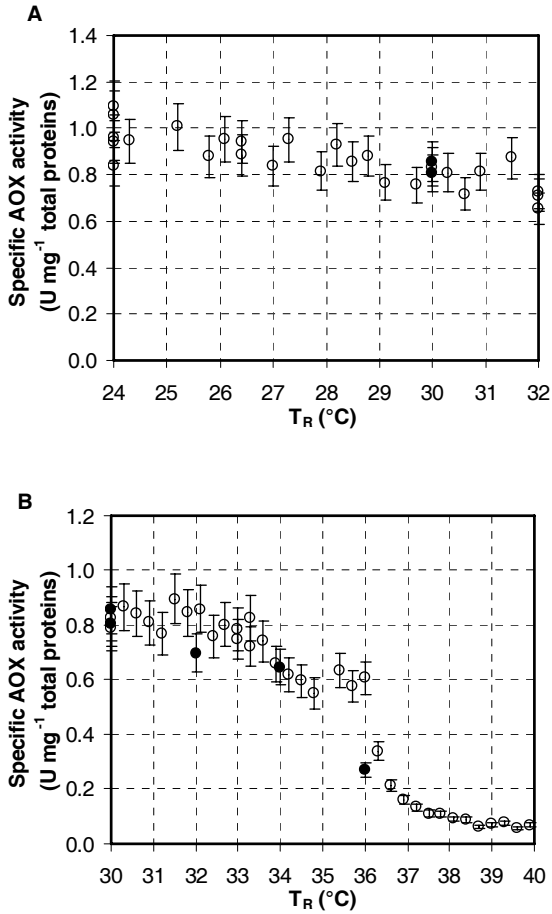

Figure 12: Specific (○) alcohol oxidase activities according to culture temperature during two A-stat cultures at a dilution rate of 0.06 h^{-1}. Figure A: linear increase of temperature from 24 to 32 °C. Figure B: linear increase of temperature from 30 to 40 °C. Specific (●) alcohol oxidase activities according to culture temperature during chemostat cultures.

5. CONCLUSIONS

The growth stoichiometry of a *P. pastoris* Mut$^+$ strain on methanol was only slightly influenced by temperature between 24 and 36°C (change in yield coefficients of less than 8%) because the substrate consumption for maintenance purposes is rather low. Cultivation at temperatures lower than the optimal growth temperature of 30°C did not lead to higher specific recombinant avidin productivity. However, an increase in recombinant avidin productivity at higher dilution rates was confirmed.

Yield coefficients, recombinant avidin production rates and specific alcohol oxidase activities obtained during pseudo steady-state continuous cultures with a linear increase of culture temperature at a rate of 0.1°C h^{-1} were in very good agreement with results obtained at steady state during chemostat cultures. Hence, continuous cultures with linear increase in culture temperature can be used as a tool for the rapid and rational characterization of host cells, in particular for the analysis of the relationship between culture temperature and recombinant protein productivity, which is often strain and protein specific. Experiments at higher acceleration rates of culture temperature should be performed in order to determine the highest acceleration rate, which allows the determination of culture characteristics comparable with those obtained during chemostat cultures. Also would it be more interesting to test this A-stat technique with changes in temperature for species whose growth stoichiometry strongly depends on temperature so that deviations from steady-state growth can easily be detected.

6. ACKNOWLEDGEMENTS

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7. NOMENCLATURE

AOX	Alcohol oxidase
a_0	Model parameter independent of dilution rate or temperature
a_D	Model parameter describing the influence of

	dilution rate	
a_T	Model parameter describing the influence of temperature	
a_{DT}	Model parameter describing the influence of the interaction between dilution rate and temperature	
S	Substrate concentration	C-mol L^{-1}
D	Dilution rate	h^{-1}
F	Variance ratio	
h	Statistical test value	
m	Maintenance coefficient	C-mol C-mol^{-1}h^{-1}
P	Variance-covariance matrix	
p-value	Probability that F would be obtained by chance	
q_P	Biomass-specific avidin productivity	mg g^{-1} h^{-1}
r	Correlation coefficient	
R_i	Volumetric production or consumption rate of i	C-mol L^{-1} h^{-1}
T	Temperature	
VIF	Variance inflation factors	
X	Cell dry weight	C-mol L^{-1}
x_H	Atomic coefficient of H of one C-mol of biomass	
x_O	Atomic coefficient of O of one C-mol of biomass	
x_N	Atomic coefficient of N of one C-mol of biomass	
$Y_{j/i}$	Yield coefficient of substance j on substance i	C-mol C-mol^{-1}
$Y_{j/i}^{true}$	True yield coefficient	C-mol C-mol^{-1}
β	Avidin formation proportional to the cell density	μg g^{-1} h^{-1}
ε	residuals	
μ	Specific growth rate	h^{-1}

Subscripts

i	Refers to compound i
j	Refers to compound j
max	Maximal
min	Minimal

opt	Optimal
P	Refers to the produced recombinant avidin
R	Refers to the reactor
S	Refers to the limiting carbon source

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CHAPTER 5

REGULATION OF ALCOHOL OXIDASE OF A RECOMBINANT *PICHIA PASTORIS* MUT$^+$ STRAIN IN TRANSIENT CONTINUOUS CULTURES

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1. ABSTRACT

In the methylotrophic yeast *Pichia pastoris,* alcohol oxidase (AOX) is a key enzyme involved in the dissimilation of methanol. Heterologous proteins are usually expressed under the control of the AOX1 promoter, which drives the expression of alcohol oxidase 1 in the wild-type strain. This study investigates the regulation of the alcohol oxidase enzyme of a recombinant *P. pastoris* Mut$^+$ strain in cultures on glycerol and methanol as sole carbon sources and in mixed substrate cultures on both substrates. The aim was to have a better insight in the transition from growth on glycerol to growth on methanol, which is a key step in standard high cell density *P. pastoris* cultures for the production of foreign proteins. Nutrient shifts in chemostat cultures showed that after growth on glycerol use of mixed feeds of glycerol and methanol allowed faster induction of alcohol oxidase and faster adaptation of cellular metabolism than with a feed containing methanol as sole carbon source.

The results of this study showed also how critical it is to avoid transient methanol accumulation during *P. pastoris* cultures operated at low residual methanol concentrations. Indeed, pulse experiments during chemostat cultures showed that sudden increase in methanol concentrations in cultures performed under methanol-limited or dual methanol and glycerol-limited growth conditions leads to wash-out of the culture because of too high consumption rate of methanol, which leads to excretion of toxic intermediates. High rate of methanol consumption was due to high specific AOX activities observed at low residual methanol concentrations.

Keywords: *Pichia pastoris*; Alcohol oxidase; Pulse; Mixed substrate; Calorimetry.

2. INTRODUCTION

The methylotrophic yeast *Pichia pastoris* has become a popular host for the production of recombinant proteins (Cregg et al. (1993); Hollenberg and Gellissen (1997); Cereghino and Cregg (2000); Gellissen (2000); Daly and Hearn (2005)). Heterologous proteins are usually expressed under the control of the strong, tightly regulated, and methanol-induced alcohol oxidase promoter AOX1. This promoter drives the expression of the enzyme alcohol oxidase 1 in the wild-type strain.

Standard high cell density fed-batch cultures with recombinant *P. pastoris* strains are usually performed in three steps. Cells are first grown on glycerol in batch culture in order to produce biomass and repress recombinant protein expression. Indeed, the AOX1 promoter is repressed by unlimited growth on glycerol (Tschopp et al. (1987)). Secondly, glycerol is fed at limiting concentrations in order to increase biomass concentration further and to derepress the methanol metabolic machinery. This transition phase is usually thought to derepress gradually the enzymes necessary for the dissimilation of methanol and reduces the time necessary for the cells to adapt to growth on methanol. Indeed, Chiruvolu et al. (1997) found that addition of a transition phase reduced the total induction time of 15 h for a *P. pastoris* Mut^+ strain. Finally, methanol is fed to the culture to induce recombinant protein production and further increase cell density.

In order to develop strategies for optimal production of recombinant proteins in cultures with *P. pastoris* strains, it is necessary to have a better insight into the regulation of methanol dissimilating enzymes, and in particular of the alcohol oxidase enzyme because it is the first enzyme involved in methanol dissimilation and expression of recombinant proteins in *P. pastoris* strains is usually driven by the AOX1 promoter.

There are two genes that encode alcohol oxidase in *P. pastoris*: AOX1 and AOX2 (Koutz et al. (1989)). AOX1 is responsible for the majority of alcohol oxidase activity in the cell (Cregg et al. (1989)). Since alcohol oxidase has a very low affinity for oxygen (Couderc and Baratti (1980); Veenhuis et al. (1983)), large amounts of this enzyme are synthesized. Indeed, in cultures on methanol as carbon source, AOX can account for over 30% of the total cell protein (Couderc and Baratti (1980)). This strong AOX1 promoter can therefore be used to drive the expression of recombinant proteins to high levels, up to 12 g L^{-1} of recombinant protein (Cregg et al. (1993)).

Expression of the AOX1 gene is controlled at the level of transcription (Ellis et al. (1985)). Several authors pointed out that the regulation of the AOX1 gene involves two mechanisms: a repression/derepression mechanism and an induction mechanism (Tschopp et al. (1987); Ozimek et al. (2005)) similar to the regulation of alcohol oxidase in the methylotrophic yeast *H. polymorpha* (Egli et al. (1980)). However, unlike for *H. polymorpha*, the presence of methanol is essential to induce high levels of transcription (Tschopp et al. (1987)). Indeed, previous studies in chemostat cultures with a recombinant *P. pastoris* strain (Jungo et al. (2006)) showed that the presence of methanol is essential to induce the synthesis of high levels of alcohol oxidase and expression of recombinant protein.

In order to have a greater insight into the dynamics of alcohol oxidase expression, transient continuous cultures have been chosen as experimental tool. Transient experiments can yield important information on the regulation of central metabolism and expression systems of the host cell (Galindez et al. (1983); Petrik et al. (1983); Käppeli and Fiechter (1985); Sonnleitner (1991); Bally and Egli (1995); Duboc et al. (1998); Flikweert et al. (1999); Herwig et al. (2001); Herwig and von Stockar (2003)).

Since the transition from growth on glycerol to growth on methanol is a key step in high cell density *P. pastoris* fed-batch cultures (Stratton et al. (1998)), the time required for transition from growth on glycerol to growth on methanol was compared with that necessary for transition from glycerol to mixed glycerol and methanol in continuous cultures at a dilution rate of 0.07 h^{-1}, which corresponds to half of the maximum specific growth rate on methanol. Adaptation is expected to be faster in the second case because glycerol might support expression of methanol dissimilating enzymes, by supplying the energy and building blocks necessary for the adaptation of cellular metabolism and in particular for the synthesis of methanol dissimilating enzymes. Indeed, Bally and Egli (1995) studied the dynamics of induction of nitrilotriacetate degrading enzymes during mixed substrate growth of *Ch. heintzii* and showed that when cells were subjected to a medium shift at constant dilution rate, the time necessary for induction of nitrilotriacetate degrading enzymes was reduced considerably with medium shift from glucose to mixtures of glucose and nitrilotriacetate than with medium shift from glucose to nitriloacetate. Moreover, during high cell density fed-batch cultures with recombinant *P. pastoris* strains, several authors reported that adaptation of the metabolism on methanol was faster when methanol was fed along with glycerol already dur-

ing the transition phase (Katakura et al. (1998); Zhang et al. (2000a); Zhang et al. (2000b); Minning et al. (2001); Zhang et al. (2005); Cos et al. (2006)).

Therefore, in order to analyze quantitatively if use of mixed feeds of glycerol and methanol (instead of methanol as sole carbon source) reduce the time necessary for the cells to synthesize the alcohol oxidase enzyme after growth on glycerol, continuous cultures with sudden changes in the composition of the feed medium were performed in this study.

On the other hand, during the induction phase, it is known that the feeding of methanol has to be tightly controlled in order to avoid methanol accumulation (Stratton et al. (1998)). Indeed, the stability of *P. pastoris* cultures on methanol can be threatened by substrate toxicity (methanol) or by the excretion of oxidative intermediates of methanol catabolism, which are toxic to growth, for instance formaldehyde, formic acid or hydrogen peroxide. Swartz and Cooney (1981) examined growth inhibition by methanol, formaldehyde, and formic acid in continuous cultures with the methylotrophic yeasts *H. polymorpha*. They concluded that in order to ensure the stability of continuous cultures on methanol with *H. polymorpha*, it is necessary to prevent transient methanol accumulation.

In this study, the transient response of continuous cultures subjected to sudden increases in methanol concentration in the culture was examined. The behaviour of cultures on glycerol or methanol as sole carbon sources and on mixed feeds of glycerol and methanol was compared by adding pulses of 2 g methanol after having attained steady-state growth conditions.

A recombinant *P. pastoris* Mut^+ strain expressing and secreting avidin was chosen as a model system.

3. MATERIALS AND METHODS

3. 1. Microorganism, inoculum preparation and media

A recombinant *Pichia pastoris* Mut^+ strain was kindly provided by Andrea Zocchi from the University of Neuchâtel (Switzerland). Zocchi et al. (2003) designed a clone of *P. pastoris* expressing and secreting a recombinant glycosylated avidin (recGAvi) with an acidic isoelectric point. Precultures were prepared in complex YPG medium at 30°C for 24h as described previously (Jungo et al. (2006)).

The complex YPG medium contained 20 g L^{-1} glycerol (Sigma-Aldrich, Steinheim, Germany), 6 g L^{-1} yeast extract (OXOID, Hampshire, England), 5 g L^{-1} Bacto Peptone (Becton, Le Pont de Claix, France). This medium was sterilized by heating at 120°C for 20 min.

The defined medium used for continuous cultures was based on the medium developed by Egli and Fiechter (1981) and adapted in a previous study (Jungo et al. (2007)) for optimal expression of recombinant avidin. This medium contained per liter: 0.652 C-mol of carbon source (either 20 g glycerol, 20.87 g methanol, or 10 g glycerol and 10.43 g methanol), 15.26 g NH_4Cl, 5.62 g KH_2PO_4, 1.18 g $MgSO_4$ $7H_2O$, 0.9 g EDTA $2H_2O$, 110 mg $CaCl_2$ $2H_2O$, 75 mg $FeCl_3$ $6H_2O$, 28 mg $MnSO_4$ H_2O, 44 mg $ZnSO_4$ $7H_2O$, 8 mg $CuSO_4$ $5H_2O$, 8 mg $CoCl_2$ $6H_2O$, 5.2 mg Na_2MoO_4 $2H_2O$, 8 mg H_3BO_3, 1.2 mg KI, 20 μg biotin, and 0.4 mL of antifoam agent (Struktol SB 2121, Schill and Seilacher, Hamburg, Germany).

The same medium containing 20 g L^{-1} glycerol and 20 g L^{-1} methanol as carbon sources was used for mixed substrate batch cultures.

All components of the defined media were obtained from Fluka, Buchs, Switzerland, with the exception of glycerol which was obtained from Sigma-Aldrich, Steinheim, Germany. The defined media were sterilized by filtration (0.22 μm, Steritop, Millipore Corporation, Billerica, USA).

3. 2. Culture conditions

Growth experiments were undertaken using a bench-scale heat-flux calorimeter RC1 (Mettler Toledo AG, Greifensee, CH) modified for biotechnological applications with a working volume of 1.6 L. Characteristics of this so called Bio-RC1 can be found in Marison et al. (1998). A culture volume of 1.050 L was used for both batch and continuous modes.

Temperature was maintained at 30°C and pH at 5.0 by the automatic addition of 2M KOH. The heat of neutralization was calculated from the consumption of KOH 2M according to Meier-Schneiders et al. (1995). Pre-saturated and thermostatted air was supplied to the reactor at a rate of 2 NL min^{-1}. Stirring rate was fixed at 800 rpm.

All liquid inlet flows were quantified gravimetrically, with the feed pump rate controlled by the on-line monitoring of the reservoir scale. The temperature of all feeds was adjusted to the reactor temperature prior to entry to the calorimeter.

Since release of CO_2 from the medium is an endothermic process, corrections for the heat flow induced by CO_2 stripping were made according to Meier-Schneiders et al. (1995). Heat losses to the environment were calculated using a heat transfer coefficient for the bioreactor of 0.133 W K^{-1}, which was determined as described in Birou (1986).

3. 3. Continuous cultures

All continuous cultures were performed on 0.652 C-mol L^{-1} of carbon source, either on 20 g L^{-1} glycerol, 20.87 g L^{-1} methanol or a mixture of 10 g L^{-1} glycerol and 10.43 g L^{-1} methanol.

To initiate chemostat cultures on glycerol, cultures were first grown in batch mode on 20 g L^{-1} glycerol, and the supply of feed medium started immediately after depletion of glycerol at the end of batch growth, as indicated by a sudden decrease of the heat signal.

Chemostat cultures on methanol or a mixture of glycerol and methanol were first grown in batch mode on a mixture of 20 g L^{-1} glycerol and 20 g L^{-1} methanol. Methanol was consumed only after all the glycerol had been consumed, since high glycerol concentrations are known to have a repressing effect on the expression of the methanol dissimilating enzymes. After all glycerol had been exhausted, methanol was consumed which allowed the induction and synthesis of methanol dissimilating enzymes and prepared the cells for chemostat growth on methanol. As soon as all the methanol had been consumed (indicated by a sudden decrease of the heat signal), the supply of feed medium was initiated.

The dilution rate was set to 0.07 h^{-1} for all experiments and all transient experiments were begun only after five liquid residence times.

In pulse experiments, 2 g of sterile methanol was added using a syringe after having attained steady-state growth conditions. When conducting experiments with sudden changes of nutrient supply, a change in medium composition was performed by switching the feed line from a feed reservoir containing 20 g L^{-1} glycerol to a reservoir containing 20.87 g L^{-1} methanol, or to a reservoir containing a mixture of 10 g L^{-1} glycerol and 10.43 g L^{-1} methanol as carbon sources. In this way, the total feed rate in C-moles per unit time stayed constant.

3. 4. Substrate and metabolite analysis

Culture samples (about 10 mL) were collected using a purpose built auto-sampler (Cannizzaro (2002)) and kept at 2°C for up to 12h before handling.

The concentration of biomass (dry cell weight) was determined gravimetrically. Samples were centrifuged in pre-weighed tubes, the cell pellet washed twice with ultrapure water followed by drying at 100°C to constant weight.

Glycerol and methanol were determined by HPLC analysis (1100 series, Agilent Technologies, Palo Alto, USA). An ion exchange chromatography column (Supelcogel H 300 mm, Supelco, Bellefonte, USA) with a guard column (Superlguard C610H, Supelco, Bellefonte, USA) was used at 60 °C. A 5 mM sulphuric acid solution in ultrapure water was applied at a constant eluent flow rate of 0.6 mL min^{-1}. Metabolites were measured using a refractive index detector. The detection limit was of 0.01 g L^{-1} for glycerol and 0.05 g L^{-1} for methanol.

Formaldehyde was determined according to the method described in Werringloer (1978), which is based on the method developed by Nash (1953). Samples were diluted to formaldehyde concentrations within the range 0.01 to 0.7 mM. The lower limit of formaldehyde detection was approximately 0.01 mM.

Formic acid was determined using a commercially available enzyme assay (Formic acid, R-Biopharm AG, Darmstadt, Germany) by monitoring spectrophotometrically the production of NADH at 340 nm at 32°C (COBAS MIRA, Roche, Basel, Switzerland).

The oxygen uptake rate (R_{O2}) and carbon dioxide production rate (R_{CO2}) in the bioreactor off-gas were determined using paramagnetic (serie 2210, Servomex, Crowborough, UK) and infrared (serie 2500, Servomex, Crowborough, UK) analysers, respectively. The measured values of oxygen and carbon dioxide were corrected for water vapour according to Duboc and von Stockar (1998).

3. 5. Preparation of cell-free extracts and alcohol oxidase activity measurement

The procedures for the preparation of cell-free extracts and the measurement of specific alcohol oxidase activity were carried out as described previously (Jungo et al. (2006)).

The specific activity of alcohol oxidase was expressed in U mg^{-1} total proteins. One unit (U) of AOX corresponds to the oxidation of 1.0 µmole of methanol to formaldehyde per min at pH 7.5 at 25°C.

4. RESULTS AND DISCUSSION

4. 1. Batch culture of *P. pastoris* on methanol and glycerol

The results for mixed substrate growth of *P. pastoris* on glycerol and methanol in batch culture are presented in figure 1. Sequential utilization of substrates with two-phase growth was observed. The decrease of methanol concentration during the first 14 h of culture was due to methanol stripping and not to methanol consumption. This was confirmed by measuring a similar decrease of residual methanol concentration in batch experiments prior to inoculation. Methanol consumption began only after all glycerol was exhausted (14 h after inoculation), at which time, specific AOX activity of cell free extracts began to increase rapidly. As soon as methanol was depleted the specific AOX activity decreased rapidly.

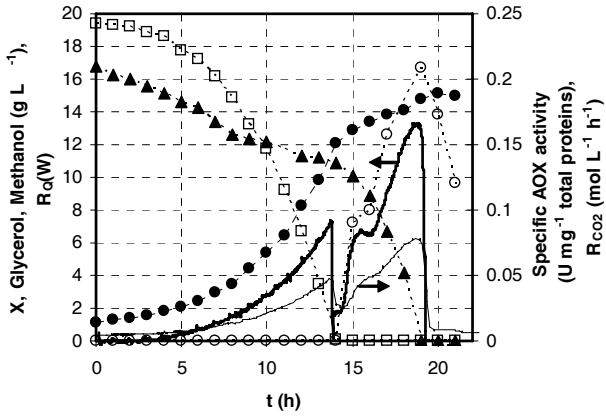

Figure 1: Dry cell weight X (●), methanol (▲) and glycerol (□) concentrations, carbon dioxide production rate (R_{CO2}, thin line), heat production rate (R_Q, bold line) and specific alcohol oxidase activities (○) as a function of time in batch culture on 20 g L^{-1} glycerol and 20 g L^{-1} methanol.

Similar sequential substrate utilization has been observed for batch growth of the methylotrophic yeast *C. boidinii* on glucose and methanol (Sakai et al. (1987)). However, with a mixture of glycerol and methanol, both carbon sources were utilized simultaneously by the methylotrophic yeast *H. polymorpha* due to sufficient derepression of methanol dissimilating enzymes (Wanner and Egli (1990)).

Specific growth rates were calculated from dry cell weight measurements and attained 0.22 h^{-1} during growth on glycerol and 0.03 h^{-1} during growth on methanol. The maximum specific growth rates on these two substrates as sole carbon source were determined in single carbon source batch cultures to be 0.24 h^{-1} and 0.14 h^{-1} on glycerol and methanol respectively. The error on the determination of the maximal specific growth rate was of about 6%. Hence, it can be considered that during the mixed substrate batch culture, the specific growth rate on glycerol was not significantly lower than during growth on glycerol as sole carbon source. A slight reduction of the specific growth rate on glycerol in the presence of methanol could be due to growth inhibition by methanol (Katakura et al. (1998)). The specific growth rate during growth on methanol in mixed substrate batch culture was 4-fold lower than in a culture on methanol as sole carbon source. In all probability this is due to the fact that cells need more time in order to synthesize methanol dissimilating and assimilating enzymes and to adapt to the new growth conditions.

The carbon dioxide production rate (R_{CO2}) and the heat production rate (R_Q) increased exponentially during the consumption of glycerol, decreased suddenly as glycerol was exhausted and increased again during the consumption of methanol, but not exponentially. The two-phase catabolism during methanol consumption could not be explained on the basis of our measurements. However, the fact that R_{CO2} and R_Q increased linearly during methanol consumption (and not exponentially) is probably due the fact that the cells did not have enough time in order to adapt to the new growth conditions. In all probability, all the enzymes necessary for methanol dissimilation and assimilation at maximal specific growth rate were not already synthesized. A nutrient limitation can be excluded because previous experiments showed that carbon is the limiting nutrient until at least 15.65 g C L^{-1}, which corresponds for instance to 40 g L^{-1} glycerol (Jungo et al. (2006)).

4. 2. Sudden change of nutrient supply

Since induction of alcohol oxidase is necessary for the dissimilation of methanol and for the expression of many recombinant proteins in *P. pastoris* strains, it is of high interest to know how fast this enzyme can be synthesized as soon as methanol is provided to the culture, either as sole carbon source or as a mixture of for instance methanol and glycerol. One problem at the beginning of the induction phase in high cell density fed-batch cultures with *P. pastoris* strains is accumulation of methanol because the cells lack the enzymes necessary for its consumption. Once methanol dissimilating enzymes are synthesized, a high rate of methanol consumption can lead to accumulation of toxic intermediates such as formaldehyde, formic acid and hydrogen peroxide (Swartz and Cooney (1981)).

Transition from growth on glycerol to growth on methanol

The results for the transition from 20 g L^{-1} glycerol (0.652 C-mol L^{-1}) to 20.87 g L^{-1} methanol (0.652 C-mol L^{-1}) at a dilution rate of 0.07 h^{-1} are presented in figures 2A, 2B and 4A. In all the graphs showing the results of transient continuous cultures, the time zero corresponds to the change in nutrient supply or to a pulse of methanol.

Once the feed medium was switched from the one containing glycerol as carbon source to the one containing methanol, a transient accumulation of methanol was observed during the first hour after the shift (figure 2A). In fact during the first hour, the measured residual methanol concentrations corresponded well with the theoretical curve of methanol accumulation in the chemostat $S = S_{in}(1-e^{-Dt})$, if no methanol would be consumed (figure 2A, dotted curve). During this first hour after the shift in nutrient supply, methanol was thus not significantly consumed and it can be calculated that the decrease of biomass concentration was below the dilution rate. However, half an hour after methanol was supplied to the cells, the specific AOX activities increased (figure 4A). This increase in specific AOX activity allowed the consumption of all the fed amount of methanol. Indeed, seven hours after the shift, the residual methanol concentration in the culture medium was lower than the detection limit of the HPLC method and steady-state growth on methanol was reached about 10h after the shift in nutrient supply.

Under glycerol-limited growth conditions, basal levels of AOX of 0.02 U mg^{-1} total proteins could be detected in the cells (figure 4A). During the first 30 minutes after the shift of nutrient, the specific AOX activity remained constant. However, after 30 minutes, the

specific AOX activity increased linearly at a rate of 0.05 U mg^{-1} total proteins h^{-1} (determined with linear regression for measurements between 1 and 8 h after the nutrient shift). For some reason, the specific AOX activity continued to rise although methanol had already decreased to limiting concentrations. After this «overshoot», steady-state values of 0.6 U mg^{-1} total proteins were reached about 13 h after the shift. It can be noted that maximal values of AOX activities were reached very late, about 4 hours later as compared with maximal gas exchange and heat production rates.

Carbon dioxide production and oxygen consumption rates (figure 2B) and heat production rate (figure 2A) decreased during the first 40 minutes after the shift (probably because methanol dissimilating enzymes needed first to be synthesized) and then increased over 6 h, as long as non-limiting concentrations of methanol were present in the culture. It can be noted that 2.5 h after the shift, methanol accumulation stopped because a sufficient amount of alcohol oxidase had been produced to consume the fed methanol which is also reflected for instance in the achieved level of oxygen consumption rate. Indeed, the steady-state catabolic rates on methanol (R_Q, R_{CO2}, R_{O2}) are exactly at the same level as the ones that can be read on the figures 2A and 2B, 2.5 h after the shift when the methanol concentration was at its peak value. A similar behaviour has been observed during transient experiments with *S. cerevisiae* subjected to sudden increase in dilution rate (Duboc et al. (1998)). Finally, as accumulated methanol was exhausted 6 hours after the shift of nutrient supply, the gas exchange rates and the heat production rate decreased rapidly, though not immediately, and then stabilized to new steady-state values.

Transition from growth on glycerol to mixed substrate growth on glycerol and methanol

The results for the transition from growth on 20 g L^{-1} glycerol (0.652 C-mol L^{-1}) to mixed substrate growth on 10 g L^{-1} glycerol (0.326 C-mol L^{-1}) and 10.43 g L^{-1} methanol (0.326 C-mol L^{-1}) are shown in figures 3A, 3B and 4B.

A similar behaviour was observed as for the transition from growth on glycerol to growth on methanol, however, adaptation was faster.

Transient accumulation of methanol was also observed during the first hour after the shift. Methanol was not consumed significantly during this period of time, since the measured residual methanol concentrations corresponded with the theoretical curve of methanol accumulation in the chemostat $S = S_{in}(1-e^{-Dt})$, if no methanol would be con-

sumed (figure 3A, dotted curve). The total amount of accumulated methanol was lower for the transition to mixed substrate growth, which is advantageous since high residual methanol concentrations are known to be toxic for methylotrophic yeasts (Katakura et al. (1998); Swartz and Cooney (1981)). Indeed, during high cell density fed-batch cultures with *P. pastoris* strains, cells are therefore less likely to be intoxicated by methanol accumulation at the beginning of the induction phase (after growth on glycerol) when both glycerol and methanol are fed to the culture instead of methanol as sole carbon source.

Specific AOX activity increased also linearly half an hour after the nutrient shift, but 3 times faster than for the transition from glycerol to methanol as sole carbon source (figure 4B). Indeed, specific activity increased at a rate of 0.16 U mg^{-1} total proteins h^{-1} (determined from linear regression of measurements between 1 and 6 h after the nutrient shift). Similarly, an unexplained and late «overshoot» of AOX activity was observed after residual methanol had reached limiting concentrations. Indeed, maximal values of AOX activities were only reached several hours (about 3.5 h) after maximal gas exchange and heat production rates were achieved.

During mixed substrate growth, specific AOX activities were 1.33-fold higher than during growth on methanol as sole carbon source (0.8 and 0.6 U mg^{-1} total proteins respectively in figures 4A and 4B). A two- to threefold increase in productivity of alcohol oxidase enzyme was also reported for mixed substrate cultures on methanol and glucose with the methylotrophic yeast *H. polymorpha* (Egli et al. (1986)). The authors pointed out that the higher specific AOX activities during mixed substrate growth were in all probability due to the lower residual concentration of methanol during mixed substrate growth than during growth with methanol as sole carbon source (Egli (1995)). Indeed, if methanol oxidation is considered as the growth rate-limiting reaction (Brinkmann et al. (1990)), the cells will maintain a high rate of substrate oxidation by increasing the amount of the rate limiting enzyme, when the residual methanol concentration decreases. Similarly, it has been shown for *H. polymorpha* and for *P. pastoris* that specific AOX activities increase when dilution rate is decreased in chemostat cultures on methanol as sole carbon source (Dijken et al. (1976); Jungo et al. (2006)).

It can be noted that during the whole experiment residual glycerol concentration remained lower than the detection limit of the HPLC method (< 0.01 g L^{-1}). During mixed

substrate growth, residual glycerol concentrations were sufficiently low to avoid AOX repression.

In carbon-limited continuous cultures, where residual concentrations of carbon sources are usually in the μg L^{-1} range (Lendenmann et al. (1996)), combinations of carbon sources can be consumed simultaneously even if they are consumed sequentially in batch cultures. Examples include *E. coli* growing on mixtures of up to six sugars (Lendenmann et al. (1996)) or *H. polymorpha* and *Kloeckera* sp. 2201 growing on glucose and methanol (Egli et al. (1982b); Egli et al. (1982a)). At low residual substrate concentrations, such as found in chemostat cultures, enzymes are derepressed and simultaneous assimilation of several substrates occurs.

These results suggest a positive influence of alternative carbon substrates such as glycerol on the expression of alcohol oxidase and the adaptation of the culture under mixed substrate growth conditions. Indeed, according to theses results, use of mixed substrate feeds during the induction phase contributes to accelerate induction of cells because their adaptation from growth on glycerol is facilitated. Moreover, this analysis in continuous cultivation confirms the observation of several authors during high cell density fed-batch cultures that co-feeding of methanol and glycerol during the transition phase facilitates the adaptation of cells on methanol (Katakura et al. (1998); Zhang et al. (2000a); Zhang et al. (2000b); Minning et al. (2001); Zhang et al. (2005); Cos et al. (2006)).

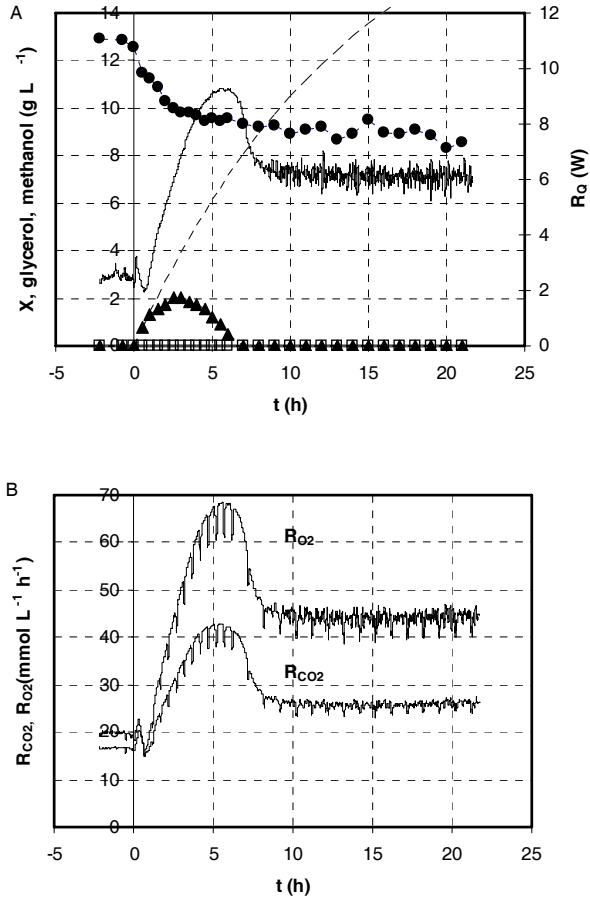

Figure 2: Continuous culture with change of nutrient supply at time zero from 20 g L^{-1} glycerol to 20.87 g L^{-1} methanol. A: Dry cell weight X (●), methanol (▲) and glycerol (□) concentrations, heat production rate (R_Q, continuous line), theoretical curve of methanol accumulation if methanol is not consumed by cells according to the equation $S = S_{in} (1- e^{-Dt})$ (dotted line); B: carbon dioxide production rate (R_{CO2}) and oxygen consumption rate (R_{O2}).

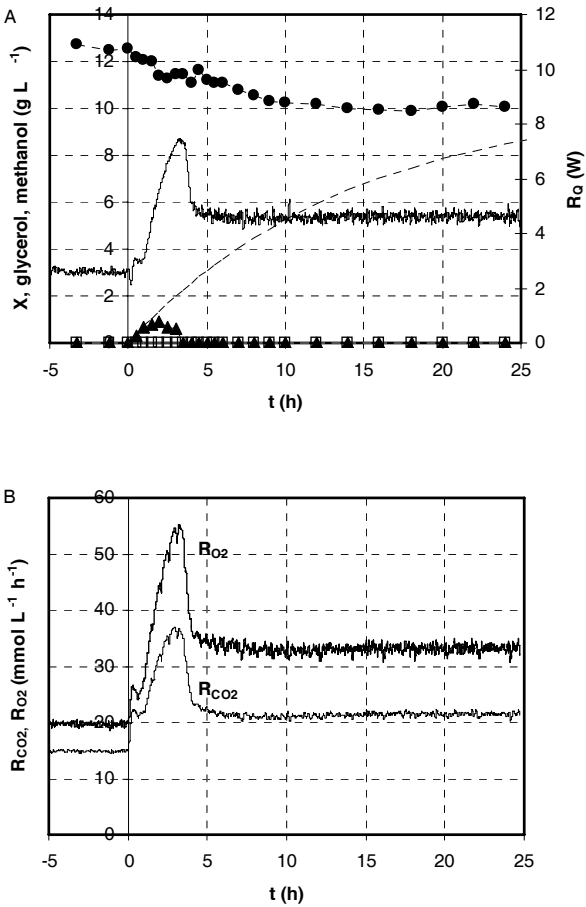

Figure 3: Continuous culture with change of nutrient supply at time zero from 20 g L^{-1} glycerol to a mixture of 10 g L^{-1} glycerol and 10.43 g L^{-1} methanol. A: Dry cell weight X (●), methanol (▲) and glycerol (□) concentrations, heat production rate (R_Q, continuous line), theoretical curve of methanol accumulation if methanol is not consumed by cells according to the equation $S = S_{in}(1- e^{-Dt})$ (dotted line); B: carbon dioxide production rate (R_{CO2}) and oxygen consumption rate (R_{O2}).

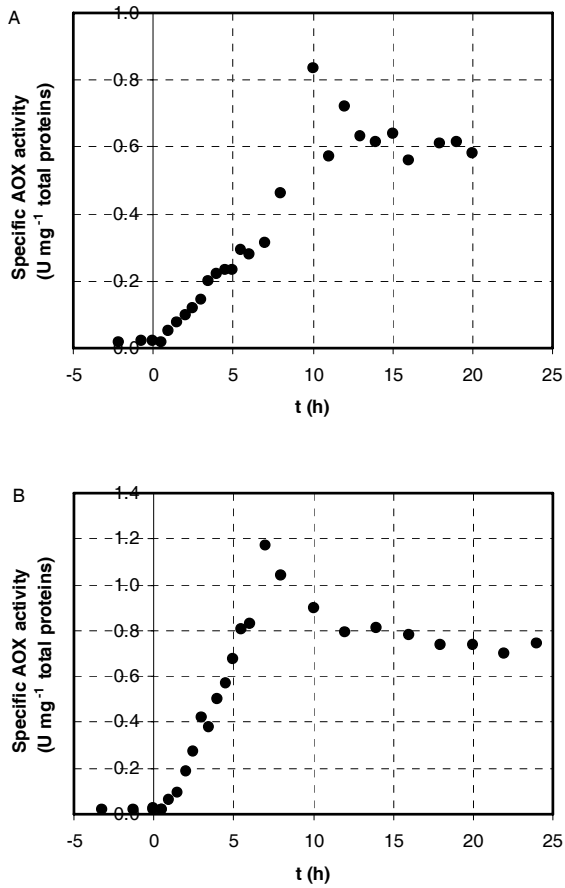

Figure 4: Specific alcohol oxidase activities during continuous culture with change of nutrient supply at time zero, A: from 20 g L^{-1} glycerol to 20.87 g L^{-1} methanol, B: from 20 g L^{-1} glycerol to a mixture of 10 g L^{-1} glycerol and 10.43 g L^{-1} methanol.

4. 3. Pulse experiments

In order to examine the effect of a transient accumulation of methanol in carbon-limited *P. pastoris* cultures, the transient response of continuous cultures subjected to a pulse of 2 g methanol was compared for cultures on either glycerol, methanol or a mixed substrate culture on glycerol and methanol.

Pulse of methanol in chemostat culture on glycerol

The results for the transient adaptation of a chemostat culture on 20 g L^{-1} glycerol (0.652 C-mol L^{-1}) after a pulse of 2 g methanol are presented in figures 5A, 5B and 8A. The oxygen consumption rate, the carbon dioxide production rate and the heat production rate increased immediately as methanol was added to the culture (figures 5A and 5B) and increased again about 1.5 h after the pulse to higher values than those attained just after the pulse. The gas exchange rates and the heat production rate increased as long as residual methanol was present in the culture, and decreased immediately as methanol was exhausted, 3.7 h after the pulse of methanol and attained the same steady-state values as before the pulse.

During the first 40 minutes after the pulse, methanol was not significantly consumed. Indeed, the measured residual methanol concentrations corresponded well with the theoretical wash-out curve of methanol $S = S_0 \exp^{-Dt}$, if no methanol is consumed (dotted line in figure 5A). During this periode of time, the specific AOX activity remained constant at basal levels of 0.02 U mg^{-1} total proteins, characteristic of chemostat cultures on glycerol at this dilution rate (figure 8A). The AOX induction pattern was very similar to the one observed after a sudden change of nutrient supply from chemostat growth on glycerol to chemostat growth on methanol. Indeed, after one hour, the specific AOX activity increased at a rate of 0.04 U mg^{-1} total proteins h^{-1} (determined from linear regression of measurements between 1 and 2.7 h after the pulse, figure 8A) and methanol was consumed by the cells (figure 5A). This similarity can be explained by the fact that the history of the cells was the same before the pulse of methanol or before the nutrient shift and by the fact that induction was provoked in both experiments with the same substrate and at the same dilution rate.

As soon as all methanol had been consumed (3.7 h after the pulse of methanol) the specific AOX activity decreased, similarly as after the exhaustion of methanol during batch culture. Finally, 16 h after the pulse, the specific AOX activity attained again the same steady-state values as prior to the pulse. The fine dotted line in figure 8A represents the theoretical wash-out curve. Since the decrease of specific AOX activity proceeded faster than the theoretical wash-out curve, synthesis of AOX not only ceased as soon as methanol was exhausted, but AOX was also subjected to degradation in addition to being diluted among the newly formed cells. An «apparent» exponential coefficient of -0.17 h^{-1} was determined with linear regression of the logarithm of specific

AOX activity as a function of time for t > 3.67 h. The bold dotted line in figure 8A simulates the exponential decrease of specific AOX activity with this coefficient. Because of wash-out of the cells, the «true» exponential coefficient associated to degradation of AOX was therefore 0.17 - 0.07 = 0.10 h^{-1}.

Active degradation of alcohol oxidase when *P. pastoris* cells growing on methanol are suddenly transferred to media containing ethanol or glucose was reported by Tuttle and Dunn (1995). They showed that this degradation is due to fusion of peroxisomes with vacuolar vesicles followed by proteolysis.

During this transient experiment, no residual glycerol, neither overflow metabolites, such as formaldehyde or formic acid, were detected. Residual glycerol concentrations always remained below the detection limit of the HPLC method used for its quantification.

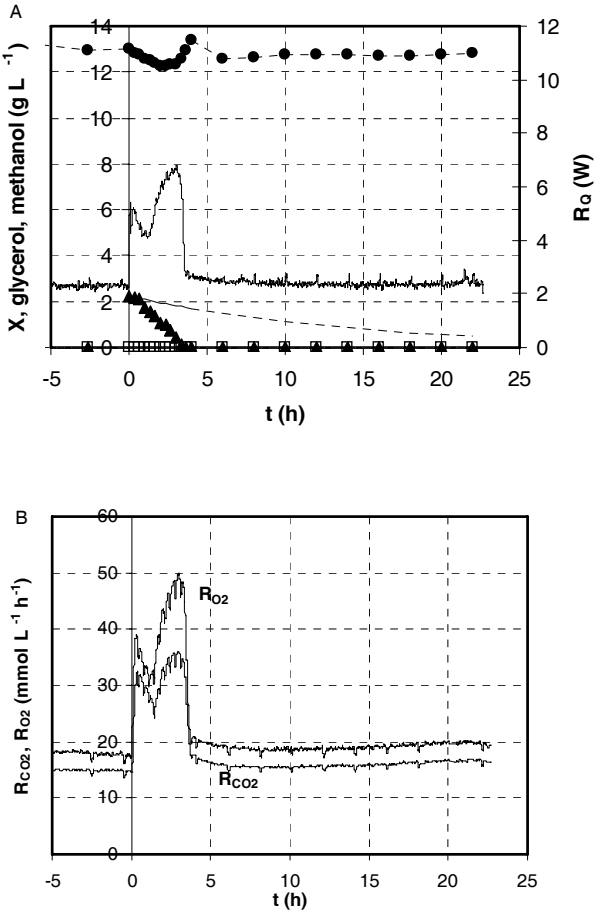

Figure 5: Continuous culture on 20 g L^{-1} glycerol with pulse of 2 g of methanol at time zero. A: Dry cell weight X (●), methanol (▲) and glycerol (□) concentrations, heat production rate (R_Q, continuous line), theoretical wash-out curve of methanol if methanol is not consumed by cells according to the equation $S = S_0 \exp^{-Dt}$ (dotted line); B: carbon dioxide production rate (R_{CO2}) and oxygen consumption rate (R_{O2}).

Pulse of methanol in chemostat culture on methanol

The results for the transient adaptation of a chemostat culture on 20.87 g L^{-1} methanol (0.652 C-mol L^{-1}) after a pulse of 2 g methanol are shown in figures 6A, 6B and 8B. There is a fundamental difference with the chemostat culture on glycerol because the pulse of methanol led to wash-out of the culture. Looking at the details, we can see that the pulse of methanol led first to an immediate sharp peak and then to fast exponential decrease in gas exchange rates (figure 6B) and heat production rate (figure 6B), which happened in two unexplained distinct phases.

Methanol accumulated in the culture (figure 6A) after a transient decrease of methanol concentration during the first hour after the pulse. Since cells were grown in a chemostat culture on methanol before the pulse, they already contained all the necessary enzymes for methanol dissimilation. Hence, methanol was consumed at a higher rate (up to 3% higher volumetric rate than during steady-state growth) during the first hour after the pulse. This sudden increase in the rate of methanol consumption led to transient accumulation of metabolic intermediates like formaldehyde and formic acid (figure 6A), which are known to be toxic for methylotrophic yeasts (Swartz and Cooney (1981)). The excretion of these toxic intermediates finally led to wash-out of the culture.

The specific AOX activity was 0.73 U mg^{-1} total proteins on average during chemostat growth on methanol. The results in figure 8B show that the pulse of methanol led to an exponential decrease of specific AOX activity. An «apparent» exponential coefficient of -0.25 h^{-1} was determined with linear regression of the logarithm of specific AOX activity as a function of time (for $0 < t < 13.5$ h). The bold dotted line in figure 8B simulates the exponential decrease of specific AOX activity with this coefficient. The theoretical decrease of specific AOX activity due only to wash-out of cells is also shown in figure 8B. It can be concluded that the alcohol oxidase enzyme was subjected to degradation. This degradation was exponential and the «true» exponential coefficient was therefore $0.25 - 0.07 = 0.18$ h^{-1}.

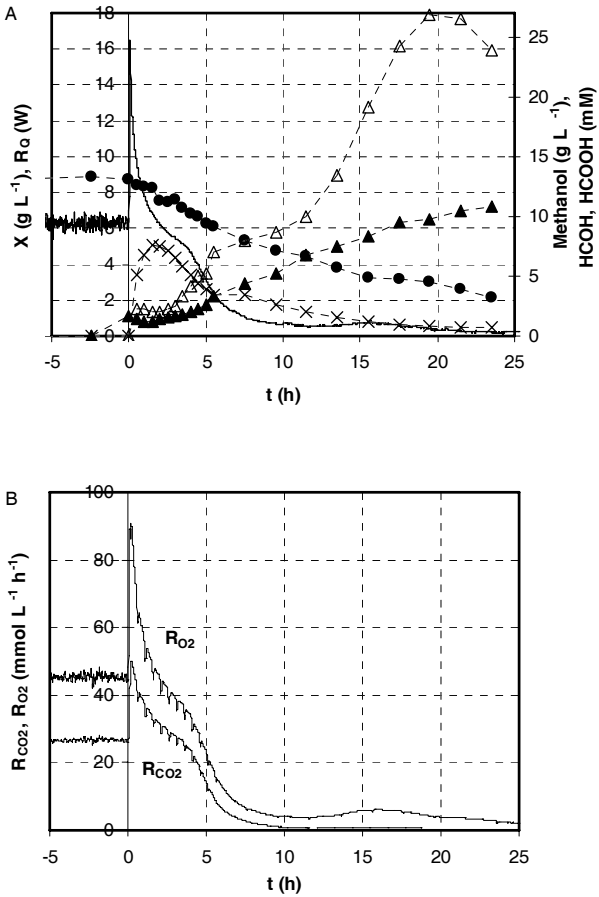

Figure 6: Continuous culture on 20.87 g L^{-1} methanol with pulse of 2 g of methanol at time zero. A: Dry cell weight X (●), methanol (▲), formaldehyde (△) and formic acid (x) concentrations, heat production rate (R_Q, continuous line); B: carbon dioxide production rate (R_{CO2}) and oxygen consumption rate (R_{O2}).

Pulse of methanol in mixed substrate chemostat culture on glycerol and methanol

The results for the transient adaptation of a mixed substrate chemostat culture on 10 g L^{-1} glycerol (0.326 C-mol L^{-1}) and 10.43 g L^{-1} methanol (0.326 C-mol L^{-1}) after a pulse of 2 g methanol are presented in figures 7A, 7B and 8C.

After an initial jump to higher values immediately after the pulse of methanol, the carbon dioxide production rate, the oxygen consumption rate (figure 7B) and the heat production rate (figure 7A) decreased exponentially after the pulse of methanol. Indeed, as in chemostat culture on methanol, a pulse of 2 g of methanol led to wash-out of the culture. During the first 2.5 h after the pulse of methanol, residual methanol concentration decreased because methanol was consumed by the cells. During mixed substrate culture on glycerol and methanol, the methanol dissimilating enzymes were synthesized and excess methanol could therefore be consumed by the cells. However, this sudden increase in methanol consumption rate (up to 14 % higher volumetric rate than during steady-state growth) led to accumulation of toxic intermediates. Figure 7A shows the profiles of formaldehyde and formic acid concentrations after the pulse of methanol.

After the pulse of methanol, glycerol consumption ceased. The theoretical wash-in curve (bold dotted line in figure 7A) corresponded well with the measured glycerol concentrations. A slight discrepancy might be due to an underestimation of dilution rate.

The average specific AOX activity was 1.0 U mg^{-1} total proteins during steady-state mixed substrate growth. After a sudden initial drop to 0.54 U mg^{-1} total proteins, an exponential decrease of specific AOX activity was observed (figure 8C). An «apparent» exponential coefficient of -0.21 h^{-1} was determined with linear regression of the logarithm of specific AOX activity as a function of time (for $t > 0.5$ h). In figure 8C, the bold dotted line simulates the exponential decrease of specific AOX activity with this coefficient. Since the decrease of specific AOX activity was faster than the theoretical wash-out curve (dotted line), the AOX enzyme was subjected to degradation. The «true» exponential coefficient characterizing this degradation was $0.21 - 0.07 = 0.14$ h^{-1}.

Hence, during carbon-limited cultures on methanol or on methanol and glycerol, methanol accumulation led to sudden wash-out because of excretion of toxic intermediates. However no overflow metabolites, such as formaldehyde or formic acid, had been detected after the pulse of methanol during chemostat culture on glycerol, neither during

the nutrient shifts from chemostat growth on glycerol to chemostat growth on methanol or to mixed substrate growth on glycerol and methanol. In our view this is related to the amount of AOX present in the cells at the time when the pulse of methanol is performed. During chemostat growth on glycerol, residual specific AOX activity is very low, about 0.02 U mg^{-1} total proteins. After a pulse of methanol during chemostat growth on glycerol, methanol cannot be consumed rapidly because the cells lack the necessary enzymes for the efficient and fast consumption of methanol.

In chemostat cultures on methanol or in mixed substrate chemostat cultures, specific AOX activity was high, 0.7 and 1.0 U mg^{-1} total proteins, respectively. Hence, immediately after the pulse of methanol, methanol was consumed at high rate, which led to the excretion of metabolic intermediates like formaldehyde and formic acid. The toxic effect of these intermediates led to wash-out of the culture.

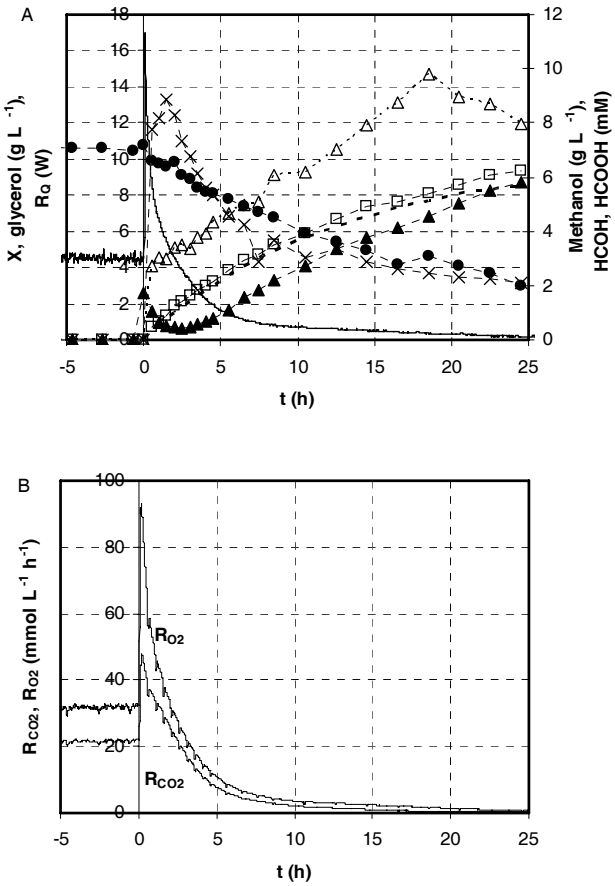

Figure 7: Mixed substrate continuous culture on 10 g L^{-1} glycerol and 10.43 g L^{-1} methanol with pulse of 2 g of methanol at time zero. A: Dry cell weight X (●), methanol (▲), glycerol (□), formaldehyde (△) and formic acid (x) concentrations, heat production rate (R_Q, continuous line), theoretical wash-in curve of glycerol if glycerol is not consumed by cells according to the equation $S = S_{in} - (S_{in}-S_0) \exp^{-Dt}$ (bold dotted line); B: carbon dioxide production rate (R_{CO2}) and oxygen consumption rate (R_{O2}).

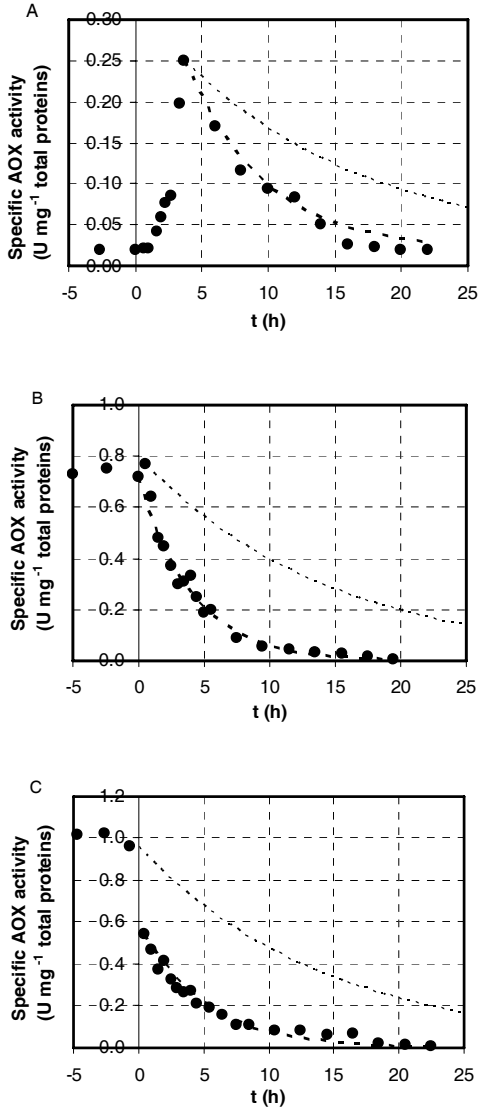

Figure 8: Specific AOX activities after pulse of 2 g methanol at time zero, A: during chemostat on 20 g L^{-1} glycerol, B: during chemostat on 20.87 g L^{-1} methanol, C: during chemostat on 10 g L^{-1} glycerol and 10.43 g L^{-1} methanol. Dotted curves represent theoretical wash-out curves. Bold dotted curves are simulations of specific AOX activity after pulse of methanol.

5. CONCLUSIONS

The results of this study suggest that the use of glycerol along with methanol during the induction phase has a strong influence on the dynamics of expression of alcohol oxidase and on the dynamics of metabolic rates. Transition from growth on glycerol to growth on a mixture of glycerol and methanol (50-50% C-mol C-mol^{-1}) allowed to reduce the time needed for induction of alcohol oxidase compared with transition from glycerol to methanol as sole carbon source. Hence, the strategy of high cell density fed-batch cultures with *P. pastoris* strains can be improved by use of mixed substrate feeds during the induction phase. Indeed, feeding of mixed substrates during the induction phase will shorten the time cells need to adapt to new growth conditions and reduce transient methanol accumulation, which can lead to excretion of toxic metabolic intermediates. Moreover, co-feeding of methanol and glycerol already during the transition phase, which is usually performed on glycerol, is also a means to facilitate the adaptation of methylotrophic yeasts to growth on methanol during the induction phase of high cell density fed-batch cultures for the production of recombinant proteins.

Pulses of methanol in chemostat cultures on either glycerol, methanol or mixed substrate cultures on methanol and glycerol contributed to a better understanding of the regulation of the alcohol oxidase enzyme after transient methanol accumlation. Results showed that in cultures on methanol or on mixed methanol and glycerol, sudden increase in the consumption rate of methanol after a transient increase of methanol led to excretion of toxic intermediates. The intoxication of cells resulted in wash-out of the culture. This sudden increase in the consumption rate of methanol is due to the high amount of alcohol oxidase present in the cells during growth at low residual methanol concentrations. Indeed, a pulse of methanol during chemostat growth on glycerol or a transition from growth on glycerol to growth on methanol as sole carbon source did not lead to wash-out because methanol consumption was delayed and the methanol consumption rate increased progressively as methanol dissimilating enzymes were synthesized. During high cell density fed-batch cultures for the production of recombinant proteins with *P. pastoris* strains, induction is usually performed at low residual methanol concentrations. Hence, methanol feeding, mixing and aeration of the culture have to be tightly controlled in order to avoid transient methanol accumulation, which can lead to intoxication of cells by excretion of metabolic intermediates.

6. ACKNOWLEDGEMENTS

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7. NOMENCLATURE

AOX	Alcohol oxidase	
S	Substrate concentration in bioreactor	C-mol L^{-1}
D	Dilution rate	h^{-1}
R_{CO2}	Carbon dioxide production rate	mol L^{-1} h^{-1}
R_{O2}	Oxygen consumption rate	mol L^{-1} h^{-1}
R_Q	Heat production rate	W
X	Cell dry weight	C-mol L^{-1}

Subscripts

0	Initial condition
in	Inlet

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CHAPTER 6

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

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1. ABSTRACT

Transient continuous cultures constitute a means to speed up strain characterization, by avoiding the need for many time-consuming steady-state experiments. In this study, mixed substrate growth on glycerol and methanol of a *Pichia pastoris* strain expressing and secreting recombinant avidin was characterized quantitatively by performing a nutrient gradient with linear increase of the methanol fraction in the feed medium from 0.5 to 0.93 C-mol C-mol^{-1} at a dilution rate of 0.06 h^{-1}. The influence of the methanol fraction in the feed medium on recombinant avidin productivity and on specific alcohol oxidase activity were also examined.

Results showed that, compared with cultures on methanol as sole carbon source, the specific recombinant avidin production rate was the same provided the methanol fraction in the feed medium was higher than 0.6 C-mol C-mol^{-1}. The volumetric avidin production rate was even 1.1-fold higher with a methanol fraction in the feed medium of 0.62 C-mol C-mol^{-1} as a result of the higher biomass yield on mixed substrate growth compared with methanol alone. Moreover, since heat production and oxygen uptake rates are lower during mixed substrate growth on glycerol and methanol, mixed substrate cultures present technical advantages for the performance of high cell density *P. pastoris* cultures.

Results obtained in a high cell density fed-batch culture with a mixed feed of 0.65 C-mol C-mol^{-1} methanol and 0.35 C-mol C-mol^{-1} glycerol were in agreement with results obtained during the transient nutrient gradient.
Keywords: *Pichia pastoris*; avidin; transient; mixed substrate; calorimetry.

2. INTRODUCTION

The methylotrophic yeast *Pichia pastoris* is increasingly used for the expression of recombinant proteins. A commonly used approach for heterologous protein expression with this species is to express the gene of interest under the control of the AOX1 promoter. In the wild-type *P. pastoris* strain, the AOX1 promoter controls expression of alcohol oxidase 1 which is the first enzyme in methanol metabolism (Ellis et al. (1985); Cregg et al. (1989)). This promoter is reported to be induced by methanol, but repressed in the presence of excess glycerol (Tschopp et al. (1987)).

In high cell density processes, recombinant *P. pastoris* is usually grown in a multi-stage process. The first stage is a batch culture on glycerol to achieve high cell densities rapidly while repressing foreign gene expression. This is followed by a fed-batch phase on glycerol to raise biomass concentrations to even higher levels while derepressing the AOX promoter. The final stage usually involves the induction of recombinant protein expression through the fed-batch feeding of methanol (Stratton et al. (1998)).

As a result of the high heat of combustion of methanol (-727 kJ C-mol^{-1}, Weast (1980)), considerable heat is generated during the fed-batch stage on methanol. This requires rapid and efficient cooling systems, particularly at large scale where heat losses through the bioreactor walls may be limiting due to the small surface area to volume ratio. Failure to remove this heat may result in reactor temperature increase and affect the productivity and quality of the recombinant protein. The enthalpy of combustion of glycerol (-549.5 kJ C-mol^{-1}, von Stockar et al. (1993)) is lower than that of methanol, therefore, for a given growth rate, less heat will be released during supply of a mixed feed of glycerol and methanol compared with methanol alone. Moreover, oxygen consumption will also be reduced with a mixed feed since less oxygen is necessary for the oxidation of glycerol. This is also important since oxygen transfer is often a problem with high cell density *P. pastoris* cultures (Jenzsch et al. (2004)). Consequently, any method which reduces the oxygen consumption rate without affecting productivity would clearly be advantageous.

However, the influence of the methanol-glycerol ratio in the feed medium on the expression of recombinant protein has to be examined since glycerol is reported to repress the expression of alcohol oxidase (Tschopp et al. (1987)).

Several reports in the literature describe mixed cultures on glycerol and methanol with the *P. pastoris* expression system. A number of authors showed that the presence of glycerol during the induction phase in fed-batch processes can lead to improved productivities (Cregg et al. (1993); Loewen et al. (1997); McGrew et al. (1997); Katakura et al. (1998); Zhang et al. (2003)). According to the literature, the specific rates of methanol consumption and protein expression are not lower, providing the residual glycerol concentration in the culture medium does not reach levels sufficient to repress the AOX promoter or cause the excretion of inhibitory metabolites such as ethanol or acetate (Mardon (1995); Inan and Meagher (2001)). However, there is no consensus on this, with some authors claiming that the presence of glycerol results in partial repression of the AOX1 promoter (Sreekrishna et al. (1997); Hellwig et al. (2001)).

Traditionally, physiological strain characterization is performed using continuous cultures at steady state. Such experiments are very time-consuming since many steady states have to be attained to achieve an exhaustive picture of the strain under physiologically adapted conditions. On the other hand, the performance of transient experiments in continuous cultures allows a more rapid strain characterization and hence process development. In this study, the influence of the methanol-glyerol ratio in the feed medium was investigated by performing a transient nutrient gradient during continuous cultivation. More precisely, the methanol fraction in the feed medium was increased linearly from 0.5 to 0.93 C-mol C-mol^{-1} in 80 hours in order to investigate quantitatively the influence of the methanol-glycerol ratio in the feed medium on the growth stoichiometry and recombinant protein productivity for a *P. pastoris* Mut^{+} strain expressing and secreting a recombinant glycosylated avidin. Similar transient experiments have been reported for other microorganisms using linear C/N gradients of the feed medium (Zinn (1998); Maskow and Babel (2000); Maskow and Babel (2002)).

A high cell density fed-batch culture was subsequently performed at the optimum methanol fraction in the feed medium, determined from the transient experiment in order to validate the results at high cell density.

3. MATERIALS AND METHODS

3. 1. Microorganism, inoculum preparation and media

A recombinant *Pichia pastoris* Mut^+ strain expressing and secreting a recombinant glycosylated avidin with an acidic isoelectric point was used in this work (Zocchi et al. (2003)). Precultures were prepared in complex YPG medium at 30°C for 24h as described previously (Jungo et al. (2006)).

The complex YPG medium contained 20 g L^{-1} glycerol (Sigma-Aldrich, Steinheim, Germany), 6 g L^{-1} yeast extract (OXOID, Hampshire, England), 5 g L^{-1} Bacto Peptone (Becton, Le Pont de Claix, France). This medium was sterilized by heating at 120°C for 20 min.

The defined medium used for continuous cultures at low cell densities was based on the medium developed by Egli and Fiechter (1981) and was adapted as described previously (Jungo et al. (2007)) for optimal expression of recombinant avidin. This medium contained per liter: 10 g glycerol and 10.43 g methanol, 15.26 g NH_4Cl, 5.62 g KH_2PO_4, 1.18 g $MgSO_4$ $7H_2O$, 0.9 g EDTA $2H_2O$, 110 mg $CaCl_2$ $2H_2O$, 75 mg $FeCl_3$ $6H_2O$, 28 mg $MnSO_4$ H_2O, 44 mg $ZnSO_4$ $7H_2O$, 8 mg $CuSO_4$ $5H_2O$, 8 mg $CoCl_2$ $6H_2O$, 5.2 mg Na_2MoO_4 $2H_2O$, 8 mg H_3BO_3, 1.2 mg KI, 20 μg biotin, and 0.4 mL of antifoam agent (Struktol SB 2121, Schill and Seilacher, Hamburg, Germany). The pH was adjusted to 5.0 with KOH 4M. For the transient nutrient gradient, the mixing flask initially contained 10 g L^{-1} glycerol and 10.43 g L^{-1} methanol as carbon and energy sources, and the reservoir flask contained 20.87 g L^{-1} methanol and no glycerol. The mixing and reservoir flasks also contained all other nutrients at the same concentrations.

The defined medium used for the fed-batch culture at high cell densities contained per liter: 20 g glycerol, 26.7 mL H_3PO_4 85%, 0.93 g $CaSO_4$ $2H_2O$, 18.2 g K_2SO_4, 14.9 g $MgSO_4$ $7H_2O$, 4.13 g KOH, 0.8 g EDTA $2H_2O$, 4.35 mL of trace elements solution, 400 μL of a solution containing 1g L^{-1} biotin in NaOH 1M and 0.4 mL of antifoam agent (Struktol SB 2121, Schill and Seilacher, Hamburg, Germany). The trace elements solution contained per liter: 5 mL H_2SO_4 97%, 0.02 g H_3BO_3, 6 g $CuSO_4$ 5 H_2O, 0.08 g NaI, 3 g $MnSO_4$ H_2O, 0.2 g Na_2MoO_4 $2H_2O$, 0.5 g $CaSO_4$ 2 H_2O, 20 g $ZnCl_2$, 65 g $FeSO_4$ 7 H_2O.

All components of the defined media were obtained from Fluka, Buchs, Switzerland, with the exception of glycerol which was obtained from Sigma-Aldrich, Steinheim, Ger-

many. The defined media were sterilized by filtration (0.22 μm, Steritop, Millipore Corporation, Billerica, USA).

3. 2. Culture conditions

Growth experiments were undertaken using a bench-scale reaction calorimeter RC1 (Mettler Toledo AG, Greifensee, CH) modified for biotechnological applications with a working volume of 1.6 L. Characteristics of this so called Bio-RC1 can be found in Marison et al. (1998). The steady-state culture volume was 1.050 L. Reaction calorimeters are equipped and can be operated like standard bench-scale bioreactors.

Temperature was maintained at 30°C and pH at 5.0 by the automatic addition of 2M KOH. The heat of neutralization was calculated from the consumption of KOH 2M according to Meier-Schneiders et al. (1995). A polarographic pO_2 probe (InPro 6800, Mettler Toledo, Greifensee, Switzerland) was used to monitor dissolved oxygen, with respect to air-saturated medium. The dissolved oxygen in the culture medium was maintained above 40% air saturation, through the addition of oxygen to the inlet gas flow. The total inlet gas flow was maintained constant (2 L min^{-1}). The gas was saturated with water immediately prior to entry in the bioreactor. A constant stirring rate of 800 rpm was employed throughout the experiments.

All liquid inlet flows were quantified gravimetrically and used to control continuously and on-line the feed pump rate. The temperature of all liquid feeds was adjusted to the reactor temperature immediately prior to entry in the bioreactor.

Torque measurements were used to detect baseline shifts of the heat flux (Menoud et al. (1995)). As the release of CO_2 from the medium is an endothermic process, the heat flow induced by CO_2 stripping was taken into account according to Meier-Schneiders et al. (1995). The heat losses to the environment were calculated using a heat transfer coefficient for the bioreactor of 0.133 W K^{-1}, which was determined as described in Birou (1986).

3. 3. Transient nutrient gradient in continuous culture

The culture was first grown in batch phase on a mixture of 20 g L^{-1} glycerol and 20 g L^{-1} methanol. Methanol was consumed only after all the glycerol had been consumed since high glycerol concentations are known to have a repressing effect on the expression of the enzymes necessary for the dissimilation of methanol. After all glycerol had been exhausted, methanol was consumed which allowed the induction and synthesis

of methanol dissimilating enzymes and prepared the cells for chemostat growth on methanol.

As soon as all the methanol had been consumed (indicated by a sudden increase in the dissolved oxygen level), the supply of the feed-medium containing 10 g L^{-1} glycerol and 10.43 g L^{-1} methanol as carbon sources was activated to provide a dilution rate of 0.06 h^{-1}.

The transient nutrient gradient was begun after approximately five liquid residence times. The exact time was determined from both on-line analysis (heat signal, CO_2 and O_2 in the exhaust gas) and off-line analysis (dry cell weight) to ensure that steady-state conditions had been achieved. The methanol fraction in the feed medium was linearly increased, without changing the concentration of any other nutrient, by means of a special feeding system presented in figure 1 (Maskow and Babel (2000)).

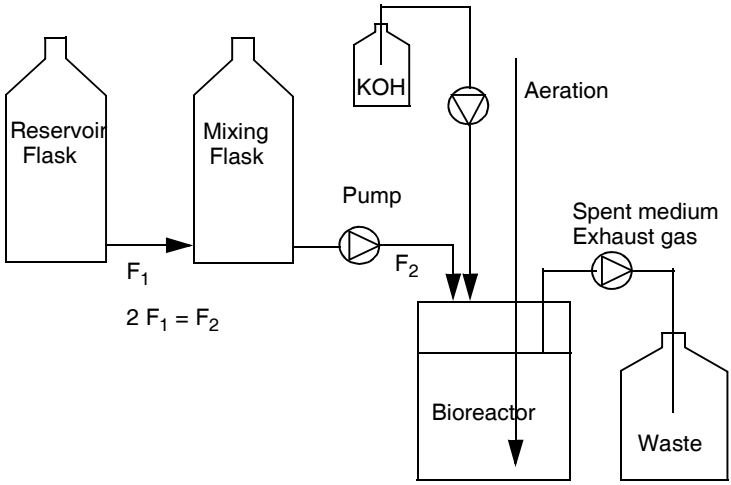

Figure 1: Experimental set-up used to create a linearly increasing methanol fraction in the feed medium at a constant dilution rate and a constant supply of all nutrients other than methanol and glycerol.

By integration of the differential mass balance equation describing the amount of substrate (methanol or glycerol) in the mixing flask, the time course of the concentration of

substrate in the mixing flask and entering the bioreactor can be shown to follow equation (1), Maskow and Babel (2000):

$$C_S{}^M = C_S{}^R - (C_S{}^R - C_{S(0)}{}^M) \cdot \left(\frac{V_0 + (F_1 - F_2) \cdot t}{V_0}\right)^{\frac{F1}{F2 - F1}} \quad (1)$$

where $C_S{}^R$ and $C_S{}^M$ are the substrate concentrations in the reservoir and mixing flasks respectively, $C_{S(0)}{}^M$ the initial substrate concentrations in the mixing flask, V_0 the volume of the mixing and reservoir flasks at time zero, and F_1 and F_2 are the constant medium flows from the reservoir and mixing flasks respectively.

In order to perform a linear nutrient gradient (Maskow and Babel (2002)), only one pump was necessary to supply the medium, at a constant rate, from the mixing flask to the bioreactor (figure 1). Since the reservoir and mixing flasks are connected to each other at the same height, medium from the reservoir flask flows into the mixing flask at the medium flow F_1 which is exactly half of the medium flow F_2, the latter being chosen to achieve a dilution rate of 0.06 h^{-1}. Since 2 $F_1 = F_2$, equation (1) can be re-written in the following simplified form (equation 2):

$$C_S{}^M = C_{S(0)}{}^M + (C_S{}^R - C_{S(0)}{}^M) \cdot \frac{F_1}{V_0} \cdot t \quad (2)$$

Choosing an initial volume of medium in the mixing and reservoir flasks of 3 L and working at a dilution rate of 0.06 h^{-1} allowed a linear increase of the methanol fraction in the mixing flask from 0.5 to 0.93 C-mol C-mol^{-1} in 80 hours.

3. 4. *A priori* evaluation of pseudo steady-state growth during the transient nutrient gradient

In order to achieve pseudo steady-state growth conditions, the nutrient gradient has to be designed such that the cells are able to adapt to the changing substrate concentration in the feed medium and to avoid an increase of the specific growth rate. Too fast a change of substrate concentration in the mixing flask could lead to substrate accumulation in the bioreactor, which could lead to an increase of the specific growth rate. In order to distinguish *a priori* the pseudo steady-state from a real steady-state, the residual methanol concentration in the bioreactor and the specific growth rate were estimated during the nutrient gradient. In order to estimate the residual methanol

concentration, equation 3 describing the balance of methanol over the whole bioreactor has to be integrated.

$$\frac{dC_S}{dt} = (C_{S(0)}{}^{M} + \alpha \cdot t - C_S) \cdot D - q_S \cdot X \quad (3)$$

where D is the dilution rate (h^{-1}), X the cell dry weight (C-mol L^{-1}), C_S the residual methanol concentration in the bioreactor (C-mol L^{-1}), q_S the biomass-specific methanol consumption rate (C-mol C-mol^{-1} h^{-1}) and α the rate of the nutrient gradient (C-mol L^{-1} h^{-1}), which can be defined by equation 4, on the basis of equation 2 .

$$\alpha = (C_S{}^{R} - C_{S(0)}{}^{M}) \cdot \frac{F_1}{V_0} \quad (4)$$

In this study, the increase in methanol concentration was performed at a rate of α = 0.0034 C-mol methanol L^{-1} h^{-1}.
In equation 10, q_S and X can be described by equations 5 and 6, respectively, taking into account substrate consumption due to maintenance.

$$q_S = q_S^{max} \cdot \frac{C_S}{K_S + C_S} - m \quad (5)$$

$$X = \frac{Y_{X/S}^{true} \cdot D}{D + Y_{X/S}^{true} \cdot m} \cdot (C_{S(0)}{}^{M} + \alpha \cdot t - C_S) \quad (6)$$

where $q_S{}^{max}$ is the maximum specific methanol consumption rate, K_S the Monod substrate affinity constant, m the maintenance coefficient and $Y_{X/S}{}^{true}$ the true yield coefficient. The maintenance coefficient and the true yield coefficient for growth on methanol were determined previously for the strain used (Jungo et al. (2006)). The residual methanol concentration in the bioreactor at the beginning of the nutrient gradient corresponds to the residual methanol concentration during mixed substrate chemostat growth on 50% C-mol C-mol^{-1} methanol and 50% C-mol C-mol^{-1} glycerol and was estimated using the Monod model and the Monod constant K_S = 0.22 g L^{-1} determined by Curvers et al. (2001) for growth on methanol as sole carbon source. It has to be pointed out that the influence on the Monod substrate affinity constant during mixed substrate

growth was not taken into account in this estimation but was in all probability lowered during mixed substrate growth if a Monod model is applied (Kovarova-Kovar and Egli (1998)). A detailed review on growth kinetics of single and mixed substrate cultures was done by these authors.

Figure 2 presents the estimated residual methanol concentration and the corresponding estimated specific growth rate with the Monod model for growth on methanol as sole carbon source as a function of time during the first eight hours of the nutrient gradient. For the rest of the nutrient gradient, simulated values stayed constant. Integration of equation 3 was carried out numerically using the program Matlab and a Runge-Kutta algorithm.

As reviewed by Kovarova-Kovar and Egli (1998) more complex kinetic models than the Monod model should be applied in order to predict more accurately the residual methanol concentration during mixed substrate growth, for instance the model proposed by Lendenmann and Egli (1998). However because of lack of kinetic parameters (substrate saturation constant K_S in glycerol-limited chemostat culture), we chose to apply the Monod model for growth on methanol as sole carbon source as a first approximation. According to these simplified calculations the increase of methanol in the feed medium is sufficiently slow to avoid significant substrate accumulation and increase of specific growth rate (figure 2). Preliminary calculations even showed that the rate of the nutrient gradient (described by the constant α) could be increased 10-fold without significant accumulation of methanol in the bioreactor (bold dotted curve in figure 2). However, in this case, it would not be possible to take many samples during the nutrient gradient and less information on the influence of the methanol-glycerol ratio would finally be obtained during this faster experiment. Since the withdraw of samples represents a perturbation for the pseudo steady-state growth condition, sampling was performed only every 2 hours during the nutrient gradient.

The fact that the amount of glycerol fed to the culture decreases linearly during the transient nutrient gradient was not taken into account in this estimation. A rapid decrease of a limiting nutrient could lead to an increase of endogenous metabolism (consumption of own mass in the absence of an energy source). This effect was not taken into ac-

count because glycerol is not the sole limiting carbon source and because decrease of glycerol concentration in the feed medium was performed at a very slow rate.

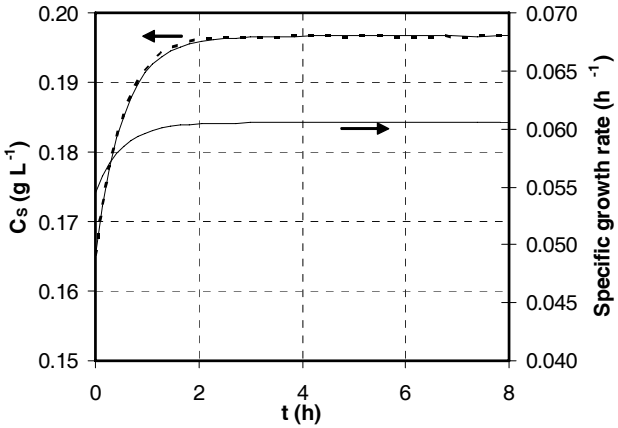

Figure 2: *A priori* estimation of residual methanol concentration C_S and specific growth rate during the first eight hours of the nutrient gradient. The bold dotted curve represents the estimation of residual methanol concentration for a 10-fold faster nutrient gradient.

3. 5. Fed-Batch operation

The culture was run in batch phase on 20 g L^{-1} glycerol for approximately 13 h. Two hours after all glycerol had been consumed an exponential feed, at a growth rate of 0.06 h^{-1}, was begun. The feed solution contained 65% C-mol C-mol^{-1} methanol and 35% C-mol C-mol^{-1} glycerol, supplemented with 12 mL of trace element solution per liter of feed solution.

For a constant specific growth rate, the feed rate, F(t) in g h^{-1}, was varied according to the following equation (D'Anjou and Daugulis (2000)):

$$F(t) = \frac{\mu \cdot X(t_B) \cdot V(t_B)}{Y_{XS}} \cdot e^{\mu \cdot t} \qquad (7)$$

where μ is the specific growth rate, $Y_{X/S}$ the biomass yield (determined during the transient continuous culture at a methanol fraction of 0.65 C-mol C-mol^{-1} in the feed medium), and $X(t_B)$ and $V(t_B)$ are the dry cell weight and the culture volume at the end of the

batch phase respectively. In equation 7, time zero corresponds to the time when the exponential feed is begun.

The culture was stopped about 48 h after induction, which corresponds to approximately 63 h total culture time.

3. 6. Substrate and metabolite analysis

Culture samples (about 10 mL) were collected using a purpose built auto-sampler (Cannizzaro (2002)) and kept at 2°C for up to 12h before handling.

Glycerol and methanol as well as dry cell weight concentrations were determined as described previously (Jungo et al. (2007)).

The oxygen uptake rate and carbon dioxide evolution rate in the bioreactor off-gas were determined using paramagnetic (series 2210, Servomex, Crowborough, UK) and infrared (series 2500, Servomex, Crowborough, UK) analysers, respectively. The measured values of oxygen and carbon dioxide were corrected for water vapour according to Duboc and von Stockar (1998).

3. 7. Preparation of cell-free extracts and alcohol oxidase activity measurement

The procedures for the preparation of cell-free extracts and the measurement of specific alcohol oxidase activity were carried out as described previously (Jungo et al. (2006)).

The specific activity of alcohol oxidase was expressed as U mg^{-1} total proteins. One unit (U) of AOX corresponds to the oxidation of 1.0 µmole of methanol to formaldehyde per min at pH 7.5 at 25°C.

3. 8. Recombinant avidin quantification

Recombinant avidin was quantified by biotin-4-fluorescein titration of binding sites (Kada et al. (1999)), assuming a tetrameric form of recombinant avidin, with four active-binding sites (Zocchi et al. (2003)).

Since the detection limit of this method is about 10 mg L^{-1} of avidin, it was necessary to concentrate certain samples by up to 15-fold by ultrafiltration (Centriplus, 30 kDa, Millipore Corporation, Bedford, USA) prior to titration with biotin-4-fluorescein.

At high cell densities (above 50 g L^{-1} dry cell weight) the total cell volume was not negligible compared with the culture volume, however, the amount of recombinant avidin was calculated using the volume of the culture supernatant (equation 8).

$$V_{Sup} = V - a \cdot V \cdot X \quad (8)$$

where V and V_{Sup} are the culture volume and the volume of the culture supernatant, X the cell dry weight (g L^{-1}) and «a» is the cell volume per dry cell (0.0032 L g dry-$cell^{-1}$) under the studied experimental conditions.
The parameter «a» was determined at 115 g L^{-1} cell dry weight by measuring the volume of supernatant for a 10 mL culture sample.

3. 9. Check of data consistency and reconciliation of yield coefficients

On a C-molar basis, the black box stoichiometry was described by the following equation for mixed substrate growth on glycerol and methanol, at a methanol fraction of 0.5 C-mol $C\text{-}mol^{-1}$:

$$0.5\ CH_{8/3}O + 0.5\ CH_4O + Y_{O2/S}\ O_2 + Y_{NH3/S}\ NH_3 \rightarrow Y_{X/S}\ CH_{1.706}O_{0.459}N_{0.168} + Y_{CO2/S}\ CO_2;\ \Delta_r H_S^{*\circ} \quad (9)$$

$Y_{i/S}$ are the stoichiometric coefficients in mole or C-mole per C-mole of substrate, where i can be S (total amount of carbon source (glycerol and methanol)), O_2, CO_2, NH_3 or X (biomass). $\Delta_r H_S^{*\circ}$ is the standard molar enthalpy change caused by the reaction described in equation 9, with CO_2, H_2O and NH_3 as reference state.
The C-molar composition of biomass was determined by elemental analysis as described in Gurakan et al. (1990). Cultures samples were collected at steady state during the chemostat culture on 10 g L^{-1} glycerol and 10.43 g L^{-1} of methanol. Samples were centrifuged and washed three times with deionized water. After the final washing/ centrifugation step, the cell pellets were spread as a thin layer of about 2 mm on Petri dishes and stored at -20 °C. The frozen cell pellets were freeze-dried for 24 hours. Analysis of carbon, nitrogen and hydrogen content was made using a CHN analyzer (Carlo Erba Instruments, Rodano, Italy). Oxygen content was calculated by difference (Gurakan et al. (1990)). It was assumed that the elemental composition of biomass did not change significantly during the performance of the nutrient gradient.

The yield of substance j on substance i was calculated as

$$Y_{j/i} = \frac{R_j}{R_i} \quad (C-mol/C-mol) \tag{10}$$

where R_i and R_j are the volumetric production or consumption rates of substance i and j respectively.

The heat yield $Y_{Q/S}$ represents the molar enthalpy change caused by the reaction described in equation 9 per C-mole of substrate and is defined by equation 11.

$$Y_{Q/S} = \frac{R_Q}{R_S} \quad (kJ/C-mol) \tag{11}$$

During continuous cultivation, measurements of dry cell weight, carbon source concentrations, carbon dioxide and oxygen in the exhaust gas and heat production rate were used in order to calculate four of the five yields of the black box stoichiometry, namely biomass, carbon dioxide, oxygen and heat yields.

For the fed-batch culture, the monitoring of oxygen in the exhaust gas was not used in calculations because of interference by the high amounts of oxygen provided to the culture in order to avoid oxygen limitations.

Together with the constraints provided by the carbon, nitrogen, degree of reduction and enthalpy balances a redundancy of 3 and 2 resulted for the continuous and the fed-batch cultures respectively. The redundancy of a system can be calculated from:

$$L = -(N - J - M) \tag{12}$$

where N is the number of total yields in the black box stoichiometric equation, J the number of constraints (4 balances) and M the number of measured yields.

The redundancy of the system was used in order to estimate the unknown yields and to reconcile the data.

The yields were checked for consistency and reconciled based on a χ^2-test as proposed by Wang and Stephanopoulos (1983) and Stephanopoulos et al. (1998). The obtained reconciled yield coefficients are the best yield estimates that minimize the magnitude of the residuals ε and are determined by minimizing the sum of squared er-

rors scaled according to their variance. The test value h is given by the sum of the weighted squares of the residuals ε:

$$h = \varepsilon \cdot P^{-1} \cdot \varepsilon \quad (13)$$

where P is the variance-covariance matrix. Assuming measurement errors of 5%, a test value h lower than 5.99 for a redundancy of 2 (fed-batch culture) and lower than 7.81 for a redundancy of 3 (continuous culture) permitted to assume a 95% significance level that no gross errors and no metabolites other than the ones appearing in equation 9 had affected the balances.

4. RESULTS AND DISCUSSION

4. 1. Mixed substrate growth of *P. pastoris* in carbon-limited chemostat cultures

The studied *P. pastoris* strain was grown in chemostat culture at a dilution rate of 0.06 h^{-1} with 10 g L^{-1} glycerol and 10.43 g L^{-1} methanol as carbon sources in the feed medium. The dilution rate was chosen to be less than the maximal specific growth rate on glycerol and on methanol as sole carbon sources (0.24 and 0.14 h^{-1} respectively).

Albeit glycerol and methanol are known to be consumed sequentially in batch cultures (Inan and Meagher (2001)), under carbon-limited growth conditions the two substrates were consumed simultaneously and completely, as described previously by D'Anjou and Daugulis (2001) for a recombinant *P. pastoris* Mut^S strain during mixed substrate chemostat growth on glycerol and methanol. The residual substrate concentrations were less than the detection limit of the HPLC method used for their quantification (0.01 g L^{-1} for glycerol and 0.05 g L^{-1} for methanol). Moreover, no fermentation by-products, such as ethanol or acetate, were detected.

At steady state, the reconciled yield coefficients were $Y_{X/S}$ = 0.56 C-mol C-mol^{-1}, $Y_{CO2/S}$ = 0.48 mol C-mol^{-1}, $Y_{O2/S}$ = 0.78 mol C-mol^{-1}, $Y_{NH3/S}$ = 0.09 mol C-mol^{-1} and $Y_{Q/S}$ = -383.8 kJ C-mol^{-1}. The test value h was 1.39 which is lower than the threshold value of 7.81 for a redundancy of 3 and a confidence level of 95% and therefore validates the proposed black box stoichiometry presented in equation 9.

Although glycerol was present in the feed medium, methanol was consumed and recombinant avidin was expressed. The volumetric and specific productivities for recom-

binant avidin were 0.121 mg L^{-1} h^{-1} and 0.012 mg g^{-1} h^{-1} respectively, which is lower than the values found with methanol as sole carbon source under the same growth conditions. In chemostat cultures on methanol, the specific productivity was 0.022 mg g^{-1} h^{-1} at a dilution rate of 0.06 h^{-1} (Jungo et al. (2006)).

The average specific AOX activity of cell free extracts was 1.3 ± 0.1 U mg^{-1} total proteins at steady state, which is 1.6-fold higher than the specific AOX activities in chemostat cultures on methanol at the same dilution rate (Jungo et al. (2006)).

Depending on the glucose to methanol ratio and on the dilution rate, a two- to threefold increase in productivity of alcohol oxidase enzyme was also found in mixed substrate cultures with the methylotrophic yeast *H. polymorpha* (Egli et al. (1986)). The same authors pointed out that the residual concentration of methanol was always lower during mixed substrate growth than during growth with methanol as sole carbon source, which is in all probability the reason why specific AOX activities were higher for *P. pastoris* cells growing on methanol and glycerol. One explanation is that if methanol oxidation is considered as the growth rate-limiting reaction (Brinkmann et al. (1990)), the cells will maintain a high rate of substrate oxidation by increasing the amount of the rate limiting enzyme, when the residual methanol concentration decreases. Similarly, it has been shown that specific AOX activities increase when dilution rate is decreased in chemostat cultures on methanol as sole carbon source (Dijken et al. (1976); Jungo et al. (2006)).

4. 2. Mixed substrate continuous culture with linear increase of methanol fraction in the feed

In order to determine the influence of the methanol fraction in the feed medium on the specific recombinant avidin productivity, a transient continuous culture with a linearly increasing methanol fraction in the feed medium was realised. Since specific recombinant avidin productivity was already lower with a methanol fraction of 0.5 C-mol C-mol^{-1} in the feed medium than with methanol as sole carbon source the gradient was performed between 0.5 and 1.0 C-mol C-mol^{-1} of methanol in the feed medium and not at lower methanol fractions.

Figure 3 shows the profiles of dry cell weight concentration, carbon dioxide production rate, oxygen uptake rate and heat production rate as a function of time during the linear nutrient gradient, which was started at time zero. With an increasing methanol fraction

in the feed medium, oxygen uptake rate, carbon dioxide production rate and heat production rate increased, which corresponded to our expectations, since these rates are known to be higher in cultures on methanol than on glycerol. Dry cell weight decreased with increasing methanol fraction in the feed medium because biomass yields are lower on methanol compared with glycerol.

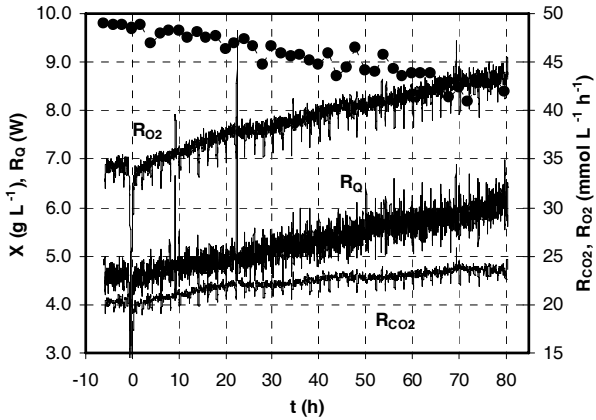

Figure 3: Cell dry weight (X, ●), heat production rate (R_Q), carbon dioxide production rate (R_{CO2}) and oxygen uptake rate (R_{O2}) as a function of time during transient continuous culture.

The reconciled yield coefficients are shown in figure 4 according to the methanol fraction in the feed medium. The corresponding test values h calculated with reconciliation procedures for all the analyzed samples were always lower than the threshold value of 7.81, which signifies that the measurements and the proposed black box stoichiometry (equation 9) are reliable considering a confidence level of 95%.

In figure 4 the reconciled yield coefficients for a chemostat culture on methanol as sole carbon source at the same dilution rate are also represented (Jungo et al. (2006)). It

can be seen that at the end of the transient, the yield coefficients approached the ones found at steady state on methanol.

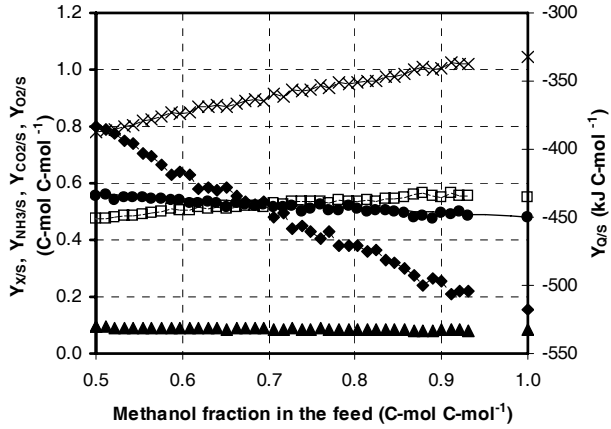

Figure 4: Reconciled biomass ($Y_{X/S}$, ●), ammonia ($Y_{NH3/S}$, ▲), carbon dioxide ($Y_{CO2/S}$, □), oxygen ($Y_{O2/S}$, x) and heat ($Y_{Q/S}$, ◆) yield coefficients as a function of the methanol fraction in the feed medium. Theoretical biomass yield coefficients (straight line) were calculated as the sum of the individual biomass yield coefficients on each substrate multiplied by the C-molar fraction of each substrate in the feed medium. At a C-molar fraction of 1.0 of methanol, the results of a chemostat culture on methanol as sole carbon source were also represented (Jungo et al., 2006).

The biomass yield coefficients were found to be about the same as the sum of the individual biomass yield coefficients theoretically possible for each substrate (given in Jungo et al. (2006)) multiplied by the C-molar fraction of each substrate in the feed. Indeed, in figure 4, the theoretical biomass yield coefficients calculated in this way (straight line) were very close to the experimentally determined yield coefficients. Therefore, in all probability, the individual yield coefficients on glycerol and methanol did not change significantly during mixed substrate growth. However, the contribution of each substrate to growth can only be stricktly determined by the use of labelled substrates, which was carried out by Egli et al. (1982) for mixed substrate growth of *H. polymorpha* and *Kloeckera sp.* 2201 on glucose and methanol. These authors showed that the individual growth yields for glucose and methanol were the same during mixed substrate conditions as during growth with glucose and methanol as sole carbon sources.

No «auxilliary substrate» effect could be observed for either the two methylotrophic yeasts studied by Egli et al. (1982), or for the *P. pastoris* strain studied here. The «auxilliary substrate» effect has been described in detail by Babel et al. (1993) and can be defined as the increase in growth yield due to the simultaneous utilization of physiologically similar substrates. As a result, growth yields can actually increase on mixed substrates due to a selective use, i.e. a more effective use, of substrates. Moreover, the possibility to achieve increased specific growth rates (higher than the maximal specific growth rate for the substrate on which cells are gowing at a lower specific growth rate) is rather the rule than the exception in mixed substrate utilization. This property was not examined as part of the present study, although from the results of Egli et al. (1986), *P. pastoris* cells would be expected to grow on glycerol-methanol mixtures at higher dilution rates than the maximal specific growth rate on methanol (0.14 h^{-1}). This property might actually be used to increase recombinant protein productivity, since it has been previously shown that recombinant avidin productivity increased with dilution rate in cultures on methanol as sole carbon source (Jungo et al. (2006)).

The volumetric and specific recombinant avidin production rates are shown in figure 5 according to the methanol fraction in the feed medium. The values at methanol fractions of 0.5 and 1.0 C-mol C-mol^{-1} are average values at steady state and the others were measured during the transient nutrient gradient. The chemostat culture on methanol as sole carbon source was performed three times and the mixed substrate chemostat culture on 50% C-mol C-mol^{-1} methanol was performed twice (once immediately prior to the transient nutrient gradient). Taking into account the errors on the determination of avidin production rates (vertical error bars), the results show that the specific avidin production rate remained constant for methanol fractions ranging from 0.6 to 1.0 C-mol C-mol^{-1} methanol in the feed medium. However, the volumetric avidin production rate increased in this range due to the higher biomass yields during mixed substrate growth. For instance, with a methanol fraction in the feed medium of 0.62 C-mol C-mol^{-1}, the volumetric avidin production rate was 1.1-fold higher than with methanol as sole carbon source. According to these results, it is preferable to work with a feed solution consisting of 60% C-mol C-mol^{-1} methanol and 40% C-mol C-mol^{-1} glycerol than with pure methanol. In this way the specific avidin production rate will not be affected, the volumetric avidin production rate will be increased by about 10%, and both

heat production and oxygen consumption rates will be lowered (figure 3), features which are very interesting for high cell density cultures at large scale.

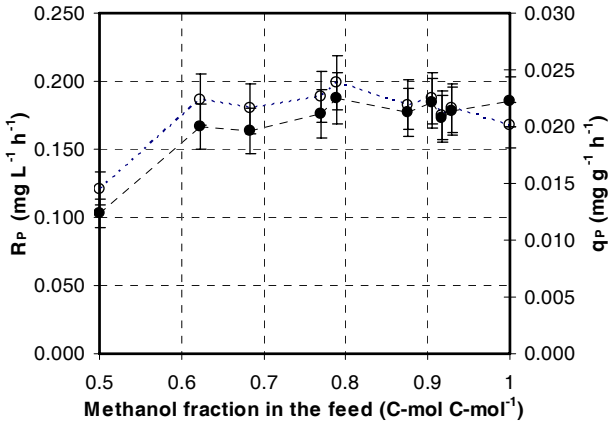

Figure 5: Volumetric (R_P, ○) and specific (q_P, ●) recombinant avidin production rates as a function of the methanol fraction in the feed medium.

Specific AOX activities of cell-free extracts of samples collected during the transient nutrient gradient were also determined and are presented in figure 6. The results for two chemostat mixed substrate cultures on 10 g L^{-1} glycerol and 10.43 g L^{-1} methanol and during two chemostat cultures on 20 g L^{-1} of methanol are also shown in this figure.

In the studied range of methanol fractions, an increase of methanol in the feed medium led to a decrease of the specific AOX activity in the cells. The specific AOX activity decreased from 1.3 to 0.8 U mg^{-1} total protein when the fraction of methanol in the feed medium was increased from 0.5 to 0.65 C-mol C-mol^{-1}. Above this value, the specific AOX activity was constant and the same as in chemostat cultures on methanol as sole carbon source.

For methanol fractions in the feed medium lower than 0.6 C-mol C-mol^{-1}, specific AOX activity was higher than with cultures on methanol as sole carbon source but a decay in recombinant avidin productivity was observed. As pointed out in a previous study with the same recombinant strain (Jungo et al. (2006)) it seems that once recombinant protein production is induced there is no proportional correlation between the specific AOX activity and the specific recombinant avidin production rate, although the recom-

binant protein is expressed under the control of the AOX promoter. However, previous results with this strain (Jungo et al. (2006)) showed that specific recombinant avidin production rate was correlated with metabolic fluxes of central metabolism. Indeed, specific recombinant avidin productivity increased linearly with dilution rate and therefore also with specific methanol consumption rate. The lower specific recombinant avidin production rates at methanol fractions below 0.6 C-mol C-mol^{-1} could be correlated with lower specific methanol consumption rate.

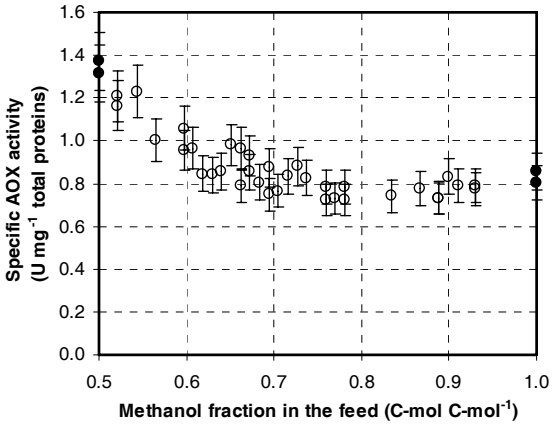

Figure 6: Specific AOX activities as a function of the methanol fraction in the feed medium during transient continuous culture (❍) and comparison with specific AOX activities for chemostat cultures at steady state (●).

4. 3. Mixed substrate high cell density fed-batch culture

Since production of recombinant proteins with *P. pastoris* strains is usually performed with high cell density fed-batch cultures (Stratton et al. (1998)), an experiment was carried out to verify whether the results obtained with the transient nutrient gradient in low cell density continuous cultivation were valid at high cell densities in mixed substrate fed-batch culture. According to the results from the transient nutrient culture, mixed substrate cultures should be performed on a mixture containing more than 60% C-mol C-mol^{-1} methanol in the feed medium. Hence a high cell density fed-batch culture was performed with 65% C-mol C-mol^{-1} of methanol in the feed medium, with an exponential feed at a constant specific growth rate of 0.06 h^{-1}.

The dry cell weight, recombinant avidin concentration and heat production rate are plotted according to time in figure 7A. The residual glycerol and methanol concentrations and the carbon dioxide production rate are shown in figure 7B.

The biomass concentration, the power generated by the culture and the carbon dioxide production rate increased exponentially according to the exponential feed at a specific growth rate of 0.06 h^{-1}, which was begun 15.5 h after inoculation. The power generated by the culture increased exponentially only 2.5 h after the beginning of the fed-batch phase on methanol and glycerol and gives a relatively accurate estimation of the time the cells need to adapt to the new environmental conditions (figure 7A). During this adaptation phase, the increase and the slight overshoot of the heat signal was due to methanol accumulation. Indeed about half an hour after the beginning of the exponential feed the residual methanol concentration was 0.5 g L^{-1}. A similar effect can be observed with the carbon dioxide production rate (figure 7B). Residual glycerol and methanol concentrations remained below the detection limit of the HPLC method during the rest of the fed-batch phase as was previously observed for the continuous culture. Indeed, under carbon-limited growth conditions methanol and glycerol are consumed simultaneously and completely.

Avidin concentration increased to 110 mg per liter of supernatant and the specific avidin production rate was 0.022 mg g^{-1} h^{-1} during the exponential fed-batch phase. The measured specific avidin production rate was therefore in good agreement with the values found during the transient continuous culture (figure 5).

During the exponential growth phase on glycerol and methanol, the reconciled yield coefficients were $Y_{X/S}$ = 0.44 C-mol C-mol^{-1}, $Y_{CO2/S}$ = 0.55 mol C-mol^{-1}, $Y_{O2/S}$ = 0.89 mol C-mol^{-1}, $Y_{NH3/S}$ = 0.07 mol C-mol^{-1} and $Y_{Q/S}$= -438.6 kJ C-mol^{-1}, which is very close to yield coefficients obtained during the nutrient gradient at a methanol fraction of 0.65 C-mol C-mol^{-1}, except for the biomass yield, which was slightly lower during the high cell density fed-batch culture. The statistical test value h was 5.67 which is below the threshold value of 5.99 for a redundancy of 2 and a confidence level of 95%, which sig-

nifies that the measurements and the proposed black box stoichiometry (equation 9) are reliable.

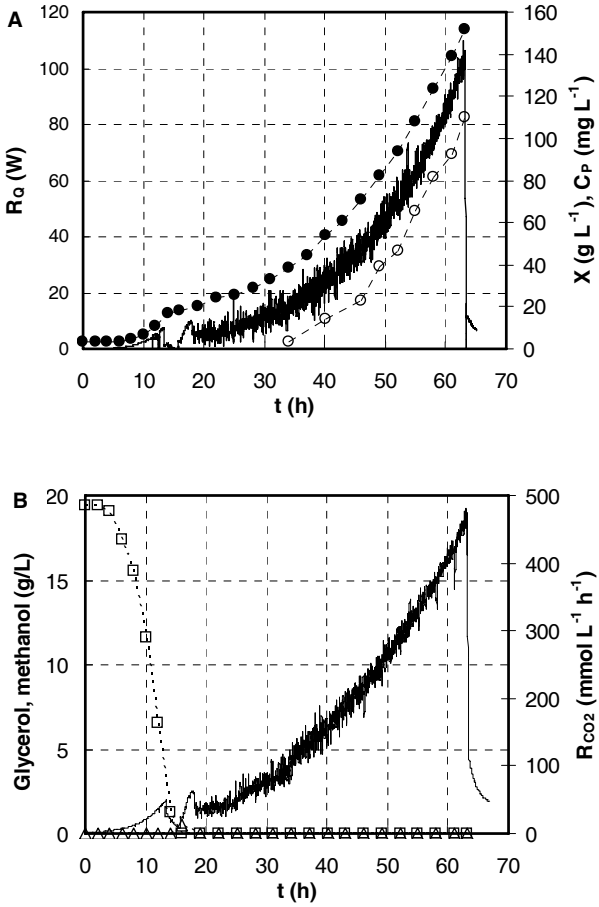

Figure 7: High cell density fed-batch culture. Figure A: Heat production rate (R_Q, continuous line), cell dry weight (X, ●) and avidin concentrations in supernatant (C_P, ○) as a function of time; Figure B: Glycerol (□) and methanol (△) concentrations, carbon dioxide production rate, (R_{CO2}, continuous line) as a function of time.

5. CONCLUSIONS

In carbon-limited continuous and fed-batch cultures, glycerol and methanol were consumed simultaneously and completely, which allowed the production of recombinant avidin with a *P. pastoris* Mut^+ strain in the presence of glycerol. Low residual concentrations of glycerol did not repress the synthesis of alcohol oxidase. Specific AOX activities were even 1.6-fold higher in mixed substrate cultures with a methanol fraction of 0.5 C-mol C-mol^{-1} than in cultures on methanol as sole carbon source.

No proportional correlation between the specific AOX activity and the specific avidin production rate could be established in mixed substrate cultures for the studied range of methanol fractions, albeit the promoter of the AOX enzyme was used in this strain for the expression of recombinant avidin. Instead, the specific recombinant avidin production rate seemed to be a function of the specific methanol consumption rate. However, the specific recombinant avidin production rate did not increase above methanol fractions of 0.6 C-mol C-mol^{-1}, but was the same as with cultures on methanol as sole carbon source.

Since reduction of the methanol fraction in the feed medium from 100% to 60% C-mol C-mol^{-1} allowed to produce recombinant avidin at the same specific productivity and a slightly higher volumetric productivity (1.1-fold higher for a methanol fraction of 0.62 C-mol C-mol^{-1}), and with significant lowering of the heat production rate and oxygen consumption rate, use of mixed feeds of glycerol and methanol during the induction phase presents interesting advantages over standard high cell density fed-batch cultures performed with methanol as sole carbon source during the induction phase. With a mixture of 60 % C-mol C-mol^{-1} methanol and 40 % C-mol C-mol^{-1} glycerol and at a specific growth rate of 0.06 h^{-1}, oxygen consumption rate and heat production rate will be diminished by about 28% for a fixed dry cell weight concentration. Mixed substrate feeds of glycerol and methanol during the induction phase therefore present major advantages for the performance of high cell density fed-batch cultures with recombinant *P. pastoris* strains given the fact that technical adaptations such as use of pure oxygen, high performance stirrers and special cooling facilities will still be necessary in order to control reactor cooling and aeration of the culture properly.

6. ACKNOWLEDGEMENTS

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7. NOMENCLATURE

AOX	Alcohol oxidase	
a	Cell volume per dry cell	L g dry-cell^{-1}
C_P	Concentration of avidin	mg L^{-1}
C_S	Substrate concentration in bioreactor	C-mol L^{-1}
C_S^M	Substrate concentration in the mixing flask	C-mol L^{-1}
$C_{S(0)}^M$	Initial substrate concentration in the mixing flask	C-mol L^{-1}
C_S^R	Substrate concentration in the reservoir flask	C-mol L^{-1}
D	Dilution rate	h^{-1}
F	Feeding rate of medium during fed-batch phase	g h^{-1}
F_1	Medium flow from the reservoir flask	L h^{-1}
F_2	Medium flow from the mixing flask	L h^{-1}
h	Statistical test value	
$\Delta_r H_S^{*\circ}$	Standard molar enthalpy change caused by reaction r per mole of subtrate S	kJ C-mol^{-1}
J	Number of constraints	
K_S	Monod substrate saturation constant	C-mol L^{-1}
L	Redundancy	
M	Number of measured yields	
N	Number of total yields in black box stoichiometric equation	
m	Maintenance coefficient	C-mol C-mol^{-1}h^{-1}
P	Variance-covariance matrix	
q_P	Biomass-specific avidin productivity	mg g^{-1} h^{-1}
q_S	Biomass-specific substrate consumption rate	C-mol C-mol^{-1}h^{-1}
q_S^{max}	Maximum biomass-specific substrate consumption rate	C-mol C-mol^{-1}h^{-1}
R_{CO2}	Carbon dioxide production rate	mol L^{-1} h^{-1}

R_{O2}	Oxygen consumption rate	mol L^{-1} h^{-1}
R_Q	Heat production rate	W
R_P	Volumetric avidin productivity	mg L^{-1} h^{-1}
t_B	Time at end of batch phase	h
V	Culture volume	L
V_{Sup}	Volume of culture supernatant	L
V_0	Initial volume in reservoir and mixing flasks	L
X	Cell dry weight	C-mol L^{-1}
$Y_{j/i}$	Yield coefficient of substance j on substance i	C-mol C-mol^{-1}
$Y_{j/i}^{true}$	True yield coefficient	C-mol C-mol^{-1}
α	Rate of nutrient gradient	C-mol L^{-1} h^{-1}
ε	residuals	
μ	Specific growth rate	h^{-1}

Subscripts

B	End of batch phase
i	Refers to compound i
j	Refers to compound j
S	Refers to the limiting nutrient
sup	Refers to the culture supernatant
P	Refers to the produced recombinant avidin
Q	Refers to heat
X	Refers to biomass
0	Initial condition

Superscripts

M	Refers to mixing flask
R	Refers to reservoir flask

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CHAPTER 7

A QUANTITATIVE ANALYSIS OF THE BENEFITS OF MIXED FEEDS OF SORBITOL AND METHANOL FOR THE PRODUCTION OF RECOMBINANT AVIDIN WITH *PICHIA PASTORIS*

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1. ABSTRACT

The advantages of mixed feeds of sorbitol and methanol for the production of recombinant proteins with *Pichia pastoris* were analysed quantitatively. The influence of the methanol-sorbitol ratio in the feed medium was investigated by performing transient nutrient gradients in continuous cultures at constant dilution rates of 0.03 and 0.06 h^{-1}, below and above the maximal specific growth rate on sorbitol. The analysis of the influence of the methanol-sorbitol ratio on growth stoichiometry, specific alcohol oxidase activity and recombinant protein productivity was performed with a *P. pastoris* Mut^+ strain secreting avidin.

Results showed that mixed feeds of sorbitol and methanol instead of methanol as sole carbon source can improve the productivity in recombinant avidin due to increased biomass yields during mixed substrate growth. At a dilution rate of 0.03 h^{-1}, the highest volumetric avidin productivity was achieved at a methanol fraction of 43% C-mol C-mol^{-1} in the feed medium: the volumetric avidin productivity was 1.3-fold higher than during chemostat culture on methanol. The heat production and the oxygen consumption rates were reduced by about 38% for a given dry cell weight concentration at this methanol fraction, features which are very useful for high cell density cultures. Moreover, results showed that sorbitol is a «non-repressing» carbon source with respect to alcohol oxidase expression. As a matter of fact, at a dilution rate of 0.06 h^{-1}, sorbitol accumu-

lation in the culture medium was observed for methanol fractions lower than 90 % C-mol C-mol^{-1} but did not affect specific avidin productivity.

Keywords: *Pichia pastoris*; Avidin; Mixed substrate; Sorbitol; High cell density.

2. INTRODUCTION

The methylotrophic yeast *P. pastoris* is an attractive host for the expression of recombinant proteins. Advantages with this expression system include achievement of high cell densities (up to 160 g L^{-1} dry cell weight), the possibility to secrete recombinant proteins, the availability of the expression system as a commercial kit (Invitrogen Corporation) and expression linked to the strong, tightly regulated alcohol oxidase promoter AOX1. Numerous examples of recombinant proteins expressed with *P. pastoris* strains can be found in the literature (Cregg et al. (1993); Hollenberg and Gellissen (1997); Cereghino and Cregg (2000); Gellissen (2000); Cereghino et al. (2002); Gerngross (2004); Daly and Hearn (2005); Macauley-Patrick et al. (2005)).

Recombinant *P. pastoris* cells are usually first grown in batch culture on glycerol to achieve high cell densities rapidly while repressing foreign gene expression. After a glycerol-limited fed-batch phase to further increase cell density and derepress methanol dissimilating enzymes, recombinant protein expression is usually induced by feeding methanol as sole carbon source.

Several authors pointed out that use of mixed feeds of glycerol and methanol during the induction phase could increase the volumetric recombinant protein productivitiy as a result of higher cell densities and feeding rates possible with growth on glycerol (Cregg et al. (1993); Loewen et al. (1997); McGrew et al. (1997); Katakura et al. (1998); Zhang et al. (2003)).

Recently, some authors pointed out that carbon sources other than glycerol might further improve the fed-batch strategy with recombinant *P. pastoris* strains. For instance, sorbitol is an interesting additional carbon source to methanol for the production of recombinant proteins with *P. pastoris* strains. According to the literature (Sreekrishna et al. (1997); Thorpe et al. (1999); Boze et al. (2001); Inan and Meagher (2001); Xie et al. (2005)), sorbitol is a «non-repressing» carbon source with respect to the AOX1 promoter, usually used for the expression of recombinant proteins in *P. pastoris*. Thorpe et al. (1999) pointed out that by contrast with glycerol, one advantage with mixed feeds of sorbitol and methanol is that sorbitol accumulation during the induction phase does not

affect the expression level of recombinant protein. Hence, control of residual sorbitol concentration during the induction phase is less critical than with mixed feeds of glycerol and methanol.

Use of mixed feeds of sorbitol and methanol present also other advantages, including the lower heat production rate and the lower oxygen consumption rate for growth on sorbitol than for growth on glycerol or methanol. Indeed, since the enthalpy of combustion of sorbitol is about 8% lower than that of glycerol and about 30% lower than that of methanol, for a given growth rate less heat will be released in cultures with mixed feeds of sorbitol and methanol than with mixed feeds of glycerol and methanol or with feeds of methanol alone. The enthalpies of combustion of methanol, glycerol and mannitol are -727 kJ C-mol^{-1} (Weast (1980)), -549.5 kJ C-mol^{-1}(von Stockar et al. (1993)) and -507.8 kJ C-mol^{-1} (Weast (1980)), respectively. The enthalpy of combustion of dissolved sorbitol was not found in the literature but can be assumed to be very close to the enthalpy of combustion for mannitol, which is an isomer of sorbitol. Since the degree of reduction of sorbitol is lower than those of glycerol and methanol, less oxygen will be consumed during mixed substrate growth on sorbitol and methanol than on mixed feeds of glycerol and methanol or on methanol as sole carbon source.

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 scale. Indeed, oxygen transfer and reactor cooling frequently represent the major technical limitations for high cell density operations with *P. pastoris* strains (Hensing et al. (1995); Schilling et al. (2001); Jenzsch et al. (2004)). Therefore, any method which reduces the oxygen consumption rate and the heat production rate without affecting recombinant protein productivity is welcomed.

To our knowledge, no detailed quantitative study on the influence of methanol-sorbitol ratio in the feed medium on recombinant protein productivity in *P. pastoris* cultures has been reported. Studies have been performed in batch or fed-batch cultures with changing ratios of substrates, changing feed rates and changing specific growth rates (Thorpe et al. (1999); Boze et al. (2001); Inan and Meagher (2001); Xie et al. (2005)).

In this study, we investigated the influence of the methanol-sorbitol ratio in the feed medium on the the growth stoichiometry, the specific activity of alcohol oxidase enzyme and the productivity of a recombinant glycosylated avidin secreted by a *P. pastoris* Mut^{+} strain (Zocchi et al. (2003)).

The influence of methanol-sorbitol ratio in the feed medium was investigated by performing cultures in continuous mode since this allows for good control of cultivation conditions and hence of cell physiology. However, instead of performing several chemostat cultures at various ratios of methanol and sorbitol, transient continuous cultures on methanol and/or sorbitol as carbon sources, with linearly increasing or decreasing methanol fractions in the feed medium, were performed at constant dilution rate. This technique has been applied and validated previously (Jungo et al. (2007a)) with mixtures of glycerol and methanol as carbon sources.

More precisely, two nutrient gradients with linearly changing methanol-sorbitol ratios in the feed medium were performed, one at 0.03 h^{-1}, a value slightly below the maximum specific growth rate on sorbitol, and one at 0.06 h^{-1}, which is above the maximum specific growth rate on sorbitol, in order to further analyse the influence of high residual sorbitol concentrations on recombinant protein productivity.

3. MATERIALS AND METHODS

3. 1. Microorganism, inoculum preparation and media

A recombinant *Pichia pastoris* Mut^+ strain was kindly provided by Andrea Zocchi from the University of Neuchâtel (Switzerland). Zocchi et al. (2003) designed a clone of *P. pastoris* expressing and secreting a recombinant glycosylated avidin (recGAvi) with an acidic isoelectric point (pI = 5.4). Precultures were prepared in complex medium on 20 g L^{-1} glycerol at 30°C for 24h as described previously (Jungo et al. (2006)).

The defined medium used for continuous cultures was developed by Egli and Fiechter (1981) and was modified as described previously (Jungo et al. (2007b)) for optimal expression of recombinant avidin. It contained per liter: 15.26 g NH_4Cl, 5.62 g KH_2PO_4, 1.18 g $MgSO_4$ $7H_2O$, 0.9 g EDTA $2H_2O$, 110 mg $CaCl_2$ $2H_2O$, 75 mg $FeCl_3$ $6H_2O$, 28 mg $MnSO_4$ H_2O, 44 mg $ZnSO_4$ $7H_2O$, 8 mg $CuSO_4$ $5H_2O$, 8 mg $CoCl_2$ $6H_2O$, 5.2 mg Na_2MoO_4 $2H_2O$, 8 mg H_3BO_3, 1.2 mg KI, 20 μg biotin, and 0.4 mL of antifoam agent (Struktol SB 2121, Schill and Seilacher, Hamburg, Germany). Methanol and/or sorbitol were used as carbon sources with the total amount of carbon source constant (0.652 C-mol L^{-1}).

During transient nutrient gradients, the concentrations of carbon sources (methanol and sorbitol) in the feed medium were varied by use of a special feeding system de-

scribed previously (Jungo et al. (2007a)), involving two flasks, called reservoir flask and mixing flask. All other nutrients were present at the same concentrations in these two flasks used to perform the transient nutrient gradient. The medium in the reservoir flask was flowing into the mixing flask in order to obtain a linearly increasing or decreasing methanol fraction in the mixing flask. The medium of the mixing flask was fed to the bioreactor. Detailed explanations are given in Jungo et al. (2007a).

All components of the defined media were obtained from Fluka, Buchs, Switzerland. The media were sterilized by filtration (0.22 μm, Steritop, Millipore Corporation, Billerica, USA).

3. 2. Culture conditions

Growth experiments were undertaken using a bench-scale heat-flux calorimeter RC1 (Mettler Toledo AG, Greifensee, CH) modified for biotechnological applications with a working volume of 1.6 L. The design and operation of this so called Bio-RC1 can be found in Marison et al. (1998a), Marison et al. (1998b). The culture volume was 1.050 L in continuous mode.

Temperature was maintained at 30°C and pH at 5.0 by the automatic addition of 2M KOH. The heat of neutralization was calculated from the consumption of KOH 2M according to Meier-Schneiders et al. (1995). A polarographic pO_2 probe (In Pro 6800, Mettler Toledo, Greifensee, Switzerland) was used to monitor dissolved oxygen, with respect to air-saturated medium. The reactor was aerated with air at 2 L min^{-1} using thermal mass flow controllers (5850E, Rosemount Brooks, Veenendaal, The Netherlands). The air was saturated with water before its entry in the calorimeter. Stirring rate was fixed at 800 rpm.

All liquid inlet flows were quantified gravimetrically and used to control continuously and on-line the feed pump rate. The temperature of all liquid feeds was adjusted to the reactor temperature immediately prior to entry in the bioreactor.

As the release of CO_2 from the medium is an endothermic process, the heat flow induced by CO_2 stripping was taken into account according to Meier-Schneiders et al. (1995). The heat losses to the environment were calculated using a heat transfer coefficient for the bioreactor of 0.133 W K^{-1}, which was determined as described in Birou (1986).

3. 3. Transient nutrient gradients in continuous cultures

Two nutrient gradients were performed with the same set up as described previously (Jungo et al. (2007a)), one beginning with chemostat growth on sorbtiol at a dilution rate of 0.03 h^{-1} and with a linearly increasing methanol fraction in the feed medium, the other beginning with chemostat growth on methanol at a dilution rate of 0.06 h^{-1} and with a linearly decreasing methanol fraction in the feed medium.

Linear increase of methanol fraction in the feed medium at a dilution rate of 0.03 h^{-1}

Cells were first grown in batch culture on 20 g L^{-1} glycerol. As soon as all the glycerol had been consumed (indicated by a sudden decrease of the heat signal), the supply of the feed-medium containing 0.652 C-mol L^{-1} (19.80 g L^{-1}) sorbitol as carbon source was initiated and the dilution rate set to 0.03 h^{-1}.

The transient nutrient gradient in order to increase linearly the methanol fraction in the feed medium was begun after five liquid residence times.

The time course of the concentration of substrates (methanol and sorbitol) in the mixing flask and entering the bioreactor has been shown (Jungo et al. (2007a)) to follow equation (1).

$$C_S{}^M = C_{S(0)}{}^M + (C_S{}^R - C_{S(0)}{}^M) \cdot \frac{F_1}{V_0} \cdot t \qquad (1)$$

where $C_S{}^R$ and $C_S{}^M$ are the substrate (methanol or sorbitol) concentrations in the reservoir and mixing flasks respectively, $C_{S(0)}{}^M$ the initial substrate concentrations in the mixing flask, V_0 the volume of the mixing and reservoir flasks at time zero, and F_1 is the constant medium flow in L h^{-1} from the mixing flask.

Initially, the mixing and reservoir flasks contained 1.5 L of medium each, the mixing flask contained 0.652 C-mol L^{-1} (19.80 g L^{-1}) sorbitol and the reservoir flask 0.652 C-mol L^{-1} (20.87 g L^{-1}) methanol as carbon source.

The methanol concentration in the mixing flask changed at a rate of α = 0.006 C-mol methanol L^{-1} h^{-1}, which can be defined by equation (2), according to Jungo et al. (2007a).

$$\alpha = (C_S{}^R - C_{S(0)}{}^M) \cdot \frac{F_1}{V_0} \qquad (2)$$

Linear decrease of methanol fraction in the feed medium at a dilution rate of 0.06 h^{-1}

Cells were first grown in batch culture on a mixture of 20 g L^{-1} glycerol and 20 g L^{-1} methanol. Methanol was consumed only after all the glycerol had been exhausted, since high glycerol concentrations are known to have a repressing effect on the expression of the enzymes necessary for methanol dissimilation. This resulted in the induction and synthesis of methanol dissimilating enzymes and prepared the cells for chemostat growth on methanol.

As soon as all the methanol had been consumed (indicated by a sudden decrease of the heat signal), the supply of the feed-medium containing 0.652 C-mol L^{-1} (20.87 g L^{-1}) methanol as carbon source was initiated and the dilution rate set to 0.06 h^{-1}.

The transient nutrient gradient was begun after five liquid residence times. The methanol fraction in the feed medium was decreased linearly at the same rate as for the first transient nutrient gradient (α = 0.006 C-mol methanol L^{-1} h^{-1}).

Initially, the mixing and reservoir flasks contained 3.0 L of medium each, the mixing flask contained 0.652 C-mol L^{-1} (20.87 g L^{-1}) methanol and the reservoir flask 0.652 C-mol L^{-1} (19.80 g L^{-1}) sorbitol as carbon source.

3. 4. Substrate and metabolite analysis

Culture samples (about 10 mL) were collected using a purpose built auto-sampler (Cannizzaro (2002)) and kept at 2°C for up to 12h before handling.

The concentration of biomass was determined gravimetrically as dry cell weight. Samples were centrifuged in pre-weighed tubes, washed twice with ultrapure water and the pellet dried at 100°C to constant weight.

Sorbitol and methanol were determined by HPLC analysis (1100 series, Agilent Technologies, Palo Alto, USA). An ion exchange chromatography column (Supelcogel H 300 mm, Supelco, Bellefonte, USA) with a guard column (Superlguard C610H, Supelco, Bellefonte, USA) was used at 60 °C. A 5 mM sulphuric acid solution in ultrapure water was applied at a constant eluent flow rate of 0.6 mL min^{-1}. Metabolites were measured using a refractive index detector. The detection limit was 0.01 g L^{-1} for sorbitol and 0.05 g L^{-1} for methanol.

The carbon dioxide evolution rate in the bioreactor off-gas was determined using an infrared analyser (series 2500, Servomex, Crowborough, UK). The measured values were corrected for water vapour according to Duboc and von Stockar (1998).

3. 5. Preparation of cell-free extracts and alcohol oxidase activity measurement

The procedures for the preparation of cell-free extracts and the measurement of specific alcohol oxidase activity have been described previously (Jungo et al. (2006)).
The specific activity of alcohol oxidase was expressed in U mg^{-1} total protein. One unit (U) of AOX corresponds to the oxidation of 1.0 µmole of methanol to formaldehyde per min at pH 7.5 at 25°C.

3. 6. Recombinant avidin quantification

Recombinant avidin was quantified by biotin-4-fluorescein titration of binding sites (Kada et al. (1999)), assuming a tetrameric form of recombinant avidin, with four active-binding sites.
Since the detection limit of this method is about 10 mg L^{-1} of avidin, it was necessary to concentrate the samples by up to 15-fold by ultrafiltration (Centriplus, 30 kDa, Millipore Corporation, Bedford, USA) prior to titration with biotin-4-fluorescein.

3. 7. Check of data consistency

On a C-molar basis, the black box stoichiometry was described by equation 3 for growth on methanol and by equation 4 for growth on sorbitol.

$$CH_4O + Y_{O2/S}\,O_2 + Y_{NH3/S}\,NH_3 \rightarrow Y_{X/S}\,CH_{1.691}O_{0.502}N_{0.176} + Y_{CO2/S}\,CO_2;\ \Delta_rH_S^{*\circ} \quad (3)$$

$$CH_{7/3}O + Y_{O2/S}\,O_2 + Y_{NH3/S}\,NH_3 \rightarrow Y_{X/S}\,CH_{1.757}O_{0.629}N_{0.105} + Y_{CO2/S}\,CO_2;\ \Delta_rH_S^{*\circ} \quad (4)$$

S represents the limiting carbon source (methanol and/or sorbitol), $Y_{i/S}$ the stoichiometric coefficients in mole or C-mole per C-mole of substrate, where i can be S (substrate), O_2, CO_2, NH_3 or X (biomass). $\Delta_rH_S^{*\circ}$ is the standard molar enthalpy change caused by reactions described in equations 3 or 4 with CO_2, H_2O and NH_3 as reference state. The C-molar composition of biomass during growth on sorbitol was determined by elemental analysis (Gurakan et al. (1990)). Culture samples were collected at steady state during the chemostat culture on 19.80 g L^{-1} sorbitol. Samples were centrifuged and washed three times with ultrapure water. After the final washing/centrifugation step, the cell pellets were spread as a thin layer of about 2 mm on Petri dishes and stored at -20

°C. The frozen cell pellets were freeze-dried for 24 hours. Analysis of carbon, nitrogen and hydrogen content was made using a CHN analyser (Carlo Erba Instruments, Rodano, Italy). Oxygen content was calculated by difference (Gurakan et al. (1990)). The C-molar composition of biomass during growth on methanol was determined previously (Jungo et al. (2006)).

The yield of substance j on substance i was calculated as

$$Y_{j/i} = \frac{R_j}{R_i} \quad (C-mol/C-mol) \tag{5}$$

where R_i and R_j are the volumetric production or consumption rates of substance i and j respectively.

The heat yield $Y_{Q/S}$ represents the molar enthalpy change caused by the reactions described in equations 3 or 4 per C-mole of substrate and is defined by equation 6.

$$Y_{Q/S} = \frac{R_Q}{R_S} \quad (kJ/C-mol) \tag{6}$$

The measurements of dry cell weight, carbon source concentrations, carbon dioxide in the exhaust gas and heat production were used in order to calculate three of the five yields of the black box stoichiometry, namely biomass, carbon dioxide and heat yields. Together with the constraints provided by the carbon, nitrogen, degree of reduction and enthalpy balances a redundancy of 2 resulted. The redundancy of the system was used in order to estimate the unknown yields and to reconcile the data.

The yields were checked for consistency and reconciled based on a χ^2-test (Wang and Stephanopoulos (1983); Stephanopoulos et al. (1998)). The resulting reconciled yield coefficients are the best yield estimates that minimize the magnitude of the residuals ε and are determined by minimizing the sum of squared errors scaled according to their variance. The test value h is given by the sum of the weighted squares of the residuals ε:

$$h = \varepsilon \cdot P^{-1} \cdot \varepsilon \tag{7}$$

where P is the variance-covariance matrix. Assuming measurement errors of 5%, a test value h lower than 5.99 for a redundancy of 2 permitted to assume a 95% significance

level that no gross errors and no metabolites other than the ones appearing in equations 3 or 4 had affected the balances.

During mixed substrate growth on methanol and sorbitol, reconciliation of data was done by assuming that the C-molar composition of biomass was the sum of the individual biomass compositions on each substrate multiplied by the C-molar fraction of each substrate in the feed.

4. RESULTS AND DISCUSSION

4. 1. Linear increase of methanol fraction in the feed medium at a dilution rate of 0.03 h^{-1}

The influence of the methanol-sorbitol ratio in the feed medium on specific recombinant avidin productivity and stoichiometric yields was examined at a constant dilution rate of 0.03 h^{-1}, slightly below the maximal specific growth rate on sorbitol (0.032 ± 0.002 h^{-1}). A nutrient gradient with a linearly increasing methanol fraction in the feed medium was performed after having achieved steady-state growth conditions in a chemostat culture on sorbitol as sole carbon source.

The steady-state reconciled yield coefficients during chemostat growth on sorbitol were $Y_{X/S}$ = 0.62 C-mol C-mol^{-1}, $Y_{CO2/S}$ = 0.41 mol C-mol^{-1}, $Y_{O2/S}$ = 0.47 mol C-mol^{-1}, $Y_{NH3/S}$ = 0.07 mol C-mol^{-1} and $Y_{Q/S}$ = -224 kJ C-mol^{-1}. The test value h was 1.69 which is lower than the threshold value of 5.99 for a redundancy of 2 and a confidence level of 95%. A statistical test value h exceeding the χ^2-distribution would either indicate the presence of a gross error in measurement or of an unidentified metabolite not accounted for in the stoichiometric black box description. According to these results, cell growth is as efficient on sorbtiol as on glycerol (Jungo et al. (2006)).

During chemostat growth, the average residual sorbitol concentration determined by HPLC was 85 mg L^{-1}. This relatively high residual limiting-substrate concentration is due to the fact that the dilution rate was set very close to the maximal specific growth rate on sorbitol, which was determined in a batch culture on sorbitol to be slightly higher than 0.03 h^{-1} (0.032 ± 0.002 h^{-1}).

No recombinant avidin could be detected during chemostat growth on sorbitol. However, a partial derepression of the AOX enzyme was observed. Indeed, a specific AOX

activity of 0.07 U mg^{-1} total protein was determined during steady-state growth on sorbitol.

The time course of dry cell weight concentration, heat production rate and carbon dioxide production rate during the nutrient gradient are displayed in figure 1. Time zero corresponds to the beginning of the nutrient gradient. Only one hour after the beginning of the nutrient gradient, a sudden increase in the carbon dioxide production rate and in the heat production rate was observed because cells needed roughly one hour in order to induce the synthesis of the enzymes necessary for the dissimilation of methanol. Values of carbon dioxide and heat production rates remained relatively high during the first 10 hours after the beginning of the nutrient gradient until the metabolism of *P. pastoris* was fully adapted to mixed substrate growth on methanol and sorbitol.

With increasing methanol fractions in the feed medium, the dry cell weight concentration decreased because biomass yields are lower on methanol than on sorbitol as carbon source. On the other hand, carbon dioxide and heat production rates increased with increasing methanol fraction in the feed medium since these rates are higher in cultures on methanol than on sorbitol.

The straight line in figure 1 represents the theoretical dry cell weight concentration calculated as the sum of the individual biomass yield coefficients theoretically possible for each substrate (determined during chemostat cultures on either sorbitol or methanol as sole carbon source) multiplied by the C-molar fraction of each substrate in the feed medium and by the C-molar mass of biomass. Very good agreement between the measured and the calculated values can be observed in figure 1, which means that biomass yields can be considered to be additive during mixed substrate growth on sorbitol and methanol.

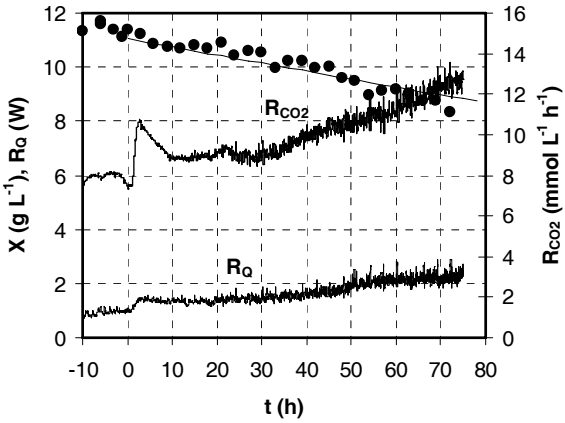

Figure 1: Cell dry weight (X, ●), heat production rate (R_Q) and carbon dioxide production rate (R_{CO2}) as a function of time during the nutrient gradient. Theoretical dry cell weight concentrations (straight line) calculated as the sum of the individual biomass yields on each substrate multiplied by the C-molar fraction of each substrate in the feed medium and by the C-molar mass of biomass. After chemostat growth on 0.652 C-mol L^{-1} sorbitol, the methanol fraction in the feed medium was increased linearly (at t = 0 h) at a rate of 0.006 C-mol L^{-1} h^{-1}. The dilution rate was fixed at 0.03 h^{-1}.

The measured residual sorbitol concentrations during the nutrient gradient are presented in figure 2. The residual methanol concentrations could not be determined because they were below the detection limit of the HPLC method used for methanol quantification. Figure 2 shows that residual sorbitol concentrations decreased during the nutrient gradient, as the sorbitol fraction in the feed medium decreased. These results are in agreement with the findings of Lendenmann et al. (1996), who studied the kinetics of simultaneous utilization of sugar mixtures by *E. coli* in continuous cultures. They showed that the steady-state residual concentrations of the simultaneously consumed sugars were proportional to the ratio of these sugars in the inflowing medium and did not depend on the total substrate concentration in the inflowing medium. The bold dotted line in figure 2 represents the theoretical residual sorbitol concentrations assuming a linear relationship between the steady-state concentrations of sorbitol and methanol

and the corresponding proportions of these sugars in the inflowing medium (Lendenmann et al. (1996)).

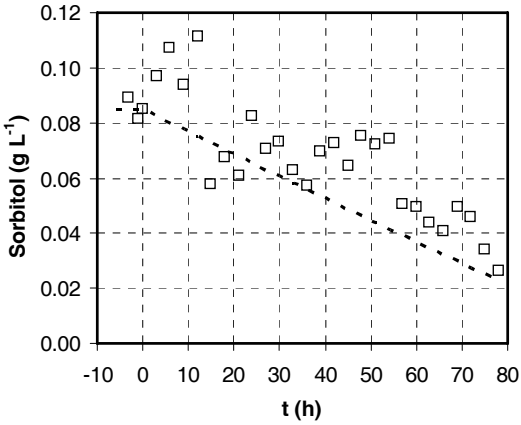

Figure 2: Residual sorbitol (□) concentration as a function of time during the nutrient gradient. After chemostat growth on 0.652 C-mol L^{-1} sorbitol, the methanol fraction in the feed medium was increased linearly (at t = 0 h) at a rate of 0.006 C-mol L^{-1} h^{-1}. The dilution rate was fixed at 0.03 h^{-1}. The bold dotted line represents the theoretical residual sorbitol concentrations as a function of time during the nutrient gradient according to Lendenmann et al. (1996).

The reconciled yield coefficients are presented in figure 3 according to the methanol fraction in the feed medium. The corresponding test values h are shown in figure 4. In the first 10 hours one can see very well the excursion towards more maintenance in $Y_{O2/S}$, $Y_{CO2/S}$ and $Y_{Q/S}$. The statistical test was not passed during the first 10 hours after the beginning of the nutrient gradient because cells had to adapt their metabolism to grow on a new additional substrate, methanol, and grew with an untypical low biomass yield. Except during the first 10 hours after the beginning of the transient nutrient gradient, the h test values were always lower than the threshold value of 5.99, which signifies that no gross measurement errors and no metabolites other than the ones appearing in equations 3 and 4 had affected the balances with a confidence level of 95%.

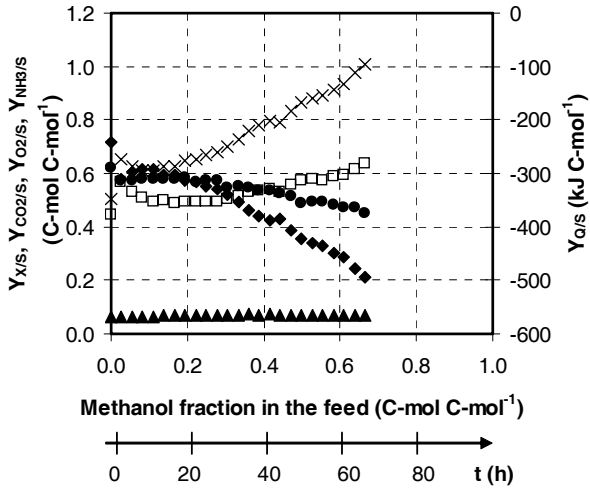

Figure 3: Reconciled biomass (●), carbon dioxide (□), oxygen (x), ammonia (▲) and heat (◆) yield coefficients as a function of the methanol fraction in the feed medium and as a function of time during the nutrient gradient. After chemostat growth on 0.652 C-mol L^{-1} sorbitol, the methanol fraction in the feed medium was increased linearly at a rate of 0.006 C-mol L^{-1} h^{-1}. The dilution rate was fixed at 0.03 h^{-1}.

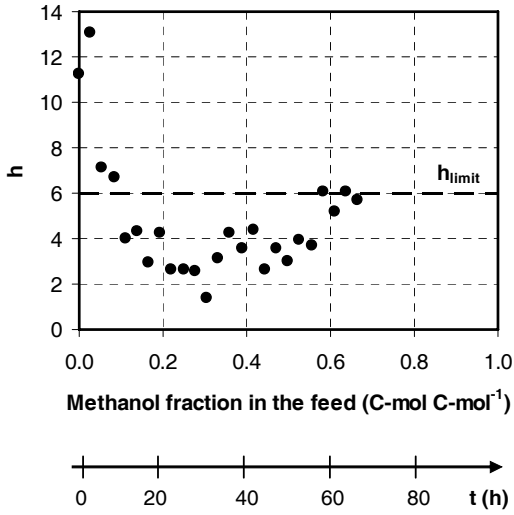

Figure 4: Test values h calculated with reconciliation procedures for all the analysed samples at the various methanol fractions in the feed medium and as a function of time during the nutrient gradient with a linear increase of the methanol fraction in the feed medium at a rate of 0.006 C-mol L^{-1} h^{-1}, at a dilution rate of 0.03 h^{-1}. The threshold value to reject hypothesis with a 95% confidence level and a redundancy of 2 is 5.99 (h_{limit}).

Specific AOX activities of cell-free extracts of samples collected during the nutrient gradient are presented in figure 5. Specific AOX activities increased at a high rate during the first 12 hours after the beginning of the nutrient gradient (slope = 0.10 U mg^{-1} total protein h^{-1} for methanol fractions lower than 0.11 C-mol C-mol^{-1}) because feeding of methanol induced synthesis of methanol dissimilating enzymes. Maximal specific AOX activities of 1.46 U mg^{-1} total proteins were achieved 27 hours after the beginning of the nutrient gradient at a methanol fraction of 0.25 C-mol C-mol^{-1}, above which the specific AOX activities decreased. In all probability, this decrease in specific AOX activity is due to an increase of the residual methanol concentration as the methanol fraction in the feed medium is increased. According to Lendenmann et al. (1996), the residual methanol concentration is expected to increase linearly with linearly increasing methanol fractions in the feed medium. As pointed out in earlier studies (Jungo et al. (2006); Jungo et al. (2007a)), if methanol oxidation is considered to be the growth rate-limiting reaction, cells will maintain a high rate of substrate oxidation by increasing the amount

of the rate limiting enzyme when the residual methanol concentration decreases. Hence, if residual methanol concentration is increasing, specific AOX activities will decrease.

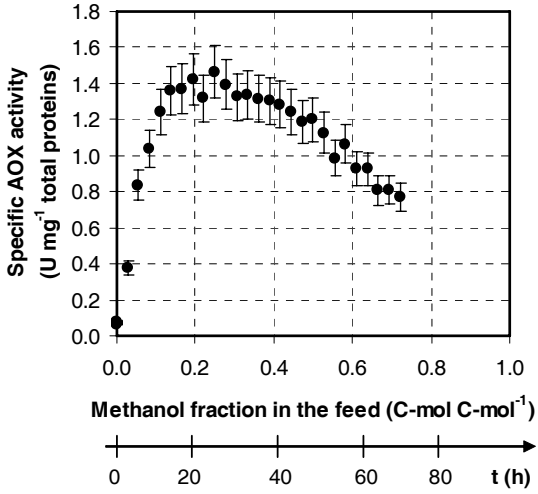

Figure 5: Specific AOX activities as a function of the methanol fraction in the feed medium and a as a function of time during the nutrient gradient. After chemostat growth on 0.652 C-mol L^{-1} sorbitol, the methanol fraction in the feed medium was increased linearly at a rate of 0.006 C-mol L^{-1} h^{-1}. The dilution rate was fixed at 0.03 h^{-1}.

The concentrations of avidin measured in the culture supernatant during the nutrient gradient and the calculated volumetric and specific avidin production rates are presented in figures 6A and 6B, respectively. The results of the average volumetric and specific avidin production rates during chemostat growth on methanol as sole carbon source are also presented in figure 6B for a dilution rate of 0.03 h^{-1} (Jungo et al. (2006)). The avidin concentration and the specific avidin production rate increased as the methanol fraction in the feed medium was increased, up to a methanol fraction of 43% C-mol C-mol^{-1} methanol (t = 46 h). This increase in specific avidin production rate can be correlated with the increase in specific methanol consumption rate (Jungo et al. (2006); Jungo et al. (2007a)). However, for methanol fractions higher than 0.43 C-mol C-mol^{-1}, the specific avidin production rate remained constant and was the same as

with methanol as sole carbon source. The concentration of avidin decreased above a methanol fraction of 0.43 C-mol C-mol^{-1} because of the decrease in biomass concentration. The volumetric avidin production rate was the highest for methanol fractions between 0.43 and 0.47 C-mol C-mol^{-1}. Indeed, the volumetric avidin production rate was 1.3- to 1.4-fold higher than for chemostat cultures on methanol as sole carbon source because of the higher biomass yields during mixed substrate growth. According to these results it is preferable to work with a mixed feed solution of sorbitol and methanol containing 40-45% C-mol C-mol^{-1} methanol than with methanol alone. In this way, the specific avidin production rate will not be affected compared with a culture with methanol as sole carbon source and the volumetric avidin production rate will be increased by about 30 %. Moreover, both oxygen consumption rate and heat production rate will be diminished during mixed substrate growth on sorbitol and methanol (figure 3), which will facilitate the performance of cultures at high cell density. For instance, with a feed containing 43% C-mol C-mol^{-1} methanol and 57% C-mol C-mol^{-1} sorbitol, oxygen consumption and heat production rates were diminished by about 38 % for a given dry cell weight concentration (figure 3).

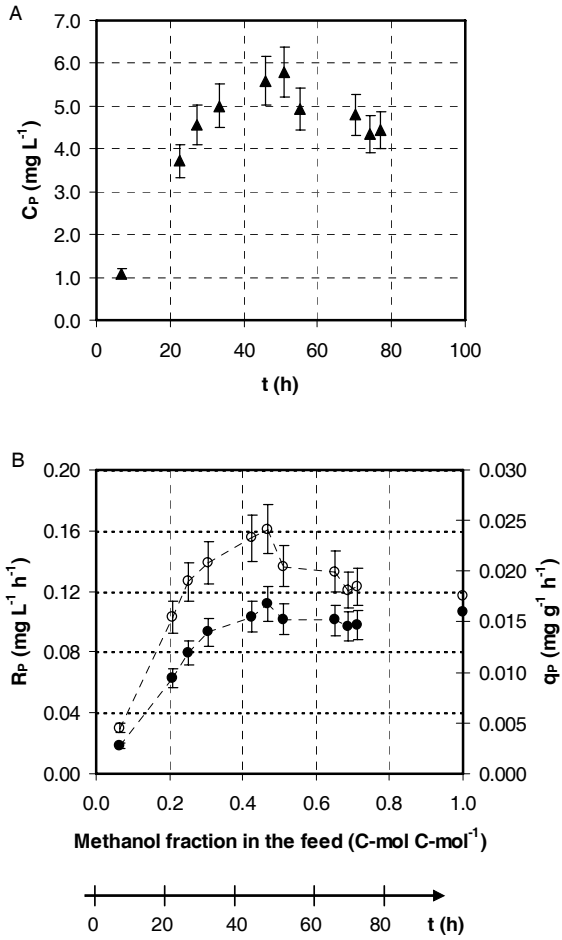

Figure 6: A: Concentration of avidin in culture supernatant (C_P, ▲) as a function of time during nutrient gradient. B: Volumetric (R_P, ○) and specific (q_P, ●) recombinant avidin production rates as a function of the methanol fraction in the feed medium and as a function of time during the nutrient gradient. After chemostat growth on 0.652 C-mol L^{-1} sorbitol, the methanol fraction in the feed medium was increased linearly at a rate of 0.006 C-mol L^{-1} h^{-1}. The dilution rate was fixed at 0.03 h^{-1}. The volumetric and specific avidin production rates achieved during chemostat growth on methanol at 0.03 h^{-1} were also represented (Jungo et al. (2006)).

4. 2. Linear decrease of methanol fraction in the feed medium at a dilution rate of 0.06 h^{-1}

The influence of the methanol-sorbitol ratio in the feed medium on specific recombinant avidin production rate and stoichiometric yields was also examined at a constant dilution rate of 0.06 h^{-1}, which is between the maximal specific growth rates on sorbitol and methanol, 0.032 ± 0.002 h^{-1} and 0.140 h^{-1} ± 0.007 h^{-1} respectively. Mixed substrate growth with consumption of both methanol and sorbitol is expected to occur at a dilution rate of 0.06 h^{-1} for a certain range of methanol fractions in the feed medium. As a matter of fact, growth at specific growth rates higher than the maximal specific growth rate on the substrate at which cells are growing at a lower rate is the rule rather than the exception in mixed substrate utilisation (Egli et al. (1986)). A dilution rate higher than the maximal specific growth rate on sorbtiol (and lower than the maximal specific growth rate on methanol) allows determination of the range of methanol fractions in the feed medium where both substrates are consumed simultaneously at this dilution rate, as well as studies of the influence of residual sorbitol concentration on specific recombinant avidin productivity at a constant growth rate and a constant total amount of carbon source in the feed medium.

To our knowledge, all previous studies with mixed feeds of methanol and sorbitol were performed in fed-batch cultures with changing specific growth rates during the induction phase and/or with changing amounts of carbon sources fed to the culture (Thorpe et al. (1999); Boze et al. (2001); Xie et al. (2005)). Changing several parameters at a time makes it difficult to draw conclusions on the influence of mixed feeds of sorbitol and methanol and the influence of residual sorbitol in the culture on recombinant protein production rate.

A nutrient gradient with linearly decreasing methanol fractions in the feed medium was performed after having achieved steady-state growth conditions in a chemostat culture on methanol as sole carbon source. The experiment was begun with a chemostat culture on methanol because wash-out would be observed during chemostat culture on sorbitol since the dilution rate was higher than the maximal specific growth rate on sorbitol.

Cells were grown in chemostat culture on 0.652 C-mol L^{-1} (20.87 g L^{-1}) methanol at a dilution rate of 0.06 h^{-1}. The steady-state reconciled yield coefficients for growth on methanol were $Y_{X/S}$ = 0.44 C-mol C-mol^{-1}, $Y_{CO2/S}$ = 0.58 mol C-mol^{-1}, $Y_{O2/S}$ = 1.07 mol

C-mol^{-1}, $Y_{NH3/S}$ = 0.08 mol C-mol^{-1} and $Y_{Q/S}$ = -528 kJ C-mol^{-1}. The test value h was 3.99 which is lower than the threshold value of 5.99 for a redundancy of 2 and a confidence level of 95%. At steady state, the volumetric and specific productivities of recombinant avidin were on average 0.158 mg L^{-1} h^{-1} and 0.022 mg g^{-1} h^{-1} respectively. Figure 7 shows the profiles of dry cell weight concentration, carbon dioxide production rate and heat production rate during the nutrient gradient, which was started at time t = 0 h.

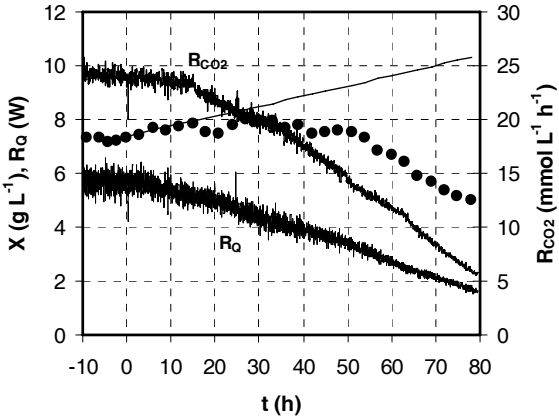

Figure 7: Cell dry weight (X, ●), heat production rate (R_Q) and carbon dioxide production rate (R_{CO2}) as a function of time during the nutrient gradient. Theoretical dry cell weight concentrations (straight line) calculated as the sum of the individual biomass yields on each substrate multiplied by the C-molar fraction of each substrate in the feed medium and by the C-molar mass of biomass. After chemostat growth on 0.652 C-mol L^{-1} methanol, the methanol fraction in the feed medium was decreased linearly (at t = 0 h) at a rate of 0.006 C-mol L^{-1} h^{-1}. The dilution rate was fixed at 0.06 h^{-1}.

The residual sorbitol concentrations in the culture medium are displayed in figure 8. The residual methanol concentration always remained under the detection limit of the HPLC method. During the first 6 hours after the beginning of the nutrient gradient, the residual sorbitol concentration was under the detection limit of the HPLC method used for its determination, but subsequently sorbitol began to accumulate in the culture. The bold dotted line represents the theoretical curve of sorbitol accumulation if sorbitol is not consumed by the cells according to equation 9, which was obtained by integration

of equation 8. This equation describes the balance of sorbitol over the whole bioreactor during the nutrient gradient assuming that sorbitol is not consumed by the cells.

$$\frac{dC_S}{dt} = ({C_S}_{(0)}{}^{M} + \alpha \cdot t - C_S) \cdot D \quad (8)$$

$$C_S(t) = \alpha \cdot \left(t - \frac{1}{D}\right) + \left({C_S}_{(0)} + \frac{\alpha}{D}\right) \cdot e^{-Dt} \quad (9)$$

where D is the dilution rate (h^{-1}), C_S the residual sorbitol concentration in the bioreactor (C-mol L^{-1}), ${C_S}_{(0)}$ the residual sorbitol concentration in the bioreactor at the beginning of the nutrient gradient (C-mol L^{-1}), ${C_S}_{(0)}{}^{M}$ the concentration of sorbitol in the mixing flask at the beginning of the nutrient gradient (C-mol L^{-1}) and α the rate of the nutrient gradient (C-mol L^{-1} h^{-1}), which is defined by equation 2.

An increase in dry cell weight concentration was observed at the beginning of the nutrient gradient (figure 7) because biomass yields are higher for growth on sorbtiol than on methanol. However, as the methanol fraction in the feed medium decreased, the increase in biomass yield was counterbalanced by the limited ability of the cells to consume sorbitol. Indeed, the higher the sorbitol content in the feed medium, the lower will be the dilution rate at which a certain sorbitol consumption rate will be reached by the cells (Egli et al. (1986)). This is due to the fact that the dilution rate applied is higher than the maximal specific growth rate on sorbitol. For methanol fractions higher than 0.50 C-mol C-mol^{-1} ($t < 51h$), the dry cell weight concentrations remained more or less constant because, although biomass yields were increasing (figure 9), less sorbitol could be consumed by the cells.

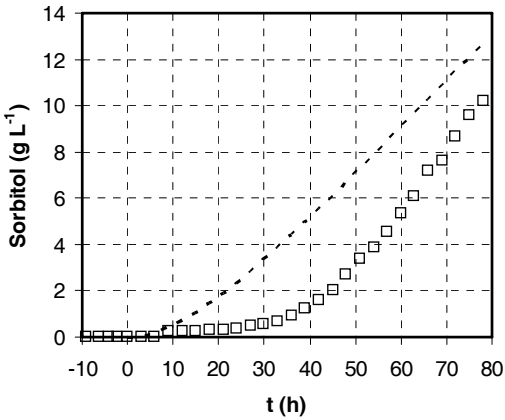

Figure 8: Residual sorbitol (□) concentration as a function of time during the nutrient gradient. The bold dotted line represents the theoretical curve of sorbitol accumulation if sorbitol is not consumed by the cells. After chemostat growth on 0.652 C-mol L^{-1} methanol, the methanol fraction in the feed medium was decreased linearly (at t = 0 h) at a rate of 0.006 C-mol L^{-1} h^{-1}. The dilution rate was fixed at 0.06 h^{-1}.

All reconciled yield coefficients are presented in figure 9 as a function of the methanol fraction in the feed medium. The corresponding test values h are shown in figure 10. The test values h were always lower than the threshold value of 5.99, except for methanol fractions lower than 0.30 C-mol C-mol^{-1}. The test values h increased for methanol fractions in the feed medium lower than 0.33 C-mol C-mol^{-1} (t > 63 h) because of washout of the culture.

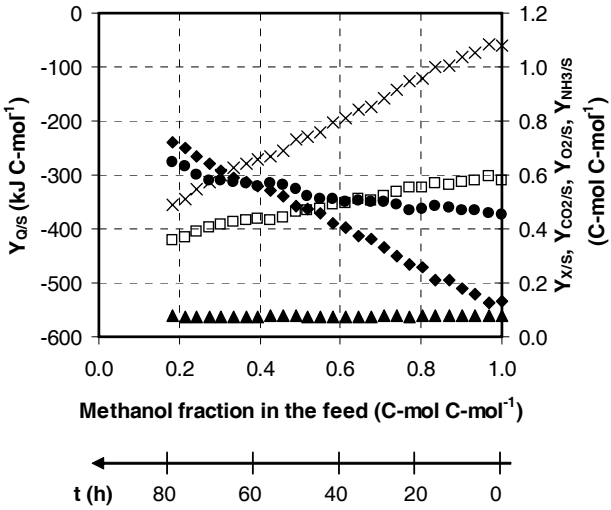

Figure 9: Reconciled biomass (●), carbon dioxide (□), oxygen (x), ammonia (▲) and heat (◆) yield coefficients as a function of the methanol fraction in the feed medium. After chemostat growth on 0.652 C-mol L^{-1} methanol, the methanol fraction in the feed medium was decreased linearly at a rate of 0.006 C-mol L^{-1} h^{-1}. The dilution rate was fixed at 0.06 h^{-1}.

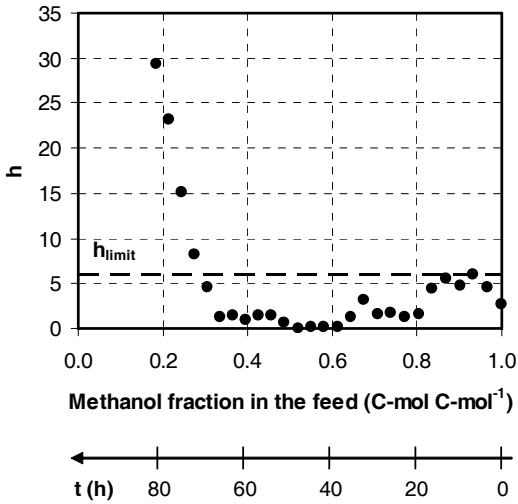

Figure 10: Test values h calculated with reconciliation procedures for all the analysed samples at the various methanol fractions in the feed medium during the transient culture with a linear decrease of the methanol fraction in the feed medium at a rate of 0.006 C-mol L^{-1} h^{-1}, at a dilution rate of 0.06 h^{-1}. The threshold value to reject hypothesis with a 95% confidence level and a redundancy of 2 is 5.99 (h_{limit}).

The straight line in figure 7 represents the theoretical dry cell weight concentration calculated as the sum of the individual biomass yield coefficients theoretically possible for each substrate multiplied by the C-molar fraction of each substrate in the feed medium and by the C-molar mass of biomass. During the first 15 hours after the beginning of the nutrient gradient, for methanol fractions in the feed medium higher than 0.84 C-mol C-mol^{-1}, good agreement between the measured and the theoretical values was obtained. However, this was not the case for lower methanol fractions in the feed medium and is due the the fact that not all of the fed sorbitol could be consumed and assimilated by the cells. Moreover, for $t > 54$ h, which corresponds to methanol fractions lower than 0.43 C-mol C-mol^{-1}, the difference between the measured dry cell weight concentrations and the theoretical values was more marked because of wash-out of the culture.

Three distinct growth regimes can be distinguished during this transient nutrient gradient in continuous culture at 0.06 h^{-1}. Indeed, for methanol fractions higher than 0.90 C-mol C-mol^{-1}, the culture was dually limited in methanol and sorbitol. Both carbon sources were consumed simultaneously to completion (figure 8). For methanol fractions between 0.43 and 0.90 C-mol C-mol^{-1}, the culture was only limited by methanol. Sorbitol could not be consumed to completion for this range of methanol fractions and began to accumulate in the culture medium (figure 8). Finally, for methanol fractions lower than 0.43 C-mol C-mol^{-1}, wash-out of the culture was observed. This was reflected by faster decrease in biomass concentration (figure 7) and by the fast increase of the statistical test value h above the threshold limit (figure 10).

During the nutrient gradient, specific AOX activities increased from 0.8 U mg^{-1} total proteins on methanol as sole carbon source up to 1.2 U mg^{-1} total proteins at a methanol fraction in the feed medium of 0.2 C-mol C-mol^{-1} (figure 11). As explained earlier, the observed increase in specific AOX activity as the methanol fraction in the feed medium is decreased is in all probability due to a decrease in the residual methanol concentration in the reactor.

It can be noted that for a given methanol fraction in the feed medium, the specific AOX activity is higher for mixed substrate growth at 0.03 h^{-1} than for growth at 0.06 h^{-1}. An increase in the specific AOX activity has also been reported for lower dilution rates during chemostat cultures on methanol (Jungo et al. (2006)). The reason is again that at lower dilution rate, the residual methanol concentration is lower and higher amounts of alcohol oxidase are synthesized in order to maintain a high limiting substrate oxidation rate.

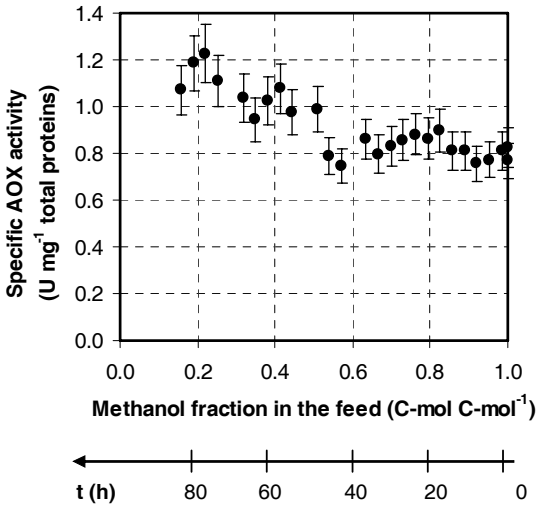

Figure 11: Specific AOX activities as a function of the methanol fraction in the feed medium. After chemostat growth on 0.652 C-mol L^{-1} methanol, the methanol fraction in the feed medium was decreased linearly at a rate of 0.006 C-mol L^{-1} h^{-1}. The dilution rate was fixed at 0.06 h^{-1}.

The concentrations of avidin measured in the culture supernatant during the nutrient gradient and the calculated volumetric and specific avidin productivities are shown in figures 12A and 12B, respectively. The concentration of avidin began to decrease about 50 h after the beginning of the nutrient gradient (figure 12A). This corresponds to the beginning of the decrease in biomass concentration due to wash-out of the culture (figure 7). For methanol fractions higher than 0.50 C-mol C-mol^{-1}, the avidin concentration and the specific and volumetric avidin production rates did not change significantly. Since sorbitol accumulation was observed for methanol fractions lower than 0.90 C-mol C-mol^{-1} (figure 8), it can be concluded that sorbitol accumulation does not repress foreign gene expression. This confirms that sorbitol is a «non-repressive» carbon source with respect to the AOX1 promoter (Thorpe et al. (1999); Boze et al. (2001)). By contrast with sorbitol, transient accumulation of non-limiting concentrations

of glycerol would have led to a decrease in recombinant protein productivity (Tschopp et al. (1987)).

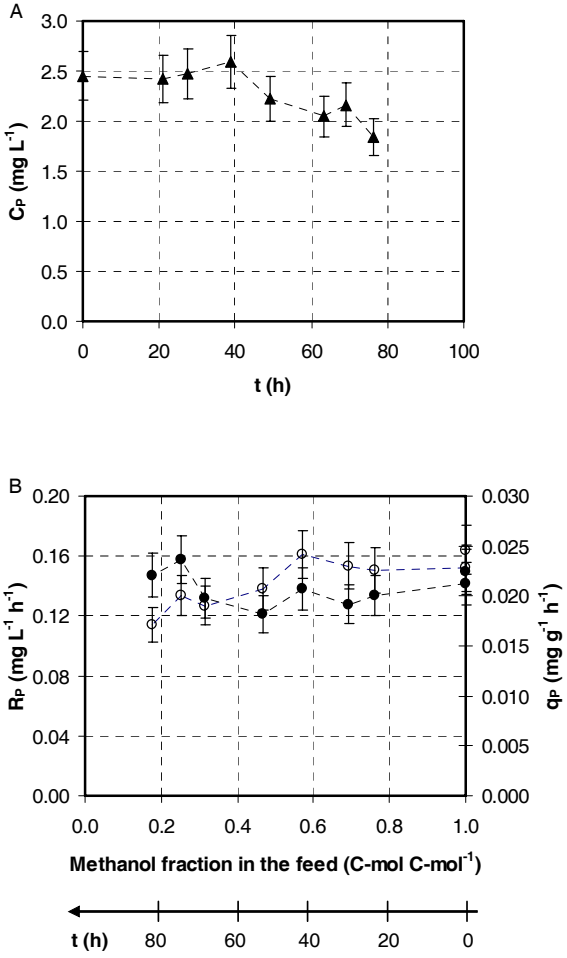

Figure 12: A: Concentration of avidin in culture supernatant (C_P, ▲) as a function of time during nutrient gradient. B: Volumetric (R_P, ❍) and specific (q_P, ●) recombinant avidin production rates as a function of the methanol fraction in the feed medium. After chemostat growth on 0.652 C-mol L^{-1} methanol, the methanol fraction in the feed medium was decreased linearly at a rate of 0.006 C-mol L^{-1} h^{-1}. The dilution rate was fixed at 0.06 h^{-1}.

5. CONCLUSIONS

The performance of transient nutrient gradients in continuous cultures with linearly increasing or decreasing methanol fractions in the feed medium allowed to study rapidly and rationally the influence of the methanol-sorbitol ratio in the feed medium on growth stoichiometry, specific AOX activity and recombinant avidin productivity at constant dilution rates.

Results showed that the specific avidin productivity achieved in mixed substrate cultures on methanol and sorbitol could be as high as in cultures on methanol as sole carbon source. For instance, at a dilution rate of 0.03 h^{-1}, the specific avidin productivity was the same with a mixed feed of methanol and sorbitol as with a feed of methanol as sole carbon source, if the methanol fraction in the feed medium was higher than 43 % C-mol C-mol^{-1}. Moreover, since biomass yields are higher for growth on sorbitol than for growth on methanol, the volumetric avidin productivity was about 1.3-fold higher with a mixed feed of sorbitol and methanol, at a methanol fraction of 43 % C-mol C-mol^{-1}, than with a culture on methanol as sole carbon source.

Compared with mixed feeds of glycerol and methanol, mixed feeds of sorbitol and methanol have the advantage that accumulation of sorbitol in the culture medium does not significantly affect specific recombinant protein productivity because sorbitol is a «non-repressive» carbon source with respect to alcohol oxidase expression. By contrast with glycerol, control of residual sorbitol concentration is not critical for cultures of recombinant *P. pastoris* strains.

Moreover, oxygen consumption and heat production rates were indeed lower during mixed substrate growth because the enthalpy of combustion and the degree of reduction of sorbitol are lower than those of methanol. For a given biomass concentration, oxygen consumption and heat production rates can for instance be diminished by 38% for a methanol fraction of 43% C-mol C-mol^{-1} at a dilution rate of 0.03 h^{-1}.

6. ACKNOWLEDGEMENTS

Financial support from the Swiss National Science Fundation is gratefully acknowledged. We also gratefully acknowledge the laboratory of Prof. T. Ward at the university of Neuchâtel for having kindly provided the recombinant *P. pastoris* strain expressing and secreting avidin.

7. NOMENCLATURE

AOX	Alcohol oxidase	
C_P	Concentration of avidin	mg L^{-1}
C_S	Substrate concentration in bioreactor	C-mol L^{-1}
C_S^M	Substrate concentration in the mixing flask	C-mol L^{-1}
$C_{S(0)}^M$	Initial substrate concentration in the mixing flask	C-mol L^{-1}
C_S^R	Substrate concentration in the reservoir flask	C-mol L^{-1}
D	Dilution rate	h^{-1}
F_1	Medium flow from the reservoir flask	L h^{-1}
F_2	Medium flow from the mixing flask	L h^{-1}
h	Statistical test value	
$\Delta_r H_S^{*\circ}$	Standard molar enthalpy change caused by reaction r per mole of subtrate S	kJ C-mol^{-1}
K_S	Monod substrate saturation constant	g L^{-1}
P	Variance-covariance matrix	
q_P	Biomass-specific avidin productivity	mg g^{-1} h^{-1}
R_{CO2}	Carbon dioxide production rate	mol L^{-1} h^{-1}
R_i	Volumetric production or consumption rate of substance i	C-mol L^{-1} h^{-1}
R_Q	Heat production rate	W
R_P	Volumetric avidin productivity	mg L^{-1} h^{-1}
V	Culture volume	L
V_{Sup}	Volume of culture supernatant	L
V_0	Initial volume in reservoir and mixing flasks	L
X	Cell dry weight	C-mol L^{-1}
$Y_{j/i}$	Yield coefficient of substance j on substance i	C-mol C-mol^{-1}
α	Rate of nutrient gradient	C-mol L^{-1} h^{-1}
ε	Residuals	
μ	Specific growth rate	h^{-1}

Subscripts

i	Refers to compound i
j	Refers to compound j
S	Refers to the limiting nutrient
P	Refers to the produced recombinant avidin
Q	Refers to heat
X	Refers to the biomass
0	Initial condition

Superscripts

M	Refers to mixing flask
R	Refers to reservoir flask

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CHAPTER 8

OPTIMAL FED-BATCH STRATEGY FOR THE PRODUCTION OF RECOMBINANT AVIDIN EXPRESSED AND SECRETED BY A *PICHIA PASTORIS* MUT$^+$ STRAIN

1. ABSTRACT

The high cell density fed-batch strategy for the production of recombinant avidin with a *Pichia pastoris* Mut$^+$ strain was optimized with respect to the specific growth rate applied during the induction phase and the composition of the feed medium, using methanol or a mixture of methanol and sorbitol as carbon and energy sources. The objective was to maximize the total accumulated amount of avidin per unit operation time, performing the induction phase with an exponential feed at a constant specific growth rate. Simulations of the performance of avidin in high cell density fed-batch cultures were realized with data presented in the preceding chapters of this thesis. Simulations of the performance of avidin in fed-batch cultures as a function of the specific growth rate during the induction phase showed that the maximal performance of avidin is achieved at low specific growth rates (about 0.03 h^{-1}). Simulations were compared with results obtained during high cell density fed-batch cultures with a feed of methanol or a mixed feed of methanol and sorbitol during the induction phase.

The highest performance of avidin in fed-batch cultures was achieved with a mixed feed of sorbitol and methanol (at a methanol fraction of 43% C-mol C-mol^{-1}) at a specific growth rate of 0.03 h^{-1} during the induction phase.

Keywords: *Pichia pastoris*; Avidin; Mixed substrate; Sorbitol; High cell density.

2. INTRODUCTION

Host cells need to be cultured in an environment that provides optimal conditions for maximal protein expression while taking into account technical constraints. For example, with recombinant *Pichia pastoris* strains, factors such as temperature (Whittaker and Whittaker (2000); Li et al. (2001); Jahic et al. (2003); Li et al. (2003); Shi et al.

(2003)), pH (Sreekrishna et al. (1997); Inan et al. (1999)), composition of the feed medium (Boze et al. (2001); Xie et al. (2005); Jungo et al. (2007)) and specific growth rate (D'Anjou and Daugulis (2001)) have to be optimized in order to achieve a high performance of recombinant protein (total accumulated amount of recombinant protein per unit operation time). Technical constraints such as reactor cooling capacity and oxygen transfer rate have to be taken into account because high heat production and oxygen consumption rates often represent major technical challenges with high cell density *P. pastoris* cultures (Schilling et al. (2001); Jenzsch et al. (2004); Hoeks et al. (2005)). Heat production and oxygen consumption rates are closely correlated; with 460 kJ of heat generated per mole of oxygen consumed during aerobic growth (Roels (1983)).
Since production of recombinant proteins with *P. pastoris* strains is usually performed in substrate-limited high cell density fed-batch cultures, the substrate feed rate determines the specific growth rate. In order to maximize the amount of recombinant protein produced, the specific growth rate and therefore the substrate feeding rate have to be optimized because the amount of recombinant protein depends on the time integral of the product of the amount of biomass and the specific protein productivity according to the following equation:

$$m_P = q_P \cdot \int_0^t X \cdot V dt \qquad (1)$$

where m_P is the amount of recombinant protein produced (mg), q_P the specific recombinant protein production rate (mg g^{-1} h^{-1}), X the dry cell weight concentration (g L^{-1}) and V the reactor volume (L).
For *P. pastoris*, both the biomass and the specific recombinant protein production rate usually depend on the specific growth rate. Indeed, several authors (D'Anjou and Daugulis (2001); Curvers et al. (2001); Jungo et al. (2006)) reported that the specific recombinant protein productivity increased with the specific growth rate. Hence, after a given induction time, a higher specific growth rate will lead to a higher biomass concentration and to a higher amount of recombinant protein. However, in high cell density fed-batch cultures, the highest achievable specific growth rate is not necessarily the optimal one because of technical constraints such as reactor cooling capacity or oxygen transfer rate. Indeed, high cell density cultures performed at high specific growth rates often

have to be interrupted due to insufficient reactor cooling or oxygen transfer rate (Schilling et al. (2001); Jenzsch et al. (2004)). Hence, for maximal recombinant protein expression, it is necessary to optimize and to control the specific growth rate during the induction phase.

Interestingly a higher productivity of recombinant protein can be achieved with mixed feeds, such as methanol and glycerol (Cregg et al. (1993); Loewen et al. (1997); McGrew et al. (1997); Katakura et al. (1998); Zhang et al. (2003)) or methanol and sorbitol (Thorpe et al. (1999); Boze et al. (2001); Xie et al. (2005)) than with methanol as sole carbon source as a result of higher biomass yields on mixed feeds. Furthermore, use of mixed feeds significantly diminishes the heat production and the oxygen consumption rates (Jungo et al. (2007)). For instance, results presented in the preceding chapter of this thesis showed that a mixed feed of 43% C-mol C-mol^{-1} methanol and 57% C-mol C-mol^{-1} sorbitol allowed to diminish the heat production and the oxygen consumption rates by about 38 % for a given dry cell weight concentration and allowed to achieve a 1.3-fold higher volumetric avidin productivity compared with methanol as sole carbon source. High heat production and oxygen consumption rates frequently represent the major technical limitations for high cell density operations with *P. pastoris* strains (Hensing et al. (1995); Schilling et al. (2001); Jenzsch et al. (2004); Hoeks et al. (2005); Meyer and Klein (2006)). Hence, the reduction of heat production and oxygen consumption rates and the simultaneous increase in recombinant protein productivities with mixed substrate feeds are very advantageous during high cell density cultures with *P. pastoris* strains.

Moreover, it was pointed out in the preceding chapter of this thesis that one advantage with sorbitol as additional carbon source is that operation with mixed feeds of methanol and sorbitol is less constrained by the need for tight control of substrate concentration in high cell density fed-batch cultures than with glycerol as second carbon source. By contrast with glycerol, it was shown that sorbitol is not a repressive carbon source with respect to the alcohol oxidase promoter.

However, one drawback with sorbitol as additional carbon source is the low maximum-specific growth rate (0.032 h^{-1}). Since the productivity of recombinant avidin increases with the specific growth rate (Jungo et al. (2006)), higher specific productivities are achieved at higher growth rates. For comparison, the maximum specific growth rates on methanol and glycerol are 0.14 and 0.24 h^{-1} respectively (Jungo et al. (2006)).

Therefore, higher specific growth rates can be achieved with methanol as sole carbon source or with mixed feeds of glycerol and methanol than with mixed feeds of sorbitol and methanol.

In this study, we investigated the influence of the specific growth rate and composition of the feed medium (methanol or a mixture containing 43% methanol and 57% sorbitol C-mol C-mol^{-1} as carbon sources) on the performance of recombinant avidin secreted by a *P. pastoris* Mut^{+} strain (Zocchi et al. (2003)). The aim was to optimize the production of recombinant avidin at laboratory scale by taking into account technical constraints, namely reactor scale and reactor cooling capacity.

First of all, the influence of the specific growth rate during the induction phase of high cell density fed-batch cultures, on the performance of avidin was investigated with simulations based on the results presented in chapter 3 of this thesis, obtained in chemostat cultures on methanol (Jungo et al. (2006)) and on the results obtained by Schenk et al. (2007) in fed-batch cultures at low specific growth rates. Simulations were performed for constant specific growth rates during the induction phase (exponential substrate feeding).

Results were compared with high cell density fed-batch cultures with a feed of methanol during the induction phase. Comparisons with a mixed feed containing 43% methanol and 57% sorbitol C-mol C-mol^{-1} were done in order to evaluate quantitatively the advantages of mixed feeds of sorbitol and methanol.

3. MATERIALS AND METHODS

3. 1. Microorganism, inoculum preparation and media

A recombinant *Pichia pastoris* Mut^{+} strain was kindly provided by Andrea Zocchi from the University of Neuchâtel (Switzerland). Zocchi et al. (2003) designed a clone of *P. pastoris* expressing and secreting a recombinant glycosylated avidin (recGAvi) with an acidic isoelectric point (pI = 5.4). Precultures were prepared in complex medium on 20 g L^{-1} glycerol at 30°C for 24h as described previously (Jungo et al. (2006)).

The defined medium used for fed-batch cultures contained per liter: 40 g glycerol, 26.7 mL H_3PO_4 85%, 0.93 g $CaSO_4$ $2H_2O$, 18.2 g K_2SO_4, 14.9 g $MgSO_4$ $7H_2O$, 4.13 g KOH, 0.8 g EDTA $2H_2O$, 4.35 mL of trace elements solution, 400 μL of a solution containing 1g L^{-1} biotin in NaOH 1M and 0.6 mL of antifoam agent (Struktol SB 2121, Schill and

Seilacher, Hamburg, Germany). The trace element solution contained per liter: 5 mL H_2SO_4 97%, 0.02 g H_3BO_3, 6 g $CuSO_4$ 5 H_2O, 0.08 g NaI, 3 g $MnSO_4$ H_2O, 0.2 g Na_2MoO_4 $2H_2O$, 0.5 g $CaSO_4$ 2 H_2O, 20 g $ZnCl_2$, 65 g $FeSO_4$ 7 H_2O.
All components of the defined media were obtained from Fluka, Buchs, Switzerland. The media were sterilized by filtration (0.22 μm, Steritop, Millipore Corporation, Billerica, USA).

3. 2. Culture conditions

Growth experiments were undertaken using a bench-scale heat-flux calorimeter RC1 (Mettler Toledo AG, Greifensee, CH) modified for biotechnological applications with a working volume of 1.6 L. The design and operation of this so called Bio-RC1 can be found in Marison et al. (1998a), Marison et al. (1998b).
Temperature was maintained at 30°C and pH at 5.0 by the automatic addition of 28% NH_4OH. A polarographic pO_2 probe (InPro 6800, Mettler Toledo, Greifensee, Switzerland) was used to monitor dissolved oxygen, with respect to air-saturated medium. The dissolved oxygen in the culture medium was maintained above 40% air saturation, through the addition of oxygen to the inlet gas flow. The total inlet gas flow was maintained constant (2 L min^{-1}) using thermal mass flow controllers (5850E, Rosemount Brooks, Veenendaal, The Netherlands). The gas was saturated with water immediately prior to entry in the bioreactor. A constant stirring rate of 800 rpm was employed throughout the experiments.
All liquid inlet flows were quantified gravimetrically and used to control continuously and on-line the feed pump rate.

3. 3. High cell density fed-batch cultures

Cultures were initially operated in batch mode on 40 g L^{-1} glycerol in 1.0 L of defined medium. After 15.5 h, all glycerol had been consumed (indicated by a sudden increase of the pO_2 signal) and the transition fed-batch phase on glycerol was performed according to Zhang et al. (2000). A 50% (g g^{-1}) glycerol solution supplemented with 12 mL of trace element solution per liter was fed at a constant rate of 20 g h^{-1} during one hour. Subsequently a pulse of 1.5 g of methanol was added and the glycerol feed rate decreased linearly from 20 g h^{-1} to 0 over 3 hours. Methanol consumption began about one hour after the pulse of methanol (indicated by a decrease of the pO_2 signal). As soon as all the pulsed methanol had been consumed (about 2.5 h after the pulse), an

exponential feed, determined to provide a constant growth rate, was begun. The feeding solution contained either methanol or a mixture of 43% C-mol C-mol^{-1} methanol and 57% C-mol C-mol^{-1} sorbitol in water (250 g sorbitol, 198.8 g methanol and 250 g water), supplemented with 12 mL of trace elements solution per liter of feeding solution. Previous results (chapter 7) showed indeed that at a specific growth rate of 0.03 h^{-1} the highest productivity in recombinant avidin is achieved with a methanol fraction of 43% C-mol C-mol^{-1} if a mixture of methanol and sorbitol is fed to the culture.
For a constant specific growth rate, the feeding rate, F(t) in g h^{-1}, was varied according to the following equation (Shuler and Kargi (2002)):

$$F(t) = \frac{\mu \cdot X_0 \cdot V_0}{S_f \cdot Y_{X/S}} \cdot e^{\mu \cdot t} \qquad (2)$$

where μ is the specific growth rate (h^{-1}), $Y_{X/S}$ the biomass yield (g g^{-1}), S_f the carbon source content in the feeding solution (g g^{-1}) and X_0 and V_0 are the dry cell weight and the culture volume at the beginning of the induction phase, respectively. In equation 2, time zero corresponds to the time when the exponential feed was begun.

3. 4. Substrate and metabolite analysis

Culture samples (about 10 mL) were collected using a purpose built auto-sampler (Cannizzaro (2002)) and kept at 2°C for up to 12h before handling.
The concentration of biomass was determined gravimetrically as dry cell weight. Samples were centrifuged in pre-weighed tubes, washed twice with ultrapure water and the pellet dried at 100°C to constant weight.
Glycerol, sorbitol and methanol were determined by HPLC analysis (1100 series, Agilent Technologies, Palo Alto, USA). An ion exchange chromatography column (Supelcogel H 300 mm, Supelco, Bellefonte, USA) with a guard column (Superlguard C610H, Supelco, Bellefonte, USA) was used at 60 °C. A 5 mM sulphuric acid solution in ultrapure water was applied at a constant eluent flow rate of 0.6 mL min^{-1}. Metabolites were measured using a refractive index detector. The detection limit was 0.01 g L^{-1} for glycerol and for sorbitol and 0.05 g L^{-1} for methanol.
The carbon dioxide evolution rate in the bioreactor off-gas was determined using an infrared analyser (series 2500, Servomex, Crowborough, UK). The measured values were corrected for water vapour according to Duboc and von Stockar (1998).

3. 5. Recombinant avidin quantification

Recombinant avidin was quantified by biotin-4-fluorescein titration of binding sites (Kada et al. (1999)), assuming a tetrameric form of recombinant avidin, with four active-binding sites.

Since the detection limit of this method is about 10 mg L^{-1} of avidin, it was necessary to concentrate certain samples by up to 15-fold by ultrafiltration (Centriplus, 30 kDa, Millipore Corporation, Bedford, USA) prior to titration with biotin-4-fluorescein.

At high cell densities (above 50 g L^{-1} dry cell weight) the total cell volume was not negligible compared with the culture volume. Therefore, the concentrations of recombinant avidin were calculated taking into account that the measured avidin concentrations during titration of biding sites were determined per volume of culture supernatant (equation 3) and not per total culture volume.

$$V_{Sup} = V - a \cdot V \cdot X \tag{3}$$

where V and V_{Sup} are the culture volume and the volume of the culture supernatant, X the cell dry weight (g L^{-1}). «a» is the cell volume per dry cell (0.0032 L g dry $cell^{-1}$) and was determined previously (Jungo et al. (2007)).

3. 6. Check of data consistency

On a C-molar basis, the black box stoichiometry was described by equation 4 for growth on methanol and by equation 5 for mixed substrate growth on 43% methanol and 57% sorbitol C-mol $C\text{-}mol^{-1}$ as carbon sources.

$$CH_4O + Y_{O2/S}\, O_2 + Y_{NH3/S}\, NH_3 \rightarrow Y_{X/S}\, CH_{1.691}O_{0.502}N_{0.176} + Y_{CO2/S}\, CO_2;\ \Delta_r H_S^{*\circ} \tag{4}$$

$$0.43\, CH_4O + 0.57\, CH_{14/3}O + Y_{O2/S}\, O_2 + Y_{NH3/S}\, NH_3 \rightarrow Y_{X/S}\, CH_{1.483}O_{0.574}N_{0.136} + Y_{CO2/S}\, CO_2;\ \Delta_r H_S^{*\circ} \tag{5}$$

S represents the limiting carbon source (methanol and/or sorbitol), $Y_{i/S}$ the stoichiometric coefficients in mole or C-mole per C-mole of substrate, where i can be S (substrate), O_2, CO_2, NH_3 or X (biomass). $\Delta_r H_S^{*\circ}$ is the standard molar enthalpy change caused by reactions described in equations 4 or 5 with CO_2, H_2O and NH_3 as reference state.

The C-molar composition of biomass was determined previously by elemental analysis (chapter 7).
The yield of substance j on substance i was calculated as

$$Y_{j/i} = \frac{n_j}{n_i} \quad (C-mol/C-mol) \tag{6}$$

where n_i and n_j are the total cumulated quantities of substances i and j respectively.
In order to calculate the total cumulated quantity n_i of substance i each time a sample was taken, mass balances for each measured component were set-up according to (Duboc and von Stockar (1995)) arround the system boundary corresponding to the reactor envelope:

$$\text{In - Out + Reaction = Accumulation} \tag{7}$$

$$Q_{in} \cdot C_{i,\,in} - Q_{out} \cdot C_{i,\,out} + R_i \cdot V = \frac{d(C_{i,\,out} \cdot V)}{dt} \tag{8}$$

where Q_{in} and Q_{out} are the liquid flows entering and leaving the bioreactor respectively in L h^{-1}, $C_{i,in}$ and $C_{i,out}$ are the concentrations of substance i entering and leaving the bioreactor in C-mol L^{-1}, R_i is the volumetric production or consumption rate of substance i in C-mol L^{-1} h^{-1} and V is the culture volume in L. During fed-batch culture, the outlet term can be left out.
During fed-batch experiments, the accumulation term between the $(k-1)^{th}$ and the k^{th} observation is not negligible. The mass balance equation was integrated between two observations in order to account for the cumulated amount of compound i produced or consumed between two observations. The following equation is obtained between two observations at times t_{k-1} and t_k (Duboc and von Stockar (1995)):

$$\Delta n_i(t_{k-1} \rightarrow t_k) = (V \cdot C_{i,\,out})_{t_k} - (V \cdot C_{i,\,out})_{t_{k-1}} - \int_{t_{k-1}}^{t_k} (Q_{in} \cdot C_{i,\,in} \cdot dt) \tag{9}$$

The inlet term of equation 9 was calculated by the trapezoidal rule.

Increments Δn_i were summed up between the first observation k = 1 (corresponding to the beginning of the induction phase) and the last observation k, leading to the total cumulated quantity $n_i(k)$ expressed in C-mol.

$$n_i(k) = \Delta n_i(t_1 \rightarrow t_2) + \Delta n_i(t_2 \rightarrow t_3) + \ldots + \Delta n_i(t_{k-1} \rightarrow t_k) \quad (10)$$

It has to be noted that the total cumulated quantity n_i accounts for the amount of compound i that has been consumed or produced during the induction phase and not to the number of moles of i present in the reactor.

The total cumulated quantity of produced carbon dioxide was calculated by integration of the carbon dioxide production rate according to the following equation:

$$n_{CO_2} = \int_{t_0}^{t_k} V \cdot R_{CO_2} dt \quad (11)$$

The measurements of dry cell weight, carbon source concentrations and carbon dioxide in the exhaust gas were used in order to calculate two of the five yields of the black box stoichiometry ($Y_{NH3/S}$, $Y_{O2/S}$, $Y_{X/S}$, $Y_{CO2/S}$ and $Y_{Q/S}$), namely biomass and carbon dioxide yields ($Y_{X/S}$, $Y_{CO2/S}$). Together with the constraints provided by the carbon, nitrogen, degree of reduction and enthalpy balances a redundancy of 1 resulted. The redundancy of the system was used in order to estimate the unknown yields and to reconcile the data. $Y_{Q/S}$ represents the molar enthalpy change caused by reactions described in equations 4 and 5 per C-moles of substrate.

The yields were checked for consistency and reconciled based on a χ^2-test (Wang and Stephanopoulos (1983); Stephanopoulos et al. (1998)). The resulting reconciled yield coefficients are the best yield estimates that minimize the magnitude of the residuals ε and are determined by minimizing the sum of squared errors scaled according to their variance. The test value h is given by the sum of the weighted squares of the residuals ε:

$$h = \varepsilon \cdot P^{-1} \cdot \varepsilon \quad (12)$$

where P is the variance-covariance matrix. Assuming measurement errors of 5%, a test value h lower than 3.84 for a redundancy of 1 permitted to assume a 95% significance

level that no gross errors and no metabolites other than the ones appearing in equations 4 and 5 had affected the balances.

3. 7. Model development for calculation of the performance of recombinant avidin in fed-batch cultures

During mixed substrate growth, the contribution of both methanol and sorbitol to cell growth must be considered. In order to predict the amount of recombinant avidin produced during the culture, the biomass concentration was calculated according to the following equation:

$$X(t) = \frac{X_0 \cdot V_0 + m_X{}^{MeOH} + m_X{}^{Sorbitol}}{V(t)} \tag{13}$$

where $m_X{}^{Methanol}$ and $m_X{}^{Sorbitol}$ are the produced amounts of biomass due to growth on methanol and sorbitol, respectively, and can be defined by the following equations:

$$m_X{}^{MeOH} = Y_{X/MeOH} \cdot \left(f_{MeOH,\,in} \cdot \int_{t_0}^{t} F(t)dt - C_{MeOH}(t) \cdot V(t) \right) \tag{14}$$

$$m_X{}^{Sorbitol} = Y_{X/Sorbitol} \cdot \left(f_{Sorbitol,\,in} \cdot \int_{t_0}^{t} F(t)dt - C_{Sorbitol}(t) \cdot V(t) \right) \tag{15}$$

where $Y_{X/MeOH}$ and $Y_{X/Sorbitol}$ are the biomass yields for growth on methanol and sorbitol, respectively, $f_{MeOH,in}$ and $f_{Sorbitol,in}$ are the fractions of methanol and sorbitol in the feeding solution (g g^{-1}), respectively, C_{MeOH} and $C_{Sorbitol}$ are the residual concentrations of methanol and sorbitol in the bioreactor, respectively. The amount of recombinant avidin produced during the induction phase can be calculated according to equation 1.

In equations 13 to 15, time zero corresponds to the beginning of the induction phase. The performance of avidin was calculated in mg h^{-1} according to the following equation:

$$L_P = \frac{m_P}{t_d + t_{culture}} \tag{16}$$

where m_P is the amount of avidin produced (equation 1), $t_{culture}$ is the total culture time in h, t_d the down time. A down time of 24 h was assumed in calculations.

4. RESULTS AND DISCUSSION

4. 1. Influence of specific growth rate on the performance of avidin

Previous investigations with the studied strain showed that the specific recombinant avidin production rate increased linearly with the specific growth rate in chemostat cultures on methanol (Jungo et al. (2006)). A growth associated linear model was established for a range of specific growth rates from 0.03 to 0.12 h^{-1}. The results obtained during this previous study and the corresponding linear regression are represented in figure 1 as a function of the specific growth rate (solid circles and straight line, respectively). The influence of the specific growth rate on the specific avidin productivity has also been investigated at low specific growth rates with the same strain in fed-batch cultures (Schenk et al. (2007)). The results obtained at specific growth rates of 0.001 and 0.002 h^{-1} are also shown in figure 1 (open circles) and a logarithmic regression (figure 1) was applied taking into account the result obtained in chemostat culture on methanol at a specific growth rate of 0.03 h^{-1} (Jungo et al. (2006)).

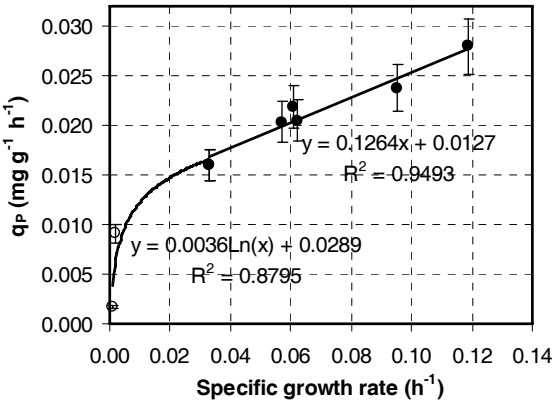

Figure 1: Specific avidin productivity q_P according to specific growth rate. ●: Data measured during chemostat cultures on methanol (Jungo et al. (2006)), ○: Data determined at low specific growth rate during fed-batch cultures on methanol (Schenk et al. (2007)). Logarithmic and linear regressions have been applied in order to describe the specific avidin productivity as a function of the specific growth rate below and above 0.03 h^{-1} respectively.

The logarithmic and linear regressions presented in figure 1 for specific growth rates below and above 0.03 h^{-1}, respectively, were used in order to calculate the performance of avidin (equation 16), which would be achieved in high cell density fed-batch cultures operated as described in materials and methods. Simulations were performed considering a reactor of 2 liters with a working volume of 1.6 L with methanol as sole carbon source during the induction phase. The yield coefficients determined previously in chemostat cultures (Jungo et al. (2006)) were used for calculations. The simulated performances of avidin are shown in figure 2 as a function of the specific growth rate during the induction phase on methanol. For comparison, results for a down time of 12, 24 and 36 hours are represented in figure 2. It can be noted that the optimal specific growth rate at which the maximal performance of avidin is achieved does not significantly depend on the down time. Maximal performance of avidin was always achieved for specific growth rates between 0.02 and 0.04 h^{-1}.

The end of the simulated fed-batch cultures was determined by technical constraints resulting from the design of the bioreactor used in this study. One constraint was the

maximal culture volume, which was 1.6 L. Another constraint was the maximal cooling capacity of the bioreactor, which was 130 W L^{-1}. Since the heat produced by stirring was approximately 10 W L^{-1} under the studied experimental conditions, the maximal heat production rate due to cellular growth was set at 120 W L^{-1} for simulations. As a first approximation, the assumption was made that the heat production can be considered to be due to cellular growth and stirring only.

Although the specific avidin production rate is increasing with the specific growth rate (figure 1), figure 2 shows that the performance of avidin is the highest for a specific growth rate close to 0.03 h^{-1} during the induction phase. Indeed, at low specific growth rates during the induction phase on methanol, technical constraints (such as maximal cooling capacity, maximal culture volume) are achieved later. Therefore, the induction phase lasts longer and according to equation 1, higher amounts of avidin can be produced. At high specific growth rates during the induction phase on methanol the culture has to be stopped after a few hours because of, for instance, the achievement of maximal cooling capacity (or another technical constraint) and it results in the production of lower amounts of recombinant protein per unit time of culture process.

For simulations presented in figure 2, the end of the culture was determined by the maximal cooling capacity for specific growth rates higher than 0.06 h^{-1}, by the maximal culture volume of 1.6 L for specific growth rates between 0.04 and 0.06 h^{-1}, and by the maximal dry cell weight concentration of 160 g L^{-1} for specific growth rates lower than 0.04 h^{-1}. As a matter of fact, the achievement of 160 g L^{-1} dry cell weight was considered as an additional constraint in simulations, because to our knowledge, this is the highest biomass concentration reported in literature with *P. pastoris* cultures (Zocchi et al. (2003); Jahic et al. (2002)).

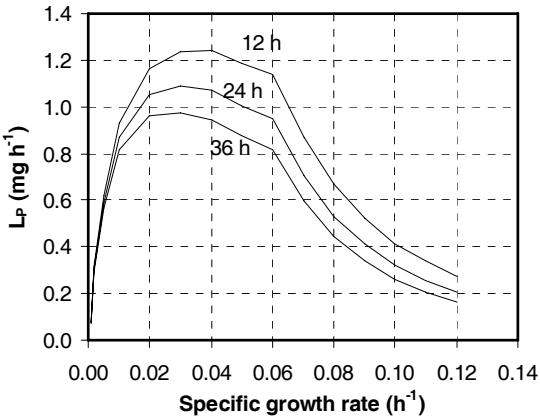

Figure 2: Performance of avidin (L_P) achieved in high cell density fed-batch cultures, calculated according to data obtained in Jungo et al. (2006) and Schenk et al. (2006) as a function of the specific growth rate set during the induction phase with an exponential feed of methanol. Simulations were done for down times of 12, 24 and 36 hours.

In order to confirm the results based on simulations with data obtained during chemostat cultivation and presented in figure 2, high cell density fed-batch cultures were performed at a specific growth rate of 0.03 and 0.01 h^{-1} during the induction phase. Experiments were performed at the optimal specific growth rate of 0.03 h^{-1} and also at 0.01 h^{-1} in order to verify the logarithmic regression obtained with only 3 measurements from two different sources (Jungo et al. (2006); Schenk et al. (2007))

In order to examine the advantages of mixed feeds of methanol and sorbitol during the induction phase in place of methanol alone as carbon source, induction phases were performed on either methanol or on a mixed feed containing 43% C-mol C-mol^{-1} methanol and 57% C-mol C-mol^{-1} sorbitol. This methanol fraction was chosen because results obtained in chapter 7 of this thesis showed that maximal recombinant avidin productivity was achieved at a methanol fraction of 43% C-mol C-mol^{-1} for a dilution rate of 0.03 h^{-1}.

4. 2. High cell density fed-batch cultures with exponential feed of methanol or a mixed feed of methanol and sorbitol during the induction phase

The profiles of dry cell weight, glycerol and methanol concentrations and amount of recombinant avidin per volume of culture supernatant are presented in figures 3A and 3B for a specific growth rate of 0.03 h^{-1} during the induction phase on methanol and on a mixed feed containing 43% C-mol C-mol^{-1} methanol and 57% C-mol C-mol^{-1} sorbitol respectively. Figures 4A and 4B show the corresponding results obtained at a specific growth rate of 0.01 h^{-1} during the induction phase. During mixed substrate growth on methanol and sorbitol, both substrates were always consumed simultaneously to completion.

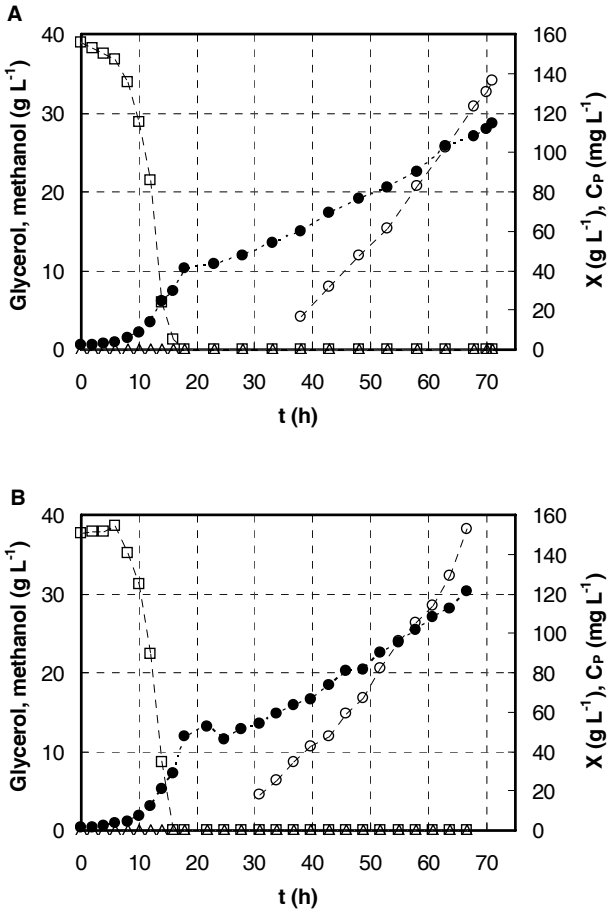

Figure 3: Concentrations of dry cell weight X (●), glycerol (□) and methanol (△), and amount of avidin (○) per volume of cell-free culture supernatant during high cell density fed-batch cultures at a specific growth rate of 0.03 h^{-1} during the induction phase. Figure A: with a feed of methanol during the induction phase. Figure B: with a feed containing 43% methanol and 57% sorbitol C-mol C-mol^{-1}.

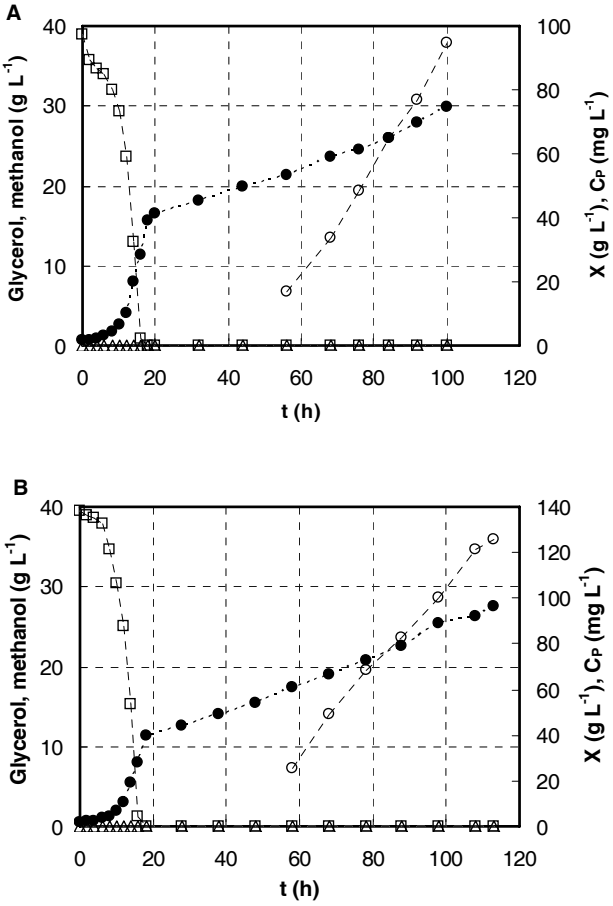

Figure 4: Concentrations of dry cell weight X (●), glycerol (□) and methanol (△), and amount of avidin (○) per volume of cell-free culture supernatant during high cell density fed-batch cultures at a specific growth rate of 0.01 h^{-1} during the induction phase. Figure A: with a feed of methanol during the induction phase. Figure B: with a feed containing 43% methanol and 57% sorbitol C-mol C-mol^{-1}.

The effective specific growth rate, the reconciled yield coefficients and the statistical test values h calculated with reconciliation procedures during the induction phase are summarized in table 1 for the four high cell density fed-batch cultures. The statistical

test was always passed for a confidence level of 95%, the test values h being always lower than the threshold value of 3.84.

Table 1: Specific growth rate, reconciled yields and test values h calculated with reconciliation procedures during induction phase of fed-batch cultures on methanol (MeOH) or on a mixture of 43% C-mol C-mol^{-1} methanol and 57% C-mol C-mol^{-1} sorbitol (MeOH/Sorbitol). The threshold value to reject hypothesis with a 95% confidence level and a redundancy of 1 is of 3.84.

Substrate for induction phase	Specific growth rate (h^{-1})	$Y_{X/S}$	$Y_{CO2/S}$	$Y_{O2/S}$	$Y_{NH3/S}$	$Y_{Q/S}$	h
			(C-mol C-mol^{-1})			(kJ C-mol^{-1})	
MeOH	0.029	0.31	0.70	1.21	0.05	-585	0.06
MeOH	0.009	0.18	0.83	1.34	0.03	-650	2.93
MeOH/Sorbitol	0.028	0.39	0.53	0.86	0.05	-369	2.93
MeOH/Sorbitol	0.011	0.29	0.77	1.15	0.04	-497	2.31

The specific recombinant avidin production rates determined during the induction phase of these four high cell density fed-batch cultures are represented in figure 5 (open symbols). It has to be realized that the avidin concentration measured in the cell-free supernatant is significantly higher than in the culture medium because, in high cell density cultures, a substantial fraction of the culture volume is occupied by biomass. The specific avidin production rate was calculated taking into account that the measured avidin concentrations were determined per volume of culture supernatant and not per total culture volume. Equation 3 was applied in order to estimate the volume of the culture supernatant at a given dry cell weight concentration.

Good agreement was obtained between specific avidin productivities determined during high cell density fed-batch cultures and the logarithmic and linear regressions presented in figure 5, obtained with data from chemostat cultures on methanol (Jungo et al. (2006)) and from data obtained by Schenk et al. (2007). Moreover, both at 0.01 and 0.03 h^{-1}, good agreement was obtained between data obtained with a feed of methanol as sole carbon source and with a mixed feed of methanol and sorbitol during the induction phase.

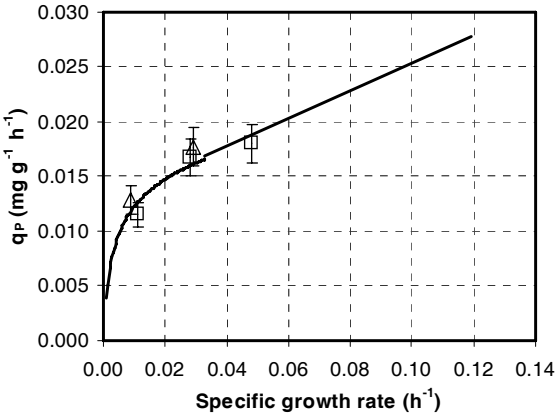

Figure 5: Specific avidin productivity q_P according to specific growth rate. The continuous lines represent the logarithmic and linear regressions obtained with data from Schenk et al. (2007) and Jungo et al. (2006). Open symbols: Data obtained during the induction phase of high cell density-fed batch cultures on methanol (△) or on a mixed feed containing 43% C-mol C-mol^{-1} methanol and 57% C-mol C-mol^{-1} sorbitol (□).

The performance of recombinant avidin, achieved with the bioreactor system used in this study if cultures would all have been performed until the achievement of one of the technical constraints (maximal heat production rate of 120 W L^{-1}, maximal culture volume of 1.6 L or maximal dry cell weight concentration of 160 g L^{-1}) were calculated with data summarized in table 1 and the specific avidin productivities measured during fed-batch cultures (open symbols in figure 5). The results of the calculated performances of avidin are shown in figure 6 for comparison. A down time of 24 hours was assumed.

For mixed substrate feeds of methanol and sorbitol, the amount of water in the feed medium was minimized in order to achieve the highest performance of avidin. For a mixed feed containing 43% C-mol C-mol^{-1} methanol and 57% C-mol C-mol^{-1} sorbitol, the minimal fraction of water in order to dissolve all the sorbitol was of 0.1 g g^{-1}.

Since the cooling capacity was quite high, the constraint on culture volume was achieved first for the two cultures on methanol during the induction phase. For the two mixed

substrate cultures, the end of the culture was considered to correspond to the achievement of 160 g L^{-1} dry cell weight, which corresponds to the highest biomass concentration reported in literature with *P. pastoris* cultures (Zocchi et al. (2003); Jahic et al. (2002)).

It can be noted that the performance of avidin calculated with data from high cell density fed-batch cultures on methanol (figure 6) is about 10% lower than with data from chemostat cultures on methanol (figure 2). This difference is due to the fact that the measured biomass yields were about 30% lower during high cell density fed-batch cultures than during chemostat cultures (Jungo et al. (2006)). A nutrient limitation can be excluded because addition of higher amounts of trace elements did not increase biomass yield coefficients and it was verified that basal salts were present in excess. A higher substrate consumption rate for maintenance purposes at high cell densities could explain the lower biomass yields obtained during high cell density cultures.

According to the results presented in figure 6, the performance of recombinant avidin is higher at a specific growth rate of 0.03 h^{-1} than at 0.01 h^{-1}, as was predicted with simulations presented in figure 2 for a down time of 24 hours. Moreover, it can be noted in figure 6 that the performance of recombinant avidin is indeed higher with a mixed feed of methanol and sorbitol than with a feed of methanol as sole carbone source.

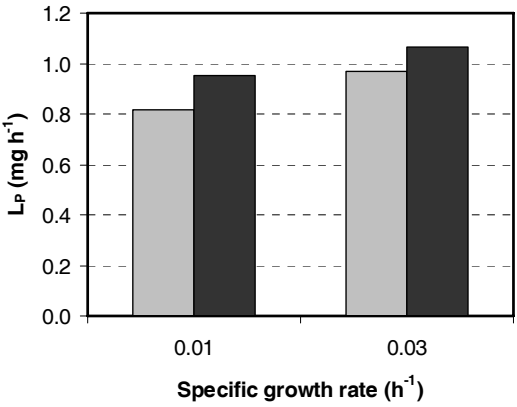

Figure 6: Performance of avidin (L_P) achieved during high cell density fed-batch cultures with a feed of methanol (grey) or a mixed feed containing 43% C-mol C-mol^{-1} methanol and 57% C-mol C-mol^{-1} sorbitol during the induction phase (black). Results are presented for specific growth rates during the induction phase of 0.01 and 0.03 h^{-1}.

Although the increase in the performance of recombinant avidin is not very significant (1.10-fold increase at 0.03 h^{-1} and 1.17-fold increase at 0.01 h^{-1}) with a mixed feed compared with methanol as sole carbon source, use of mixed feeds of methanol and sorbitol present technical advantages. Indeed, heat production and oxygen consumption rates, which are proportional in aerobic cultures (Roels (1983)), were about 40 % lower during mixed substrate growth than on methanol as sole carbon source for a given dry cell weight concentration. For example, the heat production rate due to growth achieved at the end of the high cell density fed-batch cultures presented in figures 3A and 3B at a specific growth rate of 0.03 h^{-1} during the induction phase were 87 W L^{-1} with a feed of methanol and 36 W L^{-1} with a mixed feed containing 43% C-mol C-mol^{-1} methanol and 57% C-mol C-mol^{-1} sorbitol. This reduction in heat production rate is especially useful at large scale since heat losses through the bioreactor walls may be limiting at large scale due to the smaller surface area to volume ratio. Oxygen transfer rate is also often a limiting factor in high cell density *P. pastoris* cultures, even at small scale. Although addition of pure oxygen was still necessary during the induction phase

on methanol and sorbitol at high cell densities, oxygen consumption rate could be significantly diminished without affecting recombinant avidin productivity.

Recently, the annual market of recombinant avidin has been estimated at 2 kg by the avidin producing company Belovo SA, Belgium (personal communication). The market size is the key factor that determines the industrial scale on which a heterologous protein is produced. Usually, high-added value proteins (> \$1000 kg^{-1}) such as recombinant avidin are produced at 0.1-5 m^3 scale (Hensing et al. (1995)). Even at this rather small industrial scale, maximal cooling capacities through the reactor jacket rarely exceed 20 kW m^{-3} (Kieran (2001)). In the last ten years, the scale of industrial reactors increased with high cell density microbial cultures for the production of foreign proteins of up to 75 m^3 (Hoeks et al. (2005)). At this scale, maximum cooling capacities through the reactor jacket is about 7.5 kW m^{-3} (Kieran (2001)). Although special cooling facilities can be installed to control the reactor temperature properly, any reduction in heat production rate without reduction of the recombinant protein productivity represents an interesting technical advantage.

Lower heat production and oxygen consumption rates can also be obtained with mixed feeds of methanol and glycerol during the induction phase (Jungo et al. (2007)) without reduction of specific avidin production rate. However, since the enthalpy of combustion and the degree of reduction of sorbitol are about 8% lower than the ones on glycerol (Weast (1980)), heat production and oxygen consumption rates are further reduced on mixed feeds with methanol and sorbitol. Moreover, by contrast with glycerol, sorbitol is a non-repressive carbon source with respect to AOX promoter (Thorpe et al. (1999); Boze et al. (2001); Inan and Meagher (2001a); Xie et al. (2005)). Hence, transient accumulation of sorbitol is not critical with respect to recombinant protein productivity.

In order to verify that sorbitol accumulation in the culture medium does not alter the recombinant protein productivity, a high cell density fed-batch culture on a mixed feed containing 43% methanol and 57% C-mol C-mol^{-1} sorbitol was performed at a specific growth rate of 0.05 h^{-1} during the induction phase. Since the maximal specific growth rate on sorbitol is 0.032 h^{-1}, sorbitol should only be consumed partially and should accumulate in the culture medium.

The profiles of dry cell weight, glycerol, methanol and sorbitol concentrations and of the amount of avidin per volume of culture supernatant are shown in figure 7. The theoretical curve of sorbitol accumulation, if sorbitol was not consumed at all by the cells was

also represented in figure 7 (continuous line). It shows clearly that sorbitol was consumed by the cells but not completely. Methanol was on the other hand consumed to completion. The residual methanol concentrations were always below the detection limit of the HPLC method used for the quantification of methanol. The culture was therefore single-limited in methanol as carbon source.

The specific avidin production rate during the induction phase was 0.018 mg g^{-1} h^{-1}. This value was also represented in figure 5 for comparison with results obtained during methanol-limited growth conditions in chemostat cultures on methanol without sorbitol. The measured specific avidin production rate was in agreeement with the one predicted by the linear regression obtained with data from chemostat cultures on methanol. It can therefore be concluded that sorbitol is indeed a non-repressing carbon source with respect to the AOX1 promoter. Accumulation of sorbitol in the culture medium does not affect recombinant protein productivity. This confirms the results obtained by Thorpe et al. (1999) and the conclusions of the preceding chapter of this thesis.

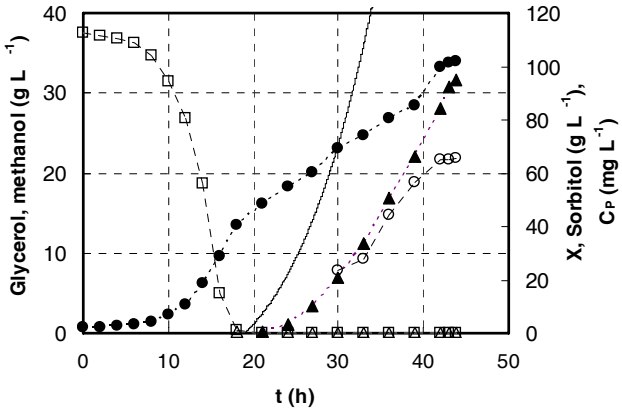

Figure 7: Concentrations of dry cell weight X (●), glycerol (□) methanol (△) and sorbitol (▲), and amount of avidin (❍) per volume of cell-free culture supernatant during high cell density fed-batch culture at a specific growth rate of 0.05 h^{-1} during the induction phase, with a feed containing 43% methanol and 57% sorbitol C-mol $C\text{-mol}^{-1}$. The continuous line represents the theoretical curve of sorbitol accumulation, if sorbitol was not consumed at all.

4. 3. Influence of the transition phase on glycerol on the performance of avidin

The fed-batch phase on glycerol is a transition phase in order to further increase biomass concentration after batch growth on glycerol. Moreover, growth at limiting concentrations of glycerol allows derepression of methanol dissimilating enzymes and reduces the time necessary for the cells to adapt to growth on methanol. As a matter of fact, Chiruvolu et al. (1997) found that addition of a transition phase reduced the total induction time of 15 h for a *P. pastoris* Mut^+ strain.

All fed-batch experiments presented in this study were performed with a transition phase proposed by Zhang et al. (2000), at the end of which about 42 g L^{-1} of dry cell weight were achieved. In order to evaluate the influence of the length of the transition phase on the performance of recombinant protein, the performance of avidin was calculated for various final biomass concentrations achieved at the end of the transition phase. It was assumed that the transition phase was performed with an exponential feed of glycerol (50% w w^{-1}) at a specific growth rate of 0.22 h^{-1}, close to the maximal specific growth rate on glycerol (0.24 h^{-1}). A 4 h adaptation time was assumed at the beginning of the induction phase since in these calculations it is assumed that the traditionnal method of complete glycerol exhaustion followed by a feed containing methanol is used (Stratton et al. (1998)).

The performance of avidin was calculated for an induction phase performed on a mixed feed of 43% C-mol C-mol^{-1} methanol and 57% C-mol C- mol^{-1} sorbitol at a constant specific growth rate of 0.03 h^{-1} during the induction phase since the preceding simula-

tions and experiments showed that optimal performances are achieved in this way. The results are presented in figure 8.

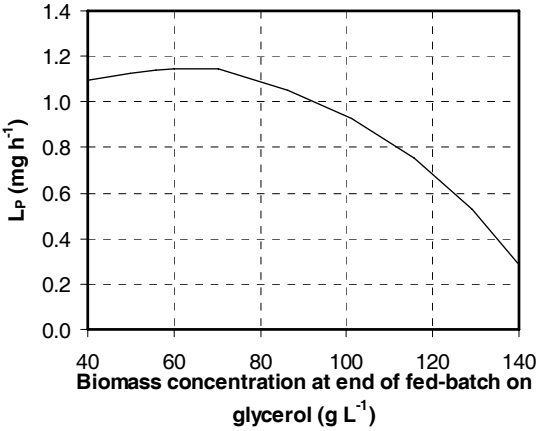

Figure 8: Simulated performance of avidin achieved in high cell density fed-batch cultures as a function of the biomass concentration achieved at the end of the transition fed-batch phase on glycerol. It was assumed that the fed-batch phase on glycerol is performed with an exponential feed of glycerol (50% w w^{-1}) at a constant specific growth rate of 0.22 h^{-1}.

The constraints were the same as during previous simulations: maximal heat production rate of 120 W L^{-1}, maximal culture volume 1.6 L and maximal dry cell weight concentrations of 160 g L^{-1}. For dry cell weight concentrations lower than 85 g L^{-1} at the end of the transition phase, the biomass concentration was the first limiting factor and for dry cell weight concentrations higher than 85 g L^{-1} at the end of the transition phase, the culture volume was the first limiting factor. The maximal reactor cooling capacity was never reached during simulations.

Figure 8 shows that the performance of avidin is increased by about 4% if the transition phase on glycerol is performed until 70 g L^{-1} dry cell weight instead until 40 g L^{-1}. If higher biomass concentrations are achieved at the end of the transition phase, lower performances in avidin are obtained. As the length of the transition phase is increased, higher biomass concentrations are achieved at the beginning of the induction phase. Hence, more cells can synthesize the recombinant protein. However, the length of the

induction phase is shortened and the overall performance in avidin is reduced if higher biomass concentrations are achieved during the transition phase on glycerol.

It has to be pointed out that in this analysis the influence of performing the transition phase on glycerol at a relatively high specific growth rate was not considered. According to the literature (Inan et al. (1999)), feeding of glycerol at high specific growth rates can lead to accumulation of ethanol. It was shown that residual ethanol concentrations repress the alcohol oxidase promoter, even at levels of around 10-50 mg L^{-1} (Inan and Meagher (2001b)). This could significantly increase the adaptation time at the beginning of the induction phase.

For this reason and since the estimated increase in the performance of avidin was only of about 4%, a transition phase on glycerol up to 70 g L^{-1} biomass at a high specific growth rate does not seem advantageous. The transition phase proposed by Zhang et al. (2000) and used in this study during fed-batch experiments seems a good strategy for the production of recombinant proteins with *P. pastoris* strains in fed-batch cultures. In particular, the addition of methanol already during the transition phase with a pulse of methanol allows faster adaptation of the cells at the beginning of the induction phase. Indeed, as was shown in chapter 5, during mixed substrate growth, the alcohol oxidase enzyme is not only derepressed but also induced, which will allow faster adaptation at the beginning of the induction phase for the production of recombinant protein.

5. CONCLUSIONS

The influence of the specific growth rate and the composition of the feed medium (methanol or a mixed feed of methanol and sorbitol) was investigated on the performance of recombinant avidin. Simulations of the performance of avidin in high cell density fed-batch cultures and verification with a few experiments in fed-batch cultures showed that higher performances of recombinant avidin are achieved at low specific growth rates (about 0.03 h^{-1}). Indeed, although the specific avidin production rate increases with the specific growth rate, at high growth rates technical constraints such as maximal cooling capacity, maximal oxygen transfer rate, maximal reactor volume or maximal cell density are achieved earlier during the culture, which has therefore to be stopped. At low specific growth rates, the induction phase lasts longer and higher performances of recombinant avidin can be achieved.

Moreover, it was shown that mixed feeds of methanol and sorbitol are more advantageous than methanol as sole carbon source because of lower heat production rate and lower oxygen consumption rate. In addition, the performance of avidin was slightly increased with mixed feeds (about 10% increase for a given specific growth rate) due to higher biomass yields on mixed methanol and sorbitol than on methanol.

Finally, since higher performances in recombinant avidin are achieved at low specific growth rates (close to 0.03 h^{-1}), the low maximal specific growth rate on sorbitol is not a drawback compared to glycerol, with which higher specific growth rates can be achieved if used as additional carbon source to methanol. Furthermore, mixed feeds of methanol and sorbitol are more advantageous than mixed feeds of methanol and glycerol because, by contrast with glycerol, sorbitol is a non-repressing carbon source. Indeed, it was shown that accumulation of sorbitol during the induction phase did not affect the specific avidin production rate. This is especially interesting for large-scale *P. pastoris* cultures where transient substrate accumulation can result from imperfect mixing.

6. ACKNOWLEDGEMENTS

Financial support from the Swiss National Science Fundation is gratefully acknowledged.

7. NOMENCLATURE

AOX	Alcohol oxidase	
a	Cell volume per dry cell	L g dry-cell^{-1}
C_i	Concentration of i in bioreactor	C-mol L^{-1}
C_{MeOH}	Methanol concentration in bioreactor	C-mol L^{-1}
$C_{Sorbitol}$	Sorbitol concentration in bioreactor	C-mol L^{-1}
C_P	Concentration of avidin	mg L^{-1}
D	Dilution rate	h^{-1}
F	Feeding rate of medium during fed-batch phase	g h^{-1}
$f_{MeOH,in}$	Fractions of methanol in the feed during fed-batch cultures	g g^{-1}
$f_{Sorbitol,in}$	Fractions of sorbitol in the feed during	

	fed-batch cultures	$g\ g^{-1}$
h	Statistical test value	
$\Delta_r H_S^{*\circ}$	Standard molar enthalpy change caused by reaction r per mole of subtrate S	$kJ\ C\text{-}mol^{-1}$
L_P	Performance of avidin	$mg\ h^{-1}$
n_i	Cumulated quantity of compound i	C-mol
m_P	Amount of recombinant avidin	mg
$m_X^{Methanol}$	Amount of biomass due to growth on methanol	g
$m_X^{Sorbitol}$	Amount of biomass due to growth on methanol	g
P	Variance-covariance matrix	
Q_{in}	Liquid flow entering the bioreactor	$L\ h^{-1}$
Q_{out}	Liquid flow leaving the bioreactor	$L\ h^{-1}$
q_P	Biomass-specific avidin productivity	$mg\ g^{-1}\ h^{-1}$
R_i	Volumetric production or consumption rate of i	$C\text{-}mol\ L^{-1}\ h^{-1}$
S_f	Carbon source content in the feeding solution	$g\ g^{-1}$
$t_{culture}$	Total culture time	h
t_d	Down time	h
V	Culture volume	L
V_{Sup}	Volume of culture supernatant	L
X	Cell dry weight	$C\text{-}mol\ L^{-1}$
$Y_{j/i}$	Yield coefficient of substance j on substance i	$C\text{-}mol\ C\text{-}mol^{-1}$
ε	Residuals	
μ	Specific growth rate	h^{-1}

Subscripts

i	Refers to compound i
j	Refers to compound j
S	Refers to the limiting nutrient
P	Refers to the produced recombinant avidin
Q	Refers to heat
X	Refers to the biomass
0	Initial condition

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CHAPTER 9

GENERAL CONCLUSIONS AND PERSPECTIVES

The present work has characterized the growth stoichiometry, the specific recombinant avidin production rate and the regulation of the alcohol oxidase enzyme of a *Pichia pastoris* Mut$^+$ strain. In particular, the influence of the specific growth rate, of the culture temperature and the use of mixed substrates during the induction phase was studied in order to optimize the productivity in recombinant avidin. Methodological aspects for the quantitative, rapid and rational characterization of host cells have been addressed. Strain characterization was performed in continuous cultures under steady-state or transient growth conditions. The performance of chemostat and transient continuous cultures enabled better insights into the physiology of the *Pichia pastoris* expression system. In particular, this study pointed out several advantages of feeding mixed substrates during the induction phase.

1. CONCLUSIONS OF THIS THESIS

1. 1. Methodology

Control of environmental conditions is essential to physiological experimentation: growth conditions have to be clearly defined in order to be able to carry out well-defined and reproducible experiments. Physiological studies are often performed in batch cultures, but even if the batch culture is convenient and looks easy, from a physiological point of view, it is highly dynamic and difficult to control. Continuous cultures, on the other hand, allow to grow cells under defined conditions and to a different extent by varing the dilution rate. Moreover, continuous cultures are highly suited for the study of cell adaptation to changes in environmental parameters. They allow a tight control of cultivation conditions and hence of cell physiology and permit the careful design of transient experiments.

In the present work, pseudo-steady state continuous cultures with linear changes in cultivation temperature (chapter 4), or in nutrient concentrations in the feed medium (chapters 6 and 7), were performed and evaluated for the characterization of host cells.

Comparison with results obtained during chemostat cultures or during high cell density fed-batch cultures and evaluation of the specific growth rate during transient experiments confirmed that pseudo-steady state continuous cultures are efficient tools for the accurate and rapid characterization of host cells.

Moreover, dynamic transient continuous cultures (chapter 5) with sudden changes in carbon sources (glycerol/methanol) and pulses of methanol enabled a better insight into the regulation of the expression of alcohol oxidase, a key enzyme during growth on methanol, which is involved in the first oxidation step of methanol metabolism.

Based on data obtained during transient continuous cultures under pseudo-steady state or dynamic growth conditions, the strategy of fed-batch cultures for the production of recombinant proteins with *P. pastoris* could be improved (chapter 8).

A further important point is that the literature relating to recombinant *P. pastoris* strains, suggests that optimal growth conditions for maximal recombinant protein productivity is strain specific and, in particular, recombinant protein specific. With this in mind, pseudo-steady state continuous cultures are efficient tools to find the optimal value of a culture parameter rapidly. For the optimization of several culture parameters at a time, the use of statistical experimental designs would be an added tool for rational and rapid determination of optimal growth conditions.

1. 2. Advantages of mixed substrates

In many natural environments the growth of microorganisms takes place in the presence of low concentrations of a diversity of mixed substrates. Simultaneous assimilation of several substrates is usually observed at low substrate concentrations (substrate-limiting growth conditions) as a result of relief of catabolite repression (derepression) and/or induction of the synthesis of catabolic enzymes. Thus, the ability to utilize several substrates simultaneously at low residual substrate concentrations confers a competitive advantage in natural environments.

In biotechnological production processes, the feed of a single limiting nutrient is usually controlled. Feeding of mixed substrates could however be used to combine and take advantage of the features of several substrates simultaneously. The present work (chapters 5, 6, 7 and 8) showed that feeding of mixed substrates during the induction phase with recombinant *P. pastoris* strains presented many advantages (higher productivities of recombinant protein, lower heat production and oxygen consumption rates, faster adaptation of cellular metabolism at the beginning of the induction phase).

Egli and coworkers have pointed out some advantages of using mixed substrates with methylotrophic yeasts in the 1980's. However, industrial applications taking advantage of mixed substrates are still scarce to our knowledge. This is simply because laboratory experiments are usually performed with one limiting substrate, while forgetting that in nature the growth conditions microorganisms meet involve mixed substrates requiring a high flexibility and adaptation capacity of the cellular metabolism.

One important part of the present work focused on the investigation of the advantages of mixed substrates for the production of recombinant proteins with the *P. pastoris* expression system. The feeding of glycerol and methanol and the feeding of sorbitol and methanol in place of methanol as sole carbon source were evaluated.

Although sequential utilisation of glycerol and methanol was observed during batch culture (chapter 5), both substates were consumed simultaneously and completely during carbon-limited continuous and fed-batch cultivation (chapter 6). Sorbitol and methanol were also consumed simultaneously to completion during carbon-limited continuous and fed-batch cultures (chapters 7 and 8). In carbon-limited continuous or fed-batch cultures, residual concentrations of carbon sources are usually in the $\mu g\ L^{-1}$ range, depending on the dilution rate applied. At such low substrate concentrations, catabolite repression (for instance by glycerol) of methanol dissimilating enzymes is relieved and simultaneous assimilation of substrates is possible, even if carbon sources are consumed sequentially in batch cultures. Mixed substrate growth on sorbitol and methanol in batch culture was not investigated, but we expect simultaneous substrate consumption because sorbitol is a non-repressing carbon source with respect to AOX promoter. As a matter of fact, in chapters 7 and 8 it was shown that methanol consumption is not affected by sorbitol accumulation in the culture medium.

In chapters 6 and 7, it was shown that use of mixed substrates did not affect the specific avidin productivity down to a certain percentage of methanol in the feed medium. During growth on methanol and glycerol at a dilution rate of 0.06 h^{-1}, and during growth on methanol and sorbitol at a dilution rate of 0.03 h^{-1}, the specific avidin production rate was indeed the same as during growth on methanol if the methanol fraction in the feed medium was higher than about 60% and 40 % C-mol C-mol^{-1}, respectively. Due to higher biomass yields achieved during mixed substrate growth than during growth on methanol as sole carbon source, use of mixed substrates improved slightly the volumetric productivity of *P. pastoris* cultures. The biomass yields during mixed substrate

growth were always equal to the sum of the individual biomass yields on each substrate multiplied by the C-molar fraction of each substrate in the feed medium. The biomass yields on sorbitol and glycerol were the same and about 30% higher than on methanol as carbon source. Depending on the methanol fraction in the feed medium and the dilution rate, the volumetric productivity in recombinant avidin was increased by up to 30% with mixed feeds of sorbitol and methanol at a methanol fraction of 43% C-mol C-mol^{-1} in the feed medium at 0.03 h^{-1} (chapter 7).

Use of mixed substrates resulted in a significant reduction in the heat production and oxygen consumption rates. Because of the high enthalpy of combustion and the high degree of reduction of methanol, high heat production and oxygen consumption rates are achieved during the induction phase in high cell density fed-batch *P. pastoris* cultures. Use of mixed feeds takes advantage of the lower enthalpies of combustion and lower degrees of reduction of either glycerol or sorbitol. For a given dry cell weight concentration, the heat production rate and the oxygen consumption rate were diminished by 28% with a mixed feed of 60% methanol and 40% C-mol C-mol^{-1} glycerol at a dilution rate of 0.06 h^{-1} (chapter 6) and by 38% with a mixed feed of 43% methanol and 57% C-mol C-mol^{-1} sorbitol at a dilution rate of 0.03 h^{-1} (chapter 7). At other dilution rates, heat production and oxygen consumption rates will be diminished by roughly the same values since maintenance requirements for *P. pastoris* are low. As a result the stoichiometric yield coefficients do not vary significantly with dilution rate. These reductions in heat production and oxygen consumption rates are significant and facilitate the performance of high cell density fed-batch cultures with *P. pastoris* strains. However, we have to point out that technical adaptations such as use of high performance stirrers, use of pure oxygen and high cooling capacities are still necessary to achieve high cell densities (up to 160 g L^{-1}), even at laboratory scale.

It was also shown in chapter 5 that use of mixed feeds of glycerol and methanol after growth on glycerol allowed faster adaptation of cellular metabolism due to faster rates of synthesis of methanol dissimilating enzymes on mixed substrates. Indeed, transient continuous cultures with sudden changes in nutrient supply showed that the specific AOX activity increased about three times faster after a transition from growth on glycerol to mixed substrate growth on glycerol and methanol (with a methanol fraction of 50% C-mol C-mol^{-1}) compared with a transition to growth on methanol as sole carbon source. The presence of glycerol supports the synthesis of new enzymes by providing

energy and building blocks necessary for cellular adaption. Faster adaptation of metabolism at the beginning of the induction phase is important because, if methanol consumption is delayed, methanol accumulation can lead to intoxication of cells due to the excretion of toxic intermediates. Pulses of methanol during chemostat cultures on methanol or on mixed methanol and glycerol showed that sudden increase in the consumption rate of methanol after a transient increase of methanol in the culture medium led to excretion of toxic intermediates (formaldehyde, formic acid). The intoxication of cells resulted always in wash-out of the culture. It was pointed out in chapter 5 that the sudden increase in the consumption rate of methanol was due to the high amount of alcohol oxidase present in the cells during growth at low residual methanol concentrations. Indeed, a pulse of methanol during chemostat growth on glycerol did not lead to wash-out because methanol consumption was delayed and the methanol consumption rate increased progressively as methanol dissimilating enzymes were synthesized.

Finally, results presented in chapters 7 and 8 showed that mixed feeds of sorbitol and methanol are more advantageous than mixed feeds of glycerol and methanol for the expression of recombinant proteins with *P. pastoris* Mut^+ strains. Biomass yields are roughly the same on glycerol and sorbitol but the reduction in heat production and oxygen consumption rates are higher with sorbitol as additional carbon source due to a lower enthalpy of combustion of sorbitol than of glycerol. Moreover, by contrast with glycerol, sorbitol is a non-repressive carbon source with respect to the AOX1 promoter, so that transient substrate accumulation does not affect foreign gene expression with mixed feeds of sorbitol and methanol. Higher specific growth rates can be achieved with mixed feeds of glycerol and methanol than with mixed feeds of sorbitol and methanol because the maximal specific growth rate on glycerol is higher than on sorbitol (0.24 and 0.03 h^{-1} respectively). However, simulations of the performance of avidin (ratio of final amount of avidin and of culture time and dead time) in high cell density fed-batch cultures as a function of the specific growth rate during the induction phase showed that the highest performances of avidin are achieved at low specific growth rates (about 0.03 h^{-1}). Indeed, at high specific growth rates, the constraints (maximal reactor volume, maximal cooling capacity, maximal oxygen transfer rate or maximal dry cell weight concentration) are achieved earlier and the culture has to be stopped earlier. At low specific growth rates, the induction phase lasts longer and a higher performance in recombinant protein can be achieved. The low maximal specific growth rate

on sorbitol is therefore not a drawback for the achievement of high recombinant protein performance in high cell density fed-batch cultures.

1. 3. Optimal fed-batch strategy for the production of recombinant avidin

The following optimal fed-batch strategy was suggested in chapter 8 for the production of recombinant avidin with a *P. pastoris* Mut^+ strain:

- Batch phase on 40 g L^{-1} glycerol.
- Fed-batch phase on 50% w w^{-1} glycerol solution.
- Fed-batch phase with an exponential mixed feed containing 43% methanol and 57% C-mol $C\text{-}mol^{-1}$ sorbitol at a specific growth rate of 0.03 h^{-1}.

The batch and fed-batch cultures on glycerol are performed in order to achieve high cell densities rapidly while repressing avidin expression. The fed-batch phase on glycerol is a transition phase in order to derepress methanol dissimilating enzymes.

Simulations showed that the duration of the transition phase on glycerol does not influence significantly the performance of the process. In the present work, a constant feed of 20 g h^{-1} during one hour followed by a linearly decreasing feed of glycerol from 20 to 0 g h^{-1} during 3 hours were performed. A pulse of 1.5 g methanol was added after one hour of fed-batch cultivation on glycerol in order to facilitate and accelerate the adaptation of the cells at the beginning of the induction phase. As soon as all the added methanol was consumed, the induction phase was begun.

Temperature was set at 30°C and pH at 5.0. Neither parameter had a significant influence on the productivity of recombinant avidin (chapter 4 and Schenk et al. (2007)).

In all probability, the use of mixed feeds of sorbitol and methanol should also improve the strategy of other recombinant *P. pastoris* strains for the production of foreign proteins under the control of the AOX1 promoter since higher biomass yields and lower heat production and oxygen consumption rates are observed with mixed feeds of sorbitol and methanol than with methanol as sole carbon source. The optimal methanol fraction in the feed medium, the optimal specific growth rate, and the optimal pH and temperature, are in all probability strain and recombinant protein specific.

1. 4. Production of recombinant avidin in continuous cultures

Production of recombinant proteins with *P. pastoris* strains is traditionnally performed in high cell density fed-batch cultures. It can however be calculated that higher perfor-

mances in recombinant avidin could be achieved in high cell density continuous cultivation. The performance of recombinant avidin can be calculated according to the following formula during continuous cultivation:

$$L_P = q_P \cdot X \cdot V \quad (\text{mg h}^{-1}) \qquad (1)$$

where q_P is the biomass-specific avidin production rate (mg g^{-1} h^{-1}), X the cell dry weight (g L^{-1}) and V the culture volume (L).

For example, at a cell density of 120 g L^{-1} and with a feed medium containing 43% methanol and 57% sorbitol C-mol C-mol^{-1}, it can be calculated on the basis of the results obtained in chapter 7 that the performance of avidin in continuous culture at 0.03 h^{-1} will be 3.07 mg h^{-1} for a cell culture volume of 1.6 L. This is about 3-fold higher than with a high cell density fed-batch culture up to 160 g L^{-1} dry cell weight with an exponential feed containing 43% methanol and 57% sorbitol C-mol C-mol^{-1} at 0.03 h^{-1} (chapter 8) if a down time of 24 h is considered.

Use of mixed feeds of glycerol and methanol could be advantageous in order to increase this performance by working at higher dilution rates because the maximum specific growth rate on glycerol is quite high (0.24 h^{-1}). Moreover, it was shown in chapter 3 that the specific productivity of avidin increases linearly with the specific growth rate. One advantage however with fed-batch cultures versus continuous cultures is that the final product concentration is higher, which facilitates the purification of the recombinant protein.

1. 5. *Pichia pastoris* as an expression system

This work contributes to the further understanding of the metabolism of the methylotrophic yeast *Pichia pastoris*, which has become a commonly used expression system for recombinant protein production. Although many studies have been published on this expression system in the last three decades, quantitative and well-defined physiological studies are necessary in order to have a better insight into the regulation of the metabolism of this yeast and to develop optimal production processes for maximum expression of recombinant proteins.

The present work focused on a *P. pastoris* Mut^+ strain secreting recombinant avidin. High growth rates on methanol can be achieved with Mut^+ strains as with wild-type *P. pastoris* strains, which can be advantageous during continuous processes since the re-

combinant avidin productivity has been shown to be growth-associated (chapter 3). However, like any wild-type *P. pastoris* strain grown on methanol, *P. pastoris* Mut^+ strains are sensitive to high residual methanol concentrations. Indeed, in chapter 5, it was shown with pulses of methanol during chemostat growth on methanol or during mixed substrate growth on methanol and glycerol that sudden changes in residual methanol levels resulted in intoxication of cells due to excretion of toxic intermediates. These problems may be overcome by the use of either Mut^S strains, which are less sensitive to high residual methanol concentration due to low methanol consumption rate, or Mut^- strains which cannot consume methanol at all and require only small amounts of methanol (about 0.5%) for protein induction. It is not clear from the literature if higher expression levels can be achieved with Mut^S or Mut^- strains compared with Mut^+ strains because many studies were performed using shake-flasks. In such cultures, Mut^S strains have an advantage over Mut^+ strains because the oxygen transfer rate is low and Mut^+ strains are more likely to become oxygen-limited. As already pointed out, chapter 8 showed that in fed-batch cultures there is no advantage to be gained by working at high specific growth rates during the induction phase on methanol with Mut^+ strains. Higher avidin performances and lower maximal heat production and oxygen consumption rates are achieved at low specific growth rates. Moreover, the specific growth rate is known to be a means of influencing product quality and with a low specific growth rate promoting better folding of recombinant proteins. For these reasons, the fact that the maximum specific growth rate of Mut^S strains is only about 0.04 h^{-1} means that this is not necessarily a drawback. Moreover, if necessary, feeding of mixed substrates can be used in order to achieve higher specific growth rates with Mut^S strains. The use of Mut^- strains constitutes also an interesting case study with mixed substrates since methanol has to be fed in order to induce recombinant protein expression while another carbon source is necessary for growth.

2. PERSPECTIVES

2. 1. Transient experiments

Many culture parameters other, than dilution rate, nutrient concentration or temperature, can be varied during transient pseudo-steady state continuous cultures for the fast and accurate analysis of the influence of a culture parameter. For instance, the influen-

ce of pH of the culture medium on recombinant protein productivity could be analyzed for the studied strain by performing slow linear changes of culture pH at constant dilution rate. This type of analysis was not performed with the studied strain, secreting avidin, since preliminary studies showed that pH did not have a significant effect on recombinant avidin productivity (Schenk et al. (2007)).

Moreover, many designs for transient experiments can be imagined. Instead of linear changes, sudden shifts or pulses of nutrients, an exponential ramp (one culture parameter is increased exponentially as a function of time), a stair (one culture parameter is changed stepwise to a new value in the same direction), a step-up-and-down, a repeated step-up-and-down, in order to reproduce periodically changing culture parameters etc could be designed depending on which transient growth conditions need to be analyzed.

2. 2. Monitoring tools

On-line monitoring tools are a prerequisite for the analysis of transient experiments, because they enable to quantitatively follow the microbial activity as a function of time, in particular if the metabolic changes are fast. This has been done in the present work with on-line calorimetry and on-line monitoring of carbon dioxide and oxygen in the exhaust gas. Nowadays, non-invasive and on-line spectroscopic techniques, such as FT-IR, Raman, 2D-fluorescence or dielectric spectroscopy are under development for the continuous monitoring of bioprocesses. Spectroscopic instruments are well suited for on-line monitoring of bioprocesses because they are non-destructive and can be used *in situ*. Hence, the drawbacks related to sampling and sample pre-treatment are avoided. Moreover, they are sterilizable, have the capacity for simultaneous multiple analyte analysis and they provide continuous, real-time data. Development of reliable, accurate and on-line techniques will facilitate the monitoring of fast metabolic changes during transient experiments.

For example, the continuous monitoring of fast changes of substrate or product concentrations by FTIR spectroscopy or the continuous monitoring of changes in biomass concentrations by dielectric spectroscopy could allow the calculation of instantaneous substrate consumption rates or product formation rates during transient growth conditions with fast changes of one culture parameter. Such data could allow the calculation of metabolic fluxes under transient growth conditions. Nowadays spectroscopic techniques are fast enough to follow metabolic changes after sudden changes of a culture

parameter (for instance a spectrum can be taken every 30 s with FTIR spectroscopy). However, the maximum resolution of in-process spectroscopic techniques rarely exceeds 0.5 g L^{-1} and has therefore to be improved to follow secretion of metabolites during transient growth conditions. For instance, in this study, the concentrations of formaldehyde and formic acid resulting from pulses of methanol did not exceed 0.8 g L^{-1} and 0.5 g L^{-1} and were monitored off-line.

The off-line analytical techniques used in the present work include quantification of dry cell weight, of substrates and secreted products by HPLC or enzyme analysis, of the secreted recombinant avidin protein and of intracellular alcohol oxidase activity after cell lysis. For complete physiological studies, these measurements should be combined with molecular biology methods such as DNA microarray analysis techniques to detect RNAs that may or may not be translated into active proteins under various conditions, two-dimentional gel electrophoresis to characterize the whole protein expression pattern, measurements of storage carbohydrates to reflect changes in biomass composition etc. The combination of molecular biology techniques and bioprocess engineering analysis would give a better insight into the regulation of host cells and would in all probability contribute to the design of optimal culture strategies. For instance, the combination of molecular methods with the quantification of the produced recombinant protein might result in a better insight into the regulation of the host cell expression system. In the present work, it was shown that there is no obvious relationship between specific recombinant avidin production rate and specific alcohol oxidase activity, although avidin was expressed under the control of the AOX1 promoter. Transcriptional analysis of the alcohol oxidase gene and the foreign gene might give useful additional information on the regulation of this expression system. Moreover, quantification of the intracellular amount of avidin (soluble cellular extracts and extracts from cellular membranes) could provide interesting information on the eventual saturation of the secretory system of *P. pastoris*.

2. 3. Mixed substrates

Alternative carbon sources to glycerol and sorbitol could be tested as additional carbon sources to methanol during the induction phase of recombinant *P. pastoris* cultures. These might include mannitol, lactic acid, alanine or trehalose, which are known to be non-repressing carbon sources with respect to the AOX1 promoter. Determination of the maximum specific growth rate and growth stoichiometry in batch cultures is neces-

sary in order to evaluate the potential advantages of these substrates. In particular, high biomass yields and low oxygen and heat yields are interesting features because higher recombinant protein productivities may be achieved with reduced heat production and oxygen consumption rates during the induction phase. According to the literature, the growth stoichiometry of these substrates is less interesting than that for sorbitol. A high maximum specific growth rate is less important since the present study (chapter 8) showed that in fed-batch cultures it is more advantageous to perform the induction phase at a low specific growth rate. The performance of avidin was indeed lower at high specific growth rates because technical constraints (maximum reactor volume, maximum cooling capacity, maximum oxygen transfer rate or maximum dry cell weight concentration) were achieved earlier at high specific growth rates and the culture had to be stopped.

2. 4. *P. pastoris* as an expression system

Many examples in literature have shown that *P. pastoris* is an interesting and highly efficient expression system for the production of recombinant proteins. The present work showed that optimization of culture conditions allows to achieve maximal productivities in recombinant protein. However, one challenge with the *P. pastoris* expression system is also to select the most appropriate strain, selection maker, secretion signal and promoter for the expression of a particular foreign protein because this can have an enormous effect on the productivity and the correct folding of the recombinant protein. The design of an «efficient» strain constitutes indeed the basis in order to achieve high titres in recombinant proteins. Often several strains, selection makers, secretion signals and promoters are tested for the production of a particular recombinant protein. Systematic analysis of their influence on productivity and protein quality for various types of proteins may help in future to design host cells more rationnally and more rapidly.

Moreover, as pointed out in the Introduction of this thesis (chapter 1), use of alternative promoters for the expression of recombinant proteins, for instance the GAP, PGK1 or FLD1 promoters, has not yet been explored in detail, although these promoters represent interesting alternatives to the AOX1 promoter. The main advantage with these promoters is that induction of recombinant protein expression does not rely on the use of methanol as carbon source. Optimal protocols for the production of recombinant proteins under the control of these alterative promoters at high cell density have to be developed and evaluated.

Finally, since most therapeutic recombinant proteins contain post-translational modifications, including glycosylation, required for biological activity, the success of the *P. pastoris* expression system will also depend in future on the advances in glycoengineering for the production of «humanized» glycoproteins in yeasts. In the last years, advances in genetic engineering of human glycosylation pathways into yeasts, and in particular in the yeast *P. pastoris*, have shown significant promise to produce therapeutic proteins in yeasts with human glycosylation patterns. Maybe, further developments in the expression of glycoproteins in humanized yeasts will challenge the current dominance of therapeutic protein production based on mammalian cell culture.

3. TAKE-HOME MESSAGES

On the basis of the work and the results obtained during this thesis I would like to state the two following *take-home messages*.

The advantages of several substrates can be combined if residual substrate concentrations are low due to simultaneous assimilation of substrates during substrate-limiting conditions.

Transient experiments in continuous cultures are efficient tools in order to have insights into the regulation of an expression system or/and in order to speed up strain characterization.

4. REFERENCES

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CURICULUM VITAE

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EDUCATION

2003-2007 **Ph.D. at the Laboratory of Chemical and Biological Engineering**
Swiss Federal Institute of Technology, Lausanne (EPFL)

1998-2003 **Master's Degree in Chemical Engineering (Dipl. Ing. Chimiste EPFL)**
Swiss Federal Institute of Technology, Lausanne (EPFL)

1994-1998 **High School Degree, *Maturité Type C* (scientific section)**
Collège Sainte-Croix, Fribourg
Obtaining the prize Louis Wantz, rewarding the best average results in natural science

PROFESSIONAL EXPERIENCE

Since 2003 **Swiss Federal Institute of Technology (EPFL), Laboratory of Chemical and Biological Engineering, Lausanne**
Research project leader
Development and optimization of production processes in biochemical engineering for the production of recombinant proteins in yeasts (*Pichia pastoris*) at a 2 L scale
Training and coaching of four graduate students for their Master thesis and of one 4th year student for semester project
Supervision and training of two apprentices, one year each
Teaching assistant
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Coaching of laboratory experiments for 3rd and 4th year students

2003 **Agroscope Liebefeld-Posieux, PilotPlant Biotechnology, Bern**

(10 months) **Master Thesis and Internship**

Research on probiotic lactic acid bacteria for the design of new dairy fermented products

Performance of batch cultures in bioreactors at a 2 L scale

Development of a milk-based medium for the growth of bifidobacteria

2001 **Laboratoire Agroalimentaire Fribourgeois, Posieux**

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Quality control of marketed milk and other dairy products, within the departments of microbiology and chemistry

LANGUAGES

French Mother tongue (All schooling in French)

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English Fluency in reading, writing and speaking (working language)

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PUBLICATIONS AND ORAL PRESENTATIONS

Understanding regulation of recombinant protein expression in *Pichia pastoris*. Oral contribution at Biotech 2005 and 3rd Swiss-Czech Symposium, 18 may 2005, Wädenswil, Switzerland.

Jungo, C., Rérat, C., Marison, I. W. and von Stockar, U. (2006). 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* 39 (4) 936-944.

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PERSONAL INTERESTS

Music Member of the Brass Band of Ecuvillens-Posieux (trombone)

Sport Running (Finisher of the following running events in 2006: Kerzerslauf, Aletsch Semi-marathon, Neirivue-Moléson, Greifensee Semi-marathon, Morat-Fribourg), Skiing and Ski touring.