A Dynamic and Steady State Metabolome Study of Central Metabolism and Its Relation with the Penicillin Biosynthesis Pathway in *Penicillium chrysogenum*

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A Dynamic and Steady State Metabolome Study of Central Metabolism and Its Relation with the Penicillin Biosynthesis Pathway in *Penicillium chrysogenum*

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Chapter 1

Introduction

1.1 Background and Motivation

Penicillin production in submerged culture of *Penicillium chrysogenum* was initiated in 1941 by a research group of the Northern Regional Research Laboratory (NRRL) in Peoria, Illinois, USA. The major contribution of this research group has been the development of a fermentation process for large-scale production of penicillin (Nielsen, 1997). The NRRL was also involved in early strain improvement programs in order to develop new strains with increased penicillin yield. Later on, the strain development programs were carried out mainly at the University of Wisconsin. Many techniques were applied to obtain strains with higher penicillin yield, such as X-ray and UV radiation followed by selection of high producing mutants, which has resulted in increased yields (140 and 200 fold increase compared to the original strain found by Fleming in 1928) (Nielsen 1997).

Nowadays, strain improvement programs are also carried out by the penicillin producing companies. β-lactam molecules are still major compounds used as precursors for different semi-synthetic antibiotics (such as amoxicillin and ampicillin) which are obtained by attachment of different side chains to the β-lactam nucleus. The demand of these semi-synthetic antibiotics is still high, with a world market of more than 60,000 tons annually. Therefore, optimization of industrial production and increasing yields are of great economical importance for the β-lactam producing companies (Thykaer et al. 2002).

Although penicillin yields have continuously been increased, the latest studies showed that penicillin yield can still further be improved (Jorgensen et al. 1995; van Gulik et al. 2000). Traditional strain improvement has relied largely on whole cell mutagenesis followed by screening, which has increased product yields remarkably throughout the years, especially
until the end of the nineties. The drawbacks of this methodology is, however, the time
consuming screening of a large number of mutants and the accumulation of undesirable
genetic changes together with the beneficial ones (Wu et al, 2005).

An alternative for the traditional approach of strain improvement is metabolic
engineering (Bailey, 1991) which is defined as precise modification of cells at the genetic
level, leading to defined alterations in enzyme amounts and properties, gene regulation and or
biochemical reaction network structure. Such targeted genetic changes with the aim of
enhancing cellular functionalities are nowadays possible because of the availability of modern
DNA technology. Several successful applications of metabolic engineering in different
microorganisms, to increase product yield or introduce a new product, have been reported.
Some examples are: overproduction of lysine in *Corynebacterium glutamicum* (Vallino and
Stephanopoulos, 1993; Vallino and Stephanopoulos, 1994; Vallino and Stephanopoulos,
2000), metabolic engineering for pentose metabolism in *Saccharomyces cerevisiae*
(Sonderegger and Sauer, 2003; Pronk et al., 2005) and pyruvate overproduction in
*Saccharomyces cerevisiae* (van Maris et al. 2004). Xylitol production by recombinant
*Saccharomyces cerevisiae* (Hallborn et al. 1991), the novo biosynthesis of indigo *E. coli*
(Murdock et al. 1993) and metabolic engineering of propanediol pathways (Cameron et al.
1998) are examples for introduction of new product pathways in microorganisms.

A recent example of metabolic engineering of an industrial strain, which has been
carried out by DSM, is the introduction of a new pathway in *Penicillium chrysogenum* leading
to production of Adipoyl-7-ADCA antibiotics (Thykaer et al. 2002; Thykaer and Nielsen,
2003). This clearly showed the applicability of this technique in a β-lactam producing
microorganism. A very promising extension of metabolic engineering in this area will be the
construction of strains capable of the direct complete biological production of, at present,
semi synthetic, antibiotics from glucose. This would lead to a large reduction of the co-
production of waste material and a reduction of the cost price.

1.2 Metabolic Engineering in Microorganisms Producing β-lactams,
Biosynthesis of Penicillin and Its Byproducts

The biosynthesis pathway of penicillin has been completely elucidated. A schematic
representation of the pathway is depicted in Figure 1.1. The first step is the condensation of
the intermediate of the lysine pathway, L-α-amino adipic acid (α-AAA), and two amino acids,
L-cysteine and L-valine, to form a tripeptide, L-α-amino adipoyl-L-cyste inyl-D-valine (LLD-
In the second step the tripeptide ACV is converted to isopenicillin-N. Finally, if the side chain precursor phenylacetic acid (PAA) is supplied to the medium, α-AAA is exchanged with PAA thereby converting isopenicillin-N into penicillin-G. The released α-AAA can be used again for the synthesis of LLD-ACV and therefore penicillin-G synthesis in principle does not lead to a net consumption of α-AAA. However, usually there is some byproduct formation, which consumes α-AAA. This byproduct is the cyclic product of α-AAA called OPC (6-oxopiperide-2-carboxylic acid) (Nielsen 1997). Other byproducts that could be formed are 6-aminopenicillanic acid (6-APA) produced form IPN or penicillinG by the enzyme AT, 8-hydroxypenicilllic acid (8-HPA) from 6APA in reaction with CO₂ (Henriksen et al. 1997) and penicilloid acid (PIO) from penicillinG. All these by-products are excreted by the cell. An obvious approach to increase penicillin production is to reduce the production of these by-products (Henriksen et al., 1996, 1998).

Figure 1.1 Biosynthesis pathway of penicillinG by *Penicillium chrysogenum*.

The uptake of the side chain precursor phenylacetic acid (PAA) was studied (Eriksen et al., 1995; Hillenga et al., 1995) in order to find out whether there is an energy uncoupling effect of weak acid, which would result in a decrease of the penicillin yield on glucose due to a
reduction of the available energy for growth and penicillin synthesis. However, it has been found that byproduct formation and uncoupling have only a small effect on the penicillin yield in industrial fermentation processes (Henriksen et al., 1998).

Stoichiometry and black box kinetics of growth and product formation

One of the first black box models for the description of growth and penicillin production in *P. chrysogenum* was developed by Heijnen et al. (1979). Later, the first metabolic models were presented and an extensive chemostat based validation of such model for penicillinV producing *P. chrysogenum* was presented by Henriksen et al. (1996). In this study specific penicillin production rates ($q_{pen}$) were measured at different growth rates, and yield and maintenance coefficient were estimated. Furthermore, the biochemical composition of the biomass, in terms of protein, carbohydrate, lipid, DNA and RNA contents as well as the amino acid composition of biomass protein and the free amino acids pool. Growth energetics was also studied with respect to ATP needed for growth. In order to obtain detailed flux distributions, a stoichiometric model of *Penicillium chrysogenum* was proposed (Henriksen et al., 1996).

An intensive stoichiometry study of growth and product formation of penicillinG in *Penicillium chrysogenum* was performed by van Gulik (van Gulik et al., 2000, 2001). Using the same starting point as the work of Henriksen et al (1996), van Gulik et al. expanded the analysis by cultivating *Penicillium chrysogenum* under different conditions, including different carbon sources and different growth rates. Together with a stoichiometric model for growth and product formation, van Gulik found, for a high producing industrial strain of *P. chrysogenum*, that the supply of carbon precursors by the central metabolic pathways could not be the limiting factor for penicillinG formation. Furthermore, from an analysis of ATP production and consumption, it was found that much larger amounts of ATP appeared to be needed for penicillinG formation than could be anticipated from the theoretical value calculated from the known biochemical pathway. It was also found that the specific penicillin production rate is dependent on the growth rate, as shown in Figure 1.2. These studies indicated that the penicillin flux was not sensitive to the supply of carbon precursors by primary metabolism; but much more sensitive to the availability of the the cofactors NADPH and ATP.
**Figure 1.2** Measured specific production of penicillin-G in glucose (●), ethanol (▲) and acetate (□) limited cultures of *Penicillium chrysogenum* DS12975. Points indicated with arrows represent no stable steady states but observed maximum specific penicillin production rates during the experiments. Solid line: qPen - \( \mu \) relation for the glucose limited chemostats (from van Gulik et al., 2000).

**Metabolic control analysis of the penicillin biosynthesis pathway**

The penicillin biosynthesis pathway is an obvious target for metabolic engineering. Metabolic control analysis can be applied to find the most important steps in the regulation of the pathway. This information can be used to tune the levels of the corresponding enzymes of the penicillin biosynthesis pathway by means of genetic modification with the aim to increase the flux towards penicillin synthesis. A metabolic control analysis of penicillin production in a penicillinV production strain has been carried out using data obtained from fed batch fermentations (Nielsen and Jorgensen, 1995; Pissara et al. 1996). Their analysis indicated that in this strain, the flux through the product pathway is determined by both \( \alpha \)-aminoadipoyl-cysteinyl-valine synthetase (ACVS) and Isopenicillin N synthetase (IPNS). However, from a later analysis, using additional measurements of the reduced dimer of the tripeptide ACV (bis ACV), performed with the same strain it was concluded that the flux control resides entirely in the enzyme IPNS (Theilgaard and Nielsen, 1999). The role of IPNS is to relieve feed back inhibition of ACV on ACVS.
A possible limitation of the supply of one of the three precursor amino acids (cysteine, valine and $\alpha$AAA) on the penicillin production rate was investigated by supplying them to the feed medium of a fed batch fermentation (Jorgensen et al. 1995). Addition of three amino acids during fedbatch cultivation resulted in a 10-20% increased production. However the contribution of each of the individual the amino acids was not investigated. According to these results the biosynthesis pathways of the precursor amino acids could be important targets for improvement of penicillin production by means of metabolic engineering.

Some attempts have been made to investigate the molecular genetic basis of the high penicillin titers resulting from the classical strain development programs. In *Penicillium chrysogenum* an elevation of the copy number of the whole penicillin gene cluster appears to be one of the most plausible explanations for the increased penicillin production (Barredo et al. 1989, Newbert et al., 1997; Theilgaard et al., 2001). Four mutants were constructed in which the following genes were over expressed,: 1) pcbAB (encoding for ACVS), 2) pcbC (encoding for IPNS), 3) penC-penDE (encoding for both IPNS and AT) and 4) pcbAB-pcbC-penDE (encoding for all three enzymes of the pathway; ACVS, IPNS and AT). Two times transformation with the whole gene cluster showed a significant increase in penicillin productivity, 124% and 176% - respectively (Theilgaard et al., 2001). Transformation with the pcbC-penDE gene fragment resulted in a decrease of 9% relative to the mother strain (Wis54-1224).

In *Aspergillus niger* (Penalave et al., 1998), the same results were also obtained when the three structural genes were all amplified using replacement of the promoters with the strong inducible ethanol dehydrogenase promoter (alcA). In the above studies, overexpression was done in a single copy organism instead of a multiple copy number strain due to difficulties with the transformation of a large copy number structural gene. However, there seems to be an upper limit to the linearity of the dose response relationship ($q_p$ versus gene copy number). At some point, increasing gene copies did not lead to increased penicillin production (Skatrud 1992; Verdoes et al., 1995; Theilgaard et al. 2001)

In general, both MCA and genetic studies point to the importance of the increase of the activities of IPNS and ACVS for the increase of penicillin production, but also that there will be a limit to the effect of a further increase of the activity of these enzymes, because at a certain point the supply of carbon precursors and cofactors will become the rate limiting step.
Relation of penicillin biosynthesis with primary metabolism

Significant over production of many metabolites requires significant redirection of the flux distribution in central metabolism. This may not readily occur simply as a result of amplification of the enzymes of the product pathway because primary metabolic pathways have evolved elaborate control architectures that resist flux alterations at the branch points (rigid branch point concept). (Stephanopoulos and Valino, 1991). This might explain the observed saturation relation between increased gene copy number and the resulting penicillin production rate.

In penicillin biosynthesis, the required precursor amino acid are synthesized from carbon precursors from central metabolism: For the amino acids cysteine, valine and αAAA, they are 3-phosphoglycerate, pyruvate and αKG., respectively (van Gulik et al. 2000) (see Figure 1.3). Furthermore, the production of penicillin requires energy in the form of adenosine triphosphate (ATP) and reduction equivalents, either nicotinamide adenine dinucleotide (NADH) or nicotinamide adenine dinucleotide phosphate (NADPH). Especially the demand for NADPH is high (8-10 moles/mol penicillin), due to the required reduction of SO₄²⁻ to H₂S for cysteine biosynthesis. The high demand for both energy and reduction equivalents could cause a competition between growth and product formation. Increasing the rate of product formation by increasing the levels of the enzymes in the product pathway, at some point, inevitably leads to bottlenecks in primary metabolism, i.e. the supply of ATP, NADPH or carbon precursors for product formation in primary metabolism (van Gulik et al. 2000). In a further study, using a high producing industrial strain of *P. chrysogenum*, van Gulik et al., (2000) reported that NADPH supply/degeneration seems to be a limiting factor in penicillin biosynthesis. NADPH demand was increased stepwise by cultivating the cells in glucose or xylose as the carbon source combined with either ammonia or nitrate as the nitrogen source, which resulted in a stepwise decrease of penicillin biosynthesis, probably due to a limiting NADPH supply.
A major finding by van Gulik et al. (2001) was that there is high additional energy demand associated with penicillin synthesis, leading to a rather low maximal theoretical penicillin yield. This makes ATP-supply from primary metabolism an obvious additional candidate limiting factor for penicillin biosynthesis. Nearly all this work on the relation of primary metabolism and penicillin synthesis has been performed on fluxome level, and until now no studies have been performed at the metabolome level.

1.3 Rational methods in metabolic engineering

With the availability of recombinant DNA and genomic technology, changing and redirecting certain pathways to increase product formation becomes possible. The challenge is to find the appropriate genetic targets to increase product formation. Application of metabolic pathway engineering followed by in vivo evolution to obtain high product formation in β-lactams producing microorganism is not as straightforward as for a product of which the rate of production is coupled to the rate of energy production of the cell (Kuyper et al., 2005; Sonderegger and Sauer, 2003). Production of a secondary metabolite like penicillin, however, leads to lower yield of biomass on substrate. Therefore in vitro evolution where a higher biomass yield on the substrate, resulting in a higher growth rate, is beneficial, leads to degradation of penicillin production. This degeneration has often been observed (van Gulik et al., 2000; Jorgensen et al., 1995) in prolonged chemostat experiments. Therefore either advanced in-vitro evolution techniques have to be applied where in some way or another...
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Penicillin production is coupled to increased fitness of the cells or, alternatively, increased penicillin production has to be achieved by means of rational metabolic engineering. Finding the appropriate targets for increased penicillin production, however, requires detailed knowledge about the relevant metabolic pathways and their in-vivo regulation. This knowledge can be obtained from dedicated experiments. Subsequent mathematical modeling is then applied to identify the targets for metabolic engineering.

**Enzyme kinetics**  The metabolic flux or metabolic conversion rate of enzymatic reactions in the cell depends on the intracellular (x) and extracellular (c) metabolite concentrations and the enzyme activity (e) as presented below

\[ \nu = f(x, e, c) \]  

(1.1)

A well known relation which describes the rate of an enzymatic reaction as a function of the amount of enzyme and the substrate concentration is the Michaelis Menten equation (Michaelis and Menten 1913) (see Eq. 1.2).

\[ \nu = \nu_{\text{max}} \frac{x}{K_m + x} \]  

(1.2)

It can be inferred from this hyperbolic relation that at sufficiently high substrate concentrations the reaction rate is independent of the substrate concentration, while at substrate concentrations well below the Km value the reaction rate becomes proportional to the substrate concentration.

In early work on the elucidation of the kinetic properties of the enzymes of the penicillin biosynthesis pathway, the effects of relevant metabolites were tested on the purified enzymes in vitro. Studies of the kinetic properties of ACV synthetase have been performed by (Zhang et al. 1989). They reported that G6P and G3P are inhibitors of ACV synthetase. Furthermore they reported that glucose repression of the enzymes of the penicillin pathway appears to be one of the genetic mechanisms involved in the negative glucose regulation of β-lactam biosynthesis. However, one should be careful in drawing conclusions on in-vivo regulation of metabolic pathways based on data from in-vitro experiments. Recently, it has become apparent that in vitro kinetics properties often do not apply to in vivo conditions (Teusink et al. 2000).

Instead of first purifying the enzyme and subsequently investigating the kinetic influence of effectors one by one, a whole cell approach (in vivo kinetics) therefore has received more attention in the last decade (Theobald et al., 1997).
Stephanopoulos et al., (1998) mentioned that reaction kinetics in metabolic networks can be divided in different time regimes. An important conclusion from such time scale properties is that in rapid pulse experiments (at a time scale of 300 seconds) enzyme levels are not expected to change. The measured changes of the intracellular metabolite levels can then directly be used to evaluate in vivo reaction kinetics.

**Approximative kinetics**

Usually, equations describing enzyme reaction kinetics are highly nonlinear functions, which makes parameter estimation difficult (Visser and Heijnen, 2002). Amongst the various different approximate kinetic approaches that have been developed are linear approximations (Heinrich and Rapoport, 1974; Kascer and Burns, 1973), log-linear kinetics (Hatzimatikatis and Bailey, 1996), biochemical systems theory (Savageau, 1976; Voit, 2000), the thermokinetic format (Nielsen 1997) and linear logarithmic kinetics (Visser and Heijnen, 2002, 2003). In a recent review about approximative kinetics, Heijnen (2004) concluded that the linear logarithmic (lin-log) approach has the considerable advantage of being able to obtain an analytical solution, yielding the steady state metabolite levels and fluxes as a function of the enzyme levels and the concentrations of independent metabolites. This solution was shown to be valid for relatively large changes in enzyme levels and concentration of independent metabolites and for any given metabolic network containing branches, cycles and conserved moieties. This solution allows the formulation of a general metabolic design equation, which provides the changes in enzyme levels that are required to obtain the desired changes in fluxes and metabolite levels (Visser and Heijnen, 2002). Furthermore, the lin-log kinetic equation is linear in the kinetic parameters (which are the well known elasticities as defined in MCA), which greatly simplifies their identification.

The Lin-log kinetic rate equation, in reference based format (Visser and Heijnen, 2003), is written as

\[
\frac{v}{J} = \left( \frac{e}{e^o} \right) \left[ 1 + e^{x^o} \ln \left( \frac{x}{x^o} \right) + e^{c^o} \ln \left( \frac{c}{c^o} \right) + \epsilon_x \frac{x^o}{x} \ln \left( \frac{x}{x^o} \right) + \epsilon_c \frac{c^o}{c} \ln \left( \frac{c}{c^o} \right) \right]
\]

where the entries in \( e^{x^o} \) and \( e^{c^o} \) are the elasticities, defined for the reference state \( (J, e^o, x^o \) and \( c^o) \) as scaled local sensitivities of the reaction rates \( v \) towards the metabolite concentrations \( x \) and \( c \), which are defined as:
\[ \varepsilon^x = \frac{x^0}{v^0} \left( \frac{\delta v}{\delta x} \right)^0 \]  
\[ \varepsilon^c = \frac{c^0}{v^0} \left( \frac{\delta v}{\delta c} \right)^0 \]  
(1.4)  
(1.5)

The number of elasticities in eq. 1.3 is equal to the number of metabolites that affect the reaction rate of enzyme. A bigger influence of a metabolite on the reaction rate corresponds to a bigger absolute value of the corresponding elasticity and vice versa.; For activating compounds the elasticity has a positive sign (\( \varepsilon > 0 \)), for inhibitors \( \varepsilon < 0 \).

**Parameter identification**

The values of the parameters (elasticities) of the lin-log kinetic equation can be obtained from perturbation of metabolic fluxes and subsequent measurement of intracellular and extracellular concentrations and enzyme activities, either for different steady state conditions or under dynamic conditions. Whether all elasticities are identifiable or not depends on the number of elasticities and the structure of the pathways in combination with the set of perturbations applied. Wu et al., (2004) demonstrated the identification of elasticities of an *in vitro* reconstituted pathway from 3PG to pyruvate (using steady state data from (Giersch, 1995)), which included the enzymes Phosphoglucomutase, Enolase and Pyruvate kinase. A reference condition was chosen and 18 quasi steady states were obtained by varying the concentrations of 2,3-bisphosphoglycerate (BPG) (a positive effector for PGM), ADP (an effector of PK) and varying the three enzyme level one by one.

The application of lin-log kinetics to steady state perturbations in the penicillin pathway was demonstrated by van Gulik et al. (2003) using fed batch data published by Nielsen et al., (1995). Using lin-log kinetics the flux control coefficient (\( C^k \)) could be directly obtained. However, to obtain the elasticities, measurement of the intracellular metabolite levels are needed. Alternatively, elasticity parameters can be obtained from dynamic experiments, in which dynamic metabolite responses are measured (Kresnowati et al., 2005).

**1.4 Experimental Tools**

To obtain an *in vivo* kinetic model of metabolism, whole cell perturbations (steady state and/or dynamic) of the metabolic (steady) state are needed. Hereby it is very important to start from a well defined and reproducible steady state condition because this allows accurate
quantification of the changes that follow the perturbation. A chemostat culture is then the preferred experimental set up (van Gulik et al., 2000). A sufficiently high steady state biomass concentration is then needed to ensure measurable intracellular metabolite concentrations (Lange et al. 2001; Visser et al. 2002; Visser et al. 2004; Mashego et al. 2003; Mashego et al. 2005).

The prerequisite for the construction of meaningful kinetic models, needed to identify genetics targets for metabolic engineering of metabolism, is therefore that perturbation of metabolites around the relevant enzymes are achieved and measured under in vivo conditions. Since the method was introduced by (Theobald et al. 1993), elucidation of in vivo kinetics using stimulus response techniques has been increasingly used for studying primary metabolism (Cassagnole et al., 2002; Theobald et al. 1997; Vashegi et al., 1999, 2001; Visser et al., 2004), product pathways (Oldiges et al. 2004; Schmitz et al., 2002; Ostergaard et al. 2001). In a stimulus response experiment a steady state culture is perturbed by rapid injection of a certain perturbing agent (e.g. a substrate, activator or inhibitor) and the subsequent transient behaviour of metabolism is recorded by measuring the concentrations of intra and extracellular metabolites within a short time interval (< 300 seconds). Within this short time frame it is allowed to assume that enzyme level have not changed during the transient.

In order to capture the metabolite concentrations at the time of sampling, procedures for rapid sampling and subsequent immediate quenching of all metabolic activity are required. Theobald et al. 1997 developed a method for rapid sampling and quenching of Saccharomyces cerevisiae, which was improved later on by Lange et al., (2001). Visser et al., developed a fully controlled rapid sampling apparatus, the BioScope (Visser et al. 2002).

After sampling and quenching of the broth, separation and subsequent washing of the cells is needed to remove all extracellular metabolites. To separate the cells from the supernatant, centrifugation at a low temperature (<-20°C) is reported to prevent further conversion of intracellular metabolites.

After washing, the intracellular cell metabolites must be extracted from the cell pellet. Many different methods for extraction of metabolites from microbial cells have been reported in literature, either based on cold chloroform and cold methanol (de Koning and van Dam, 1992), boiling ethanol (Gonzales et al., 1997) and cold acid/alkaline (Theobald et al. 1997).

(Hajjaj et al. 1998) reported that the boiling ethanol method was also successfully applied for filamentous fungi.

The quantitative analysis of the extracted metabolites is the final step in the experimental procedure. In some cases enzymatic assays, specific for each metabolite have
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been used (Theobald et al. 1997; Lange et al. 2001; Visser et al. 2002). However, because the use of enzymatic assays is very laborious in case a large number of metabolites have to be measured, high throughput analysis, using LC-MS/MS, recently became a favorable alternative (Van Dam et al. 2002). However, both methods require laborious standard addition and spiking experiments to verify for each metabolite that no (partial) degradation occurs during the sample processing and that the sample matrix does not interfere with the analysis. The impacts of these effects on the analysis result can vary in different experiments, which might lead to a variation of measured absolute concentrations of metabolites.

Wu et al., (2005) and Mashego et al., (2004) demonstrated that addition of U-13C metabolites (present in an extract from cells grown on a U-13C labeled carbon source), as internal standards to the cell pellet, prior to metabolite extraction, did successfully solve this problem in pulse response experiments with *Saccharomyces cerevisiae*.

1.5 Aim and outline of the thesis

In this thesis the results are presented of an *in-vivo* dynamic and steady state metabolome study of central metabolism and it’s relation with the penicillin biosynthesis pathway in a high producing industrial strain of *Penicillium chrysogenum*.

The main goal of the work was to study, in a quantitative way, the *in vivo* kinetics of central metabolism and the interrelation of central metabolism, as the supplier of carbon precursors, energy and reduction equivalents, with the penicillin synthesis pathway.

The first requirement for *in vivo* kinetic studies is a well defined and reproducible reference condition. In this study glucose limited steady state chemostat cultivation at a dilution rate of 0.05 h\(^{-1}\) is chosen as the reference condition, because chemostat cultivation offers the possibility to grow the cells at a constant growth rate under well defined conditions and enables to monitor almost all macroscopic fluxes (van Gulik et al., 2000).

In order to study the interrelation between central metabolism and penicillin biosynthesis, intracellular metabolites, CO\(_2\), energy (ATP) and reduction equivalents (NADPH), which are produced in primary metabolism and which are known as important precursors for penicillin synthesis need to be measured. Chapter 2 of this thesis describes the development of a chemostat protocol for steady state glucose limited chemostat cultivation of *Penicillium chrysogenum*, suited for pulse response experiments and rapid sampling. It was verified that the developed chemostat system ensures homogenous mixing that allows to observe a dynamic response in a time interval of 5 seconds. Subsequently, protocols for rapid
sampling, quenching and metabolite extraction, which were previously developed for Saccharomyces cerevisiae, were adapted to be suitable for filamentous fungi. Furthermore the MIRACLE technique (Mashego et al., 2004; Wu et al., 2005) was applied for the first time for Penicillium chrysogenum and was shown to improve significantly the accuracy of the measurement of in vivo metabolite and nucleotide concentrations using LC-MS/MS.

The first in vivo pulse response experiment in Penicillium chrysogenum is reported in Chapter 3. The experiment was first carried out in the newly developed BioScope mini reactor (Visser et al., 2002; Mashego et al., 2006) coupled to a steady state chemostat and subsequently directly in the chemostat itself. In this chapter a comparison is presented between the results of both pulse experiments and also with the results of a similar pulse response experiment with a different micro-organism, namely Saccharomyces cerevisiae.

Chapter 4 describes a quantitative analysis of the highly dynamic in vivo pulse response experiment in Penicillium chrysogenum, based on the dynamic carbon, electron and the energy balances, during a period of 300 seconds after addition of the glucose pulse. The interaction between primary and secondary metabolism is analyzed using additional measurements of the intracellular free amino acid pool. A comparison is presented between the dynamic behavior of the amino acids and their precursors in central metabolism. An important factor appears to be the turnover time of each free amino acid pool.

The results of different steady state perturbations both in primary metabolism and in the penicillin biosynthesis pathway are reported in Chapter 5. Perturbation of primary metabolism is performed by replacing glucose with ethanol as sole carbon source, while perturbation in secondary metabolism was accomplished by eliminating penicillin production, either by omission of the side chain precursor PAA or by removal of all gene clusters coding for the enzymes of the penicillin biosynthesis pathway. The perturbations were performed in carbon limited chemostats and analyzed with respect to uptake and secretion fluxes, metabolite and adenine nucleotide levels and free amino acid concentrations. The results of these steady state perturbations are compared with the dynamic perturbations with respect to the relation between central metabolism, amino acid biosynthesis and penicillin production.
References


Chapter 1


Chapter 1


Chapter 2

Measurement of intracellular metabolites of primary metabolism and adenine nucleotides in chemostat cultivated *Penicillium chrysogenum*

**Abstract**  An experimental platform has been developed for rapid sampling and quenching of chemostat cultivated *Penicillium chrysogenum* broth for metabolome analysis in highly dynamic experiments, aimed at the elucidation of the *in-vivo* kinetic properties of metabolism. The sampling and quenching protocol available from *Saccharomyces cerevisiae* had to be modified for *Penicillium chrysogenum* mainly because of its filamentous character. Intracellular metabolites of glycolysis, TCA cycle and adenine nucleotides were measured with isotope dilution mass spectrometry (IDMS) using a U-$^{13}$C labeled metabolite mix produced from yeast cells as internal standard. By addition of the U-$^{13}$C internal standard mix prior to the metabolite extraction procedure, partial degradation of metabolites as well as non-linearity and drift of the LC-MS/MS could be successfully compensated for. It was found that there is a serious matrix effect on metabolite extraction between different organisms, which is however completely corrected for by the IDMS approach. Intracellular metabolites could be analyzed with standard deviations of around 5%.

A comparison of the metabolite levels between *Saccharomyces cerevisiae* and *Penicillium chrysogenum* showed both significant similarities and large differences which seem to be related to the presence of the penicillin pathway.

2.1 Introduction

Efficient application of microbial cells for the production of chemicals and pharmaceuticals often requires modification of the microbial metabolism. It has been shown recently that metabolic pathway engineering followed by in vivo evolution is a successful procedure to obtain high production/consumption levels of compounds, which are directly related to the primary metabolism of the cell. An example is the construction of a xylose fermenting mutant of Saccharomyces cerevisiae (Kuyper et al., 2005; Sonderegger and Sauer, 2003). However, improvement of rates and yields of products of which the synthesis is not directly related to primary metabolism, i.e. when coupling between growth and product formation is absent, is less straightforward because in-vivo evolution is often not applicable. In this case detailed knowledge on the stoichiometry, in-vivo kinetics and regulation of the relevant metabolic pathways is required in order to identify the targets for metabolic engineering (Bino et al., 2004; Thykaer and Nielsen, 2003; Visser and Heijnen, 2003). It has been shown that information on the in-vivo kinetic properties of primary metabolism can be obtained from measurement of transient intracellular metabolites in short-term pulse experiments (Theobald et al., 1997; Visser et al., 2004). However, so far these methods have not been applied to micro-organisms producing significant amounts of secondary products and micro organism which are filamentous. Here we wish to apply these methods to a high producing industrial strain of the filamentous fungus Penicillium chrysogenum. This paper describes the development and application of an experimental metabolome platform for elucidation of the in-vivo kinetic properties of the fungus through pulse response experiments. The platform consists of a dedicated chemostat system enabling rapid sampling and quenching of Penicillium chrysogenum broth for metabolome analysis steady states and in highly dynamic experiments, an optimized metabolite extraction procedure and metabolite analysis with isotope dilution mass spectrometry (IDMS).

2.2 Material and Methods

Strain A high-yielding strain of Penicillium chrysogenum (code name DS17690) was used for all experiments. This strain was kindly donated by DSM Anti-Infectives (Delft – The Netherlands).
Measurement of intracellular metabolites of primary metabolism

Medium composition Two defined chemostat media were used, which were both derived from a previously described chemostat medium for *P. chrysogenum* (van Gulik et al., 2000). The composition of medium 1 was 33 g/L glucose.H₂O, 1.6 g/L KH₂PO₄, 8.75 g/L (NH₄)₂SO₄, 1 g/L MgSO₄.7H₂O, and 10 ml/L trace element solution. The trace metal solution contained 15 g/L Na₂-EDTA⋅2H₂O, 0.5 g/L CuSO₄·5H₂O, 2 g/L ZnSO₄·7H₂O, 2 g/L MnSO₄·H₂O, 4 g/L FeSO₄·7H₂O and 0.5 g/L CaCl₂·2H₂O.

Medium 2 contained 16.5 g/L glucose.H₂O, 1 g/L KH₂PO₄, 5 g/L (NH₄)₂SO₄, 0.5 g/L MgSO₄.7H₂O and 10ml/L trace element solution.

The phenylacetic acid (PAA) concentration of the feed medium was adapted to the fermentation conditions, i.e. dilution rate and biomass density such that the residual concentration during steady state conditions was approximately 3 mmol/L.

During the batch phase preceding chemostat cultivation, medium without PAA was used.

Medium preparation The appropriate amount of phenylacetic acid was dissolved in 4 liter of a KOH solution, with a molar ratio of PAA:KOH of 2:1. After neutralizing the pH with 2N H₂SO₄ the medium vessel containing the PAA solution was autoclaved for 40 min. at 121 °C. The other medium components were dissolved in 46 liter of demineralised water. This solution was filter sterilized using Supor DCF 0.2 µm filters (Pall Gelman Sciences, East Hills, USA) and added to the PAA solution in the medium vessel. Medium vessels were placed on a magnetic stirrer and were allowed to mix for at least 12 h. before they were connected to the reactor. Also during the experiments all medium vessels were continuously mixed.

Chemostat system The chemostat system was based on a 7 L turbine-stirred bioreactor (Applikon, Schiedam, The Netherlands) with a working volume of 4L. The reactor was equipped with two six bladed Rushton turbine impellers with a diameter of 8.5 cm and sensors for pH, dissolved oxygen (DO) and temperature. The complete reactor was placed on an electronic balance (Mettler Toledo) for accurate volume control. During chemostat cultivation the feed was pumped in continuously by means of a peristaltic pump. Effluent was removed discontinuously into an effluent vessel if the broth weight exceeded 4000 g, by means of a weight controlled pneumatic valve placed at the bottom of the reactor combined with a peristaltic pump. The effluent vessel was placed on an electronic balance for accurate measurement of the dilution rate. During cultivation the temperature was kept at 25.0 +/- 0.1 °C by means of a thermo circulator which was controlled via the internal temperature sensor.
of the reactor. The pH of the culture was maintained at 6.50 +/- 0.05 with 4N KOH by an automatic pH-control system (Applikon) connected to the internal pH probe of the reactor. The stirrer speed was 500 rpm and the aeration rate was 0.925vvm. The reactor was operated at an overpressure of 0.3 bar to facilitate rapid removal of sample. The dissolved oxygen tension was monitored but not controlled. During the experiments the dissolved oxygen tension never dropped below 50% of air saturation.

**Rapid sampling for extracellular glucose analysis**  
Rapid sampling for measurement of the extracellular glucose concentration was performed using the cold steel ball method described by Mashego et al. (2003). Glucose analysis was performed using an enzymatic method (Enzytec™, Scil Diagnostic GmBH, Germany).

**Rapid sampling and quenching of biomass**  
Rapid sampling was performed with an automated sampling system with minimal dead volume (Lange et al., 2001) originally developed for rapid sampling of yeast broth. With this system, 1 mL (+/- 0.05) of broth was withdrawn from the fermentor in ~0.7 seconds and injected directly into 5mL of 60% (v/v) methanol/water at –40°C for immediate quenching of metabolic activity. The exact amount of broth withdrawn was quantified by weighing the sample tubes before and after sampling.

**Cold centrifugation and metabolite extraction**  
The quenched samples were centrifuged for 5 min in a cooled (-20°C) centrifuge (Heraeus Biofuge stratos, Heraeus Instruments, Germany) at 5,000g, using a swingout rotor. The rotor was precooled to –40°C to prevent the temperature of the samples to rise above –20°C. After decanting, the cell pellet was resuspended in 5 mL of 60% (v/v) methanol/water solution (–40°C) and again centrifuged for 5 min at 5000 g. This additional washing step served to effectively remove extracellular components. After the second centrifugation step the supernatant was decanted and the intracellular metabolites were extracted from the remaining pellet in boiling ethanol (based on Gonzales et al., 1997).

According to this method, 5 mL of boiling ethanol solution (75% v/v ethanol/water) was added to the cell pellet, the tube was vortexed for a few seconds to resuspend the pellet, and placed in a hot water bath at 95°C for a period of 3 min. Immediately thereafter, the tubes with the ethanol extracts were cooled to –40°C in a cryostat. The ethanol extracts were concentrated in a Rapid-Vap (Labconco, Kansas City, MO) for 45 min under controlled vacuum and temperature to reach a final volume of approx. 300 µL. Subsequently, the
concentrated extracts were filled up with Milli-Q water to a final volume of 500 µL, centrifuged at 3,000g (Heraeus Biofuge stratos) to remove cell debris. The resulting supernatants were stored at –80°C until analysis.

Metabolite Analysis Measurement of intracellular metabolite concentrations was carried out using ion exchange chromatography and electrospray ionization with tandem mass spectrometric detection (LC-MS/MS) (Van Dam et al., 2002). Glycolytic and TCA cycle intermediates were analyzed using ion chromatography while the adenine nucleotides were analyzed using reverse phase chromatography. In order to increase the accuracy of the analysis, U-13C labeled metabolite extract was added as internal standard. The U-13C metabolite extract used was obtained from S. cerevisiae cells, grown on 100% U-13C labeled substrate (Mashego et al., 2004; Wu et al., 2005). The factors to obtain the IDMS corrected metabolite concentrations from the ratio’s of the measured unlabeled (12C)/ U-13C peak areas for each compound were obtained as follows. First 10 dilutions (0 – 70x) of a mixture of unlabeled standards were prepared. To each dilution of the standard mix the same amount of U-13C metabolite extract was added. Subsequently the mixtures were analyzed for the amounts of unlabeled as well as U-13C-labeled metabolites in terms of their peak areas. From the slope of a plot of the ratio: [peak area unlabeled compound] / [peak area U-13C standard] against the concentration of the unlabeled compound in the mixture the relation between the ratio measured unlabeled (12C)/ U-13C peak area of the sample and concentration of unlabeled species was obtained for each compound. For each sample 100 µL of U-13C extract was added to the cell pellet prior to metabolite extraction in hot 75% ethanol. After completion of the extraction procedure the final extract volume was 500 µL. For one LC-MS/MS analysis 100 µL of sample was mixed with 2 µL of chloropropionic acid (generally used internal standard) prior to analysis.

2.3 Result and Discussions

Chemostat cultivation of P. chrysogenum In previous experiments, on intracellular metabolite measurements in chemostat cultures of Saccharomyces cerevisiae, it was found that a steady state biomass concentration of 14 – 15 g DW / L was sufficient to obtain quantifiable amounts of metabolites in the final cell extract from 1 mL culture samples (Lange et al., 2001). Therefore a similar cultivation protocol and a feed medium with the same amount of carbon source were developed for chemostat cultivation of P. chrysogenum.
DS17690. A previously described defined cultivation medium (van Gulik et al., 2000) was modified to sustain carbon limited growth at a glucose concentration of 30 g/L in the feed medium (see materials and methods). From previously measured yield and maintenance coefficients for growth on glucose of this \textit{P. chrysogenum} strain (van Gulik et al., 2000), it was calculated that for this medium the expected steady state biomass concentration would be between 11 and 15 g DW/L, for dilution rates between 0.03 and 0.12 h\(^{-1}\) respectively. Results from two chemostat cultivations carried out according to this protocol at two different dilution rates are shown in Table 2.1a.

**Table 2.1a** Results from two glucose limited chemostat cultures of \textit{Penicillium chrysogenum} carried out at different dilution rates.

<table>
<thead>
<tr>
<th>Dilution rate (h^{-1})</th>
<th>Mycelium concentration g DW (L^{-1})</th>
<th>Mycelium yield on glucose Cmol(\cdot)Cmol(^{-1})</th>
<th>(q_p) mmol Cmol(^{-1})(\cdot)h(^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chemostat 1 0.030 ± 0.00</td>
<td>10.6 ± 0.12</td>
<td>0.38 ± 0.01</td>
<td>0.48 ± 0.04</td>
</tr>
<tr>
<td>Chemostat 2 0.052 ± 0.00</td>
<td>11.9 ± 0.11</td>
<td>0.43 ± 0.01</td>
<td>0.38 ± 0.03</td>
</tr>
</tbody>
</table>

**Table 2.1b.** Measured specific conversion rates for \textit{Penicillium chrysogenum} and \textit{Saccharomyces cerevisiae} cultured at \(D = 0.05 \text{ h}^{-1}\) in aerobic glucose limited steady state chemostats

<table>
<thead>
<tr>
<th>Rate</th>
<th>\textit{P. chrysogenum} mmol(\cdot)Cmol(^{-1})(\cdot)h(^{-1})</th>
<th>\textit{S. cerevisiae} mmol(\cdot)Cmol(^{-1})(\cdot)h(^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>(q_s)</td>
<td>19 ± 0.47</td>
<td>14.5 ± 0.2</td>
</tr>
<tr>
<td>(q_{O2})</td>
<td>48.4 ± 1.94</td>
<td>39.0 ± 0.1</td>
</tr>
<tr>
<td>(q_{CO2})</td>
<td>49.9 ± 0.35</td>
<td>38.0 ± 0.1</td>
</tr>
<tr>
<td>(q_{pen})</td>
<td>0.41 ± 0.01</td>
<td>-</td>
</tr>
</tbody>
</table>

The obtained biomass yields in these chemostats were similar to what has been obtained previously (van Gulik et al., 2000). Also the specific penicillin production rate appeared to be similar. The specific uptake rates of glucose, \(O_2\) and \(CO_2\) are shown in Table 2.1b. It was calculated that the carbon and redox balances closed within reasonable limits.
Pulse experiments and mixing characteristics  
An important criterion for chemostats to be used to carry out intracellular metabolite measurement in pulse response experiments is sufficiently fast mixing. After disturbance of the steady state by injection of a certain substrate, intermediate or effector, it is essential that this compound is homogeneously mixed with the culture broth in a time period which is small (a few seconds) compared to the time window of observation (approximately 300 seconds). To verify this, a glucose pulse experiment was carried out. After reaching steady state, 16 mL of a 125 g/L glucose solution was injected in the chemostat within one second. Injection of this solution increased the extracellular glucose concentration from approximately 20 mg/L to 500 mg/L. Rapid sampling was carried out during a period of 300 seconds after injecting the glucose solution to determine the extracellular glucose concentration as a function of time. The results were very scattered (not shown) indicating that mixing was insufficient and/or that the sample port was positioned in a badly mixed region of the chemostat. Furthermore, calculation of the glucose consumption rate would be impossible from these measurements. These results were confirmed by injection of 16 mL of a 0.75 M phenylacetic acid (PAA) solution into a steady state chemostat, subsequent rapid sampling during a period of 300 seconds and analysis of the PAA concentration in the samples. PAA was chosen for this purpose because the uptake of this compound within this time frame is negligible. From the obtained results it was concluded that the mixing characteristics of the reactor should be improved.

To ensure that samples were taken from a well mixed region of the reactor the position of the sample port was changed in such a way that it was located further away from the baffles and the DO probe. Furthermore the diameter of the two Rushton turbine stirrers of the reactor was increased to improve the mixing. Originally two 6 bladed Rushton turbines of different diameters were mounted, a 6.5 cm diameter stirrer just above the sparger and a 7.5 cm stirrer at approx. half the reactor height. These were replaced by two 6 bladed Rushton turbines with a diameter of 8.5 cm.

Furthermore the steady state biomass density was decreased with 50% in order to decrease the viscosity of the broth. This was accomplished by decreasing the concentration of glucose in the influent from 30 g/L to 15 g/L, which resulted in a steady state biomass concentration of approximately 6 g/L at a dilution rate of 0.05 h⁻¹, which is the expected 50% of the previous medium (Table 1a). The mixing time for the modified system was calculated according to van t’ Riet and Tramper (1991) to be 1 – 2 seconds.

Pulse experiments with PAA were carried out with this modified chemostat system to verify whether the mixing was sufficiently fast. This was done by cultivating Penicillium
Chapter 2

*chrysogenum* in the modified chemostat, according to the conditions described above at D = 0.05 h\(^{-1}\). After a steady state was reached 16 mL of a 0.75 M PAA solution was injected in the fermentor within one second. Directly after injection of the PAA solution rapid sampling was performed during a period of 300 seconds. From the PAA concentrations measured in the samples it was inferred that mixing was sufficiently fast, as the PAA concentration measured in the first sample, which was taken at 5 seconds after injection of the PAA, was close to the final value (see Figure 2.1). Furthermore the measured variations in the PAA concentrations in the samples could be contributed to the analysis procedure as the standard deviation of the average PAA concentration measured in the 12 samples taken after injection of the PAA was similar to the standard deviation of the PAA analysis of a single sample analysed 12 times (results not shown).

![Figure 2. 1 Extracellular PAA concentration before and after rapid injection of 16 mL of a 0.75 M PAA solution into a steady state chemostat culture of *P. chrysogenum*.](image)

**Rapid sampling and metabolite extraction** To obtain a snapshot of the metabolome fast sampling and rapid quenching of metabolism is required. A protocol for rapid sampling, quenching and metabolite extraction for *S. cerevisiae* has been developed previously (Lange et al., 2001). It was tried to apply this protocol directly to *P. chrysogenum*. However, one of the problems encountered when applying this protocol was that unstable cell pellets were obtained during the cold centrifugation and washing step directly after quenching of the
sample in 60% cold (-40°C) methanol. This problem was solved by centrifuging the samples at 5000 g instead of 2000 g and by using a swing out instead of a fixed angle rotor. Furthermore it was checked whether any significant cell leakage occurred during the quenching and washing procedure. This was done by measurement of the amount of ATP in the combined supernatants of the 1st and 2nd centrifugation steps and comparison with the intracellular amount of ATP. For this purpose rapid sampling and quenching was performed to withdraw six samples from a steady state chemostat within a time period of less then 3 minutes. Three of these samples were used for standard addition experiments, in order to determine the recovery of ATP in this procedure. The recovery appeared to be close to 100%. From analysis of the amount of ATP in the combined supernatants of the other three samples it appeared that leakage of ATP from the cells during the cold centrifugation and washing procedure did not occur in *P. chrysogenum*.

After quenching and the subsequent cold centrifugation and washing steps to remove all extracellular components, metabolite extraction and enzyme denaturation was performed using the boiling ethanol method (Gonzalez et al., 1997). Subsequently, removal of the extraction liquid containing ethanol and traces of methanol (total about 6 ml) is required prior to metabolite analysis. In the original protocol developed for yeast the extract is evaporated to dryness under vacuum and the residue is re-dissolved in 500 µL of water (Mashego et al., 2004). However, it appeared not possible to redissolve the residues of the *P. chrysogenum* extracts completely. This was circumvented by concentrating the ethanol/water extract to a volume of approx. 300 µL and filling it up with water to a final volume of 500 µL instead of complete drying and subsequent dissolving. A complete description of the modified procedure can be found in the materials and methods section.

**Metabolite analysis** A protocol for analysis of glycolytic intermediates using LC-MS/MS was successfully applied to *S. cerevisiae* cell extract (Van Dam et al., 2002). From standard addition experiments to cell extract of *Saccharomyces cerevisiae* it was found that in *Saccharomyces cerevisiae*, for most of the compounds, the sample matrix has little or no influence on the analysis. From spiking experiments, i.e. addition of standards before the extraction step, it was found that for all measured glycolytic compounds except for pyruvate, the recovery was satisfactory (Lange et al., 2001). However, for each different microorganism the effect of the sample matrix on the metabolite recovery during the boiling ethanol extraction and separation might be different. This would imply that for each different micro-
organism standard addition and spiking experiments have to be carried out in order to determine these recoveries, which is an enormous laborious procedure.

However, addition of an appropriate internal standard for each compound prior to the metabolite extraction procedure circumvents the need for standard addition and spiking experiments. It has been shown that the application of U-$^{13}$C labeled metabolites as internal standards combined with isotope dilution mass spectrometry (IDMS) is an excellent method to correct for losses during extraction and analysis (Mashego et al., 2004; Wu et al., 2005). Here we applied the same method for the measurement of intracellular metabolite concentrations in steady state chemostat cultivated *P. chrysogenum*. The results are shown in Table 2.2. In this table also the metabolite concentrations obtained with the conventional analysis method (without applying the corrections for the $^{13}$C labeled internal standards but using the unspecific chloropropionic acid standard) are shown as well as the ratios of the results obtained with the two methods. It can be inferred from Table 2.2 that the concentrations of the glycolytic and TCA-cycle metabolites and ATP measured with IDMS are higher than when measured with the conventional method, indicating that degradation of metabolites during the extraction procedure and/or matrix effects play a significant role. The ratios between the concentrations measured with the conventional and with the IDMS method can be regarded as the overall metabolite recoveries in the complete extraction and analysis procedure. However, it should be noted that for the conventional LC-MS/MS analysis a conventional calibration curve is used. Due to ion suppression effects these calibration curves are not always linear and are therefore an additional source of errors (Mashego et al., 2004; Wu et al., 2005). Another way of calculating metabolite recoveries in the extraction and analysis procedure is from the measured peak areas of the U-$^{13}$C internal standards, which were added both to the mix of calibration standards and to the samples. From the measured differences in peak areas per amount of U-$^{13}$C compound added respectively to the calibration standard mix and the samples the recoveries can be calculated for all measured compounds. The advantage of this method is that no conventional calibration lines are needed in this procedure. It can be seen from Table 2 (fifth column) that the metabolite recoveries calculated with this method closely resemble the calculated ratios between the conventional and IDMS results.
Table 2.2: Average intracellular metabolite concentrations of *Penicillium chrysogenum* cultivated in an aerobic, glucose limited chemostat at D = 0.05 h⁻¹. AXP concentrations are averages of 16 steady state samples; all other concentrations are averages of 8 steady state samples.

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>IDMS result (µmol.g DW⁻¹)</th>
<th>Conventional LC-MS/MS result (µmol.g DW⁻¹)</th>
<th>Ratio Conv/IDMS (-)</th>
<th>Recovery of U-¹³C standards (-)</th>
</tr>
</thead>
<tbody>
<tr>
<td>G6P</td>
<td>3.29 ± 0.14</td>
<td>2.21 ± 0.30</td>
<td>0.67 ± 0.10</td>
<td>0.64 ± 0.16</td>
</tr>
<tr>
<td>G1P</td>
<td>0.61 ± 0.03</td>
<td>0.50 ± 0.07</td>
<td>0.82 ± 0.12</td>
<td>0.80 ± 0.20</td>
</tr>
<tr>
<td>F6P</td>
<td>0.81 ± 0.04</td>
<td>0.50 ± 0.14</td>
<td>0.62 ± 0.18</td>
<td>0.64 ± 0.21</td>
</tr>
<tr>
<td>F1,6bisP</td>
<td>0.74 ± 0.05</td>
<td>0.32 ± 0.03</td>
<td>0.43 ± 0.05</td>
<td>0.46 ± 0.22</td>
</tr>
<tr>
<td>2PG + 3PG</td>
<td>0.40 ± 0.02</td>
<td>0.20 ± 0.05</td>
<td>0.50 ± 0.13</td>
<td>0.46 ± 0.17</td>
</tr>
<tr>
<td>PEP</td>
<td>0.24 ± 0.01</td>
<td>0.13 ± 0.03</td>
<td>0.54 ± 0.13</td>
<td>0.56 ± 0.20</td>
</tr>
<tr>
<td>PYR</td>
<td>0.52 ± 0.05</td>
<td>0.48 ± 0.09</td>
<td>0.92 ± 0.19</td>
<td>0.95 ± 0.12</td>
</tr>
<tr>
<td>Cit + Isocit</td>
<td>5.74 ± 0.43</td>
<td>3.77 ± 0.47</td>
<td>0.66 ± 0.10</td>
<td>0.63 ± 0.13</td>
</tr>
<tr>
<td>α-KG</td>
<td>1.12 ± 0.06</td>
<td>0.58 ± 0.06</td>
<td>0.52 ± 0.06</td>
<td>0.56 ± 0.67</td>
</tr>
<tr>
<td>SUC</td>
<td>0.35 ± 0.05</td>
<td>0.24 ± 0.04</td>
<td>0.69 ± 0.15</td>
<td>0.66 ± 0.10</td>
</tr>
<tr>
<td>MAL</td>
<td>3.05 ± 0.12</td>
<td>1.82 ± 0.24</td>
<td>0.60 ± 0.08</td>
<td>0.59 ± 0.13</td>
</tr>
<tr>
<td>FUM</td>
<td>0.70 ± 0.03</td>
<td>0.40 ± 0.05</td>
<td>0.57 ± 0.08</td>
<td>0.57 ± 0.10</td>
</tr>
<tr>
<td>ATP</td>
<td>6.25 ± 0.18</td>
<td>4.92 ± 0.27</td>
<td>0.79 ± 0.05</td>
<td>0.87 ± 0.11</td>
</tr>
<tr>
<td>ADP</td>
<td>0.97 ± 0.06</td>
<td>0.93 ± 0.04</td>
<td>0.96 ± 0.07</td>
<td>0.95 ± 0.08</td>
</tr>
<tr>
<td>AMP</td>
<td>0.14 ± 0.04</td>
<td>0.14 ± 0.02</td>
<td>1.00 ± 0.32</td>
<td>1.00 ± 0.01</td>
</tr>
</tbody>
</table>

In previous work (Lange et al., 2001) standard addition experiments have been carried out to determine the recoveries of glycolytic intermediates in extracts of chemostat cultivated yeast cells. The standards were added prior to the hot ethanol extraction procedure and metabolite analysis was carried out with LC-MS/MS. It was found that the overall recovery in the extraction and analysis procedure was close to 100% for G6P, G1P and F6P, somewhat higher than 100% for F1,6bisP and 2PG+3PG and lower than 100% (92% and 60%) for PEP.
and PYR respectively. With the exception of pyruvate the recoveries of the corresponding glycolytic intermediates from penicillium extracts is significantly lower, which might be attributed to matrix effects e.g. effects of the sample matrix on ion suppression in the electrospray ionization step resulting in a different signal response of the analyte. It is clear from these results that the application of $^{13}$C labeled internal standards to correct for metabolite losses in the extraction and analysis procedures is indispensable for a correct determination of the intracellular metabolite concentrations *Penicillium chrysogenum*. This result also shows that the use of quenching and extraction protocols validated for a specific organism do not necessarily apply to other organisms. It has been shown previously that IDMS also increases the precision of the analysis (Wu et al., 2005) which is also found from our results when the standard deviations of the averages of concentration measurements of 16 steady state samples obtained with IDMS are compared with the conventional method (see Table 2.3). It appears that standard deviations can be reduced to 5 fold.

**Mass action ratios**  For enzymes which are assumed to operate near to equilibrium under physiological conditions the mass action ratios were calculated. By comparing the calculated mass action ratios with the equilibrium constant ($K_{eq}$) of the reaction the near equilibrium assumption can be verified.

Mass action ratios were calculated for myokinase, phosphogluco isomerase (PGI), the lumped reaction of enolase and phosphoglycerate mutase (PGM) and fumarase. The reason that PGM and enolase were lumped is that 2PG and 3PG could only be analyzed as a sum and not independently. The combined mass action ratio of PGM and enolase is expressed as:

$$\frac{[PEP_{eq}]}{[2PG_{eq} + 3PG_{eq}]} = \frac{1}{\frac{1}{K_{eno}} + \frac{1}{\frac{K_{eno}}{K_{pgm}}}}$$

(2.1)
The results of the calculations are presented in Table 4. It can be seen from this table that the calculated mass actions ratios for these reactions are close to the equilibrium constants, confirming that they operate close to equilibrium.
Table 2.4 Calculated mass action ratios and literature values for the corresponding equilibrium constants ($K_{eq}$).

<table>
<thead>
<tr>
<th>Enzyme(s)</th>
<th>Substrate(s)</th>
<th>Product</th>
<th>Mass action ratio</th>
<th>$K_{eq}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myokinase</td>
<td>ATP, AMP</td>
<td>2ADP</td>
<td>1.07</td>
<td>1</td>
</tr>
<tr>
<td>PGI</td>
<td>G6P</td>
<td>F6P</td>
<td>0.24</td>
<td>0.29$^b$ – 0.4$^c$</td>
</tr>
<tr>
<td>Enolase and PGM</td>
<td>2&amp;3PG</td>
<td>PEP</td>
<td>0.46</td>
<td>0.43$^c$ – 1.1$^a$</td>
</tr>
<tr>
<td>Fumarase</td>
<td>Malate</td>
<td>Fumarate</td>
<td>0.23</td>
<td>0.28</td>
</tr>
</tbody>
</table>

$PGI = \text{Phosphogluco isomerase, PGM = Phosphoglycerate mutase, G6P = Glucose-6-phosphate; F6P = Fructose-6-phosphate; G1P = Glucose-1-phosphate; 2&3PG = pool of 2-Phosphoglycerate & 3-Phosphoglycerate; PEP = Phosphoenol pyruvate.}^a$Bergmeyer (1984), $^b$Tewari (1988), $^c$Reich and Sel’kov (1981).

Comparison of metabolite profile in *Penicillium chrysogenum* and *Saccharomyces cerevisiae*  *Saccharomyces cerevisiae* and *Penicillium chrysogenum* are both eukaryotic microorganisms which have many similarities. The metabolic model for *Penicillium chrysogenum* (van Gulik et al., 2000) is largely based on knowledge of *Saccharomyces cerevisiae*. It is therefore relevant to compare the metabolite levels of both micro-organisms during chemostat cultivation under the same conditions (aerobic, glucose limited, $D = 0.05 \text{ h}^{-1}$). Table 5 shows the results of the measured intracellular metabolite concentrations for both micro-organisms. First it appears that in both organism similar mass action ratio’s for PGI, enolase and PGM, myokinase and fumarase are obtained. Also the mass action ratio of G1P over F6P is similar.
Table 2.5 Measured average metabolite concentrations of *Penicillium chrysogenum* and *Saccharomyces cerevisiae* cultivated under similar conditions in aerobic, glucose limited chemostats at a dilution rate of 0.05 h\(^{-1}\).

<table>
<thead>
<tr>
<th>Metabolite</th>
<th><em>P. chrysogenum</em> (µmol.g DW(^{-1}))</th>
<th><em>S. cerevisiae</em> (µmol.g DW(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>G6P</td>
<td>3.29 ± 0.14</td>
<td>2.10 ± 0.05</td>
</tr>
<tr>
<td>G1P</td>
<td>0.61 ± 0.03</td>
<td>0.34 ± 0.08</td>
</tr>
<tr>
<td>F6P</td>
<td>0.81 ± 0.04</td>
<td>0.45 ± 0.05</td>
</tr>
<tr>
<td>F1,6bisP</td>
<td>0.74 ± 0.05</td>
<td>0.19 ± 0.03</td>
</tr>
<tr>
<td>2PG + 3PG</td>
<td>0.40 ± 0.02</td>
<td>1.20 ± 0.02</td>
</tr>
<tr>
<td>PEP</td>
<td>0.24 ± 0.01</td>
<td>1.10 ± 0.05</td>
</tr>
<tr>
<td>PYR</td>
<td>0.52 ± 0.05</td>
<td>0.06 ± 0.00</td>
</tr>
<tr>
<td>Cit + Isocit</td>
<td>5.74 ± 0.43</td>
<td>4.40 ± 0.11</td>
</tr>
<tr>
<td>α-KG</td>
<td>1.12 ± 0.06</td>
<td>0.07 ± 0.00</td>
</tr>
<tr>
<td>SUC</td>
<td>0.35 ± 0.05</td>
<td>0.06 ± 0.00</td>
</tr>
<tr>
<td>MAL</td>
<td>3.05 ± 0.12</td>
<td>0.28 ± 0.02</td>
</tr>
<tr>
<td>FUM</td>
<td>0.70 ± 0.03</td>
<td>0.05 ± 0.00</td>
</tr>
<tr>
<td>ATP</td>
<td>6.25 ± 0.18</td>
<td>7.40 ± 0.01</td>
</tr>
<tr>
<td>ADP</td>
<td>0.97 ± 0.06</td>
<td>1.73 ± 0.06</td>
</tr>
<tr>
<td>AMP</td>
<td>0.14 ± 0.04</td>
<td>0.46 ± 0.06</td>
</tr>
</tbody>
</table>

The adenine nucleotide levels in both organisms are highly similar and the energy charges (\(\frac{[ATP]}{2[ADP]}\)) are 0.92 for *Penicillium chrysogenum* and 0.86 for *Saccharomyces cerevisiae*. Furthermore it appears that the concentrations of the glycolytic intermediates are nearly identical up to F16bP. However, the concentrations of F16bP to 2&3PG are very different. It is obvious that in *Penicillium chrysogenum* the level of F16bP is much higher and the level of 2&3PG is much lower. Usually one considers the reaction connecting these metabolites to be in equilibrium (aldolase, triose phosphate isomerase, glyceraldehyde-3-phosphate dehydrogenase, phosphoglycerate kinase and phosphoglycerate...
mutase). The overall near equilibrium reaction follows as
\[ \frac{1}{2} F^{16b}P + ADP + P_i + NAD^+ \leftrightarrow 2 \cdot 3PG + NADH + H^+ + ATP. \]

Assuming an equilibrium constant \( K \), one can write
\[ \frac{NADH}{NAD^+} = K \frac{P_i}{H^+} \left( \frac{ADP}{ATP} \right) \left( \frac{(F^{16b}P)^{1/2}}{2 \cdot 3PG} \right) \]

Using the available concentration data and assuming that in both organisms \( K \), \( H^+ \), and \( P_i \) are the same, it can be calculated that the quotient of the \( \frac{NADH}{NAD^+} \) ratio’s in *Penicillium chrysogenum* compared to *Saccharomyces cerevisiae* is
\[ \left( \frac{NADH}{NAD^+} \right)_{P.chry} = \frac{1}{4} \left( \frac{NADH}{NAD^+} \right)_{S.cere} = 4.0 \]

Furthermore, it should be considered that *Saccharomyces cerevisiae* is cultivated at pH = 5, while *Penicillium chrysogenum* is cultivated at pH = 6.5. This might lead to a higher cytosolic pH in *Penicillium chrysogenum*. A pH difference of 1 unit would change the quotient of the \( \frac{NADH}{NAD^+} \) ratio from 4 to 40.

A very remarkable other difference is the intracellular concentration of pyruvate which is very high in *Penicillium chrysogenum*, and might be associated with the presence of penicillin production. In the penicillin biosynthesis pathway pyruvate is required to produce the valine needed for the biosynthesis of the tripeptide ACV (van Gulik et al., 2000). Finally, the concentrations of nearly all TCA cycle intermediates in *Penicillium chrysogenum* are 5 to 20 times higher than in *Saccharomyces cerevisiae*. This may be associated with the high energy need in *Penicillium chrysogenum* (van Gulik et al., 2000) leading to a higher TCA-cycle fluxes. This is supported by Table 2.1a which shows that the \( q_{O_2} \) for *Penicillium chrysogenum* is significantly higher than *Saccharomyces cerevisiae*.

**2.4 Conclusions**

A dedicated chemostat system for cultivation of the filamentous fungus *Penicillium chrysogenum*, suited for rapid sampling and quenching of biomass for metabolome analysis under steady state as well as highly dynamic conditions has been developed. Using this system steady state samples where withdrawn for LC-ESI-MS/MS analysis of concentrations of intermediates of the glycolysis, the TCA cycle and the adenine nucleotides ATP, ADP and AMP. By addition of a 100% U-\(^{13}\)C labeled standard mix prior to the metabolite extraction procedure and subsequent metabolite analysis with isotope dilution mass spectrometry.
Measurement of intracellular metabolites of primary metabolism

(IDMS) partial degradation of metabolites, non-linearity and drift of the LC-MS/MS and matrix differences between *Saccharomyces cerevisiae* and *Penicillium chrysogenum* could be successfully compensated for. From calculation of the mass action ratios for myokinase, phosphogluco isomerase (PGI), the lumped reaction of enolase and phosphoglycerate mutase (PGM) and fumarase it was found that these enzymes operate close to equilibrium under the cultivation conditions applied.

A comparison of metabolome result of *Penicillium chrysogenum* and *Saccharomyces cerevisiae* showed clear similarities (mass action ratio’s, first part of glycolysis, adenenin nucleotides) but also large differences. Higher level of pyruvate and TCA cycle intermediates in *Penicillium chrysogenum* are probably caused by the penicillin product pathway causing a higher pyruvate drain and much higher energy need respectively.

### NOMENCLATURE

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>2PG</td>
<td>2-Phosphoglycerate</td>
</tr>
<tr>
<td>3PG</td>
<td>3-Phosphoglycerate</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine Diphosphate</td>
</tr>
<tr>
<td>a-KG</td>
<td>Alpha-ketoglutarate</td>
</tr>
<tr>
<td>AMP</td>
<td>Adenosine Monophosphate</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine Triphosphate</td>
</tr>
<tr>
<td>Cit + Isocit</td>
<td>Pool of Citrate and Isocitrate</td>
</tr>
<tr>
<td>F1,6bisP</td>
<td>Fructose 1,6 bisphosphate</td>
</tr>
<tr>
<td>F6P</td>
<td>Fructose-6-phosphate</td>
</tr>
<tr>
<td>FUM</td>
<td>Fumarate</td>
</tr>
<tr>
<td>G1P</td>
<td>Glucose-1-phosphate</td>
</tr>
<tr>
<td>G6P</td>
<td>Glucose-6-phosphate</td>
</tr>
<tr>
<td>MAL</td>
<td>Malate</td>
</tr>
<tr>
<td>NAD</td>
<td>Nicotinamide Adenine Dinucleotide (oxidized)</td>
</tr>
<tr>
<td>NADH</td>
<td>Nicotinamide Adenine Dinucleotide (reduced)</td>
</tr>
<tr>
<td>PEP</td>
<td>Phosphoenol pyruvate</td>
</tr>
<tr>
<td>PYR</td>
<td>Pyruvate</td>
</tr>
<tr>
<td>SUC</td>
<td>Succinate</td>
</tr>
</tbody>
</table>
Chapter 2

References


Chapter 3

Generating short-term kinetic responses of primary metabolism of *Penicillium chrysogenum* through glucose perturbation in the bioscope mini reactor

Abstract

A first study of the *in vivo* kinetic properties of primary metabolism of *Penicillium chrysogenum* is presented. Dynamic metabolite data have been generated by rapidly increasing the extracellular glucose concentration of cells cultivated under well defined conditions in an aerobic glucose limited chemostat followed by measurement of the fast dynamic response of the primary metabolite levels (glucose pulse experiment). These experiments were carried out directly in the chemostat as well as in a mini plug flow reactor (BioScope) outside the chemostat. The results of the glucose pulse experiments carried out in the chemostat and the Bioscope were highly similar. During the 90 sec time window of the pulse experiment, the glucose consumption rate increased to a value twice as high as in the steady state, a much lower increase than observed for the fermenting yeast *Saccharomyces cerevisiae* under similar conditions. Although the observed metabolite patterns in *P. chrysogenum* were comparable to *S. cerevisiae* large differences in the magnitude of the dynamic behavior were observed between both organisms. During the pulse experiment the level of glycolytic and TCA cycle intermediates, and adenine nucleotides changed between 2 to 5 fold. Furthermore a highly similar 5 fold increase in the cytocolic NADH/NAD ratio could be calculated from two independent equilibrium assumptions (fructose 1,6 bis-phosphate to the pool of 2PG and 3PG and oxaloacetate to fumarate with glutamate transaminase). It was also found that the C4 pool (aspartate, fumarate, and malate) became much more reduced due to this increase in NADH/NAD ratio. Equilibrium conditions were confirmed to exist in the hexose-P pool, the glycolysis between F16bP and 2PG+3PG and in the C4 pool of the TCA cycle (fumarate, malate, oxaloacetate and aspartate).

3.1 Introduction

Microorganisms are increasingly becoming relevant as production platform for the synthesis of a huge variety of chemicals from renewable carbon sources, due to the ever increasing possibilities of genetic modification. This area of research is called metabolic engineering, where the challenge is to identify gene targets which lead to desirable improvement of the production organism (higher yield and rate of product formation, less by-products, etc). Use of a mathematical model of microbial metabolism is regarded as a suitable method to identify such targets. Knowledge of the relevant metabolic pathways, the involved enzymes, their kinetic properties, and their genetic regulation are required to create such a mathematical model of metabolism. The pathways and specific enzymes involved can be identified from transcript and biochemical analysis. The kinetic properties of the enzymes have usually been obtained from in-vitro experiments using purified enzyme. However, it has become increasingly clear that the in vitro kinetic properties often do not apply to in vivo conditions. Main reason for this is that the in-vivo environment of the enzyme strongly deviates from the in-vitro conditions applied for the measurement of the kinetic parameters (higher substrate and lower enzyme concentrations, absence of other proteins and metabolites, different pH etc.). One of the prerequisites for the construction of meaningful kinetic models of metabolism is therefore that the kinetic parameters of the relevant enzymes are measured under in-vivo conditions.

This has been recognized and kinetic studies to elucidate metabolite interaction and control under in-vivo conditions have increasingly being used (Theobald et al., 1993, Theobald et al., 1997, Rizzi et al., 1997, Visser et al., 2002, Mashego et al., in press). In these studies the cells are cultivated under well defined conditions in a chemostat until a steady state is reached. Subsequently this steady state culture is disturbed by the addition of a certain perturbing agent (e.g. a substrate, activator or inhibitor) where after the induced transient behavior of metabolism is recorded by measuring the concentrations of intra and extracellular metabolites within a certain time interval. This method has a number of advantages. Firstly, the in vivo approach explores the whole metabolic network in one experiment, contrary to the in vitro approach where each enzyme is studied separately; secondly, dynamic data are rich in information; and thirdly, by studying the response of the metabolome within a short time interval (~300 seconds) it is allowed to assume that no changes occur in enzyme levels.
Under these conditions only the concentrations of metabolites and allosteric effectors influence the catalytic activities of the enzymes. Recently a dedicated device (BioScope) has been developed to carry out short term pulse response experiments (Visser et al. 2002). With this device perturbation of the steady state chemostat itself (the conventional approach) is avoided and only a small flow of broth is withdraw and perturbed outside the chemostat. The BioScope system has several advantages (Visser et al. 2002), e.g. a large number of different perturbation experiments can be carried out using biomass from the same chemostat and thus with identical properties; the system can be applied to study in vivo kinetic properties of cells cultivated in fed batch and large scale fermentations; only a small amount of perturbing agent is required and the sample volume is in principle unlimited.

Recently (Mashego et al., 2006), an improved BioScope has been developed and used for pulse experiments to study the in vivo kinetics of Saccharomyces cerevisiae. Another relevant industrial microorganism is the filamentous fungus Penicillium chrysogenum, which is applied for the production of β-lactam antibiotics. In a previous study metabolic flux balancing has been applied to study primary metabolism and its interaction with the penicillin production pathway (van Gulik et al. 2000, 2001). From this study it has been found that penicillin production alters the fluxes through primary metabolism especially with respect to the pentose phosphate pathway and ATP metabolism. Kinetic modeling of the relevant parts of primary metabolism and their interaction with the penicillin biosynthesis pathway should give more insight in how primary and secondary metabolism in P. chrysogenum are interconnected.

This work is a first attempt to generate dynamic data for elucidation of the in vivo kinetic properties of primary metabolism of P. chrysogenum using stimulus response experiments. Moreover these experiments are carried out in the newly developed Bioscope and the results are compared with results from similar experiments carried out in the chemostat. A comparison is made with the dynamic response of Saccharomyces cerevisiae in a similar glucose pulse experiment.

3.2 Material and Methods

Strain A high-yielding strain of Penicillium chrysogenum (code name DS17690), was used for all experiments. This strain was kindly donated by DSM Anti-Infectives, Delft, The Netherlands.
Chemostat Cultivation  The chemostat used was a 7-L turbine-stirred bioreactor (Applikon, Schiedam, The Netherlands) with a working volume of 4L. The chemostat was operated at a dilution rate \( D = 0.05 \) h\(^{-1}\). The culture was aerated with 9.33 mol of air per hour (= 0.925 vvm under the applied conditions). Mixing of the reactor content was accomplished with two six-bladed Ruston turbine impellers (diameter 8 cm) operated at a rotation speed of 500 rpm. Foam formation was suppressed by addition of approximately 70 µL/h of anti foam agent BC86/013 (Basildon Chemicals, Abingdon, UK).

The temperature of the reactor was kept at 25.0 +/- 0.1 °C by means of a thermocirculator. The pH of the culture was maintained at 6.5 with 4N NaOH by an automatic pH-control system (Applikon) using a sterilizable pH probe mounted in the reactor. The dissolved oxygen tension was monitored but not controlled. During the experiments the dissolved oxygen tension never dropped below 50% of air saturation. The concentrations of \( O_2 \) and \( CO_2 \) in the off gas were analyzed using a combined paramagnetic/infrared gas analyzer (NGA 2000 MLT 1, Fisher-Rosemount GMbH&Co Hasselroth Germany). Chemostat cultures were directly inoculated with spores grown on rice grains. This was done by gently shaking 10 g of spore containing rice grains in 100 mL of sterile demi water until most of the spores were released. After removal of the rice grains by sieving, the suspended spores were transferred to the chemostat. Chemostat cultivation was preceded by a batch phase. Shortly before the end of the batch phase, e.g. shortly before exhaustion of the carbon source, the feed was started.

Feed medium  The medium was designed for a biomass concentration of 6 g/L dry weight and contained 16.5 g/L glucose\( \cdot \)H\(_2\)O, 1 g/L KH\(_2\)PO\(_4\), 5 g/L (NH\(_4\))\(_2\)SO\(_4\), 0.5 g/L MgSO\(_4\)\( \cdot \)7H\(_2\)O, and 10ml/L of trace element solution. The trace element solution contained 15 g/L Na\(_2\)EDTA\( \cdot 2\)H\(_2\)O, 0.5 g/L CuSO\(_4\)\( \cdot 5\)H\(_2\)O, 2 g/L ZnSO\(_4\)\( \cdot 7\)H\(_2\)O, 2 g/L MnSO\(_4\)\( \cdot \)H\(_2\)O, 4 g/L FeSO\(_4\)\( \cdot 7\)H\(_2\)O and 0.5 g/L CaCl\(_2\)\( \cdot 2\)H\(_2\)O. For penicillin-G production the side chain precursor phenylacetic acid (PAA) was added at a concentration of 0.94 g/L. The preparation and sterilization of the cultivation medium was carried out as described previously (van Gulik et al. 2000).

BioScope System  The BioScope system is based on a mini plug flow reactor (Visser et al. 2002). During operation a small flow of broth is continuously withdrawn from the chemostat and is pumped into the BioScope channel where it is mixed with a continuous flow of perturbing agent, e.g. a glucose solution. In the original version of the Bioscope a silicon tube
channel was used, configured as a serpentine system. In a newer design of the device the serpentine channels were milled in a Perspex block (Mashego, et al., 2006). In this new design the circular channel was divided into two hemi-circular channels by a silicone membrane, i.e. a gas and a liquid channel. The broth from the chemostat is pumped through one of the channels, while a continuous flow of gas is introduced in the opposite channel at the other side of the membrane. O₂ and CO₂ are exchanged via the membrane with the gaseous phase in the opposite channel. This design appeared to be more robust and easy to operate, showed good plug flow characteristics and a large aeration capacity.

The hemispheric gas and liquid channels of the BioScope system used had a diameter of 1.2 mm, and a total length of 6.5 m, corresponding to a volume of 3 mL for each channel. The gas channel was flushed with normal air at a flowrate of 130 mL.min⁻¹. The broth flow rate used was 1.8 mL/min and the perturbation solution, which contained 5 g/L of glucose, had a flow rate of 0.2 mL/min. This increased the glucose concentration at the entrance of the Bioscope from 20 mg/L, which is the residual glucose level in the chemostat, to 520 mg/L. Broth samples were obtained at residence times of 2, 6.8, 10.5, 15.3, 21.6, 27.7, 37.7, 48.1, 69 and 91.7 seconds for sampling ports 2 to 11, respectively. Sample port 1 was located just before the entrance of the glucose solution, thus from this port samples from the steady state chemostat broth were obtained.

The whole BioScope system, including pumps and glucose solution bottle, was placed in a temperature-controlled cabinet kept at the same temperature as the chemostat (25°C).

**The Broth Recycle Loop** In order to avoid the entrance of air bubbles from the chemostat into the BioScope channel, a broth recycle sample loop was constructed (see Figure 2.1). This recycle loop was driven by a pump (P₁) withdrawing fermentation broth at one side and returning it into the chemostat at the other side (L₂ part in Fig. 3.1). To minimize the residence time of the broth in the recycle loop a high pumping speed was chosen and the loop was kept as short as possible. Directly behind the entrance of the recycle loop, part of the broth is fed to the BioScope via L₁ while the remainder is pumped back into the chemostat via L₂. Because the flow rate of the broth in the recycle loop is much higher than the flow rate in L₃, that is towards the BioScope, all air bubbles remain trapped in the recycle stream.

A detailed overview of the volumes, flows, and residence times in the different loops is given in Table 3.1.
Figure 3.1 Schematic drawing of the recycle loop system

Table 3.1 Dimensions, flow rates and calculated residence times for the recycle loop system.

<table>
<thead>
<tr>
<th></th>
<th>Length [cm]</th>
<th>Volume [ml]</th>
<th>Rate [ml/s]</th>
<th>Residence time [s]</th>
</tr>
</thead>
<tbody>
<tr>
<td>L₁</td>
<td>10</td>
<td>0.23</td>
<td>2.36</td>
<td>0.10</td>
</tr>
<tr>
<td>L₂</td>
<td>85</td>
<td>7.80</td>
<td>2.36</td>
<td>3.31</td>
</tr>
<tr>
<td>L₃</td>
<td>47</td>
<td>0.29</td>
<td>0.03</td>
<td>9.67</td>
</tr>
</tbody>
</table>

Chemostat pulse experiments For the pulse response experiment carried out in the chemostat 16 mL of a 125 g/L glucose solution was injected into the reactor within 1 second. This increased the residual glucose concentration to approximately 0.5 g/L. Subsequently, rapid sampling of broth for measurement of intracellular metabolites was carried out at 5, 10, 15, 20, 25, 35, 50, 70 and 90 seconds after injection of the glucose solution. Rapid sampling and quenching was carried out as described previously (Lange et al., 2001). Sampling times were chosen similar to the sampling times applied for the BioScope experiments. Rapid sampling for determination of extracellular metabolite concentrations was carried out according to Mashego et al. (Mashego et al. 2003).

BioScope pulse experiments In order to avoid clogging, water at 25°C was pumped through the BioScope channel for a period of about 3 minutes before starting the experiment.
Subsequently culture broth and glucose solution (5 g/L) were fed to the BioScope at flow rates of 1.8 mL/min and 0.2 mL/min, respectively. The sampling sequence was started after operating the BioScope for a period of at least 3-residence times (9 minutes) to let the system stabilize. Sampling was carried out from back to forth, that is from port 11 to 1 and was performed automatically by means of computer-controlled valves. In this way, 1 mL of sample was withdrawn via every sampling port and was instantaneously quenched in a 60 % (v/v) methanol/water solution at –35°C. Further details on the sampling and quenching method have been described previously (Visser et al. 2002).

**Metabolite Extraction**

Metabolite extraction was carried out with the hot ethanol method (Gonzalez et al. 1997), which was adapted for the extraction of *P. chrysogenum* cells. The adapted methods for cold centrifugation and washing of the cells, metabolite extraction and sample preparation have been described elsewhere (Nasution et al., 2005, in press).

**Analytical procedures**

The biomass dry weight concentration was measured by filtration of 10 mL of culture broth on a pre-weighed glass fiber filter (type A/E, Pall Life Sciences, Ann Arbor, Michigan, USA) and drying to constant weight (24 hours at 70°C). The concentrations of the glycolytic and TCA cycle intermediates in the cell extracts were analyzed with isotope dilution mass spectrometry (IDMS) as described in Wu et al. (2005). Intracellular nucleotides were analyzed using LC-ESI-MS/MS. The nucleotides were separated by an ion pairing reversed phase HPLC method, using a XTerra MS C18 column (100 mmx 1mm) equipped with guard column (10 mm x 2.1 mm) (both from Waters, Milford, USA) as described previously (Wu et al., 2005, submitted).

Concentrations of PAA and PenG in the culture supernatant were measured with high performance liquid chromatography (HPLC) as described in Christensen et al. (1994). The glucose concentration in the culture supernatant was analyzed spectrophotometrically (Agilent 8453 UV-Visible Spectroscopy System, Waldbronn, Germany) using a Boehringer Mannheim enzymatic bioanalysis kit according to the manufacture’s instructions. The concentrations of organic acids (citrate, pyruvate, succinate, lactate and acetate) in the culture supernatant were determined by HPLC analysis with Aminex® HPC-87H Column (Biorad, Hercules, USA) at 60°C with 5mM H2SO4 as the mobile phase.
3.3 Result and Discussion

Steady state chemostat cultures  For pulse response experiments it is essential to start from reproducible and well defined cultivation conditions. To achieve this glucose limited chemostat cultivations were carried out under standardized conditions at a growth rate $\mu = 0.05 \text{ h}^{-1}$. From the measured time patterns of the biomass dry weight and penicillin concentrations, and the O$_2$ and CO$_2$ concentrations in the offgas during chemostat cultivation of this *P. chrysogenum* strain under the standardized conditions, it was observed that all measured concentrations reached a stable value after 6 residence times and did not significantly change until the experiment was terminated (results not shown). Table 3.2 shows the steady state characteristics of the chemostat in terms of measured concentrations and calculated biomass specific conversion rates. From these rates it was calculated that the carbon and electron balances close for about 90-95%. The most probable reason for the 5 - 10% gap in the carbon and electron balances, is that not all compounds present in the supernatant have been measured, in particular polymeric byproducts (proteins and polysaccharides) and by-products of penicillin biosynthesis. From previous measurements of the concentrations of these compounds in the culture supernatant under comparable cultivation conditions (van Gulik et al. 2000) it was calculated that the excretion of these compounds indeed represents a gap of 5-10% in the carbon and electron balances. This was confirmed by performing a total organic carbon (TOC) analysis of the supernatant of our *P. chrysogenum* chemostat culture (results not shown).

Table 3.2 Example of measured concentrations and calculated specific conversion rates for a steady state glucose limited chemostat of *P. chrysogenum* operated at a dilution rate of $D = 0.05 \text{ h}^{-1}$. All specific conversion rates are expressed in mmol.Cmol biomass$^{-1}$.h$^{-1}$.

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Specific conversion rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biomass</td>
<td>$6.21 \pm 0.16 \text{ g/L}$</td>
</tr>
<tr>
<td>Penicillin G</td>
<td>$1.81 \pm 0.06 \text{ mM}$</td>
</tr>
<tr>
<td>PAA</td>
<td>$2.86 \pm 0.09 \text{ mM}$</td>
</tr>
<tr>
<td>Glucose</td>
<td>$0.15 \pm 0.06 \text{ mM}$</td>
</tr>
<tr>
<td>O$_2$ in off gas</td>
<td>$20.46 \pm 0.03% \text{ (v/v)}$</td>
</tr>
<tr>
<td>CO$_2$ in off gas</td>
<td>$0.52 \pm 0.08% \text{ (v/v)}$</td>
</tr>
</tbody>
</table>
**Chemostat recycle loop system**  
In first instance a simple direct connection between chemostat and Bioscope was used. However, from measurement of the biomass concentration in broth samples taken from the different ports of the Bioscope large differences were observed with the steady state biomass concentration in the chemostat (Figure 3.2).

![Figure 3.2](image)

**Figure 3.2** Measured biomass concentrations at the sample ports of the Bioscope at different flow rates using a direct connection between Bioscope and fermentor. The displayed biomass concentrations are averages of biomass samples from all 11 sampling ports.

It can be seen from this figure that at lower flow rates the biomass concentration in the broth from the Bioscope was significantly lower than in the fermentor. Especially at the intended flow rate of 2 mL/min the difference was largest. Apparently part of the biomass was retained in the tubing connecting the BioScope with the chemostat. In an attempt to solve this problem a recycle loop system was introduced (Figure 3.1) which should prevent retention of biomass by fast recirculation of the broth. This appeared to be a significant improvement as can be inferred from the measured biomass concentrations displayed in Table 3.3.
Table 3.3 Measured biomass concentrations at the different ports of the BioScope system with recycle loop.

<table>
<thead>
<tr>
<th>Chemostat [g/L]</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>Average [g/L]</th>
</tr>
</thead>
</table>

From a comparison of the average biomass concentration measured at the 11 BioScope sampling ports with the biomass concentration measured in the chemostat there appeared to be no significant difference. From the applied tube volumes and flow rates (see Table 3.1) it was calculated that the residence time of the broth in the tube system between the chemostat and the Bioscope would be approximately 10 seconds. Using the experimentally determined consumption rates of glucose and oxygen (Table 3.2) it was calculated that within this time period the residual glucose concentration would decrease from a value of 20 mg/L in the chemostat, to approx. 18.4 mg/L at the entrance of the BioScope. In a similar way it was calculated that the dissolved oxygen level, which is equal to 0.28 mol/m³ in the chemostat, would decrease to approx. 0.26 mol/m³ at the entrance of the BioScope. Hence, the calculated decrease in the dissolved oxygen level and residual glucose concentration are relatively small, that is 7% and 8% respectively and were not expected to have a significant influence on the physiological state of the cells. As an independent check for possible changes in intracellular metabolite concentrations of biomass directly sampled from the chemostat and biomass withdrawn from the first sampling port of the Bioscope (which is before entrance of the glucose solution,) were compared (Table 3.4). It can be seen from this table that most metabolite levels are comparable.

Comparison of Pulse Experiments in the Chemostat and the Bioscope

The Bioscope pulse experiment was carried out using broth from a chemostat which had been running for 11 residence times. Approximately 24 hours later the same chemostat was used to carry out a second pulse experiment, whereby the complete culture was perturbed by injecting the glucose directly in the fermentor. Figure 3.3 shows the comparison of the measured patterns of the extracellular glucose concentration vs time for glucose pulse experiments carried out in the BioScope and directly in the chemostat. From the relatively slow increase of the glucose concentration during the first 15 seconds after addition of the glucose pulse to the
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In a chemostat it can be concluded that only after 15 seconds complete mixing of the glucose with the broth is achieved. In contrast to this the mixing in the BioScope appears to be instantaneous, as can be inferred from the measured glucose concentration during the first 15 seconds. It can also be seen from this figure that the glucose concentration measurements for the BioScope perturbation experiment were too scattered to calculate the glucose uptake rate. Therefore, the glucose uptake rate was calculated from the chemostat perturbation experiment. Assuming a linear decrease of the glucose concentration in time, the average glucose uptake rate was calculated to be approximately 36 mmol/Cmol.h. This value is about 2 times higher than the steady state value (see Table 3.2).

Table 3.4 Steady state concentrations of intracellular metabolites (µmol/g DW) in *P. chrysogenum* cells sampled from the chemostat and from the BioScope (from sample port 1).

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>BioScope</th>
<th>Chemostat</th>
</tr>
</thead>
<tbody>
<tr>
<td>G1P</td>
<td>0.429±0.036</td>
<td>0.608±0.036</td>
</tr>
<tr>
<td>G6P</td>
<td>2.229±0.259</td>
<td>3.391±0.036</td>
</tr>
<tr>
<td>F6P</td>
<td>0.515±0.052</td>
<td>0.805±0.026</td>
</tr>
<tr>
<td>F16bP</td>
<td>0.491±0.064</td>
<td>0.346±0.003</td>
</tr>
<tr>
<td>23PG</td>
<td>0.363±0.015</td>
<td>0.340±0.019</td>
</tr>
<tr>
<td>PEP</td>
<td>0.202±0.009</td>
<td>0.227±0.006</td>
</tr>
<tr>
<td>Pyr</td>
<td>0.542±0.040</td>
<td>0.482±0.014</td>
</tr>
<tr>
<td>αKG</td>
<td>1.170±0.110</td>
<td>1.135±0.023</td>
</tr>
<tr>
<td>Succ</td>
<td>0.263±0.010</td>
<td>0.252±0.010</td>
</tr>
<tr>
<td>Fum</td>
<td>0.683±0.024</td>
<td>0.659±0.047</td>
</tr>
<tr>
<td>Mal</td>
<td>3.430±0.110</td>
<td>2.975±0.331</td>
</tr>
<tr>
<td>Cit/Isocit</td>
<td>5.179±0.161</td>
<td>4.504±0.033</td>
</tr>
<tr>
<td>ATP</td>
<td>6.107±0.025</td>
<td>-</td>
</tr>
<tr>
<td>ADP</td>
<td>0.936±0.004</td>
<td>-</td>
</tr>
<tr>
<td>AMP</td>
<td>0.405±0.120</td>
<td>-</td>
</tr>
</tbody>
</table>
No organic acids (e.g. citric, lactic, succinic, acetic) were excreted during the glucose pulse experiment as these could not be detected by means of HPLC analysis of the supernatant (data not shown).

Figure 3.4 shows the measured dynamic time patterns of the glycolytic and TCA cycle intermediates during the pulse experiments. The differences between the Bioscope and fermentor experiments appeared to be small. It can therefore be concluded that the Bioscope is a suitable platform to perform in vivo kinetic experiments with the filamentous fungus *Penicillium chrysogenum*.

Extracellular glucose concentration vs. time during glucose pulses in the chemostat (o) and BioScope (●). The linear line used to calculate $q_s$.

*In vivo kinetic response of primary metabolism of Penicillium chrysogenum to a glucose pulse* The two fold increase of the glucose uptake rate after addition of the glucose pulse led to measurable changes in intracellular metabolite levels. It can be seen from Figure 3.4 that the levels of the hexose-phosphates G6P, G1P and F6P all increased approximately with a factor 3. Also the level of F16bP increased although the increase was much more pronounced (up to a factor 6). No significant changes were observed for 2&3PG, whereas the level of PEP was found to decrease, while the level of pyruvate increased.

Figure 3.5 shows the time patterns of the adenine nucleotide levels, which were only measured for the fermentor pulse experiment. It can be seen from this figure that the ATP level decreases linearly to reach a pseudo steady state at 50 seconds after addition of the
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glucose pulse. Both the levels of AMP and ADP show a linear increase until t=30 seconds. Thereafter a linear decrease is observed for both AMP and ADP to reach a pseudo steady state, at 70 – 80 seconds after addition of the glucose pulse, at levels slightly higher than their steady state concentrations.

Calculation of the sum of the adenine nucleotides reveals that also in P. chrysogenum this sum decreases significantly, as has been observed previously in pulse experiments in S. cerevisaeae (Theobald et al. 1997; Mashego et al., in press). At 50 seconds after the glucose pulse the adenine nucleotide sum reached a pseudo steady state at a value of approximately 85% of the steady state value. The reason for this rapid net disappearance of adenine nucleotides remains unclear.

**Figure 3.4** Response of glycolytic and TCA Cycle intermediates to a glucose pulse in chemostat (○) and BioScope (●)
It can be calculated from these results that the energy charge during steady state conditions is high (0.9) and slightly decreases to a value of 0.8 at approx. 40 seconds after addition of the pulse. However, already after 60 seconds a slight recovery is observed, although the energy charge did not come back to the steady state level but reached a plateau at a value of approx 0.8. A comparable response is observed in similar glucose pulse experiments in *S. cerevisiae* (Mashego et al., in press). However, in *S. cerevisiae* the energy charge was observed to return to the steady state level, 60 seconds after addition of the glucose pulse.

![Figure 3.5](image-url)

**Figure 3.5** Upper panels: response of the adenine nucleotides (ATP, ADP, AMP) to a glucose pulse experiment carried out in the chemostat; lower panel: calculated sum of the adenine nucleotides, mass action ratio of myokinase and Energy charge vs time.

In Figure 3.4 the measured time patterns of the TCA cycle intermediates are shown. It can be seen from this figure that the pool of citrate + isocitrate, which is known to be relatively large, does not change significantly during the time window of the glucose pulse experiment. The $\alpha$KG level was found to decrease to two third of its steady state value at 50 seconds but recovered to reach its steady state value at 120 seconds after the glucose pulse. In contrast to this the levels of its C4 products (succinate, malate and fumarate) strongly increased to reach a roughly 3 fold higher pseudo steady state value at 120 seconds after the glucose pulse.
Calculation of mass action ratios  From the measured dynamic metabolite patterns a direct insight in the existence of equilibrium reactions can be obtained. In case of the hexose phosphates equilibrium was observed to exist for phosphoglucoisomerase and phosphoglucomutase, as can be inferred from the absence of significant changes in the calculated mass action ratios (see Figure 3.6). A similar conclusion can be drawn from the calculated mass action ratio for fumarase. The mass action ratio for enolase was observed to decrease slightly after addition of the glucose pulse. The calculation of the mass action ratio for myokinase from the measured concentrations (see figure 3.5) yields values between 0.6 and 0.8, thus slightly higher than the value of 0.4 during steady state chemostat cultivation. These results indicate that these reactions operated close to equilibrium during the glucose pulse experiment as the calculated mass action ratios for these enzymes appear to remain very close to the known equilibrium constants (Visser et al., 2000 and references therein).

Figure 3.6 Calculated mass action ratios of Phosphogluco-isomerase, Phosphogluco-mutase, Fumarase and Enolase in chemostat (○) and BioScope (●) and their equilibrium constant (---)

Estimation of the cytosolic NAD/NADH ratio  As the redox status of the cell directly affects the rates of many reactions it is important to be able to calculate the change of the cytosolic NADH/NAD ratio during pulse response experiments. This can be done by using
the assumption that there exists an equilibrium pool between F16bP and 2&3PG. The overall reaction of this pool is

\[ \frac{1}{2} F16bP + ADP + P_i + NAD^+ \rightleftharpoons (2 & 3PG) + NADH + H^+ + ATP \]  \hspace{1cm} (3.1)

Assuming an equilibrium constant \( K \), the cytosolic (NADH/NAD) ratio can be written as:

\[ \frac{NADH}{NAD^+} = K \frac{P_i}{H^+} \left( \frac{ADP}{ATP} \right) \left( \frac{F16bP}{2 & 3PG} \right)^{1/2} \]  \hspace{1cm} (3.2)

Using the measured metabolite levels (and assuming constant \( H^+ \) and \( P_i \)), the fold change of the cytosolic (NADH/NAD) ratio during the pulse can be calculated relative to the steady state. It can be seen from the result shown in Figure 3.7 that the cytosolic (NADH/NAD) couple becomes transiently much more reduced as a 5 fold increase of the NADH/NAD ratio is calculated. This might be an explanation for the observed transient behaviour of the TCA cycle metabolites. It is known that OAA, aspartate, malate and fumarate are connected through reversible enzymes (aspartate transaminase, malate dehydrogenase and fumarase). The overall reaction can then be written as:

\[ Asp + aKG + NADH + H^+ \rightleftharpoons Glut + Fum + NAD^+ \]  \hspace{1cm} (3.3)

**Figure 3.7** Fold change in the ratio of NADH and NAD\(^+\) during the glucose pulse experiment relative to the steady state value calculated from assumed equilibrium between F16bP and
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2&3PG (●), and calculated from assumed equilibrium between transaminase, malate dehydrogenase and fumarase (○).

Assuming equilibrium an alternative expression for the NADH/NAD ratio (with an overall equilibrium constant K') can be derived:

$$\frac{NADH}{NAD} = K' \frac{Fum \cdot Glut}{aKG \cdot Asp \cdot H^+} \quad (3.4)$$

Measurements of the levels of free intracellular amino acids during the pulse experiment (data not shown) indicate that the concentration of glutamate did not significantly change whereas the level of aspartate decreased approx. 20%. Assuming that no changes occur in the intracellular pH, the fold change of the NADH/NAD ratio can also be calculated from these metabolite data using Eq. 4. The result is also plotted in Figure 3.7 and appears to be strikingly similar to the fold change calculated from the F16bP to 2/3PG equilibrium, in spite of the time delay which is observed when using the latter reaction (Eq 4).

The similarity of both calculated patterns indicates that the increase in the C4-pool (aspartate, malate, fumarate) is caused by the increased reduction status of the NADH/NAD pool in the cytosol.

**Comparison with similar pulse experiment in Saccharomyces cerevisiae** It is interesting to compare the dynamic response of Penicillium chrysogenum to a glucose pulse to the response of Saccharomyces cerevisiae under the same conditions, i.e. growth rate, steady state biomass concentration and concentration of the glucose pulse (Mashego et al, 2006). Some clear differences have been observed between the responses of the two species to a similar glucose pulse: product secretion does not occur in P. chrysogenum, while, due to the Crabtree effect S. cerevisiae excretes large amounts of ethanol and acetate (Visser. et al., 2004, Mashego at al. in press). Consequently, the increase of the glucose uptake rate and thus the glycolytic flux observed in S. cerevisiae was higher (approx. 6 fold) than in P. chrysogenum (approx. 2 fold).

In figure 3.8 the measured dynamics of the glycolytic and TCA-cycle intermediates and the adenine nucleotides during similar glucose pulse experiments in P. chrysogenum and S. cerevisiae (data from Mashego et al., 2006), both carried out in a chemostat, are compared. It can be seen from this figure that the dynamic patterns of most of the glycolytic intermediates are qualitatively similar for both species. However, in P. chrysogenum the changes are much less pronounced, which is most probably caused by the much smaller increase of the glycolytic flux after addition of the glucose pulse. In S. cerevisiae the hexose phosphates G6P,
G1P and F6P show a very rapid 4 to 6 fold increase after the pulse and return almost to their steady state values 80 seconds after addition of the glucose pulse whereas in *P. chrysogenum* the increase is approximately twofold and the hexose phosphate levels do not return to their steady state values within the time frame of observation, but slowly increase. In *S. cerevisiae* also the increase of the FbP level and the decrease of 2PG&3PG and PEP are very rapid compared to *P. chrysogenum*, whereas the pyruvate peak observed in *S. cerevisiae* is completely absent. In contrast to the observed differences in the dynamic behaviour of the glycolytic intermediates the changes in the levels of the TCA-cycle intermediates, with the exception of $\alpha$KG, is very similar in both species.
Figure 3.8 Comparison of the short term dynamic response of the glycolytic and TCA cycle intermediates and adenine nucleotides in *P. chrysogenum* (●) and *S. cerevisiae* (○)

The reason for this might be that the changes in the TCA-cycle fluxes are probably much more comparable in both species after the addition of the glucose pulse, as can be inferred from the similar dynamics of the specific oxygen uptake rates of both species during the glucose pulse experiment (results not shown). The cause of the different behaviour of ωKG
remains unclear, but might have been caused by differences in changes of the redox status in both species during the glucose pulse experiments.

Also the dynamic patterns of the adenine nucleotides show similar responses in both species. The ATP level is observed to decrease, although the rate at which this occurs in \textit{P. chrysogenum} is lower than in \textit{S. cerevisiae}. Furthermore the ATP level reaches a pseudo steady state after 50 seconds in \textit{P. chrysogenum} where a recovery of the ATP level is observed in \textit{S. cerevisiae}. With respect to ADP and AMP the observed time patterns in both species are very similar, although in \textit{S. cerevisiae} the increase of the AMP level is much less and for ADP almost absent, whereas a pseudo steady state is reached for both ADP and AMP at a much lower levels. An explanation for the observed differences might be the difference in glycolytic flux. Because the increase of the glycolytic flux induced by the glucose pulse is significantly higher in \textit{S. cerevisiae} the rate of ATP regeneration is also higher. This would lead to the observed faster recovery of the ATP level and lower levels of ADP and AMP in \textit{S. cerevisiae} compared to \textit{P. chrysogenum}.

3.4 Conclusions
For the first time \textit{in vivo} kinetic data from rapid pulse response experiments have been obtained for the filamentous fungus \textit{Penicillium chrysogenum}. The measured responses in glucose pulse experiments carried out directly in the chemostat and in the BioScope were very similar. This demonstrates that BioScope can be applied to generate \textit{in vivo} kinetic data in \textit{P. chrysogenum}. It also demonstrated that the BioScope can be used for filamentous fungi at a considerable biomass concentration. Average changes in metabolite levels as a response to a glucose pulse were 2 to 5 fold in a time frame of 90 seconds. The measured dynamic patterns of the glycolytic and TCA cycle intermediates and the adenine nucleotides as response to a glucose pulse appeared highly similar to the patterns measured in \textit{Saccharomyces cerevisiae}, however, the magnitude of the changes was much less extreme. This is probably due to the much lower increase in the glycolytic flux (2 fold) in \textit{Penicillium chrysogenum} than in \textit{S. cerevisiae} (6 fold). In the metabolic network, equilibrium conditions appear to exist in the hexose-P pool, the glycolysis between F16bP and the pool of 2PG+3PG and in the C4 pool (Fumarate, Malate, OAA, Aspartate) of the TCA cycle. The cytosolic NADH/NAD ratio showed a rapid 5 fold increase as could be calculated from two independent equilibrium assumptions. These successful pulse response experiments are the first step towards the
construction and parameter estimation of an in-vivo kinetic model of primary metabolism of *Penicillium chrysogenum*.

References


Chapter 4

Mass balance analysis of the dynamic interactions between central metabolism and penicillin synthesis in *Penicillium chrysogenum*

**Abstract**  The fast dynamic response of chemostat cultured glucose limited (D = 0.05 h⁻¹) penicillin producing *Penicillium chrysogenum* to a glucose pulse within a time period of 300 seconds has been analyzed in terms of the recoveries of carbon, electrons and ATP, measurements of intracellular concentrations of primary metabolites, adenine nucleotides, free amino acids as well as the rates of glucose and oxygen consumption and carbon dioxide production. Highly dynamic responses of the intracellular concentrations of primary metabolites and adenine nucleotides were observed only during the first 180 seconds after addition of the glucose pulse and were followed by a new pseudo steady. During the pseudo steady state the glucose consumption rate was 2.2 fold higher compared to the steady state before the pulse, whereas the O₂ and CO₂ fluxes increased only 10% and the energy status of the cell was lower. Calculation of the cumulative balances of carbon, electrons and ATP indicated that a significant part of the increased glucose flux was directed towards low energy cost compounds, e.g. storage carbohydrates, while energy costing pathway, like penicillin synthesis are shut down. Within the time frame of the pulse experiment also changes were observed in the levels of free amino acids, in particular the ones with relatively small turnover times. Among these are the three amino acid precursors for penicillinG biosynthesis, their changes also point to a possible shut down of the penicillin synthesis during the pulse. The observed dynamic responses of the intracellular concentration of these precursors indicate a relation between primary metabolism and penicillin production with respect to competition for ATP needed for glucose phosphorylation and ACV formation by ACV synthetase in the penicillin pathway and energy needed for storage material to accommodate the increased glucose flux.
4.1 Introduction

Since the production was initiated in the 1940’s the productivity of the penicillin process has been increased dramatically by means of induction of random mutations and subsequent selection and utilization of superior producing variants of *Penicillium chrysogenum* and process optimization (Nielsen, 1997). However previous studies (Jorgensen et al. 1995; vanGulik et al. 2001) show that there is still room for improvement of the product yield, i.e., amount of penicillin produced per amount of glucose consumed, relative to its theoretical maximal value.

Improvement of antibiotic production, e.g. penicillinG, can to a certain extent be achieved by focusing on the product pathway alone (Theilgaard et al., 2001). However, at a certain point, further increase of the productivity will be limited by the supply of carbon-precursors, e.g. the amino acids cysteine and valine for the synthesis of the β-lactam nucleus, reducing equivalents in the form of NADPH and metabolic energy, ATP, as has been indicated by van Gulik et al., (2000). Their results show that penicillin production draws very heavily from the available pools of ATP, NADPH, and cysteine. The relation between primary metabolism and the penicillin production pathway is therefore of general interest. Until now, this relation has only been studied under (pseudo)steady state conditions with respect to flux only.

However, a full understanding of the relation between primary metabolism and a product pathway requires in vivo kinetic studies (Rizzi et al., 1997; Oldiges et al., 2004). In previous work the stimulus response technique has been successfully applied to elucidate the in-vivo kinetic properties of metabolic pathways in different micro-organisms (Theobald et al. 1993; Theobald et al. 1997; Visser et al. 2002; Rizzi et al. 1997). In these studies the cells are cultivated under well-defined conditions in a chemostat until a steady state is reached. Subsequently this steady state culture is disturbed by instantaneous addition of a certain perturbing agent (e.g. substrate, inhibitor or activator) whereafter the transient behavior of metabolism is recorded by measuring the concentrations of intra- and extracellular metabolites within a certain time interval. An observation window of tens to a few hundreds seconds allows to detect metabolome responses that mainly attribute to rapid (allosteric) enzyme-metabolite interactions (Theobald et al., 1997). This technique has been successfully applied to generate dynamic responses of chemostat cultured glucose limited *Penicillium chrysogenum* in glucose perturbation experiments (Nasution et al., 2006b). In the previous work, only intermediates of primary metabolism, extracellular metabolite concentrations and
adenine nucleotides were measured which allowed to analyze in primary metabolism and the identification of near equilibrium reactions.

In the present work, the time frame of the perturbation experiment was extended to 300 seconds. This allowed us to observe whether a possible pseudo-steady state would be reached following the dynamic response. Such a situation was observed in similar studies in *Saccharomyces cerevisiae* (Wu et al., 2006). Furthermore the dynamics of O$_2$-uptake and CO$_2$ production were obtained (using the approach of Wu et al (2003)). This allows setting up carbon, electron and energy balances during the pulse. These balances are highly useful to check the data consistency and also to detect the occurrence of unknown processes. Finally, in addition to the measurement of the glycolytic and TCA cycle intermediates and nucleotides now also the free intracellular amino acid concentrations were quantified during the perturbation experiment. This allowed us to study the influence of primary metabolite levels on the down stream amino acid pathways during the window of observation of 300 seconds. Because penicillin synthesis is intimately linked to amino acid metabolism, this allowed us to investigate the link between primary metabolism, amino acid pathways and penicillin biosynthesis.

4.2 Material and Methods

**Strain** A high-yielding strain of *Penicillium chrysogenum* (code name DS17690), identical to the strain which has been used in previous research (van Gulik et al. 2000; vanGulik et al. 2001), was used for all experiments. This strain was kindly donated by DSM Anti-Infectives (Delft – The Netherlands).

**Chemostat Cultivation and Medium Composition** Glucose limited, aerobic, chemostat cultivations were carried out in 7-L turbine-stirred bioreactors (Applikon, Schiedam, The Netherlands) with working volume 4.0 L at dilution rate $D = 0.05$ h$^{-1}$. Except for the aeration rate, which was 4.48 mol of air per hour (= 0.463 vvm), the cultivation conditions were the same as described previously (Nasution et al., 2006a; 2006b).

The medium composition was designed to support a biomass concentration of 6 g/L dry weight. Also the medium composition and preparation have been described previously (Nasution et al., 2006a).
The concentrations of O₂ and CO₂ in the off gas from the chemostat were analyzed using a combined paramagnetic/infrared gas analyzer (NGA 2000 MLT 1, Fisher-Rosemount GMbH&Co Hasselroth Germany).

Chemostat cultivation was preceded by a batch phase. Shortly before the end of the batch phase, e.g. shortly before exhaustion of the carbon source, the feed was started. After reaching steady state, which was usually accomplished after about 4 residence times, (as shown by constant concentration of biomass, penicillin, and off-gas O₂ and CO₂), pulse experiments were performed.

**Glucose Pulse Experiment**  The pulse experiment in the chemostat was carried out by injecting 16 mL of 62.5 g/L glucose solution into the reactor within 1-2 seconds. This increased the residual glucose concentration from about 20 mg/L to approximately 250 mg/L. Subsequently, rapid sampling of broth for measurement of intracellular metabolites was carried out at 5, 10, 15, 20, 25, 35, 50, 70, 90, 120, 150, 180, 240 and 300 seconds after injection of the glucose solution. Rapid sampling and quenching was carried out as described previously (Lange et al., 2001). For measurement of extracellular metabolite concentrations, broth samples of approximately 1.0 mL, were taken using the stainless steel ball method (Mashego et al. 2003), via a separate sampling port.

During the pulse experiment the dynamics of the concentrations of O₂ and CO₂ in the offgas from the chemostat were measured online. An hour before the pulse experiment was carried out, both O₂ and CO₂ meters were calibrated. During the pulse experiment the concentrations of O₂ and CO₂ were measured every second.

**Calculation Procedures**  Biomass specific rates ($q_s$, $q_p$, $q_{o2}$ and $q_{CO2}$) during (pseudo) steady states were obtained from the respective mass-balances and the measurements of volumes, flow rates and concentrations. Data reconciliation was carried out with the constraint that the conservation relations for the elements were satisfied according to the procedure described by van der Heijden (vanderHeijden et al. 1994).

Online measurement of the concentrations of O₂ and CO₂ in the offgas from the chemostat was carried out during the pulse experiment. However, the dynamics of these measurements were delayed because of the residence time of the offgas in the headspace of the chemostat, the connecting tubings and the offgas analyzer. In order to calculate the *in-vivo* dynamics of O₂ consumption and CO₂ production during the pulse experiment, this delay was
corrected for using the procedure developed by Wu and Bloemen, (Wu et al. 2003; Bloemen et al. 2003).

The turnover times of the free amino acid pools were calculated for each amino acid from the pool size (= measured intracellular concentration, $C_{AA}$) and the net synthesis rate, calculated from a stoichiometric model (van Gulik et al., 2000) for a specific growth rate $\mu = 0.05$ h$^{-1}$. This yields turnover time $\tau = C_{AA}/Flux_{AA}$ in seconds with $C_{AA}$ in $\mu$molAA.gDW$^{-1}$ and $Flux_{AA}$ in $\mu$molAA gDW$^{-1}$ second$^{-1}$. Similarly, the turnover time of metabolites of primary metabolism were obtained.

The carbon balances during the glucose pulse experiment were calculated based on the amount of consumed carbon relative to $t_0$, when the pulse is given, compared with the accumulation of carbon in known carbon sinks, that is glycolytic and TCA cycle intermediates, CO$_2$, biomass and penicillinG production, all expressed in $\mu$Cmol/L. In this calculation the production of biomass is assumed to be the same as during steady state conditions. The electron balances were calculated in a similar way as the carbon balances, with as only difference that all the sources and sinks are multiplied with their generalized degree of reduction. The consumption of electron source (glucose) is compared with the accumulation in known electron sinks (metabolites and O$_2$), all expressed in $\mu$mol electrons/L. The ATP balance for the glucose pulse experiment was calculated as described by Wu et al., (2006).

**Metabolite Extraction** Metabolite extraction was carried out with the hot ethanol method (Gonzalez et al. 1997), which was adapted for the extraction of $P$. chrysogenum cells. The adapted methods for cold centrifugation and washing of the cells, metabolite extraction and sample preparation have been described elsewhere (Nasution et al., 2006a).

**Analytical procedures** Measurement of the concentrations of biomass dry weight and residual glucose were carried out as described previously (Nasution, 2006b). The concentrations of penicillin-G and its side chain precursor phenylacetic acid in the culture supernatant were measured with high performance liquid chromatography (HPLC) as described in Christensen et al. (1994).

The concentrations of the glycolytic and TCA cycle intermediates in the cell extracts were analyzed with isotope dilution mass spectrometry (IDMS) as described in Wu et al. (2005).
The concentrations of the adenine nucleotides AMP, cAMP, ADP and ATP were also analyzed with IDMS, the applied LC-ESI-MS/MS procedure has been described in Wu et al., (2006).

Analysis of the concentrations of free amino acids in the cell extracts was carried out with GS-MS (Trace GC Ultra coupled to a Trace DSQ Mass-Spectrometer (Finnigan MA USA)). Solid phase extraction and derivatization of the amino acids was carried out using the EZ:faast kit (Phenomenex, Torrance, California, USA). With this method the following amino acids were detected: Alanine, Glycine, Valine, Leucine, Isoleucine, Threonine, Serine, Proline, Asparagine, Methionine, Glutamate, Phenylalanine, α-Aminoadipic acid, Cysteine, Glutamine, Homocysteine, Ornithine, Lysine, Histidine, Tyrosine and Triptophan. Also for amino acid analysis IDMS was applied, using U-\textsuperscript{13}C labeled cell extracts of \textit{S. cerevisiae} as internal standards (Wu et al. 2005). Because α-Aminoadipic-acid (which is important metabolite in the penicillin pathway) is not produced in detectable amounts in \textit{S. cerevisiae} this compound could not be analysed with IDMS. Therefore this compound was quantified using standard additions.

4.3 Result and Discussion

**Steady state chemostat cultivation of \textit{Penicillium chrysogenum}** Figure 4.1 shows the measured concentrations of penicillin-G, biomass and PAA as function of time during cultivation of \textit{P. chrysogenum} in a glucose limited chemostat at a dilution rate of 0.05 h\textsuperscript{-1}.

![Figure 4.1](image.png)

\textbf{Figure 4.1} Biomass (●, g/L), PAA (○, mM) and PenG (∇, mM) concentration in the chemostat as function of time. The arrow indicates the glucose pulse.
Steady state chemostat growth was assumed to be obtained after a period of 5 residence times, which is equal to 100 hours of chemostat growth at the applied dilution rate. Average values of measured concentrations and flow rates during steady state growth (100 - 230 hours) were used to calculate the biomass specific consumption rates of glucose and oxygen and production rates of Pen-G and carbon dioxide. Byproduct formation was calculated from the difference between the measured total organic carbon content of the culture supernatant and the carbon content in measured compounds (Pen-G and PAA). The results are shown in Table 4.1. It was calculated from the measured conversion rates that the carbon recovery was almost equal to 100%, but the electron recovery showed approx. 6% deviation (results not shown). This was most probably caused the relatively large error (visible from the relatively large standard deviation of the calculated oxygen consumption rate, see Table 4.1) in the measurement of the oxygen concentration in the offgas. Subsequently data reconciliation and gross error detection was applied according to the procedure of van der Heijden (Vanderheijden et al. 1994). The reconciled (balanced) rates are shown in Table 4.1.

Table 4.1 Measured specific conversion rates for *Penicillium chrysogenum* cultured at $D = 0.05 \, h^{-1}$ in aerobic glucose limited steady state chemostats

<table>
<thead>
<tr>
<th>Rate</th>
<th>measured</th>
<th>balanced</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mmol.Cmol$_s^{-1}$ h$^{-1}$</td>
<td>mmol.Cmol$_s^{-1}$ h$^{-1}$</td>
</tr>
<tr>
<td>$q_s$</td>
<td>19.22 ± 0.72</td>
<td>19.25 ± 0.64</td>
</tr>
<tr>
<td>$q_{O2}$</td>
<td>56.51 ± 11.52</td>
<td>50.00 ± 3.00</td>
</tr>
<tr>
<td>$q_{CO2}$</td>
<td>51.47 ± 1.11</td>
<td>51.89 ± 3.01</td>
</tr>
<tr>
<td>$q_{pen}$</td>
<td>0.33 ± 0.05</td>
<td>0.33 ± 0.05</td>
</tr>
</tbody>
</table>

There appeared no indication for errors in the calculated rates and/or the system definition. The thus obtained net balance conversion rates appeared to be very similar to the rates obtained from previous experiments carried out under the same conditions (Nasution et al. 2006a; 2006b) as well as carried out in a different chemostat system and with a lower biomass concentration (van Gulik et al., 2000). This shows that *Penicillium chrysogenum* can be reproducibly cultivated in steady state chemostats.

At two different timepoints during steady state chemostat cultivation, samples, for measurement of intracellular metabolites and adenine nucleotides were taken. Intracellular
metabolites and adenine nucleotides have been analyzed previously in similar chemostat cultures (Nasution et al., 2006a). The results, which are shown in Table 4.2, are comparable to the previously measured values. This shows that the chemostat cultures as well as the metabolite measurements yield reproducible results. In addition to the metabolites measured in previous experiments (Nasution et al., 2006a), the intracellular concentrations of fructose 2,6bis Phosphate and cAMP, have been determined. Compared to most other metabolites their concentrations are very low (in the order of nanomol per gram of dry biomass concentration). However, their levels appeared to be similar to reported values for *Saccharomyces cerevisiae* (Wu et al., 2006). Furthermore the intracellular levels of free amino acids were analyzed. The results are shown in Table 4.3. Previously, Jorgensen et al. (1995) have measured free amino acid levels of *Penicillium chrysogenum* cultivated in fedbatch fermentations. In spite of the fact that the cultivation conditions were different, a different strain was used and the product was penicillin-V instead of penicillin-G, the free amino acid levels are comparable. It appears that almost all free amino acid levels in these two different *Penicillium chrysogenum* cultures only differs a factor 2 or less. The sole exception is cystein, of which the intracellular level is four times higher in the fedbatch fermentation. From a comparison with the intracellular free amino acid levels in *Saccharomyces cerevisiae* cultivated at the same conditions (same chemostat, D = 0.05 h⁻¹, aerobic, glucose limited) (Wu et al., 2006), it appears that much lower concentrations of almost all intracellular free amino acid are observed in the yeast culture. This may be related to the generally higher level of primary metabolites in *P. chrysogenum* compared with *S. cerevisiae* (Nasution et al., 2006a).
Mass balance analysis of the dynamic interactions

Table 4.2 Average (2 samples, each in analysed in triplicate) intracellular metabolite and adenine nucleotide concentrations of *Penicillium chrysogenum* cultivated in an aerobic, glucose limited chemostat at $D = 0.05 \ h^{-1}$.

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Unit (µmol.g DW$^{-1}$)</th>
<th>Unit (µmol.g DW$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>G6P</td>
<td>4.64 ± 0.05</td>
<td>Mal</td>
</tr>
<tr>
<td>G1P</td>
<td>0.71 ± 0.05</td>
<td>Fum</td>
</tr>
<tr>
<td>F6P</td>
<td>0.71 ± 0.09</td>
<td>Glyoxylate</td>
</tr>
<tr>
<td>F1,6bisP</td>
<td>0.90 ± 0.07</td>
<td>M6P</td>
</tr>
<tr>
<td>F26bP</td>
<td>0.01 ± 0.00</td>
<td>T6P</td>
</tr>
<tr>
<td>2PG + 3PG</td>
<td>0.59 ± 0.01</td>
<td>6PG</td>
</tr>
<tr>
<td>PEP</td>
<td>0.24 ± 0.00</td>
<td>Nucleotides</td>
</tr>
<tr>
<td>PYR</td>
<td>0.22 ± 0.01</td>
<td>ATP</td>
</tr>
<tr>
<td>Cit + Isocit</td>
<td>2.91 ± 0.16</td>
<td>ADP</td>
</tr>
<tr>
<td>α-KG</td>
<td>2.05 ± 0.33</td>
<td>AMP</td>
</tr>
<tr>
<td>SUC</td>
<td>0.23 ± 0.04</td>
<td>cAMP</td>
</tr>
</tbody>
</table>

It is of general interest how the highly dynamic behaviour of the glycolytic and TCA cycle intermediates during the glucose pulse (with a time frame of 300 seconds) propagates into the associated amino acid pathways. Relevant information here is then the turnover times of the individual metabolite and amino acid pools in the perturbed steady state. Turnover times were calculated from the measured pool sizes and calculated fluxes during steady state chemostat cultivation. The results are shown in Table 4.4. It can be seen from this table that the turnover times of the primary metabolite pools (1 – 25 seconds) are much smaller compared to the turnover times of the amino acid pools (hundred to thousand seconds). This is because of the relatively small pool size and high flux for the primary metabolites. It can be expected that the dynamic behaviour of primary metabolites during the glucose pulse will propagate into derived amino acids for which the pool turnover time is not too much higher than the time frame of observation of 300 seconds. It can be inferred from the calculated turnover times that dynamic concentration changes are to be expected in cystein, methionine, phenylalanine, isoleucine, tryptophane, leucine and tyrosine, because all the other amino acids have turnover times which are much higher than 300 seconds.


Table 4.3 Free amino acid amount in µmol/gDW of *Penicillium chrysogenum*, cultivated in aerobic glucose limited chemostat at dilution rate 0.05 h\(^{-1}\) (2 samples, each in triplicate).

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Amount ± Standard Error</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ala</td>
<td>21.68 ± 0.13</td>
</tr>
<tr>
<td>Val</td>
<td>2.08 ± 0.03</td>
</tr>
<tr>
<td>Leu</td>
<td>0.73 ± 0.00</td>
</tr>
<tr>
<td>His</td>
<td>0.72 ± 0.00</td>
</tr>
<tr>
<td>Ser</td>
<td>5.69 ± 0.15</td>
</tr>
<tr>
<td>Gly</td>
<td>2.08 ± 0.04</td>
</tr>
<tr>
<td>Cys</td>
<td>0.10 ± 0.01</td>
</tr>
<tr>
<td>Tyr</td>
<td>0.26 ± 0.01</td>
</tr>
<tr>
<td>Phe</td>
<td>0.19 ± 0.00</td>
</tr>
<tr>
<td>Trp</td>
<td>0.11 ± 0.00</td>
</tr>
<tr>
<td>Asn</td>
<td>1.48 ± 0.02</td>
</tr>
<tr>
<td>Asp</td>
<td>16.27 ± 0.42</td>
</tr>
<tr>
<td>Met</td>
<td>0.14 ± 0.00</td>
</tr>
<tr>
<td>Hcys</td>
<td>0.37 ± 0.01</td>
</tr>
<tr>
<td>Thr</td>
<td>5.93 ± 0.04</td>
</tr>
<tr>
<td>Isoleu</td>
<td>0.33 ± 0.00</td>
</tr>
<tr>
<td>Glu</td>
<td>52.96 ± 0.21</td>
</tr>
<tr>
<td>Gln</td>
<td>28.7 ± 0.58</td>
</tr>
<tr>
<td>α-AAA</td>
<td>1.81 ± 0.08</td>
</tr>
<tr>
<td>Pro</td>
<td>0.95 ± 0.00</td>
</tr>
<tr>
<td>Lys</td>
<td>1.24 ± 0.02</td>
</tr>
<tr>
<td>Lys</td>
<td>1.24 ± 0.02</td>
</tr>
</tbody>
</table>

Note: α-AAA = α-Aminoadipic Acid

**General observations in the pulse experiment** As found in previous experiments (Nasution et al. 2006a, 2006b), a steady state is generally obtained after a period of 3 to 5 residence times of chemostat growth. It was observed that a stable steady state could be maintained during a period of at least another 5 residence times. In the present experiment the glucose pulse experiment was carried out after 185 hours (9 residence times) of chemostat growth, by increasing the glucose concentration from the residual value of 0.1 mol/m\(^3\) to 1.5 mol/m\(^3\) in no more than 2 seconds. At the same time that the glucose pulse was given the medium feed to the chemostat was stopped. Measurements of the residual glucose concentration during the pulse experiment show the instantaneous increase of the glucose concentration in the broth followed by a decrease in a nearly linear fashion (see Figure 4.2). Based on these measurements, the specific glucose consumption rate was calculated from linear regression to amount 41.9 mmol glucose.Cmol\(_x\)^{-1}.h\(^{-1}\). This is about 2.2 high as the specific glucose consumption rate during glucose limited steady state chemostat growth (see Table 4.1).
Mass balance analysis of the dynamic interactions

Figure 4.2 Glucose concentration in the broth (mmol/L) after a glucose pulse. Arrow indicates when the pulse is done.

In a previous glucose pulse experiment (Nasution, 2006b) whereby the extra cellular glucose concentration was increased to 3 mol/m$^3$ (twice as high as in the present experiment) but carried out under otherwise identical conditions, the same increase of the glucose consumption rate was observed. This could indicate that these values would correspond to the the maximum glucose uptake rate of this *P. chrysogenum* strain. From the batch cultivation of *Penicillium chrysogenum*, it is observed that $\mu_{\text{max}} = 0.15 \text{ h}^{-1}$, which corresponds to a $q_{\text{max}}$ of about 46 mmol.Cmol$^{-1}$.h$^{-1}$, which supports this conclusion.

**Dynamic behaviour of the primary metabolites** The doubling of the glucose uptake rate as response to the sudden increase of the extracellular glucose concentration in a glucose limited chemostat of *P. chrysogenum* leads to measurable changes in primary metabolite levels, that is, the intermediates of the glycolysis and TCA cycle, hexose phosphate pools and adenine nucleotides. The results are shown in Figures 4.3, 4.4 and 4.5, respectively. When compared to a previous glucose pulse experiment carried out under identical conditions, except that a twice as high glucose concentration was applied (Nasution et al., 2006b), the measured responses of all glycolytic and TCA cycle intermediates, as well as adenine nucleotides were very similar, except for pyruvate, $\alpha$KG and pool of citrate and isocitrate; however, their different is not more than 2 fold. This indicates that within this short time
period the dynamic metabolite response only depends on glucose uptake rate, but not on the extracellular glucose concentration as such. Also this shows the repeatability of our experimental system.

**Figure 4.3** Glycolysis and TCA Cycle intermediates. In brackets are the turnover time (in seconds) of the pools at the steady state.
Mass balance analysis of the dynamic interactions

**Figure 4.4** Hexose Phosphate

**Figure 4.5** Nucleotides
In the present experiment, samples were taken during a longer time period, that is, until 300 seconds after addition of the glucose pulse. It was observed that during approximately 180 seconds after the disturbance the metabolites showed a strong dynamic behavior. This is to be expected because the turnover time of the metabolite pools of the glycolysis, TCA cycle and Pentose Phosphate pathway (calculated from the steady state fluxes and pool sizes) are relatively short compared to the time frame of the pulse experiment, that is it ranges between 1 and 25 seconds (see Table 4.4).

**Table 4.4** Turn over time (seconds) of intracellular metabolites and intracellular free amino acid pools in *Penicillium chrysogenum*.

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>(seconds)</th>
<th>Amino acid</th>
<th>(seconds)</th>
<th>Amino acid</th>
<th>(seconds)</th>
</tr>
</thead>
<tbody>
<tr>
<td>G6P</td>
<td>23.3</td>
<td>Cys</td>
<td>18.0</td>
<td>αAAA</td>
<td>292.8</td>
</tr>
<tr>
<td>F6P</td>
<td>5.7</td>
<td>Met</td>
<td>58.8</td>
<td>Lys</td>
<td>355.8</td>
</tr>
<tr>
<td>F1,6bisP</td>
<td>7.2</td>
<td>Phe</td>
<td>61.2</td>
<td>Hcys</td>
<td>375.6</td>
</tr>
<tr>
<td>2PG + 3PG</td>
<td>2.3</td>
<td>Isoleu</td>
<td>111.3</td>
<td>His</td>
<td>432.0</td>
</tr>
<tr>
<td>PEP</td>
<td>0.9</td>
<td>Trp</td>
<td>130.0</td>
<td>Ser</td>
<td>453.2</td>
</tr>
<tr>
<td>PYR</td>
<td>0.9</td>
<td>Leu</td>
<td>131.0</td>
<td>Asn</td>
<td>458.7</td>
</tr>
<tr>
<td>Cit + Isocit</td>
<td>15.9</td>
<td>Tyr</td>
<td>144.5</td>
<td>Glu</td>
<td>657.5</td>
</tr>
<tr>
<td>α-KG</td>
<td>22.1</td>
<td>Pro</td>
<td>205.5</td>
<td>Asp</td>
<td>717</td>
</tr>
<tr>
<td>SUC</td>
<td>3.3</td>
<td>Val</td>
<td>243.0</td>
<td>Thr</td>
<td>757.5</td>
</tr>
<tr>
<td>Mal</td>
<td>19.0</td>
<td>Gly</td>
<td>243.6</td>
<td>Gln</td>
<td>1243.0</td>
</tr>
<tr>
<td>Fum</td>
<td>13.0</td>
<td>Ala</td>
<td>269.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T6P</td>
<td>47.8</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6PG</td>
<td>3.7</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

It can be seen from Figures 4.3 and 4.5 that after 180 seconds, most metabolites reached a pseudo steady state level at concentrations which were increased 1.5 to 3 fold compared to the steady state. The same can be observed for the dissolved oxygen level, which also reaches a pseudo steady state level after 180 seconds (see Figure 4.6C). Particularly interesting are the dynamics of the biomass specific rates of O₂ consumption and CO₂ production, \( q_{O2} \) and \( q_{CO2} \) (Figure 4. 6D, E) during the pulse experiment. These were calculated from the measured off-gas concentrations of O₂ and CO₂ (Figure 4.6A, B) and the dissolved O₂ concentration (Figure 4.6C) using the procedures of Wu et al., (Wu et al. 2003) and Bloemen ((Bloemen et al. 2003).
During the pseudo steady state the specific glucose uptake rate \( (q_s) \) was 2.2 times higher than during glucose limited chemostat growth. In contrast to this, the increase of \( q_{O2} \) and \( q_{CO2} \) was much less, only about 10%. This indicates that the additional glucose uptake is converted into a compound of which the synthesis requires little metabolic energy, which might either be an excreted byproduct or intracellular storage material. From analysis of the supernatant no significant amounts of secreted products were detected, suggesting that the additional glucose consumed is accumulated inside the cells in the form of a storage compound. This is supported by the strongly increased levels of T6P (Figure 4.4) which indicate increased storage metabolism.

![Figure 4.6](image)

**Figure 4.6** Mol fraction of Oxygen (A) and Carbon dioxide (B) in the off-gas, Dissolved O\(_2\) concentration in the broth during the pulse. \( q_{O2} \) (D) and \( q_{CO2} \) in mmol.Cmol\(_k\(^{-1}\).h\(^{-1}\) (E) calculated according to Wu et al. (2003). Time 0 (zero) indicates when the pulse is done.

**Dynamic behaviour of the free amino acids**  In addition to measurement of the dynamics of the intermediates of the primary metabolic pathways in response to a glucose pulse, also the dynamics of the free intracellular amino acids were measured. The results are shown in Figure 4.8, whereby the amino acids are grouped according to the primary metabolite they are derived from. It was observed that the levels of several amino acids significantly change during the glucose pulse experiment. These changes were, however, not only limited to amino acid pools with short turnover times. For the amino acids derived from
pyruvate, alanine, valine and leucine (Figure 4.8a) it can be seen that, in spite of their relatively large turnover time both the levels of alanine and valine increase together with the level of their precursor. In case of alanine this might be explained by the action of the highly reversible trans-aminase converting pyruvate and glutamate into alanine and $\alpha$KG. The high reversibility of this reaction can be inferred from the calculated mass action ratio (see Figure 4.7) which decreases shortly after the glucose pulse but returns quickly to the steady state value of approximately 1, which is close to the equilibrium constant of this transaminase.

![Figure 4.7 Mass action ratio of alanine transaminase; ([ala]$[\alpha$KG])/([pyr][glutamate])](image)

For the amino acids derived from 2PG and 3PG (Figure 4.8b), only cysteine shows a strong response, which is understandable from very short turn over time ($\approx 36$ seconds). The strong increase points to a sudden strong decrease in the demand for cysteine, because the concentration of its precursor hardly changes.

The aromatic amino acids tyrosine, phenylalanine, and tryptophane which are derived from PEP, clearly show a strong dynamic response (see Figure 4.8c) following the dynamics of its precursor. This is in agreement with the relatively short turnover times of the pools of these amino acids and indicates that the PEP concentration has a strong influence on the rate of synthesis of these aromatic amino acids. However, no significant response of the aromatic amino acid pathway to fast dynamics of the PEP concentration were observed in $S.\ cerevisiae$ (Wu et al., 2006) but was indeed observed in $E.\ coli$ (Oldiges et al. 2004).

The dynamics of the amino acids derived from oxaloacetate, that is aspartate and subsequently asparagine, threonine, methionine, homocysteine and isoleucine are shown in Figure 4.8d. Oxaloacetate was not measured but is reversibly coupled to malate and fumarate,
Mass balance analysis of the dynamic interactions

which are also shown in Figure 4.8d and are indicative for OAA dynamics. Aspartate has a very long turnover time (1434 seconds), nevertheless, its concentration shows a clear decrease in the time frame of 300 seconds. An explanation for this is the existence of a C4 equilibrium pool consisting of oxaloacetate, malate, fumarate, aspartate (Nasution et al., 2006b). It can be observed (Figure 4.8c) that the size of this pool does not change significantly during a glucose pulse experiment. However, due to the increased NADH/NAD ratio (Nasution et al. 2006b) caused by the increased glycolytic flux during the pulse experiment this pool becomes more reduced (decrease of oxaloacetate and aspartate and increase of malate and fumarate).

Except for isoleucine, no significant changes can be observed for the other amino acids derived from oxaloacetate, which is in agreement with their long turnover time. As isoleucine has a relatively short a turnover time (223 seconds) the observed decrease might be associated with a decrease in the OAA level as a result of the increased NADH/NAD ratio (see above).

Most of the amino acids derived from \( \alpha \)-ketoglutarate (\( \alpha \)KG) do not respond significantly despite the strong change in the \( \alpha \)KG level during the pulse experiment (Figure 4.8e). This agrees with the long turnover times of the associated amino acid pools. Exceptions are proline and \( \alpha \)Aminoadipic acid (\( \alpha \)AAA) which have the lowest turnover times of the amino acids derived from \( \alpha \)KG and have a clear tendency to rise during the pulse. \( \alpha \)AAA is related to the penicillin production pathway where it is consumed for the synthesis of the tripeptide ACV, but is released a few steps later when \( \alpha \)AAA is exchanged with the added side chain precursor, in this case phenyl acetic acid (PAA). It is interesting to note that the three amino acids related to penicillin synthesis all increase during the pulse (Figure 4.9).

In Figure 4.8, intracellular concentration of amino acids during glucose pulse. In brackets the turnover times of the metabolite pool in steady state. The amino acid families are grouped as obtained from their precursors (a) Pyruvate, (b) 2PG and 3PG, (c) PEP, (d) Oxaloacetate, (e) \( \alpha \)KG.

![Figure 4.8a Intracellular amounts of amino acids obtained from Pyruvate](image)
Figure 4.8b Amino acids from pool of 2PG and 3PG

Figure 4.8c Amino acids from PEP

Figure 4.8d Amino acids from Oxaloacetate (using fumarate and malate as associated precursors)

Figure 4.8e Amino acids from αKG
Cumulative Balances of Carbon, Electrons and ATP  The recoveries of carbon, electrons and ATP during the pulse experiment have been calculated from cumulative balances. The results are shown in Figure 4.10. For these calculations it was assumed that that the growth rate $\mu$ has not changed during the pulse experiment and remains equal to the the steady state value ($D = 0.05 \text{ h}^{-1}$). It is unavoidable to use an assumption for $\mu$ because, given the short time interval and the amount of biomass present, it is not possible to quantify a change in growth rate by measuring a change in biomass concentration.

In the cumulative carbon balance shown in Figure 4.10A, the carbon consumed in the form of the substrate glucose is compared to the accumulation of carbon in glycolytic and TCA cycle intermediates and in produced biomass, penicillin-G and CO$_2$. It can be seen from Figure 4.10A that, compared to the total amount of carbon consumed, the amount of carbon present in intracellular intermediates is relatively very small. The same holds for the carbon present in the amount of produced penicillin. In contrast to this the amount of produced biomass represents a significant part when using the above assumption of an unchanged growth rate.
It can be inferred from Figure 4.10A that the cumulative carbon balance contains a significant gap (other sink) of about 30% of the amount of glucose consumed. This implies that either the assumption of an unchanged growth rate is not correct or a large amount of consumed carbon disappears in unknown sinks. No secretion of by-products occurred, as was found from analysis of the supernatant (results not shown). Hence, the unknown carbon sink is either biomass itself or compounds accumulated inside the cells.

To obtain more insight in the possible destination of the additional amount of consumed carbon the electron recovery was calculated from the balance of degree of reduction (see Figure 4.10B). Also this balance showed a gap of about 30%, indicating that the degree of the reduction of the unknown compound(s) must be approximately equal to the degree of reduction of glucose, which is 4 electrons per Cmol. Finally the ATP needs for the synthesis of the unknown carbon sink(s) were estimated from an ATP balance (see Figure 4.10C). Both the rate of ATP production in primary metabolism as well as the rates of ATP consumption for biomass formation, penicillin synthesis and maintenance were calculated from the measured macroscopic rates during the pulse experiment by means of metabolic flux balancing using the stoichiometric model published by van Gulik et al. (2000), with the assumption that \( q_p \) is not changed. From the flux balancing results it appeared that of the total amount of ATP produced, 48% is used for biomass assimilation (assuming that the growth rate does not change) 22% for penicillin production and 20% for maintenance. This would mean that only 10% of the amount of ATP synthesized and 30% of the consumed glucose would have been used for the synthesis of the unknown compound(s), indicating a high yield of this compound on ATP. This would assumption make it unlikely that the additional carbon consumed is converted into biomass. Furthermore, this would imply an increase of the growth rate \( \mu \) with more than a factor 2 within approximately 180 seconds, which is highly unlikely given the observed slow response of growth rate in nutrient up-shift experiments in *Saccharomyces cerevisiae* (Duboc et al., 1998). This is also supported by the observations that no big changes occurred in the amino acid levels (see Figure 4.8) and that the ATP level has decreased (see Figure 4.5), which does not favour an increase in \( \mu \). However, the most decisive argument against an increase of the growth rate is that the amount of ATP to support such an increase in growth rate is simply not available as this would lead to a shortage gap of approximately 50% in the ATP balance.

The most likely explanation is therefore that the additional carbon consumed is converted into storage carbohydrates, i.e. trehalose and/or glycogen. This is supported by the steep increase of the cAMP concentration during the pulse experiment (see Figure 4.7) as cAMP strongly
activates storage carbohydrate synthesis (Vandercammen et al., 1989). The observation that also the concentration of T6P increases steadily during the pulse experiment (see Figure 4.6) would indicate that trehalose is the most likely candidate.

No attempts were made to quantify the amount of accumulated storage carbohydrates. During the pulse experiment, the biomass present (≈ 6 gDW/L) consumes about 100 mg/L of glucose (Figure 4.2) of which about 30% is possibly converted to storage carbohydrates. It can be calculated that this would lead to an increase of the trehalose content of about 5 mg trehalose/g biomass dry weight (≈ 14 µmol/gDW). It has been found previously that during glucose limited steady state chemostat growth of P. chrysogenum at this dilution rate the biomass contains about 40 µmol trehalose/gDW (van Gulik, unpublished data). Quantification of such a small increase therefore requires special techniques, such as addition of labelled glucose. The measurement of the isotopomer ratio of trehalose is then much more accurate to quantify this amount.

Although the amount of ATP needed for storage carbohydrate synthesis is much less than for growth, still a considerable amount of ATP is needed. The cumulative ATP recovery was calculated again for the assumption that all additional carbon consumed during the pulse experiment is converted into trehalose (see Table 4.5). It can be inferred from the ATP balance that the amount of ATP synthesized is not sufficient to support unchanged rates of growth and penicillin synthesis and conversion of the surplus of glucose into trehalose.
Table 4.5 ATP balance (mmol.L\(^{-1}\).h\(^{-1}\)) balance in steady state and pseudo steady state

<table>
<thead>
<tr>
<th></th>
<th>Steady state</th>
<th>Pseudo steady state</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Trehalose</td>
</tr>
<tr>
<td><strong>Production</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oxidative</td>
<td>32.30</td>
<td>34.03</td>
</tr>
<tr>
<td>Phosphorylation</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Consumption</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Biomass</td>
<td>16.19 (50.2%)</td>
<td>16.19  (47.6%)</td>
</tr>
<tr>
<td>Formation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PenG synthesis</td>
<td>7.54 (23.3%)</td>
<td>7.54 (21.8%)</td>
</tr>
<tr>
<td>Trehalose</td>
<td>0.38 (1.2%)</td>
<td>7.41 (22.2%)</td>
</tr>
<tr>
<td>formation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maintenance</td>
<td>7.07 (21.9%)</td>
<td>7.07 (20.8%)</td>
</tr>
<tr>
<td>Other</td>
<td>1.12 (3.5%)</td>
<td>1.12 (3.3%)</td>
</tr>
<tr>
<td></td>
<td>100 %</td>
<td>116 %</td>
</tr>
</tbody>
</table>

**Relation of penicillin synthesis to primary metabolism**  Surveying the responses of amino acids to the glucose pulse it appears that the levels of the amino acids involved in penicillin synthesis (αAAA, cysteine and valine) (see Figure 4.9) increase during the pulse. Especially the increase of αAAA is remarkable because the net consumption of this intermediate of the lysine biosynthesis pathway is relatively low, as it is used for the synthesis of the tripeptide ACV but it is regenerated in the conversion of iso-penicillinN (IPN) into penicillinG. Only small amounts of αAAA are used for lysine synthesis and are converted to the excreted byproduct OPC (van Gulik et al, 2000). The total amount of αAAA present as free αAAA and in bound forms of the tripeptide ACV and of IPN can therefore almost be considered as a conserved moiety. The sudden increase in the level of free αAAA might be an indication of a decrease in tripeptide concentration as a result of a decrease of the rate of tripeptide synthesis. This is supported by the observation that also the levels of cysteine and valine increased during the pulse experiment. The reason for a decreased rate of tripeptide synthesis might be the strongly decreased ATP concentration during the pulse (Figure 4.6). Because it is known that synthesis of ACV from its precursor amino acids requires a large
amount of ATP. It has been reported that there is a considerable amount of additional ATP consumption associated with penicillin biosynthesis (van Gulik et al., 2000). Another indication for a decrease in penicillin synthesis during the glucose pulse experiment is the observed increase of 6PG (6-phosphogluconate), Figure 4.4, which indicates to a sudden lower demand for NADPH from the pentose phosphate pathway (PPP). It is well known that the biosynthesis of penicillin requires large amounts of NADPH, and hence it is logical that the level of 6PG increases when penicillin production drops.

On setting up the balances (for carbon, electron and ATP) during the pulse, it was concluded that a very large part of the consumed glucose is directed towards storage compounds (trehalose). The ATP balance for this situation shows a clear deficit of 16% (Table 4.5). However, by taking into account that during the pulse the penicillin production strongly decreases (assumed decrease to 20% of the steady state value) changes the ATP balance such that the ATP spent in penicillin biosynthesis becomes available to enable the formation of storage carbon (trehalose). Table 4.5 shows that this leads to a strongly improved ATP balance. Instead of a deficit, the ATP balance is nearly closed.

In industrial fedbatch penicillin fermentations, glucose reaches the microorganism in a pulse wise manner (Theobald et al. 1997) due to non ideal broth mixing resulting in locally high glucose concentrations. This could trigger the response what is observed here, which would lead to a negative effect on the rate of penicillin synthesis. A decrease of the ATP level, as a response to the glucose pulse, is already observed in the beginning of the pulse experiment, after 25 seconds. In order to avoid this decrease, a mixing time less than 25 seconds is required. Another alternative would be to consider to use a mutant strain containing an ATP buffering system, e.g. adenylate kinase (Canonaco et al. 2003; Sauer and Schlattner 2004)

Comparing the steady state and the pseudo steady state The glucose pulse experiment shows that after a high dynamic phase of about 180 seconds, metabolism achieves a new pseudo steady state with modified metabolite levels and changed fluxes. The analysis of the carbon, degree of reduction and ATP balances and the observations on the relation between primary metabolism and amino acids pathways has led to the hypothesis that in pseudo steady state a major part of the consumed glucose is directed towards storage carbon accumulation while penicillin production decreases due to shortage of energy (ATP). Basically, metabolism shifts from an energy expensive product (penicillin) to a product which costs much less energy (storage carbon).
Based on these assumptions the fluxes in the pseudo-steady state can be calculated from the measured values of \( q_s, q_{O2} \) and \( q_{CO2} \) (see Table 4.6), assuming that the growth rate \( \mu \) does not change in this short time interval \( (\mu = 0.05 \text{ h}^{-1}) \), that the gap in the carbon balance can be explained by the formation of storage carbohydrates. Under these conditions the ATP balance closes at a 5 times lower specific penicillin production rate \( (qp = 0.07 \text{ mmol.Cmol}^{-1}.\text{h}^{-1}) \). Figure 4.11 shows the calculated fluxes in pseudo steady state as compared to the original steady state. All fluxes are normalized to \( q_s \) (19.2 mmol glucose.Cmol\(^{-1}.\text{h}^{-1}\)) of the steady state before the pulse.

In table 4.6 the fold changes in metabolite levels and uptake/secretion fluxes of the pseudo steady state compared to the reference steady state before the pulse are shown.

The 2.2 fold increase of the specific glucose consumption during the glucose pulse results in a similar fold change of the G6P pool, which enormously stimulates the storage flux (factor 20 up). This increased flux is probably extra stimulated by post-translational modification, because as cAMP strength increases. Although there is no big increase in flux below G6P metabolites of the glycolysis, the same fold changes are also observed in F6P, M6P and G1P. This observation could be explained by the previously observed fast equilibrium between G6P, F6P, M6P and G1P (Nasution et al., 2006a, Nasution et al., 2006b). The higher F16bP level, despite nearly the same flux of PFK, is probably related to the lower ATP level, which inhibits PFK. The observed smaller fold changes of the pools of 2PG&3PG and PEP could be related to the small increase of the flux towards the lower glycolysis, the near equilibrium between F16bP and PEP and activation of PK by F16P, which decreased PEP and increases pyruvate. Further down to the TCA cycle, one can observed a higher fold change of the C4 pool (Succinate, Malate and Fumarate), which is not supported by increasing flux toward this cycle. A higher ratio of cytosolic NADH and NAD\(^+\) is the possible driving force of high dynamics in this pool) (Nasution et al., 2006b). The TCA cycle flux is hardly increased, which might correspond with a constant citrate amount. The Pentose Phosphate Pathway (PPP) as the major NADPH producer decreases together with assumed decreased of penicillin production. According to flux calculations, using the metabolic network analysis (van Gulik et al, 2000), 15% of NADPH production is consumed for penicillin production. In the pseudo steady state, it is found that the flux towards PPP decreased about 11%. It could be concluded that decreasing flux to PPP is caused by decreasing penicillin production.
Table 4.6 Fold change of uptake, secretion fluxes and of metabolite levels in the pseudo steady state after 180 seconds

<table>
<thead>
<tr>
<th>Rate</th>
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</tr>
</thead>
<tbody>
<tr>
<td>$q_s$</td>
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</tr>
<tr>
<td>$q_{O2}$</td>
<td>1.06</td>
</tr>
<tr>
<td>$q_{CO2}$</td>
<td>1.14</td>
</tr>
<tr>
<td>$q_{pen}$</td>
<td>0.2*</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Fold Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>G6P</td>
<td>2.09</td>
</tr>
<tr>
<td>G1P</td>
<td>2.17</td>
</tr>
<tr>
<td>F6P</td>
<td>2.13</td>
</tr>
<tr>
<td>F1,6bisP</td>
<td>1.91</td>
</tr>
<tr>
<td>2PG + 3PG</td>
<td>0.98</td>
</tr>
<tr>
<td>PEP</td>
<td>0.71</td>
</tr>
<tr>
<td>PYR</td>
<td>1.82</td>
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<tr>
<td>Cit + Isocit</td>
<td>0.98</td>
</tr>
<tr>
<td>α-KG</td>
<td>1.84</td>
</tr>
<tr>
<td>SUC</td>
<td>2.43</td>
</tr>
<tr>
<td>F26bP</td>
<td>1.00</td>
</tr>
<tr>
<td>Mal</td>
<td>3.35</td>
</tr>
<tr>
<td>Fum</td>
<td>3.35</td>
</tr>
<tr>
<td>Glyoxylate</td>
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</tr>
<tr>
<td>M6P</td>
<td>1.98</td>
</tr>
<tr>
<td>T6P</td>
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</tr>
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<td>6PG</td>
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<tr>
<td>ATP</td>
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<td>ADP</td>
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<tr>
<td>AMP</td>
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</tr>
<tr>
<td>cAMP</td>
<td>3.14</td>
</tr>
</tbody>
</table>

*Assumed; not measured
Figure 4.11 Flux distribution during steady state (A) and pseudo steady state 180 seconds after pulse (B), relative to glucose uptake rate 19.22 mmol.Cmol⁻¹.h⁻¹ (100%).

4.4 Conclusions

The fast dynamic response of aerobic chemostat cultured (D = 0.05h⁻¹) glucose limited of Penicillium chrysogenum to a glucose pulse (change from 0.1 to 1.5 mM) has been analyzed further in terms of the cumulative carbon, electron and ATP balances. Most notably is that glucose uptake increases 2.2 fold but O₂ and CO₂ rates only increase 1.10 fold. This points to significant changes in metabolism.

The balances (carbon, electron and energy) suggest that there is an additional carbon sinks during carbon excess condition after the pulse, which is probably trehalose and that penicillin flux strongly decreases due to ATP-shortage and energy charge.

For intracellular metabolite concentration, the sudden increase of the residual glucose concentration caused strong dynamic responses only on the first 180 seconds, except for precursor T6P that leads to storage material, which shows monotonously increase. After this dynamic behaviour, a new pseudo steady state condition is established with changed fluxes and metabolite levels. Changes of metabolite concentration in primary metabolism do directly affect free amino acid concentration. A significant dynamic response is, as expected, shown by the free amino acid pools, which turn over time smaller than 300 seconds. This small turn over time is mainly caused by their small pool size. All three amino acid precursors of
Mass balance analysis of the dynamic interactions

penicillinG biosynthesis show significant dynamics during this pulse experiment, which points to a decreased penicillin synthesis. A possible cause of a decreased penicillin flux is the significantly lower ATP level, which is known to have significant effect on the synthesis of ACV. The lower ACV level is in agreement with the observed increased levels of αAAA, valine and cysteine.

This study has shown that small perturbations in glucose concentration to glucose limited *Penicillium chrysogenum*, which easily can occur in industrial penicillin fermentation, cause a strong metabolic response where energy costly pathways (penicillin) are decreased to favor low energy costly favorable pathways (storage).

References


Chapter 4


Chapter 5

A metabolome study of the steady state relation between central metabolism, amino acid biosynthesis and penicillin production in *Penicillium chrysogenum*

**Abstract** The relation between primary metabolism and the penicillin biosynthesis pathway in *Penicillium chrysogenum* was studied by the manipulating steady state flux in both pathways. A high producing industrial strain was cultivated at a growth rate $\mu = 0.05$ h$^{-1}$ in glucose limited chemostat cultures, both under penicillin-G producing and non-producing conditions. Non-producing conditions were accomplished in two ways: 1) by cultivation without addition of the side chain precursor phenylacetic acid and 2) by removal of all copies of the gene cluster coding for the penicillin biosynthesis pathway. Manipulation of the fluxes through primary metabolism was obtained by cultivation on either glucose or ethanol as sole carbon source. A positive relation was observed between primary metabolite concentrations and carbon flux in primary metabolism. Furthermore, in many cases a positive relation was found between the concentrations of free amino acids and their direct precursors in central metabolism. This corresponds with control of the biosynthesis of these amino acids via feed back inhibition by the end product. With respect to the penicillin production pathway, the flux seems not influenced by two of the three precursor amino acids, namely $\alpha$AAA and valine but is only influenced by cysteine, which requires a large NADPH supply, and the ATP level. An interesting observation is that the absence of penicillin production seems to stimulated storage metabolism (trehalose metabolism). This leads to the final conclusion that the penicillin production flux appears to be mostly influenced by the availability of energy and redox cofactors, where ATP is supposed to exert its influence at ACV-synthetase and NADPH at the cysteine level.
Chapter 5

5.1 Introduction

Successful improvement of the rate and yield of secondary product formation should not be limited to metabolic engineering of the product pathway alone, but should rather be applied to the product pathway in concert with the relevant parts of primary metabolism. A suitable model organism to study this approach is the penicillin producing filamentous fungus *Penicillium chrysogenum*, because the biosynthesis pathway is well known. The pathway of penicillin biosynthesis starts with the condensation of three amino acids, namely the intermediate of the lysine pathway, L-\(\alpha\)-aminoadipic acid (\(\alpha\)-AAA), L-cysteine and L-valine, to form the tripeptide, L-\(\alpha\)-aminoadipyl-L-cysteinyl-D-valine (LLD-ACV). In the second step, the tripeptide ACV is converted to isopenicillin-N. In the third and final step, which proceeds readily if a side chain precursor is added to the cultivation medium, \(\alpha\)-AAA is replaced by the supplied side chain precursor converting isopenicillin-N into Penicillin-G if phenylacetic acid is supplied or Penicillin-V if phenoxyacetic acid is supplied. However, a prerequisite for the side chain exchange is that the side chain precursor first has to be activated to a CoA ester, which is brought about by the enzyme CoA ligase. The released \(\alpha\)-AAA can be used again for the synthesis of LLD-ACV and is thus recycled. This implies that there is in principle no need for a net synthesis of \(\alpha\)-AAA. However, part of the \(\alpha\)-AAA is cyclized to 6-oxopiperide-2-carboxylic acid (OPC) and is excreted to the cultivation medium (Nielsen, 1997), leading to a net consumption of \(\alpha\)-AAA during penicillin biosynthesis.

The penicillin biosynthesis pathway is known to exhibit feedback inhibition of the first enzyme (ACV synthetase) by its own product ACV and also by the oxidized dimer bisACV (Theilgaard et al., 1999). This indicates it is important to maintain a low level of ACV in order to achieve high penicillin production fluxes and that the second enzyme (IPN synthetase), which consumes ACV, has a flux controlling influence.

Jorgensen et al. (1995) have shown from penicillin-V producing fed batch fermentations that the flux control is mainly distributed between the first two enzymes, with a shift towards IPN synthetase during fedbatch cultivation due to an increasing level of intracellular ACV (up to 6\(-15\) \(\mu\)mol/gDW). They also showed that addition of cysteine, valine and \(\alpha\)-AAA to the fedbatch culture increased the penicillin flux with about 20%. Reported typical intracellular levels (in \(\mu\)mol/gDW) of these amino acids (\(\alpha\)-AAA, cysteine and valine) (Jorgensen et al., 1995) are (1.5, 1, 2) and (1.5-3, 1, 3-5) respectively for fermentations without and with added aminoacids. This might indicate that the intracellular concentrations of the involved amino
acids could be limiting for penicillinV production, at least for the strain used in that study. This also indicates that ACV synthetase contributes to the control of the penicillin flux.

The interrelation between the penicillin pathway and primary metabolism occurs at three different levels. First, the required amino acid precursors are synthesized from primary metabolites; e.g. cysteine requires 3-phosphoglycerate (3PG), valine requires pyruvate. Increased levels of amino acid precursors in primary metabolism (3PG and pyruvate) might therefore result in increased levels of cystein and valine and to an increase of the penicillin biosynthesis flux. Furthermore, $\alpha$-AAA is synthesized from $\alpha$-ketoglutarate as an intermediate of the lysine pathways. It is reported that in older production strains lysine inhibits its synthesis from $\alpha$-AAA (Gunarson et al., 2004). Hence, a high intracellular lysine level would lead to feed back inhibition of lysine biosynthesis and supposedly to an increased level of intracellular $\alpha$-AAA, which is assumed to stimulate penicillin production (Gunarson et al., 2004).

Secondly, primary metabolism is a source of important cofactors such as NADPH and ATP. Van Gulik et al. (2000) and Kleijn et al. (2006) have shown, using stoichiometric models and chemostat experiments, that the production of NADPH for penicillin biosynthesis draws heavily on primary metabolism and might be a bottleneck for further increase of the penicillin production rate. Moreover, it was found that penicillin synthesis was accompanied by the consumption of an unexpectedly large amount of ATP. Another important result of van Gulik et al. (2000) was that large changes in fluxes through the primary metabolic pathways, which was brought about by cultivation of the fungus under well defined conditions on different carbon sources, i.e. glucose, ethanol and acetate in chemostats at the same growth rate, did not affect the biomass specific penicillin flux. Their suggestion was that different intracellular concentrations of aminoacid precursors 3PG and pyruvate did not effect penicillin production.

The relation between the penicillin biosynthesis pathway and primary metabolism has been studied previously using flux models mainly. This study presents for the first time results on their relation at the level of intracellular metabolite concentrations (glycolysis, TCA cycle, adenine nucleotides), intracellular free amino acid concentrations and fluxes. Steady state perturbations were achieved in chemostat cultivations under well defined reference conditions. As the reference culture, the industrial strain was cultivated in steady state glucose limited chemostats at a growth rate $\mu = 0.05 \, \text{h}^{-1}$. Under these conditions significant amounts of penicillin are produced. First, the penicillin production was perturbed (decreased to zero) and the effect of absence of penicillin production on primary metabolism (fluxes,
metabolite levels) and free amino acid levels was studied. This penicillin perturbation was achieved using two methods, (1) absence of the side chain precursor phenylacetic acid (PAA) and (2) by use of a genetically modified strain in which all copies of the gene cluster coding for the enzymes of the penicillin biosynthesis pathway was removed (which leads to absence of ACV synthetase, IPN synthetase and Acetyl CoA Acyltransferase (AT)). Secondly, the primary metabolism was perturbed by cultivation of the industrial production strain, under penicillin producing conditions using ethanol as the sole carbon source. It is expected that this will lead to very different levels of intermediates of primary metabolism and free amino acids.

5.2 Material and Methods

Strains

A high-producing strain of *Penicillium chrysogenum* (code name DS17690), which is the same strain as used in previous research (van Gulik et al. 2000; van Gulik et al. 2001) and a genetically modified strain derived from the DS17690 strain by removing all gene clusters coding for the enzymes of the penicillin biosynthesis pathway, indicated as Penicillin cluster-free strain (code name AFF230). Both strains were kindly donated by DSM Anti-Infectives (Delft – The Netherlands).

Chemostat Cultivation and Medium Composition

The chemostat system used was based on a 7-L turbine-stirred bioreactor (Applikon, Schiedam, The Netherlands) with a working volume 4L. All aerobic chemostat cultures were operated at a dilution rate \( D = 0.05 \) h\(^{-1}\). The cultivation conditions and operating procedure of the chemostat have been described in detail in previous papers (Nasution et al. 2006a) (Nasution et al., 2006b).

The composition of the chemostat medium has been described previously (Nasution et al., 2006b) and was designed for a biomass concentration of 6 g/L dry weight. The medium contained either 0.5 Cmol/L of glucose or ethanol (which corresponds to 16.5 g/L glucose.H\(_2\)O or 11.5 g/L ethanol absolute). The precursor of penicillin G, Phenylacetic acid (PAA), was added to the feed medium at a concentration of 4.85 mM. Under penicillin producing conditions this results in a residual PAA concentration in the chemostat of about 3 mM. For the chemostat cultivations with the penicillin clusterfree strain, 3 mM of PAA was added to the feed medium to obtain the same residual PAA concentration as in the other experiments. This residual concentration is known to have no metabolic effects (van Gulik et al. 2000). The preparation and sterilisation of the cultivation medium as well as the
inoculation and cultivation procedures have been described previously (Nasution et al., 2006a).

**Rapid sampling**
Rapid sampling, quenching and subsequent washing of cells from the chemostat for determination of intracellular metabolites was carried out as described previously (Nasution et al., 2006a)

**Metabolite Extraction** Metabolite extraction was carried out with the hot ethanol method (Gonzalez et al. 1997), which was adapted for the extraction of *P. chrysogenum* cells. The adapted methods for cold centrifugation and washing of the cells, metabolite extraction and sample preparation have been described elsewhere (Nasution et al., 2006a).

**Analytical procedures** Measurement of the concentrations of biomass dry weight and residual glucose were carried out as described previously (Nasution, 2006b). The concentrations of penicillin-G and its side chain precursor phenylacetic acid in the culture supernatant were measured with high performance liquid chromatography (HPLC) as described in Christensen et al. (1994).

The concentrations of the glycolytic and TCA cycle intermediates in the cell extracts were analyzed with isotope dilution mass spectrometry (IDMS) as described in Wu et al. (2005). The concentrations of the adenine nucleotides AMP, cAMP, ADP and ATP were also analyzed with IDMS, the applied LC-ESI-MS/MS procedure has been described in Wu et al., (2006). Measurement of the concentrations of free amino acids in the cell extracts was carried out with GS-MS as described in chapter 4 of this thesis.

**Calculation Procedures** Biomass specific rates, \(q_{B}\), \(q_{\text{pen}}\), \(q_{\text{PAA}}\), \(q_{\text{byproduct}}\), \(q_{O_2}\) and \(q_{\text{CO}_2}\), were obtained from the respective mass balances of substrate, penicillin, PAA, total carbon in supernatant (corrected for the amount of PAA and penicillin), oxygen and carbon dioxide, respectively, together with the measurements of the concentrations of these compounds in the gas and liquid phase, reactor volume and flow rates of gas and feed medium. The calculated average values of the specific rates were reconciled under the constraint that the elemental conservation relations were satisfied (Vanderheijden et al. 1994).

Metabolic fluxes were calculated from a compartmented stoichiometric model for growth and penicillin production of *P. chrysogenum* (van Gulik et al., 2000) whereby the
reconciled specific biomass growth rate, substrate uptake rate and penicillin production rate \((q_s, \mu, q_{pen})\) were used as the input.

It should be noted that in the stoichiometric model the flux through the pentose phosphate pathway (PPP) is calculated using the NADPH balance (Kleijn et al., 2005). For the chemostat culture grown on ethanol as the sole carbon source, it was assumed that the conversion of ethanol to AcetylCoA yields 1 NADPH (generated during the conversion of Acetaldehyde to AcetylCoA). This results in a relatively low PPP-flux for the ethanol culture.

For several near equilibrium reactions the mass action ratio (MAR) was calculated, either for a single reaction or for a set of near equilibrium reactions. The MAR for a single reaction, e.g. \(A \rightarrow 2B\) is defined as \([B]^2/[A]\). For near equilibrium reactions the calculated MAR is close to the equilibrium constant \((K_{eq})\). Mass action ratios were calculated for myokinase, G6P isomerase, enolase, phosphoglucomutase, fumarase, transaminase and for the reaction sequence: F16bP aldolase, triose isomerase, glyceraldehyde-P dehydrogenase and P-glycerate-mutase.

### 5.3 Results and Discussion

**Chemostat cultivations** Four different chemostat cultures were performed to study the relation between primary metabolism, penicillin pathway and amino acid pathways. One culture, further on indicated as reference culture, was performed with the high producing strain DS 17690, glucose as carbon source and with addition of the side chain precursor PAA, where penicillin production was achieved at a growth rate \(\mu=0.05\) h\(^{-1}\). In two different chemostat cultures, also operated at a dilution rate of 0.05 h\(^{-1}\) the penicillin biosynthesis pathway was negatively perturbed in two different ways: (1) by cultivating the producing strain without addition of the side chain precursor PAA and 2) by cultivating the cluster free strain (AFF230) instead of the producing strain. In both situations, penicillin-G production was absent. In the fourth chemostat culture not the penicillin pathway was perturbed but instead the fluxes through primary metabolism were altered by using ethanol as sole carbon source instead of glucose with the industrial strain.

All four chemostat cultures reached a stable steady state between 50 and 250 hours, after which they were terminated. An example is shown in Figure 5.1, where the reference culture is compared to the culture to which no PAA was added and consequently penicillin-G production is absent. It was calculated that the carbon and redox balances closed within 5% to 10% for all chemostat cultures. In all cultures to which the side chain precursor PAA was
supplied, no catabolism of PAA could be detected and nearly all consumed PAA was used for penicillin production. Only a small amount of PAA was converted to ortho-hydroxy-PAA (o-OH-PAA) (data not shown).

Figure 5.1 Concentrations of biomass (left), PAA (middle) and PenG (right) measured in glucose limited aerobic chemostat cultures of the producing strain DS17690, grown at a growth rate $\mu = 0.05 \text{ h}^{-1}$ in the presence of the side chain precursor PAA (●) and in the absence of PAA (▼).

Table 5.1. Reconciled biomass specific conversion rates for the different cultivation conditions. Specific rates of biomass growth and by-product formation are expressed in mCmol.(Cmol.h)$^{-1}$. The other rates are expressed in mmol.(Cmol.h)$^{-1}$.

<table>
<thead>
<tr>
<th>Rate</th>
<th>Reference Condition</th>
<th>Absence of penicillin production</th>
<th>Perturbation in primary metabolism</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Absence of PAA</td>
<td>Cluster free strain</td>
</tr>
<tr>
<td>$\mu$</td>
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<td>50.24</td>
<td>51.49</td>
</tr>
<tr>
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<td>0</td>
</tr>
<tr>
<td>$q_{pen}$</td>
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<td>0</td>
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<td>$q_{byproduct}$</td>
<td>13.99</td>
<td>13.79</td>
<td>22.88</td>
</tr>
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</table>

Table 5.1 shows the obtained biomass specific conversion rates for the four different conditions. A remarkable aspect is that for all conditions the supernatant of the chemostat cultures contains significant amounts of carbon which has been identified previously in similar chemostat cultures of the same *P. chrysogenum* strain to consist mainly of proteins/peptides and carbohydrates (van Gulik et al., 2000). This might partly be the result
of biomass lysis. The thus excreted carbon is quantified as $q_{\text{byproducts}}$ (mCmol/Cmol$_x$ per h). The biomass specific conversion rates obtained for the reference culture (Table 5.1) appeared to be nearly the same as obtained in a previous study by van Gulik et al., (2000) with the same *P. chrysogenum* strain, although in our study a different fermentor (4L volume instead of 1L) and different biomass concentration (6 g/L instead of 3g/L) was used. This shows the reproducibility of the chemostat studies used.

Tables 5.2, 5.3 and 5.4 show the obtained results for the intracellular concentrations of glycolytic, TCA cycle intermediates, adenine nucleotide and free amino acid concentrations, respectively, measured for the different chemostat cultures.

**Table 5.2** Intracellular concentrations of glycolytic and TCA cycle intermediates of chemostat cultivated *Penicillium chrysogenum*, with perturbation on primary metabolism and penicillin biosynthesis pathway

<table>
<thead>
<tr>
<th>Metabolite (µmol/gDW)</th>
<th>Reference DS17690 in glucose with penicillin production</th>
<th>Absence of penicillin production</th>
<th>Perturbation in primary metabolism Growth in EtOH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Absence of PAA</td>
<td>Cluster free strain</td>
</tr>
<tr>
<td>G6P</td>
<td>3.67 ± 0.24</td>
<td>3.35 ± 0.19</td>
<td>3.87 ± 0.28</td>
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<tr>
<td>F6P</td>
<td>0.62 ± 0.01</td>
<td>0.67 ± 0.13</td>
<td>0.87 ± 0.06</td>
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<td>G1P</td>
<td>0.76 ± 0.01</td>
<td>0.60 ± 0.02</td>
<td>0.95 ± 0.05</td>
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<td>M6P</td>
<td>1.57 ± 0.06</td>
<td>1.30 ± 0.02</td>
<td>1.31 ± 0.12</td>
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<tr>
<td>T6P</td>
<td>0.30 ± 0.01</td>
<td>0.39 ± 0.03</td>
<td>1.31 ± 0.12</td>
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<tr>
<td>6PG</td>
<td>0.33 ± 0.04</td>
<td>0.31 ± 0.08</td>
<td>0.33 ± 0.02</td>
</tr>
<tr>
<td>F26bP</td>
<td>0.02 ± 0.00</td>
<td>0.01 ± 0.00</td>
<td>ND</td>
</tr>
<tr>
<td>F16bP</td>
<td>0.71 ± 0.06</td>
<td>0.53 ± 0.04</td>
<td>0.67 ± 0.03</td>
</tr>
<tr>
<td>2&amp;3PG</td>
<td>0.66 ± 0.03</td>
<td>0.51 ± 0.05</td>
<td>0.54 ± 0.05</td>
</tr>
<tr>
<td>PEP</td>
<td>0.24 ± 0.00</td>
<td>0.22 ± 0.02</td>
<td>0.31 ± 0.03</td>
</tr>
<tr>
<td>Citrate</td>
<td>2.52 ± 0.09</td>
<td>1.93 ± 0.62</td>
<td>3.69 ± 0.60</td>
</tr>
<tr>
<td>PYR</td>
<td>1.28 ± 0.10</td>
<td>0.74 ± 0.09</td>
<td>0.52 ± 0.06</td>
</tr>
<tr>
<td>aKG</td>
<td>0.93 ± 0.02</td>
<td>0.63 ± 0.03</td>
<td>2.14 ± 0.45</td>
</tr>
<tr>
<td>SUCC</td>
<td>0.35 ± 0.02</td>
<td>0.40 ± 0.01</td>
<td>0.25 ± 0.03</td>
</tr>
<tr>
<td>FUM</td>
<td>0.75 ± 0.03</td>
<td>0.70 ± 0.04</td>
<td>0.66 ± 0.06</td>
</tr>
<tr>
<td>MAL</td>
<td>3.63 ± 0.21</td>
<td>3.08 ± 0.05</td>
<td>3.06 ± 0.23</td>
</tr>
<tr>
<td>Glyoxylate</td>
<td>0.16 ± 0.01</td>
<td>0.15 ± 0.01</td>
<td>0.16 ± 0.02</td>
</tr>
</tbody>
</table>
The effect of absence of penicillin synthesis on fluxes and concentration of primary metabolites

The impact of penicillin production on primary metabolism can be studied by comparing the fluxes obtained for the reference chemostat (Figure 5.2) with the fluxes obtained for the chemostat culture with the producing strain without PAA supply and thus without production of penicillin (Figure 5.3).

Table 5.3 Intracellular concentration of adenine nucleotide of standard chemostat cultivated *Penicillium chrysogenum*, with perturbation on primary metabolism and penicillin biosynthesis pathway

<table>
<thead>
<tr>
<th>Adenine nucleotide</th>
<th>Reference DS17690 in glucose with penicillin production</th>
<th>Absence of penicillin production</th>
<th>Perturbation in primary metabolism Growth in EtOH</th>
</tr>
</thead>
<tbody>
<tr>
<td>(µmol/gDW)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATP</td>
<td>7.67 ± 0.13</td>
<td>6.20 ± 0.29</td>
<td>5.95 ± 0.25</td>
</tr>
<tr>
<td>ADP</td>
<td>1.16 ± 0.05</td>
<td>1.35 ± 0.04</td>
<td>1.43 ± 0.14</td>
</tr>
<tr>
<td>AMP</td>
<td>0.34 ± 0.03</td>
<td>0.41 ± 0.04</td>
<td>0.48 ± 0.01</td>
</tr>
<tr>
<td>cAMP</td>
<td>0.0026 ± 0.003</td>
<td>0.033 ± 0.005</td>
<td>0.0033 ± 0.005</td>
</tr>
<tr>
<td>Energy Charge</td>
<td>0.90</td>
<td>0.86</td>
<td>0.85</td>
</tr>
<tr>
<td>Summation</td>
<td>9.17 ± 0.21</td>
<td>7.96 ± 0.37</td>
<td>7.86 ± 0.40</td>
</tr>
</tbody>
</table>

A remarkable difference between both conditions is that the absence of penicillin production leads to significantly lower rates of O₂ consumption and CO₂ production. The same holds for the specific rate of glucose consumption which is also significantly lower in the absence of penicillin production. A possible explanation for the observed lower rates of O₂ consumption, glucose consumption and CO₂ production in the culture without penicillin production could be the absence of PAA. It is known that the presence of the weak acid PAA can lead to energy-uncoupling due to influx of undissociated PAA and the subsequent, energy requiring export of PAA to the extracellular medium. This would result in increased energy dissipation in the presence of PAA and thus to higher biomass specific glucose consumption and respiration rates. To verify the validity of this explanation chemostat cultures were performed at different PAA levels in the feed, leading to different residual PAA concentrations in the broth. However, from these experiments it appeared that an increase of the residual PAA concentration from 2.15 to 4.45 mM did not have any significant effect on
the biomass specific conversion rates of glucose, oxygen, carbon dioxide and penicillin ($q_s$, $q_{o2}$, $q_{CO2}$ and $q_{pen}$), which excludes an uncoupling effect of PAA at the concentration level applied in our experiments. Therefore the significant decrease of the rates of glucose consumption and respiration in the absence of PAA is most likely caused by the absence of penicillin production, resulting in a lower carbon requirement and much lower energy demand. It has been reported previously that the production of penicillin-G in this strain is associated with a large additional energy requirement (van Gulik et al., 2000). The current results confirm this finding.

With respect to the metabolic flux distributions which are shown in Figure 5.3 (absence of penicillin synthesis) and Figure 5.2 the comparison shows a significant effect of absence of penicillin synthesis on the different metabolic fluxes. Note that the fluxes of central metabolites towards biomass synthesis and from the pentose phosphate pathway to F1,6bP and GAP are not shown. It can be inferred from these figures that the flux from 3PG toward cysteine is about 90% less, leading to a 40% lower flux from 3PG to serine. Also the flux from pyruvate to valine is about 40 – 50% lower. These large differences are explained by the fact that under penicillin producing condition 90% of the produced cysteine and 50% of the produced valine is converted to penicillin.

Furthermore the flux through the TCA cycle is 30% lower in the absence of penicillin production, due to the much lower ATP requirement. Another difference is the flux through the pentose phosphate pathway which is 15% lower because of the reduced need for NADPH (note that the biosynthesis of 1 mol of penicillin requires 8 – 10 mol of NADPH, depending on the biosynthesis route of cysteine). Also the glycolytic flux is approximately 20% lower in the absence of penicillin production due to the lower flux to the TCA cycle as a result of decreased energy requirement and a smaller carbon flux toward amino acid synthesis (valine and cysteine). It should be noted that the carbon need for all other amino acids is not different, because the specific growth rate $\mu$ is the same for both conditions.
**Table 5.4** Intracellular free amino acid concentration (µmol/gDW) of standard chemostat cultivated *Penicillium chrysogenum*, with perturbation on primary metabolism and penicillin biosynthesis pathway

<table>
<thead>
<tr>
<th>Amino acids and its precursors in primary metabolism (µmol/gDW)</th>
<th>Reference</th>
<th>Absence of penicillin production</th>
<th>Perturbation in primary metabolism Growth in EtOH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DS17690 in glucose with penicillin production</td>
<td>Absence of PAA</td>
<td>Cluster free strain</td>
</tr>
<tr>
<td><strong>Histidine Family</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R5P*</td>
<td>0.08</td>
<td>0.08</td>
<td>0.1</td>
</tr>
<tr>
<td>Histidine</td>
<td>1.28 ± 0.09</td>
<td>1.00 ± 0.02</td>
<td>2.32 ± 0.41</td>
</tr>
<tr>
<td><strong>Serine Family</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2&amp;3PG</td>
<td>0.66 ± 0.03</td>
<td>0.51 ± 0.05</td>
<td>0.54 ± 0.05</td>
</tr>
<tr>
<td>Cysteine</td>
<td>0.50 ± 0.14</td>
<td>0.00</td>
<td>0.14 ± 0.13</td>
</tr>
<tr>
<td>Serine</td>
<td>6.97 ± 0.46</td>
<td>6.71 ± 0.38</td>
<td>4.58 ± 0.52</td>
</tr>
<tr>
<td>Glycine</td>
<td>2.22 ± 0.08</td>
<td>2.69 ± 0.29</td>
<td>1.53 ± 0.33</td>
</tr>
<tr>
<td><strong>Aromatic Family</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PEP</td>
<td>0.24 ± 0.00</td>
<td>0.22 ± 0.02</td>
<td>0.31 ± 0.03</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>0.16 ± 0.03</td>
<td>0.12 ± 0.01</td>
<td>0.20 ± 0.02</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>0.15 ± 0.06</td>
<td>0.14 ± 0.02</td>
<td>0.23 ± 0.03</td>
</tr>
<tr>
<td><strong>Pyruvate Family</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pyruvate</td>
<td>1.28 ± 0.10</td>
<td>0.74 ± 0.09</td>
<td>0.52 ± 0.06</td>
</tr>
<tr>
<td>Alanine</td>
<td>29.81 ± 1.93</td>
<td>26.06 ± 0.08</td>
<td>21.78 ± 3.18</td>
</tr>
<tr>
<td>Valine</td>
<td>2.80 ± 0.09</td>
<td>2.36 ± 0.04</td>
<td>1.68 ± 0.18</td>
</tr>
<tr>
<td>Leucine</td>
<td>0.94 ± 0.06</td>
<td>0.77 ± 0.05</td>
<td>0.63 ± 0.05</td>
</tr>
<tr>
<td><strong>Aspartate Family</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Malate</td>
<td>3.63 ± 0.21</td>
<td>3.08 ± 0.05</td>
<td>3.06 ± 0.23</td>
</tr>
<tr>
<td>Aspartate</td>
<td>21.18 ±1.02</td>
<td>15.66±1.67</td>
<td>16.56±1.84</td>
</tr>
<tr>
<td>Asparagine</td>
<td>1.63 ± 0.08</td>
<td>1.17 ± 0.03</td>
<td>2.03 ± 0.24</td>
</tr>
<tr>
<td>Methionine</td>
<td>0.18 ± 0.01</td>
<td>0.19 ± 0.00</td>
<td>0.18 ± 0.01</td>
</tr>
<tr>
<td>Threonine</td>
<td>7.55 ± 0.40</td>
<td>6.11 ± 0.10</td>
<td>5.23±0.56</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>0.46 ± 0.02</td>
<td>0.45 ± 0.02</td>
<td>0.32 ± 0.04</td>
</tr>
<tr>
<td><strong>Glutamate Family</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>aKG</td>
<td>0.93 ± 0.02</td>
<td>0.63 ± 0.03</td>
<td>2.14 ± 0.45</td>
</tr>
<tr>
<td>Glutamate</td>
<td>58.48 ± 2.95</td>
<td>62.17 ±0.65</td>
<td>56.40±5.81</td>
</tr>
<tr>
<td>Glutamine</td>
<td>27.56 ± 3.57</td>
<td>37.69±0.82</td>
<td>38.47±4.74</td>
</tr>
<tr>
<td>Ornithin</td>
<td>1.31 ± 0.12</td>
<td>1.58 ± 0.16</td>
<td>1.74 ± 0.17</td>
</tr>
<tr>
<td>Proline</td>
<td>1.28 ± 0.27</td>
<td>1.56 ± 0.02</td>
<td>0.77 ± 0.10</td>
</tr>
<tr>
<td>α-Amino Adipicacid</td>
<td>3.41 ± 0.50</td>
<td>7.33 ± 0.08</td>
<td>0.97 ± 0.11</td>
</tr>
<tr>
<td>Lysine</td>
<td>1.92 ± 0.32</td>
<td>1.25 ± 0.11</td>
<td>1.5 ± 0.17</td>
</tr>
</tbody>
</table>

*calculated from concentration of G6P and F16bP (see Appendix 1)
Figure 5.2 Biomass specific conversion rates and calculated flux distribution for the reference condition (glucose limited chemostat culture of the producing strain in the presence of PAA)

<table>
<thead>
<tr>
<th>Specific rate</th>
<th>Reconciled value</th>
</tr>
</thead>
<tbody>
<tr>
<td>$q_s$</td>
<td>17.77 m(C)mol.(Cmol.h)$^{-1}$</td>
</tr>
<tr>
<td>$\mu$</td>
<td>50.24</td>
</tr>
<tr>
<td>$qO_2$</td>
<td>39.87</td>
</tr>
<tr>
<td>$qCO_2$</td>
<td>42.61</td>
</tr>
<tr>
<td>$qPAA$</td>
<td>0</td>
</tr>
<tr>
<td>$q_{pen}$</td>
<td>0</td>
</tr>
<tr>
<td>$q_{byproduct}$</td>
<td>13.79</td>
</tr>
</tbody>
</table>

Figure 5.3 Biomass specific conversion rates and calculated flux distribution for glucose limited chemostat cultivation of the producing strain in the absence of PAA
In Table 5.2 to 5.4 the results of the intracellular metabolite measurements are shown for all four conditions. When comparing the results for the reference conditions to the culture without penicillin production due to absence of PAA, the following general observations can be made with respect to the relation between metabolic fluxes and intracellular metabolite levels. To link metabolite levels to fluxes, it was assumed that enzyme activity have not changed, which seems acceptable because the comparison is made for the same strain, being cultivated at the same growth rate (0.05 h⁻¹) and on the same carbon source.

In the absence of penicillin production, the adenine nucleotide concentration and the calculated energy charge (Table 5.3) are lower than in the presence of penicillin production, which might be related to the lower ATP requirement if no penicillin production occurs.

The mass action ratio’s (MAR) for the reactions which are known to be close to equilibrium (Table 5.5) are nearly the same in both cultures. This shows that G6P isomerase, phosphoglucomutase, enolase and phosphoglycerate mutase, fumarase and transaminase are close to equilibrium. The altered fluxes have hardly any effect on the MAR, which agrees with the near equilibrium status of these reactions.

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>Reference DS17690 in glucose with penicillin production</th>
<th>Absence of penicillin production</th>
<th>Perturbation in primary metabolism Growth in EtOH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myokinase</td>
<td>0.52 ± 0.05</td>
<td>0.72 ± 0.08</td>
<td>0.52 ± 0.13</td>
</tr>
<tr>
<td>G6P Isomerase</td>
<td>0.17 ± 0.01</td>
<td>0.20 ± 0.04</td>
<td>0.23 ± 0.02</td>
</tr>
<tr>
<td>Phosphoglucomutase</td>
<td>0.21 ± 0.01</td>
<td>0.18 ± 0.01</td>
<td>0.30 ± 0.05</td>
</tr>
<tr>
<td>Enolase /P-Gly-Mutase</td>
<td>0.36 ± 0.02</td>
<td>0.43 ± 0.06</td>
<td>1.67 ± 0.19</td>
</tr>
<tr>
<td>Fumarase</td>
<td>0.21 ± 0.01</td>
<td>0.23 ± 0.01</td>
<td>0.16 ± 0.02</td>
</tr>
<tr>
<td>((F16bP)^2) / (2 &amp; 3PG)</td>
<td>1.28</td>
<td>1.43</td>
<td>4.44</td>
</tr>
<tr>
<td>(\text{glu} \cdot \text{pyr} / \text{aKG.ala})</td>
<td>2.70</td>
<td>2.80</td>
<td>6.90</td>
</tr>
<tr>
<td>(\text{glu.mal} / \text{aKG.asp})</td>
<td>10.77</td>
<td>19.41</td>
<td>15.03</td>
</tr>
</tbody>
</table>
In the absence of penicillin production the concentration of many glycolytic intermediates (G6P, G1P, M6P, F16bP, 2&3PG, pyruvate) (Table 5.2) do have about 10 -20 % lower values. Interestingly pyruvate is even 35% lower, which is probably due to the additional effect of decreased stimulation of pyruvate kinase by lower F16bP. All these concentrations correspond to the 20% lower glycolytic flux.

Remarkably, the concentration of T6P is higher in the absence of penicillin production. This indicates that under this condition there might be an increase in the conversion of G6P into storage carbohydrates (Chapter 4 of this thesis).

Several TCA cycle intermediates (Table 5.2); e.g. citrate, αKG and malate, as well as pyruvate are about 20% to 40% lower, respectively, which corresponds to the observed 30% lower flux in the TCA cycle in the absence of penicillin production.

The concentration of the pentose phosphate pathway (PPP) intermediate 6PG is the same for both conditions in spite of the fact that the concentration of its precursor G6P is 10% lower in the absence of penicillin production. This could be explained by the approx. 13% lower flux through the PPP in the absence of penicillin production.

In a different experiment the penicillin production was removed by completely removing all copies of the gene cluster encoding for the penicillin biosynthesis pathway (cluster-free strain). The expectation was that the experiment would yield similar results as obtained in the previous experiment where the absence of penicillin G production was achieved by cultivation on a medium without the side chain precursor PAA. The biomass specific net conversion rates as well as the metabolic fluxes for both non-producing conditions are shown in Figure 5.3 (no addition of PAA) and Figure 5.4 (cluster free strain). Surprisingly, the fluxes and metabolite levels (Table 5.2 – 5.4) are highly different. For the cluster free strain significantly higher specific rates of glucose and O₂ consumption as well as CO₂ production and by-product formation were observed (see Table 5.1). The fact that \( q_{byproduct} \) in the clusterfree strain is much higher than in the producing strain could be an indication for increased biomass lysis in the cluster-free strain. This increased lysis would then partly explain the much higher biomass specific rates of O₂ consumption, CO₂ production and glucose uptake (because if significant cell lysis occurs the clusterfree strain grows effectively at \( \mu > 0.05 \) h⁻¹). The higher fluxes in the cluster free strain lead to higher glycolytic and TCA cycle fluxes, 30% and 60%, respectively (see Figure 5.4), compared to the other experiment without penicillin production. The fluxes are even higher than for the production strain in the reference experiment. Apparently the cluster free strain has a different
metabolic behavior than the producing strain, in spite of the fact that it has been derived from this strain.

![Metabolism Diagram](image)

<table>
<thead>
<tr>
<th>Specific rate</th>
<th>Reconciled value</th>
</tr>
</thead>
<tbody>
<tr>
<td>( q_s )</td>
<td>22.93 ( \text{m(C)mol.(Cmol.h)}^{-1} )</td>
</tr>
<tr>
<td>( \mu )</td>
<td>51.49</td>
</tr>
<tr>
<td>( q_{O2} )</td>
<td>60.30</td>
</tr>
<tr>
<td>( q_{CO2} )</td>
<td>63.19</td>
</tr>
<tr>
<td>( q_{PAA} )</td>
<td>0</td>
</tr>
<tr>
<td>( q_{pen} )</td>
<td>NA</td>
</tr>
<tr>
<td>( q_{byproduct} )</td>
<td>22.88</td>
</tr>
</tbody>
</table>

**Figure 5.4** Biomass specific conversion rates and calculated flux distribution for glucose limited chemostat cultivation of the cluster free strain in the presence of PAA

The measured intracellular metabolite levels in the clusterfree strain are shown in Tables 5.2, 5.3 and 5.4. The higher fluxes in the cluster-free strain can also be recognized in the metabolite levels. It can be seen from Table 5.2 that the concentrations of most of the glycolytic intermediates that is G6P, F6P, G1P, F16bP and PEP in the cluster-free strain are significantly higher than in the experiment where no PAA was supplied to the producing strain to accomplish non-producing conditions. Several concentration levels appeared to be even higher than in the penicillin producing reference culture. This again agrees with the higher fluxes in the cluster-free strain, even when compared to the reference strain (Figure 5.3 and 5.1). The results of all three experiments where glucose was the carbon source confirm the strong positive correlation between glycolytic flux and glycolytic intermediates.
Figure 5.5 Biomass specific conversion rates and calculated flux distribution for chemostat cultivation of the production strain on ethanol as the sole carbon source in the presence of PAA.

Also the intracellular concentrations of the TCA cycle intermediates citrate and αKG were much higher in the cluster-free strain, which corresponds with the calculated higher TCA cycle fluxes. Interestingly, the concentration of trehalose-6-phosphate (T6P) is much higher in the cluster-free strain. This supports the previous notion that the absence of penicillin production stimulates the formation of storage material. Finally it is interestingly to note that in both cultures, where penicillin is not produced, the ATP concentration and energy charge are much lower than when penicillin is produced. This agrees with the notion that penicillin production requires large amounts of energy, reflecting the need for a high energy status.

Table 5.5 shows that the mass action ratio’s in this experiment are nearly the same as in the other experiment without penicillin production. Only the alanine/pyruvate transaminase mass action ratio is different, for unknown reasons.
Summarizing, the three glucose limited chemostat experiments show that in absence of penicillin production:

- There are significant changes in the fluxes through primary metabolism (10% - 60%).
  1.) For the producing strain cultivated with PAA (and thus with penicillin-G production) compared with cultivation without PAA (no penicillin-G production) this is mostly likely caused by the high energy impact of penicillin production. 2.) For the cluster free strain (no penicillin production) compared with the producing strain without PAA (also no penicillin production), this is most likely caused by increased by-product formation in the cluster free strain.

- The changes in fluxes generally lead to significant changes in concentration of primary metabolites, such that higher fluxes correspond to higher metabolite concentrations.

- For reversible reactions, the same mass action ratios are observed.

- Absence of penicillin production leads to a lower energy status (lower ATP level and lower energy charge) and possibly to increased accumulation of storage carbohydrates, which is indicated by increased levels of T6P.

**The effect of the perturbation of primary metabolism on fluxes and metabolite concentrations** Perturbation of the fluxes through primary metabolism was accomplished by cultivation of the producing strain on ethanol as the sole carbon source, under otherwise identical conditions as for the glucose limited chemostat culture (= reference condition). Remarkably, the biomass specific rate of penicillin-G production was significantly higher in the ethanol limited chemostat compared to the reference culture (see Table 5.1). Furthermore, cultivation on ethanol as carbon source led to large differences in the levels of the glycolytic intermediates which were generally much lower than with glucose as carbon source. This is not very surprising because the glycolytic fluxes are highly different for both situations (gluconeogenesis versus glycolysis). Interestingly, most of the calculated mass action ratio’s (MAR), see Table 5.5, are close to the MAR calculated for the glucose limited chemostat culture (i.e. myokinase, G6P-isomerase, phosphoglcomutase, fumarase, asp/oaa transaminase). This is expected for highly reversible reactions, and gives confidence in the measured metabolite levels. Remarkably, the MAR for enolase/P-glycerate mutase and for the F16bP to 2&3PG sequence are much higher for ethanol as substrate, which agrees with the inverted direction of fluxes under gluconeogenic conditions. Furthermore, the ATP
concentration and the energy charge for the ethanol culture are higher than the reference, which supports the observation that high penicillin production requires a higher energy status. The calculated fluxes through the primary metabolic pathways for the ethanol culture (Figure 5.5) lead to the following observations:

A five times lower PPP flux is obtained. The reason for this is the assumption that most NADPH is obtained from the conversion of acetaldehyde to acetate (van Gulik et al., 2000) which was used in the stoichiometric model for calculation of the fluxes. The 5 fold lower PPP flux corresponds to the about 4-5 lower G6P concentration in the ethanol culture compared to the reference.

The flux from PEP to pyruvate, which is only for anabolic purpose, is about 6 times lower. The intracellular pyruvate level is about 10 times lower, which is probably related to the much lower activation of pyruvate kinase due to a much lower concentration of F16bP.

Growth on ethanol as sole carbon source requires the operation of the glyoxylate cycle as anaplerotic route and thus a rearrangement of fluxes through the TCA cycle compared with growth on glucose. This results in an increased flux via citrate synthase and subsequently citrate lyase, malate synthase and malate dehydrogenase back to oxaloacetate and a decreased flux via isocitrate dehydrogenase, αKG dehydrogenase and succinyl CoA synthetase. This is partly reflected in changed metabolite levels, i.e. increase concentrations of citrate and glyoxylate and decreased concentrations of αKG. The reason for the lower concentrations of succinate, fumarate and malate is not clear, but is probably related to a much lower OAA-pool due to a low PEP level.

The perturbation of penicillin synthesis (no PAA and cluster-free strain) and primary metabolism (ethanol as substrate) has resulted in significant changes in fluxes and metabolite concentrations, where in general increased fluxes through glycolysis, TCA cycle and PPP correlate with higher concentrations of the related intracellular metabolites in these pathways. An important question is now how these changed concentrations of primary metabolites have changed the concentrations of the free amino acids which use these metabolites as precursors and how these amino acid levels relate to the rate of penicillin synthesis.

The relation between the concentrations of primary metabolites and free amino acids

It is well known that the first step of amino acid biosynthesis pathways is often an irreversible reaction which determines the amino acid flux via feed back inhibition of the first reaction by the end product. Because the four different chemostat cultures carried out in this study were
all performed at the same specific growth rate $\mu$, it can be assumed that the enzyme levels in the anabolic pathways such as amino acid synthesis are not much different in the four experiments. Moreover, because also the biomass composition is highly similar (data not shown), the fluxes through almost all amino acid pathways, except for valine and cysteine which are used for penicillin synthesis, must be very similar in the four experiments. With these assumptions it is expected that a monotonous positive relation exists between the concentrations of free amino acids and their pathway precursors from primary metabolism. In Table 5.4 the measured intracellular concentrations of free amino acids in the four cultures are shown. Remarkably, the amino acid levels of the reference condition are similar as found previously by Henriksen et al. (1996) for a different industrial *P. chrysogenum* penicillinV production strain.

In Figures 5.6 to 5.10 the relations between the free amino acid concentrations and the concentrations of their precursors in primary metabolism are shown based on the four steady states. As can be inferred from these figures there is a clear relation, for all different amino acid families (serine, aromatic, pyruvate, $\alpha$KG, aspartate and R5P) as expected. The R5P concentration could not be measured and was calculated from G6P and F16bP using equilibrium assumptions in the non-oxidative PPP (see Appendix 5.1). Similar relations between free amino acid levels and primary metabolite concentrations were also observed in *Saccharomyces cerevisiae* cultivated in glucose limited chemostats under very similar conditions and at the same specific growth rate as our reference condition in an evolutionary chemostat experiment (Mashego et al. 2005). During this evolution experiment, the concentrations of many glycolytic and TCA cycle intermediates decreased markedly with cultivation time. The observed relations between primary metabolite precursor levels and free amino acid concentrations in *Saccharomyces cerevisiae* are shown in the Appendix 5.2 in Figures 5.A2.1 to 5.A2.5. The agreements with the observed relations in *P. chrysogenum* are remarkable. Therefore the conclusion can be drawn that free amino acid concentrations are highly responsive to the levels of their precursors in primary metabolism, which must be caused by the mechanism of end product feedback inhibition.

What determines free intracellular amino acid levels? A simple kinetic rate equation for amino acid biosynthesis with end product inhibition was constructed, using the lin-log approximative kinetic format (Wu et al. 2004). With the assumption that the fluxes through the anabolic pathways as well as the levels of the anabolic enzymes are the same for the four conditions applied the following relation was derived (for details see Appendix 5.3):
\[ \ln \left( \frac{x_{AA}}{x_{AA}^o} \right) = \left[ \frac{\xi_{pm}}{-\xi_{AA}} \right] \ln \left( \frac{x_{pm}}{x_{pm}^o} \right) \]  

(5.1)

With \( x_{AA} \) the free intracellular aminoacid concentration, \( x_{pm} \) the primary metabolite precursor concentration and superscript \(^o\) indicates the reference steady state.

This assumption does not hold for amino acids, which are on the route towards amino acid precursors of penicillin synthesis. The absent of penicillin synthesis causes a different flux.

Equation 5.1 shows a double-logarithmic relation between the concentration of free amino acid and its direct precursor in primary metabolism. \( \xi_{pm} (>0) \) reflects the kinetic effect of the primary metabolite on the first reaction of the amino acid synthesis pathway, \( \xi_{AA} (<0) \) reflects the end-product feedback inhibition of the amino acid on the first reaction.

In Figures 5.6 – 5.10 the concentrations of free amino acid are plotted against the precursor concentration. From these plots it can be inferred that for nearly all amino acids this linear relation holds. Furthermore, the slope of each double-logarithmic plot represents the ratio of the elasticities \( \frac{\xi_{pm}}{-\xi_{AA}} \) for each amino acid. Table 5.8 shows that for some amino acids \( \xi_{pm} < \xi_{AA} \) and \( \xi_{pm} > \xi_{AA} \), which indicates weak and strong feedback inhibition of amino acid on its producing pathway, respectively.

For Michaelis Menten reaction, \( \xi_{pm} \approx 0 \) and \( \xi_{AA} \) is elasticity the reflects end product inhibition with a negative value between -4 and 0, as explained in Appendix 5.3. With those assumptions we expect that \( \xi_{pm}/-\xi_{AA} \) will be a positive number and \( \xi_{pm}/-\xi_{AA} < 1 \).

Most of the amino acid family (histidine, serine, aromatic and pyruvate), for both \textit{Penicillium chrysogenum} and \textit{Saccharomyces cerevisiae}, meet the expected value. The exception is shown by aspartate family, the values are bigger than 1, which indicates a weak feedback inhibition of amino acids in this family. In \textit{Penicillium chrysogenum}, asparagine even shows a negative value for an uncertain reason.

Due to the assumption that requires the same flux to derive equation 5.1, some of the \( \xi_{pm}/-\xi_{AA} \) value in \textit{Penicillium chrysogenum} culture cannot be calculated. Flux toward penicillin pathway changes in producing and nonproducing condition, such as serine family, valine and leucine.

There is no representative line can be drawn on the plot metabolite and amino acid concentrations in glutamate family, in \textit{Penicillium chrysogenum} culture, which result the value of \( \xi_{pm}/-\xi_{AA} \) can not be identified in this family.
Table 5.8. Elasticities of amino acid pathways

<table>
<thead>
<tr>
<th>Amino Acids Family</th>
<th>Precursors</th>
<th>P. chrysogenum</th>
<th>S. cerevisiae</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Histidine Family</strong></td>
<td>R5P</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Histidine</td>
<td>0.598</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td><strong>Serine Family</strong></td>
<td>2&amp;3PG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cysteine</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Serine</td>
<td>-</td>
<td>0.106</td>
<td></td>
</tr>
<tr>
<td>Glycine</td>
<td>-</td>
<td>0.306</td>
<td></td>
</tr>
<tr>
<td><strong>Aromatic Family</strong></td>
<td>PEP</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>0.830</td>
<td>0.649</td>
<td></td>
</tr>
<tr>
<td>Tyrosine</td>
<td>0.849</td>
<td>0.467</td>
<td></td>
</tr>
<tr>
<td><strong>Pyruvate Family</strong></td>
<td>Pyruvate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alanine</td>
<td>0.340</td>
<td>0.987</td>
<td></td>
</tr>
<tr>
<td>Valine</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Leucine</td>
<td>-</td>
<td>0.077</td>
<td></td>
</tr>
<tr>
<td><strong>Aspartate Family</strong></td>
<td>Malate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aspartate</td>
<td>2.055</td>
<td>1.468</td>
<td></td>
</tr>
<tr>
<td>Asparagine</td>
<td>-1.912</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Methionine</td>
<td>1.323</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Threonine</td>
<td>3.214</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Isoleucine</td>
<td>2.905</td>
<td>1.380</td>
<td></td>
</tr>
<tr>
<td><strong>Glutamate Family</strong></td>
<td>aKG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glutamate</td>
<td>-</td>
<td>1.168</td>
<td></td>
</tr>
<tr>
<td>Glutamine</td>
<td>-</td>
<td>1.629</td>
<td></td>
</tr>
<tr>
<td>Ornithin</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Proline</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>α-Amino Adipicacid</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Lysine</td>
<td>-</td>
<td>0.839</td>
<td></td>
</tr>
</tbody>
</table>
Figure 5.6 Relation of amino acids from serine family with 2&3PG, their precursors in primary metabolism. Reference culture (●), Absence of PAA (○), Penicillin Cluster-free strain (□) and Ethanol culture (■). Solid lines according to equation 5.1.

Figure 5.7. Relation of amino acids from aromatic family with PEP, their precursors in primary metabolism. Reference culture (●), Absence of PAA (○), Penicillin Cluster-free strain (□) and Ethanol culture (■). Solid lines are amino acid calculated using estimated elasticities.
Figure 5.8 Relation of amino acids from pyruvate family with pyruvate, their precursors in primary metabolism. Reference culture (●), Absence of PAA (○), Penicillin Cluster-free strain (□) and Ethanol culture (■). Solid lines are amino acid calculated using estimated elasticities.

Figure 5.9 Relation of amino acids from glutamate family with αKG, their precursors in primary metabolism. Reference culture (●), Absence of PAA (○), Penicillin Cluster-free strain (□) and Ethanol culture (■).
**Figure 5.10** Relation of amino acids from aspartate family with malate, their precursors in primary metabolism. Reference culture (●), Absence of PAA (○), Penicillin Cluster-free strain (□) and Ethanol culture (■). Solid lines are according to equation (5.1).

**What determines the penicillin flux?** Valine, αAAA and cysteine are the amino acids needed in the first step (by ACV-synthetase) of the penicillin pathway. αAAA is an intermediate of the lysine pathway. Figure 5.11b shows that the fee lysine concentration is not sensitive to a wide range of αAAA concentrations, which suggested that lysine inhibition on αAAA synthesis (as noted in older production strain) is absent in this production strain.

**Figure 5.11a** Relation of Lysine and αAAA with αKG in primary metabolism. Reference culture (●), Absence of PAA (○), Penicillin Cluster-free strain (□) and Ethanol culture (■).
The relation of $q_p$ and valine and $\alpha$AAA concentration is shown in Figure 5.12. Clearly penicillin flux is insensitive to the intracellular concentrations of these compounds. This agrees with the reported $K_m$ value of ACV synthetase for valine ($K_m = 0.55 \mu$mol/gDW) and $\alpha$AAA ($K_m = 0.17 \mu$mol/gDW) (Banko et al., 1987) which are far lower than the measured value. These results contrast to the results of Jorgensen et al., (1995) which showed (for penicillinV and other industrial strain V) a small increase in flux when slightly higher intracellular concentrations for valine, cysteine and $\alpha$AAA (by simultaneously addition of these compounds to the used fedbatch culture). However, that result does not reveal which of the three amino acids is responsible for this flux increase. The current result seems to rule out a flux determining role of $\alpha$AAA and valine. However, our four chemostat experiments reveal a clear correlation between penicillin flux and intracellular free cysteine concentration (Figure 5.11). This result is not surprising because about 90% of all cysteine goes to penicillin. It should be noted that the cysteine level in our studies is not dependent on its precursor (2&3PG) (Figure 5.6). It is therefore likely that other factors are relevant. A most probable candidate is the level of NADPH, which is consumed in high amount for cysteine production. Recently (Kleijn et al., 2006) a clear relation was established between the penicillin pathway flux and the flux in the pentose phosphate pathway. It is found that a high penicillin production corresponds to a high ATP concentration and high energy charge (Figure 5.13). This impact of ATP concentration is probably related to ACV synthetase where large amounts of ATP are used for the peptide synthesis.
5.4 Conclusion

The relation between penicillin production flux and primary metabolism has been studied by four steady state perturbations of penicillin pathway and primary metabolism. For near equilibrium reactions nearly the same mass action ratio was found for the four different steady states. These perturbations created large changes in fluxes in primary metabolism, which caused large changes in the intracellular concentration of primary metabolites (glycolysis, TCA cycle and PPP) and free intracellular amino acids, for nearly all amino acids. Positive correlations between fluxes and metabolite concentrations were found. For nearly all amino acids, unique positive relations were obtained between amino acid concentration and the concentration of their precursor of primary metabolism. These comparable relations were also
A metabolome study of the steady state relation found in comparable with *Saccharomyces cerevisiae* and appear therefore general. This relation could be simply understood and modeled using pathway activation by the precursor and amino acid feedback inhibition. Using a simple lin-log kinetic format, elasticity ratios were obtained. The penicillin production flux is not influenced by its precursor amino acids, $\alpha$AAA and valine, but mostly by the ATP level and cysteine concentration, where there is possibly a positive relation between cysteine level and NADPH level (4 NADPH are needed for cysteine production). This leads to the final conclusion that the penicillin production flux appears to be mostly influenced by energy and redox cofactors, which are set by primary metabolism. Another interesting observation is that the absence of penicillin production corresponds to high T6P levels, which suggests increased accumulation of storage metabolites.

References


Chapter 5

Appendix 5.1 Calculation of Ribose-5-Phosphate (R5P) concentration from the measurement of Glucose-6-Phosphate (G6P) and Fructose-1,6-bisPhosphate (F16bP)

The reactions taken as near equilibrium are

\[ F16bP \leftrightarrow GAP + DAHP \quad \text{by Aldolase (ald)} \]
\[ GAP \leftrightarrow DHAP \quad \text{by Triosphosphate Isomerase (tim)} \]
\[ GAP + S7P \leftrightarrow E4P + F6P \quad \text{by Transaldolase (tald)} \]
\[ GAP + S7P \leftrightarrow R5P + X5P \quad \text{by Transketolase1 (tket1)} \]
\[ F6P + GAP \leftrightarrow E4P + X5P \quad \text{by Transketolase2 (tket2)} \]
\[ R5P \leftrightarrow X5P \quad \text{by Phosphoribulose epimerase (epim)} \]
\[ R5P \leftrightarrow Ribu5P \quad \text{by Phosphopentoseisomerase (isome)} \]

Equilibrium constants are defined as

\[ K_{ald} = \frac{GAP \cdot DHAP}{F16bP} = 5.6 \times 10^{-5} \quad (\text{Veech et al., 1969}) \]
\[ K_{tim} = \frac{DHAP}{GAP} = 22 \quad (\text{Veech et al., 1969}) \]
\[ K_{tald} = \frac{E4P \cdot F6P}{GAP \cdot S7P} = 0.37 \quad (\text{Casazza et al., 1986}) \]
\[ K_{tket1} = \frac{R5P \cdot X5P}{GAP \cdot S7P} = 0.48 \quad (\text{Casazza et al., 1986}) \]
\[ K_{tket2} = \frac{E4P \cdot X5P}{GAP \cdot F6P} = 0.0337 \quad (\text{Casazza et al., 1986}) \]
\[ K_{epim} = \frac{X5P}{Ribu5P} = 1.82 \quad (\text{Casazza et al., 1986}) \]
\[ K_{isome} = \frac{Ribu5P}{R5P} = 0.833 \quad (\text{Casazza et al., 1986}) \]
While unmeasured metabolite concentrations are calculated from measured F16bP and F6P using

\[ R5P = \frac{X5P}{K_{isom} \cdot K_{ipem}} \]

\[ GAP = \left( \frac{K_{ald} \cdot F16bP}{K_{tim}} \right)^{\frac{1}{2}} \]

\[ X5P = \left( \frac{K_{epim} \cdot K_{isom} \cdot K_{tket1} \cdot K_{tket2} \cdot (F6P)^2 \cdot GAP}{K_{ald}} \right)^{\frac{1}{3}} \]
Appendix 5.2 Relation between amino acid levels and their precursor central metabolites in *Saccharomyces cerevisiae* (Mashego et al., 2005)

In the work of Mashego et al. (2005) a chemostat culture of (aerobic, glucose limited, D = 0.05 h⁻¹, 30°C, pH: 5.0) of *S. cerevisiae* was monitored over nearly 1000 hours for intracellular metabolites. Also the free intracellular aminoacids were analyzed recently in these samples. This data set was used to obtain the relation between concentration precursor metabolite and free intracellular metabolite amino acid.

**Figure 5.A2.1** Relation of amino acids from serine family with 2&3PG, their precursors in primary metabolism. Solid lines are calculated using estimated elasticities in equation 5.1. The value of slopes are listed in Table 5.8.

![Graph](image_url)
Figure 5.A2. 2 Relation of amino acids from aromatic family with PEP, their precursors in primary metabolism. Solid lines are amino acid calculated using estimated elasticities in equation 5.1. The value of slopes are listed in Table 5.8.

Figure 5.A2. 3 Relation of amino acids from pyruvate family with pyruvate, their precursors in primary metabolism. Solid lines are amino acid calculated using estimated elasticities in equation 5.1. The value of slopes are listed in Table 5.8.
Figure 5.A2. 4 Relation of amino acids from glutamate family with αKG, their precursors in primary metabolism. Solid lines are amino acid calculated using estimated elasticities in equation 5.1. The value of slopes are listed in Table 5.8.

Figure 5.A2. 5 Relation of amino acids from aspartate family with malate, their precursors in primary metabolism. Solid lines are amino acid calculated using estimated elasticities in equation 5.1. The value of slopes are listed in Table 5.8.
Appendix 5.3 Kinetic model of amino acid pathways

\[ X_{pm} \xrightarrow{\text{enzyme } e_1} X_{AA} \]

\( X_{pm} = \) precursor concentration from primary metabolism
\( X_{AA} = \) free amino acid concentration
\( e_1 = \) enzyme concentration of first committed step

\[
\frac{J}{J^0} = \frac{e_1}{e_0} \left( 1 + \xi_{pm}^o \ln \left( \frac{x_{pm}^o}{x_{pm}} \right) + \xi_{AA}^o \ln \left( \frac{x_{AA}^o}{x_{AA}} \right) \right)
\]

The amino acid pathway is assumed to have a first enzyme (\( e_1 \)), called committed step, which is positively activated by the precursor of primary metabolism (\( x_{pm} \)) and is subject to end product feedback inhibition by the amino acid concentration (\( x_{AA} \)). The flux through the pathway can be expressed relative to the reference flux as a function of the precursor and end-product concentrations using the linlog approximative kinetic format (Visser, Heijnen, 2003; Wu et al. 2004). See equation above. Herein \( J^0, e_0^o, x_{pm}^o \) and \( x_{AA}^o \) refer to the flux, enzyme level, precursor concentration and amino acid concentration for the reference condition. \( \xi_{pm} \) is the elasticity of precursor concentration to the flux of the enzyme \( e_1 \), which for Michaelis Menten reaction is a positive number with a value between 0 – 1, because it is often assumed that metabolite levels are close to the \( K_m \) value of the associate enzyme, \( \xi_{pm} \approx 0.5 \). \( \xi_{AA} \) is the elasticity which reflects the inhibitory kinetic effect of the amino acid level on the reaction rate (end product feedback inhibition). Therefore this elasticity has a negative value, between -4 and 0, because allosteric enzyme usually contains up to 4 regulation subunits. For the four different chemostat cultivations carried out in this study, all amino acid biosynthesis rates, except the ones of cysteine and valine fluxes are assumed identical (because \( \mu \) is the same), hence \( J/J^0 = 1 \). Also it is assumed that the level of the first enzyme (committed step) is the same in the four cultivations (\( e/e_0^o = 1 \)). An argument in favour of this simplifying assumption is the fact that the enzyme levels of anabolic pathways are usually determined by the specific growth rate \( \mu \), which was the same for the four chemostats. Furthermore it was shown recently (Mashego et al. 2005) for prolonged chemostat cultivation of Saccharomyces cerevisiae that a constant biomass specific glycolytic flux coincided with constant enzyme
activities of the rate determining steps (HK, PFK and PYK), while the glycolytic metabolite levels changed many fold. With these assumptions $\frac{J}{J^0}$ and $\frac{e}{e^0}$ of the lin-log rate equations are set equal to one and the following relation is obtained:

$$\ln \frac{x_{AA}}{x_{AA}^0} = \left( \frac{\varepsilon_{pm}^o}{\varepsilon_{AA}^o} - \frac{\varepsilon_{pm}^o}{\varepsilon_{AA}^o} \right) \ln \frac{x_{pm}}{x_{pm}^0},$$

which shows a linear relation in logarithmic coordinates between the concentration of free amino acid and its precursor. The slope equals to the ratio of elasticities.
Chapter 6

Conclusions and Future Directions

Conclusions

Some conclusions can be drawn from this study including technical observations in sample extraction and measurement, about analysis of dynamic and steady state metabolome study in central metabolism and its relation to penicillin biosynthesis.

On the development of sample extraction, we found that a matrix effect gives different recovery factors not only for different microorganism but also for different metabolites.

In steady state, mass action ratios for the reactions that are assumed operating near equilibrium are close to its $K_{eq}$ value, such as phosphoglucomutase, phosphoglucoisomerase and fumarase. This indicates they, indeed, operate near equilibrium. The results are also confirmed for dynamic conditions as well as for *P. chrysogenum* cultivated in different conditions, which are growth in ethanol as sole carbon source and the absence of penicillin production.

A significant difference in metabolite levels between *P. chrysogenum* and *S. cerevisiae* are observed mainly for the ones that are related to penicillin biosynthesis, pyruvate and TCA cycle intermediates. Pyruvate and TCA cycle intermediates relate to valine synthesis and high energy demand, respectively.

In dynamic experiments, when glucose concentration was increased abruptly, magnitude change of metabolite concentration depends on the change of flux, which is caused by increasing glucose uptake rate.

Detailed observation on the increasing of C4 pool (succinate, malate and fumarate) shows that their increase due to the increasing reduction status of cytosolic NADH/NAD. The change NADH/NAD during pulse is calculated using two different reactions, and there is a good agreement which confirms the proposed NADH/NAD changes after glucose pulse.
Dynamics of free amino acid concentrations during the glucose pulse experiment can be recognized mainly for the ones with small turnover times. Exceptions, such as aspartate, are observed for the ones that are involved in fast equilibrium reactions, such as transaminase.

Further analysis of the glucose pulse based on mass balance calculation shows that 30% of glucose consumed goes to an unknown sink. And formation of this compound requires very little energy; the most probable candidate is increasing storage material. This is also supported by monotonously increase of T6P, which is the precursor of storage compounds, such as trehalose.

Increasing of three amino acid precursors of penicillin synthesis suggest a decrease of penicillin flux during the pulse. This decrease is also supported by the fact of a drop in ATP level and energy charge.

This study suggests that perturbation in glucose concentration causes a strong metabolic response where energy costly pathways, such as penicillin synthesis, are decreased to favor low energy costly favorable pathway (storage).

In the experiment with flux manipulation both in primary and secondary metabolism, a positive relation is observed between primary metabolite concentration and carbon flux in primary metabolism. Positive relations are also observed in many cases between free amino acid concentrations and their direct precursor in primary metabolism.

In penicillin production pathway, the flux is not stimulated by αAA and valine but is stimulated by cysteine, and ATP level. This leads to the final conclusions that the penicillin production flux appears to be mostly influenced by the availability of energy and redox cofactors.

The absence of penicillin synthesis correlates with high T6P level which confirms the relation of penicillin and storage metabolism.

**Future directions**

All the conclusions that mentioned previously remained some questions to be answered.

In pulse experiment, glucose uptake rate increased two times higher no matter how much glucose that was added in pulse solution. This means that amount of carbon that assumed to go storage carbon cannot be increased. Hence, an accurate measurement of storage carbohydrate is needed to test the hypothesis.

With the same argument for storage carbohydrate, an accurate measurement of metabolites in penicillin metabolism is also needed. The proposed solution for these two
measurements is using MIRACLE technique that has been proved working for intracellular metabolites and nucleotides. In order to get fully 13C labeled intracellular metabolite for penicillin pathway, *Penicillin chrysogenum culture grown in fully 13C glucose labeled* is needed.

*In vivo kinetic interaction between enzymes and metabolites* is also needed to be elucidated. In the study of the relation between precursor metabolite and free amino acid concentrations to the flux toward amino acid, it was assumed only these concentration that played a role. Integrating other effectors, such as ATP, NADPH, to the pathway, by adding other elasticity parameters, could lead to different conclusion of identified elaticities from only two effectors (metabolite and free amino acid, Chapter 5).

The whole set of pulse data will be used for dynamic modeling using lin-log kinetic format as shown by Kresnowati et al. (2005).
Summary
Of the thesis:

'A Dynamic and Steady State Metabolome Study of Central Metabolism and Its Relation with the Penicillin Biosynthesis Pathway in *Penicillium chrysogenum*’
by Uly Nasution

Understanding of the functioning and disfunctioning of living cells, with the aim to cure diseases, develop new drugs or to improve product formation in industrial microorganisms, requires detailed information on the regulation of metabolism on different levels. A way to obtain this information is by means of stimulus response experiments, whereby the measured response of the metabolic network to a certain disturbance is used to elucidate the properties of the regulatory circuits.

In this thesis stimulus response experiments have been applied to a high producing industrial strain of the penicillin producing fungus *Penicillium chrysogenum* with the aim to elucidate the kinetic properties of the enzymes of central metabolism, with special focus on the relation between central metabolism and the penicillin biosynthesis pathway.

In these stimulus response experiments the cells were grown under well defined conditions in a chemostat until a steady state was reached. Thereafter the steady state was disturbed by applying a certain stimulus, e.g. by suddenly increasing the extracellular glucose concentration. The metabolic response of the cells to this stimulus can be divided into different time regimes, which correspond with different regulatory mechanisms. The fastest response of the enzyme activity, brought about by changing metabolite concentrations, is through mass action effects and allosteric interaction and occurs on a time scale of seconds. Thereafter, on a time scale of minutes, the regulation of gene expression sets in, which can be observed firstly as changes in transcript levels. Later on, after tenths of minutes, this results in changes of enzyme concentrations. In this work the time frame of observation has been a few hundreds of seconds and therefore no significant changes in enzyme levels are expected to have occurred.

Before such pulse response studies can be carried out a suitable protocol had to be developed to carry out well defined steady state chemostat cultivations with this filamentous fungus. Chapter 2 of this thesis describes the development of a dedicated chemostat system.
with sufficiently fast mixing and a sufficiently high biomass concentration to ensure measurability of all relevant intracellular metabolite concentrations. Furthermore adapted rapid sampling and rapid quenching methods have been developed based on an available protocol for rapid sampling and quenching of the yeast *Saccharomyces cerevisiae*. Adaptations were mainly required because of the filamentous morphology of *P. chrysogenum*. It was also shown that application of the MIRACLE technique (the application of uniformly $^{13}$C labeled metabolites as internal standards, which are added prior to metabolite extraction and analysis) to *P. chrysogenum* increased the measurement precision between 3 to 7 fold for all measured metabolites in central metabolism.

The successful development of these experimental techniques allowed to carry out the first pulse response studies with *P. chrysogenum* of which the results are presented in Chapter 3. In these experiments dynamic metabolome data were generated by increasing the extracellular glucose concentration in the chemostat in less than 2 seconds. Rapid sampling was performed during a time frame of 300 seconds. As alternative to perturbation of the steady state chemostat a second method was applied, whereby the glucose perturbation experiment was carried out in the BioScope plug flow minireactor. It was found that the results of similar perturbation experiments carried out using these two methods were well comparable. It was observed that in these glucose pulse experiments the specific glucose consumption rate increased two fold compared with the steady state. This sudden increase of the glucose consumption rate, and thus of the glycolytic flux, resulted in a highly dynamic response of the intracellular concentrations of the glycolytic and TCA cycle metabolites. Metabolic reactions operating close to equilibrium were identified from the observation that the calculated mass actions ratio’s during the dynamic response remained close to the equilibrium constant. With respect to the glycolysis these were e.g. phosphoglucone isomerase, the reactions between F16bP and the pool of 2PG and 3PG and for the TCA-cycle the reactions between fumarate and oxaloacetate. It was also found that, as response to the glucose pulse, the combined pool of the C4 compounds aspartate, fumarate and malate became much more reduced, probably due to an increase of the cytosolic NADH/NAD ratio.

A further analysis of the dynamic response to the glucose pulse is described in Chapter 4, and was carried out by calculation of the dynamic mass balances, using additional data of intracellular free amino acid and specific oxygen consumption rate ($q_{o2}$) and carbon dioxide evolution rate ($q_{CO2}$). From the measured time patterns of the concentrations of the intermediates of central metabolism and adenine nucleotides, as well as from the specific rates of oxygen consumption and carbon dioxide production it was observed that the highly
dynamic response only lasted for the first 200 seconds, after which a pseudo steady state condition was established. With respect to the time profiles of the concentrations of the free intracellular amino acids it was observed that only amino acids of which the intracellular pool has a relatively small turnover time, the concentration patterns follow the patterns of their direct precursors from central metabolism.

Considering all main sources and sinks of carbon, electrons, and ATP allowed the construction of the carbon, electron and ATP balances. These calculations showed that approx. 30% of the consumed glucose during the pulse experiment must have been converted into unknown compounds. However, from the relatively small increases of the oxygen consumption and carbon dioxide production rates it was inferred that apparently not much energy (in the form of ATP) is required for this conversion. Therefore it was assumed that this part of the consumed glucose was converted into storage carbohydrates, which was also indicated by a continuous increase of the trehalose 6 phosphate (T6P) concentration during the glucose pulse experiment. However, it was calculated that this could not completely explain the observations as there was still a deficit of energy during the pseudo steady state which was achieved after approx. 200 seconds. The only way to close the energy balance was to assume that the specific rate of penicillin production had decreased during the pseudo steady state. An indication for this might be the measured increase of the levels of the three precursor amino acids of penicillin (alpha amino adipic acid (αAAA), valine and cysteine).

The relation between central metabolism and the penicillin biosynthesis pathway was further analyzed by steady state perturbations of both the fluxes through central metabolism and the penicillin pathway, which is described in Chapter 5. As the reference condition, cultivation of the high producing strain in a glucose limited chemostat at a dilution rate of 0.05 h⁻¹ was chosen, under which the penicillin production rate is close to its maximum. Perturbation of the fluxes through central metabolism was achieved by carbon limited chemostat cultivation of the high producing strain on ethanol as the sole carbon source under otherwise the same conditions as the reference culture. Perturbation of penicillin production was achieved in two different ways; 1) by cultivating the high producing strain under the same conditions as the reference culture but without the side chain precursor phenylacetic acid (PAA) and 2) by deletion of all gene clusters coding for the enzymes of the penicillin biosynthesis pathway. From this study it was found that a positive relation exists between the concentrations of the central metabolites and the carbon flux through primary metabolism. Furthermore, the steady state free amino acid concentrations seem to be directly related to the concentrations of their precursors in central metabolism, which is in agreement with end
product feedback inhibition of the amino acid biosynthesis pathways. In addition it was found that the flux through the penicillin production pathway seems not significantly influenced by the concentrations of the precursor amino acids, αAAA and valine, but mostly by the ATP and cysteine concentrations, where the cysteine level is probably related to the availability of NADPH. This led to the conclusion that the penicillin production flux in the studied industrial strain of *P. chrysogenum* appears to be mostly influenced by the availability of energy and redox cofactors, which are determined by the fluxes through central metabolism.

The research project described in this thesis represents a first attempt to develop and apply experimental methods for the quantitative analysis of the in-vivo kinetic properties of the metabolic network of the antibiotic producing fungus *Penicillium chrysogenum*. Such research is indispensable for the rational development of fermentation processes for the entire biological production of bulk and fine chemicals as well as pharmaceuticals from the renewable carbon source sugar as main feedstock.
Samenvatting
Van het proefschrift:

'A Dynamic and Steady State Metabolome Study of Central Metabolism and Its Relation with the Penicillin Biosynthesis Pathway in *Penicillium chrysogenum’

door Uly Nasution

Het verkrijgen van een beter begrip van het functioneren en disfunctioneren van levende cellen op diverse regulatie niveaus is op velerlei gebied van belang, op medisch en farmaceutisch gebied bijvoorbeeld voor het genezen van ziekten en het ontwikkelen van nieuwe medicijnen en in de biotechnologie voor het verhogen van productvorming in industriële micro-organismen. Een van de methoden om meer inzicht in de regulatie van het metabole network binnen de levende cel te verkrijgen is middels zogenaamde stimulus respons experimenten. In dergelijke experimenten wordt de stabiele toestand van een metabool netwerk verstoord en wordt vervolgens aan de hand van de optredende respons getracht de eigenschappen van de regulatoire netwerken op te helderen.

In het onderzoek waarvan de resultaten zijn vastgelegd in dit proefschrift, zijn dergelijke stimulus respons experimenten toegepast op een hoog producentende industriële stam van de penicilline producerende schimmel *Penicillium chrysogenum*, met als doel om de in-vivo kinetische eigenschappen van de enzymen van het centrale metabolisme te ontrafelen. Hierbij stond de relatie tussen het centrale metabolisme en de penicilline biosynthese route centraal.

Voorafgaand aan het uitvoeren van dergelijke stimulus respons experimenten werd de schimmel gecultiveerd in een chemostaat cultuur onder constante en goed gedefinieerde condities, totdat een in de tijd onveranderlijke toestand (steady state) werd bereikt. Vervolgens werd deze steady state verstoord door het aanbieden van een stimulus, bijvoorbeeld het plotseling verhogen van de extracellulaire concentratie van het groeilimitierende substraat glucose. Bij de respons van het metabolisme op deze verstoring kan onderscheid gemaakt worden tussen verschillende tijdsregimes, die corresponderen met de verschillende regulatiemechanismen die de cel voorhanden heeft om op de verstoring te reageren. De snelst optredende verandering van de enzym activiteit, die wordt veroorzaakt door veranderingen in intracellulaire metabolite concentraties, is die via massa actie en
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allostere modificatie, dat wil zeggen veranderingen in de concentraties van substraten, effectoren en producten van een enzym leiden vrijwel onmiddellijk, dat wil zeggen binnen (fracties van) seconden tot een verandering van de snelheid van de door het enzym gecatalyseerde reactie. Daarna, binnen een tijdsbestek van minuten, zullen veranderingen in genexpressie optreden, het eerst waarneembaar in veranderingen in transcript concentraties. Dit resulteert vervolgens, na tientallen minuten, in waarneembare veranderingen in enzym concentraties. In de stimulus respons experimenten uitgevoerd in dit onderzoek bedroeg de observatiertijd slechts enkele honderden seconden, zodat aangenomen mocht worden dat er geen significante veranderingen optraden in de enzym concentraties.

Alvorens stimulus respons experimenten konden worden uitgevoerd met *P. chrysogenum* diende eerst een goed bruikbare methode voor chemostaat cultivatie van deze filamenteuze schimmel te worden ontwikkeld. De ontwikkeling hiervan wordt beschreven in hoofdstuk 2 van dit proefschrift. Belangrijke eisen hierbij zijn goede meng eigenschappen en een voldoend hoge biomassa concentratie, nodig voor betrouwbare metingen van de intracellulaire metaboliet concentraties. Verder werden aangepaste technieken voor snelle monsternamie en quenching ontwikkeld op basis van reeds beschikbare methoden voor de gist *Saccharomyces cerevisiae*. De aanpassingen waren met name noodzakelijk vanwege het filamenteuze karakter van *P. chrysogenum*. Verder werd aangetoond dat toepassing van de MIRACLE methode voor metaboliet metingen (waarbij een uniform $^{13}$C gelabeld metaboliet mengsel als interne standard wordt gebruikt, toegevoegd voor extractie en metaboliet analyse) de meetnauwkeurigheid verhoogde met een factor 3 tot 7 voor alle gemeten metabolieten uit het centrale metabolisme.

Na de succesvolle ontwikkeling van bovengenoemde technieken bleek het mogelijk om de eerste stimulus respons experimenten uit te voeren met *P. chrysogenum*, de resultaten hiervan staan beschreven in hoofdstuk 3 van dit proefschrift.

In deze experimenten werden dynamische metaboliet profielen gegenereerd door het verhogen van de extracellulaire glucose concentratie van de chemostaat binnen een tijdsbestek van 2 seconden. Direct na de verstoring werd snelle monsternamie uitgevoerd gedurende een periode van 300 seconden. Als alternatief voor het verstoren van de complete chemostaat cultuur werd een zelfde experiment uitgevoerd in de BioScope, een mini propstroomreactor special ontworpen voor het uitvoeren van stimulus respons experimenten. Uit de resultaten bleek dat de metaboliet profielen van beide experimenten goed vergelijkbaar waren. In beide experimenten werd een verhoging van de specifieke glucose opnamesnelheid waargenomen van ongeveer een factor 2. Deze verhoging van de glucose opnamesnelheid en dus van de de
glycolytische flux leidde tot dynamische veranderingen in de intracellulaire concentraties van de intermediairen van de centrale metabole routes zoals de glycolyse en de citroenzuurcyclus.

Aan de hand van de berekende massa actie ratio’s voor de diverse reacties gedurende de dynamische respons konden reacties die dicht bij het evenwicht opereren worden geïdentificeerd. Voor wat betreft de glycolyse waren dit fosfoglucose isomerase, de reacties tussen F1,6bisP en de gecombineerde pool van 2PG en 3PG en voor de citroenzuurcyclus de reacties tussen fumaraat en oxalaalazijnzuur. Verder werd gevonden dat na het toedienen van de glucose puls de gecombineerde pool van de C4 componenten aspartaat, fumaraat en malaat in een meer gereduceerde toestand kwam, wat mogelijk veroorzaakt werd door het stijgen van de NADH/NAD ratio in het cytosol als gevolg van de verhoogde glycolytische flux.

In hoofdstuk 4 wordt een verdere analyse beschreven van de dynamische respons van het metabolisme welke werd uitgevoerd aan de hand van de dynamische massa balansen en additionele gegevens omtrent de intracellulaire concentraties aan vrije aminozuren en de specifieke zuurstof consumptie en kooldioxide productiesnelheden.

Aan de hand van de gemeten tijdprofielen was duidelijk af te leiden dat het dynamische gedeelte optreedt gedurende de eerste 200 seconden na het toedienen van de verstoring (de glucose puls) waarna een pseudo steady state toestand optrad. Verder kon uit het dynamische gedrag van de metabolieten gedurende de eerste 200 seconden worden afgeleid dat de concentraties van de intracellulaire aminozuren waarvan de pool een kleine turnover tijd heeft gerelateerd blijken aan het concentratieverloop van de precursors uit het centrale metabolisme.

Aan de hand van de dynamische koolstofbalans kon worden berekend, met daarbij de aanname dat de groeisnelheid van de cellen niet verandert, dat ongeveer 30% van de geconsumeerde hoeveelheid glucose tijdens het stimulus respons experiment niet kon worden teruggevonden en blijkbaar wordt omgezet in onbekende koolstof bevatende producten. Een opmerkelijke observatie was tevens dat terwijl de specifieke glucose opnamesnelheid met een factor 2 toenam tijdens het experiment, dit bij lange na niet correspondeerde met een evenredige toename van de specifieke zuurstof consumptie en kooldioxide productie snelheden. Hieruit kan worden afgeleid dat voor het omzetten van deze extra hoeveelheid glucose blijkbaar Weinig energie, in de vorm van ATP, nodig is. Er werd daarom aangenomen dat het onbekende product, verantwoordelijk voor 30% van de glucose consumptie, waarschijnlijk reservekoolhydraten betreft. Een indicatie hiervoor is tevens de stijging van de intracellulaire concentratie van trehalose 6 fosfaat (T6P) tijdens het puls experiment. Uit het opstellen van de ATP balans tijdens de pseudo steady state bleek echter dat met bovenstaande
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aanname er meer ATP consumptie zou optreden dan ATP productie. Alleen door aan te nemen
dat de glucose puls leid tot een daling van de penicilline productie kon een sluitende ATP
balans worden verkregen. Een mogelijke indicatie voor een daling van de penicilline
productiesnelheid was de geobserveerde stijging van de precursor aminozuren alfa
aminoadipinezuur (αAAA), valine en cysteine.

Een verdere analyse van de relatie tussen het centrale metabolisme en de penicilline
biosynthese route werd uitgevoerd door een aantal steady state perturbaties van de fluxen
door de betreffende routes uit te voeren, dit wordt beschreven in hoofdstuk 5.

De referentie conditie voor deze experimenten was glucose gelimiteerde chemostaat
cultivatie van de hoog producerende industriële stam bij een verdunningssnelheid van 0.05 h\(^{-1}\). Onder deze omstandigheden wordt de, voor deze stam, vrijwel maximale penicilline
productiesnelheid verkregen. Verandering van de flux door het centrale metabolisme werd
verkregen door de koolstofbron glucose te vervangen door ethanol, onder overigens dezelfde
cultivatie condities als in geval van de referentie cultuur.

Verandering van de penicilline productie snelheid werd op 2 verschillende manieren
bewerkstelligd; 1) door cultivatie in afwezigheid van de penicilline-G precursor
phenylazijnzuur (PAA), onder overigens dezelfde condities als voor de referentie cultuur, en
2) door constructie van een niet producerende stam, op basis van de hoogproducerende stam,
door verwijderen van alle genclusters die coderen voor de enzymen van de penicilline
biosynthese route.

De meest belangrijke bevindingen die uit deze studie naar voren kwamen waren, voor wat
betreft het centrale metabolisme dat een positieve relatie werd gevonden tussen de
concentraties van de metabolieten uit het centrale metabolisme en de metabole flux. Verder
bleek er een directe relatie te bestaan tussen de concentraties van de vrije aminozuren en hun
precursors uit het centrale metabolisme, wat overeenkomt met feed back inhibitie van de
aminozuur biosyntheseroutes door het eindproduct. Tevens werd gevonden dat de flux door
de penicilline biosynthese route nauwelijks werd beïnvloed door de concentraties van de
precursors αAAA en valine. De ATP en cysteine concentraties hadden echter wel een effect
op de flux waarbij het effect van de cysteine concentratie direct herleid kan worden tot een
voldoende beschikbaarheid van reducerend vermogen in de vorm van NADPH. Hieruit kan de
conclusie worden getrokken dat in de onderzochte industriële stam van *P. chrysogenum* de
snelheid van de penicilline productie voornamelijk wordt beïnvloed door de beschikbaarheid
van energie, in de vorm van ATP, en reducerend vermogen, voornamelijk in de vorm van
NADPH.
Het in dit proefschrift beschreven onderzoek geeft een eerste aanzet, middels de ontwikkeling en toepassing van specifieke experimentele methoden, voor quantitatief onderzoek aan de in-vivo kinetische eigenschappen van het metabole network van de antibiotica producerende industriële schimmel *Penicillium chrysogenum*. Dergelijk onderzoek is onontbeerlijk voor de racionele ontwikkeling van productieprocessen voor volledige biologische productie, met suiker als belangrijkste grondstof, van zowel bulk als fijnchemicalien en farmaceutica.
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List of Publications

Journal Articles/ Proceeding


Oral Presentation


Poster Presentations


Curriculum Vitae

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After graduation, she stayed for one other year in the same group doing research for a project funded by Ministry of Culture and Education, Republic of Indonesia. In March 2000, she went to Belgium to pursue a master by research program in Catholique Universite de Louvain (UCL) - Belgium with a scholarship from Belgian Federal Government. She obtained a Diplome d’Etude Approfondie (DEA) degree from Department of Applied Mathematics, Catholique Universite de Louvain in September 2001.

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