Carbon Metabolism Limits Recombinant Protein Production in *Pichia pastoris*

Jan Heyland, Jianan Fu, Lars M. Blank, Andreas Schmid

1Department of Biochemical and Chemical Engineering, Laboratory of Chemical Biotechnology, TU Dortmund University, Emil-Figge-Str. 66, D-44227 Dortmund, Germany; telephone: +49-231-755-7383; fax: +49-231-755-7382; e-mail: lars.blank@bci.tu-dortmund.de

2Leibniz-Institut für Analytische Wissenschaften – ISAS – e.V., Dortmund, Germany

Received 1 November 2010; revision received 20 January 2011; accepted 14 February 2011

Published online 23 February 2011 in Wiley Online Library (wileyonlinelibrary.com). DOI 10.1002/bit.23114

**ABSTRACT:** The yeast *Pichia pastoris* enables efficient (high titer) recombinant protein production. As the molecular tools required are well established and gene specific optimizations of transcription and translation are becoming available, metabolism moves into focus as possible limiting factor of recombinant protein production in *P. pastoris*. To investigate the impact of recombinant protein production on metabolism systematically, we constructed strains that produced the model protein β-aminopeptidase BapA of *Sphingosinicella xenopeptidilytica* at different production yields. The impact of low to high BapA production on cell physiology was quantified. The data suggest that *P. pastoris* compensates for the additional resources required for recombinant protein synthesis by reducing by-product formation and by increasing energy generation via the TCA cycle. Notably, the activity of the TCA cycle was constant with a rate of $2.1 \pm 0.1 \text{ mmol g}^{-1}\text{CDW h}^{-1}$ irrespective of significantly reduced growth rates in high BapA producing strains, suggesting an upper limit of TCA cycle activity. The reduced growth rate could partially be restored by providing all 20 proteinogenic amino acids in the fermentation medium. Under these conditions, the rate of BapA synthesis increased twofold. The successful supplementation of the growth medium by amino acids to unburden cellular metabolism during recombinant protein production suggests that the metabolic network is a valid target for future optimization of protein production by *P. pastoris*. Biotechnol. Bioeng. 2011;108: 1942–1953. © 2011 Wiley Periodicals, Inc.

**KEYWORDS:** amino acid biosynthetic cost; Crabtree; metabolic network analysis; $^{13}$C flux analysis; yeast; off-gas analysis

**Introduction**

The yeast *Pichia pastoris* has been developed into an efficient expression system for the production of recombinant proteins (Cregg et al., 2000; Macauley-Patrick et al., 2005). At the end of 2007, *P. pastoris* has been used as expression host for more than 600 proteins (Zhang et al., 2009). This development is based on the available genetic toolbox including strong promoters like the methanol inducible promoter of the alcohol oxidase I AOX1 (Cregg et al., 1993), its eukaryotic protein synthesis pathways, disulfide bond formation and proteolytic processing (Cregg et al., 2000), and finally its extraordinary ability to be cultured to very high cell densities (Cereghino et al., 2002). As the AOX1 promoter is strongly induced in the presence of methanol, protein production on methanol or glycerol/methanol mixtures are the methods of choice (Macauley-Patrick et al., 2005; Zhang et al., 2009). Several papers, however, report drawbacks of methanol for protein production in an industrial setting, including considerable methanol induced cell lysis and proteolysis and considerable safety requirements (Macauley-Patrick et al., 2005; Mattanovich et al., 2009; Menendez et al., 2004; Zhang et al., 2007, 2009). Alternatively, the constitutive glyceroldehyde-3-phosphate dehydrogenase (GAP) promoter or AOX1 derivative promoters (Hartner et al., 2008) enable efficient protein production on the industrially preferred carbon sources glycerol or glucose (Heyland et al., 2010b; Waterham et al., 1997). It was reported that the expression strength of the GAP promoter is 30% lower on glycerol when compared to expression during growth on glucose (Waterham et al., 1997).

To optimize the performance of protein production by *P. pastoris* beyond state-of-the-art, insights into cellular metabolism during heterologous gene expression are required (Graf et al., 2009). Especially the effect of bioprocess parameters such as temperature, oxygen supply, substrate choice, growth rate, and gene expression on metabolism and its subsequent interplay with protein synthesis is here of interest (Mattanovich et al., 2004). Notably, only few physiological studies, mainly on methanol and/or glycerol, exist that elucidate the impact of recombinant protein...
production on metabolic network operation of this yeast (Baumnann et al., 2010; Celik et al., 2010; Dragoni et al., 2009; Gasser et al., 2007; Sola et al., 2004, 2007).

In a previous report, Dragoni et al. (2009) identified a correlation of recombinant protein production with an increased flux through the TCA cycle, which these authors attributed to an increased energy demand for recombinant protein during growth in a glucose limited chemostat. In general, the extra energy needed for recombinant protein production is summarized as metabolic burden (Glick, 1995). This metabolic burden might manifest itself in form of reduced growth and protein production rates (Glick, 1995; Mattanovich et al., 2004; Ramon et al., 2007). Notably, high transcription levels, which can limit precursor availability and cause energy depletion in Escherichia coli (Sanden et al., 2003) are assumed not to significantly increase the metabolic burden as the rate of recombinant gene expression in P. pastoris is moderate (Mattanovich et al., 2004). Rather it is speculated that the metabolic burden in yeasts is caused by posttranslational processes (folding and secretion) and/or protein degradation of misfolded or unfolded proteins in the endoplasmic reticulum (Mattanovich et al., 2004).

Recently, we have shown the impact of recombinant protein production on the metabolism of a high yielding P. pastoris strain using glucose as carbon and energy source in high cell-density fed-batch fermentations (Heyland et al., 2010b). As model protein we chose the bacterial β-aminopeptidase 3-2W4 BapA of Sphingosinicella xenopeptidilytica. This enzyme belongs to a new class of β-peptidases that has been recently described by Gueuke et al. (2005). These β-peptidases are unique as they catalyze degradation and synthesis of a wide variety of β-peptides (Heck et al., 2006, 2007; Heyland et al., 2010a).

In the present study, we compared the impact of protein production on metabolism by using a series of differentially BapA producing P. pastoris strains. Changes in by-product formation and intracellular flux distribution due to recombinant protein synthesis were quantified in 13C-labeling experiments during batch cultivations. The metabolic flux model was equipped with the recently published biomass composition of P. pastoris (Carnicer et al., 2009). The data suggest that P. pastoris compensates for the additional resources required for recombinant protein synthesis by reducing by-product formation and increasing energy generation via the TCA cycle. Amino acid addition to compensate the burden on cellular metabolism suggests that operation of the cellular network is one aspect that limits recombinant protein production by P. pastoris.

Materials and Methods

Strains and Growth Conditions

The haploid P. pastoris strain SMD1168H (Invitrogen, Carlsbad, NM) was used throughout this study. The SMD1168H-based recombinant protein producing P. pastoris strains produced the β-peptidyl aminopeptidase (3-2W4 BapA) from S. xenopeptidilytica (Gueuke et al., 2005). To increase recombinant protein synthesis, the DNA sequence of 3-2W4 bapA the GC composition and the codon usage were adapted to P. pastoris as described previously (Heyland et al., 2010b).

The recombinant strain contained the vector-gene construct pG3BapA, which harbored the GAP promoter (Waterham et al., 1997) for gene expression in P. pastoris. Electroporation was used for transformation of P. pastoris (Lin-Cereghino et al., 2005). Before transformation, the plasmid was linearized to facilitate genome integration (Cregg, 2007). Transformants with different BapA production performance were selected as described previously (Heyland et al., 2010b). To identify BapA expressing transformants with different expression levels, 10 independent isolates from colonies of agar plates with different antibiotic concentrations were tested for their specific in vitro BapA activity. According to their activity (low, medium, and high) a set of three strains (BapA low, BapA med, and BapA high) was selected.

Batch cultures of P. pastoris were carried out in 1,400 mL sealed Erlemeyer flasks (100 mL of growth medium) equipped with off-gas sensors in a rotary shaker at 30 °C and 200 rpm. The conditions were shown to ensure aerobic growth (Heyland et al., 2009). In addition, parallel experiments were carried out with open flasks, resulting in highly similar results. For shake flask experiments either complex medium (yeast extract, peptone, dextrose [YPD]) or minimal medium (Verduyn et al., 1992) was used. To avoid pH shifts due to ammonia uptake and acetate production, the medium was supplemented with 50 mM potassium hydrogen phthalate. The reference pH value was set to 5.0. Growth medium for investigation of amino acid uptake was either YPD or Verduyn medium supplemented with all 20 proteinogenic amino acid or as indicated in the text and figures at concentrations reported by Sherman (2002).

Analytical Procedures

Cell growth was monitored by measuring the optical density (OD) of cultures at 600 nm, while an OD value of 1 corresponds to 0.221 gCDW L−1. Glucose, acetate, and ethanol were analyzed by isocratic UV-RI-HPLC, CO2, and ethanol were measured online in shake flasks experiments with off-gas sensors as reported previously (Heyland et al., 2009). Gas chromatography and mass spectrometry (GC–MS) analysis were used for the separation of the 15 proteinogenic amino acids present in protein hydrolysates and for the identification of the 13C-labeling pattern, respectively, as described previously (Blank et al., 2005a; Heyland et al., 2009).

The activity of 3-2W4 BapA was assayed from cell crude extract after sonication by following the hydrolysis of
β-alanine-para-nitroanilide (H-β-Ala-pNA) in a spectrophotometer (Geueke et al., 2006). One unit is defined as the amount of enzyme that catalyzes the formation of 1 μmol pNA min$^{-1}$ ($c = 10,400$ M$^{-1}$ cm$^{-1}$). The specific activity of pure BapA under these conditions is 2.1 U mg$^{-1}$ (Geueke et al., 2006).

**Determination of Extracellular Fluxes**

Substrate uptake, product secretion, and ethanol/CO$_2$ production rates of *P. pastoris* were determined during exponential growth phase as reported previously (Heyland et al., 2010b). The growth model consisted of five equations including acetate, ethanol, CO$_2$, glucose, and biomass synthesis/uptake as described previously (Heyland et al., 2009). Data consistency was investigated using a simple black box model of yeast having a respiro-fermentative metabolism (Stephanopoulos et al., 1998).

**13C-Labeling Experiments**

13C-Tracer experiments were performed under pseudo-steady-state (exponential growth) conditions. The glucose used in shake flasks experiments was a mixture of 20% (n/n) uniformly labeled [U-13C]-glucose (EURISO-TOP, Gif sur Yvette, France) and 80% (n/n) naturally labeled glucose. Detection of labeling patterns of the amino acids was performed by GC-MS analysis (Blank et al., 2005a).

**13C-Constrained Metabolic Flux Analysis**

The stoichiometric model for 13C-constrained metabolic flux analysis comprises the major pathways of yeast central carbon metabolism (Blank et al., 2005a). This model contains 32 unknown fluxes and 30 metabolites. To calculate intracellular fluxes, the stoichiometric model was constrained with four extracellular flux parameters (growth rate, formation rates of ethanol and acetate, and the glucose uptake rate) and seven intracellular flux ratios (fraction of mitochondrial oxaloacetate derived through anaplerosis, fraction of phosphoenolpyruvate originating from cytosolic oxaloacetate, fraction of serine derived through glycolysis (implemented as 1—PEP derived through the pentose phosphate pathway), fraction of mitochondrial pyruvate from malate (upper and lower bound), fraction of serine from glycine, and fraction of glycine from serine). The biomass composition of *P. pastoris* was recently published (Carnicer et al., 2009). Error minimization for the flux calculations in the determined network was carried out as described by Fischer et al. (2004). Measured CO$_2$ concentrations were not added as constraint. The measured CO$_2$ formation rates were about 95 ± 3% of the calculated rates, indicating that the flux calculations and the experimental data were highly consistent under the chosen experimental conditions. The rate of NADPH regeneration was derived from the determined fluxes. Explicitly, NADPH was not used as a constraint, since enzyme specificities for redox cofactors, especially of the acetaldehyde dehydrogenase isozymes and the isocitrate dehydrogenase, are not reported for *P. pastoris*.

**Determination of De Novo Amino Acid Synthesis**

Intracellular de novo amino acid synthesis was determined for the amino acids alanine, aspartate, glutamate, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine proline, serine, threonine, tyrosine, and valine. The percentages of de novo synthesized amino acids correspond to the 13C-labeling in the amino acids derived from 13C-labeled glucose. The unlabeled fraction corresponds to the amount of unlabeled amino acid, which was taken up from the medium. GC-MS analysis based on proteinogenic amino acids is able to detect 15 of the 20 proteinogenic amino acids. Arginine was omitted because rearrangements during electron impact ionization obscure its fragmentation pattern. Cysteine and tryptophan are oxidatively destroyed during acid hydrolysis, and asparagine and glutamine are deamidated to aspartate and glutamate, respectively (Dauner and Sauer, 2000). The mixtures of asparagine/aspartate and glutamine/glutamate were subsequently referred to as ASX and GLX, respectively. Labeling patterns were analyzed using the software FiatFlux (Zamboni et al., 2005).

**Results**

**Growth and Biomass Formation of Recombinant *P. pastoris***

It is known that recombinant protein production results in a metabolic burden (Glick, 1995). This metabolic burden might manifest itself in the form of reduced growth and limited protein production and might be a combination of the energetic cost for recombinant protein production and increased cell maintenance for non-growth related processes. For *P. pastoris*, alterations in the carbon flux distribution during recombinant protein production have been shown previously (Celik et al., 2010; Dragosit et al., 2009); while the influence of the product yield on the metabolic network has not been investigated. Here, the physiology of three recombinant strains producing the β-aminopeptidase 3-2W4 BapA at different yields was investigated (BapA$_{low}$, BapA$_{med}$ and BapA$_{high}$, Table I) using 13C tracer-based metabolic flux analysis during unrestricted growth on glucose minimal medium (Fig. 1).

The specific growth rate decreased from 0.28 h$^{-1}$ for the reference strain to 0.16 h$^{-1}$ for the highest protein yielding strain BapA$_{high}$ (Table I). Interestingly, the growth rate did not change in accordance with the glucose uptake rate (Fig. 2a) as seen in previous studies using *S. cerevisiae* under
environmental stress conditions (Blank and Sauer, 2004; Heyland et al., 2009). Rather, an increase in the biomass yield was observed with decreasing growth and glucose uptake rates ranging from 0.30 gCDW g\(^{-1}\) glucose for the reference strain to 0.35 gCDW g\(^{-1}\) glucose for the highest protein yielding strain BapA\(_{\text{high}}\). Although \textit{P. pastoris} is classified as Crabtree negative yeast (Porro et al., 2005), our results agree with the literature (Inan and Meagher, 2001; Kern et al., 2007) that \textit{P. pastoris} produces small amounts of ethanol during aerobic batch cultures in the presence of excess glucose. This finding underlines that the Crabtree effect is not a qualitative characteristic (yes/no), but rather a quantitative aspect of metabolism of hemiascomycetes (Blank et al., 2005b).

By-product formation (i.e., ethanol and acetate) was affected by the change in the rate of growth and/or glucose uptake rate. The ethanol production rate dropped from 1.7 ± 0.4 mmol g\(^{-1}\) CDW h\(^{-1}\) for the reference strain to zero for the strain BapA\(_{\text{high}}\). Thus, the increase in biomass yield during recombinant protein production is a result of reduced by-product formation. Notably, the batch experiments reveal the necessity to perform cultivations for protein production under substrate limited growth conditions, that is, using continuous or fed-batch fermentations, thereby minimizing by-product formation and maximizing the yield of biomass up to approximately 0.5 gCDW g\(^{-1}\) glucose (Heyland et al., 2010b; Sola et al., 2004).

To identify if the increased biomass yield is either a direct response to recombinant protein production of \textit{P. pastoris} or to the growth and glucose uptake rate, experiments with the reference strain under environmental stress (pH) causing a decrease in growth and glucose uptake rate irrespective of recombinant protein expression were performed. It is to

---

**Table 1.** Physiological parameters of \textit{P. pastoris} strains during growth in different media.

<table>
<thead>
<tr>
<th>Cultivation condition</th>
<th>(\mu^c (\text{h}^{-1}))</th>
<th>(r_{\text{glucose}}) (mmol g(^{-1}) h(^{-1}))</th>
<th>(r_{\text{acetate}}) (mmol g(^{-1}) h(^{-1}))</th>
<th>(r_{\text{ethanol}}) (mmol g(^{-1}) h(^{-1}))</th>
<th>(^c\text{Yield}^b) (g BapA g(^{-1}) total protein)</th>
<th>(\text{C-balance}) (%)</th>
<th>BapA selectivity (mg BapA mg(^{-1}) protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Verduyn(a)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reference pH 5.0(b)</td>
<td>0.28 ± 0.0</td>
<td>5.0 ± 0.3</td>
<td>1.3 ± 0.2</td>
<td>1.7 ± 0.4</td>
<td>0.30 ± 0.02</td>
<td>99 ± 3</td>
<td>—</td>
</tr>
<tr>
<td>BapA(_{\text{low}})</td>
<td>0.26 ± 0.0</td>
<td>4.5 ± 0.2</td>
<td>1.2 ± 0.3</td>
<td>1.0 ± 0.2</td>
<td>0.32 ± 0.01</td>
<td>100 ± 2</td>
<td>22 ± 2</td>
</tr>
<tr>
<td>BapA(_{\text{med}})</td>
<td>0.22 ± 0.0</td>
<td>3.7 ± 0.2</td>
<td>0.9 ± 0.1</td>
<td>0.2 ± 0.1</td>
<td>0.34 ± 0.01</td>
<td>102 ± 1</td>
<td>27 ± 3</td>
</tr>
<tr>
<td>BapA(_{\text{high}})</td>
<td>0.16 ± 0.0</td>
<td>2.5 ± 0.1</td>
<td>0.3 ± 0.1</td>
<td>n.d.</td>
<td>0.35 ± 0.02</td>
<td>100 ± 3</td>
<td>37 ± 3</td>
</tr>
<tr>
<td>Reference pH 7.5</td>
<td>0.25 ± 0.0</td>
<td>4.4 ± 0.2</td>
<td>1.4 ± 0.3</td>
<td>0.8 ± 0.2</td>
<td>0.32 ± 0.02</td>
<td>99 ± 3</td>
<td>—</td>
</tr>
<tr>
<td>Reference pH 3.5</td>
<td>0.18 ± 0.0</td>
<td>2.9 ± 0.1</td>
<td>0.4 ± 0.1</td>
<td>n.d.</td>
<td>0.34 ± 0.01</td>
<td>100 ± 2</td>
<td>—</td>
</tr>
<tr>
<td>Verduyn(a)(c)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reference pH 5.0</td>
<td>0.30 ± 0.0</td>
<td>4.2 ± 0.3</td>
<td>0.9 ± 0.2</td>
<td>1.4 ± 0.4</td>
<td>0.39 ± 0.02</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>BapA(_{\text{low}})</td>
<td>0.28 ± 0.0</td>
<td>3.8 ± 0.2</td>
<td>0.7 ± 0.3</td>
<td>0.8 ± 0.3</td>
<td>0.40 ± 0.02</td>
<td>—</td>
<td>28 ± 2</td>
</tr>
<tr>
<td>BapA(_{\text{med}})</td>
<td>0.23 ± 0.0</td>
<td>3.0 ± 0.2</td>
<td>0.6 ± 0.1</td>
<td>0.3 ± 0.1</td>
<td>0.42 ± 0.01</td>
<td>—</td>
<td>35 ± 2</td>
</tr>
<tr>
<td>BapA(_{\text{high}})</td>
<td>0.18 ± 0.0</td>
<td>2.2 ± 0.1</td>
<td>0.4 ± 0.1</td>
<td>0.1 ± 0.1</td>
<td>0.44 ± 0.01</td>
<td>—</td>
<td>47 ± 3</td>
</tr>
<tr>
<td>Reference pH 7.5</td>
<td>0.26 ± 0.0</td>
<td>3.6 ± 0.2</td>
<td>1.2 ± 0.3</td>
<td>0.6 ± 0.1</td>
<td>0.40 ± 0.02</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Reference pH 3.5</td>
<td>0.19 ± 0.0</td>
<td>2.5 ± 0.1</td>
<td>0.4 ± 0.1</td>
<td>0.3 ± 0.1</td>
<td>0.42 ± 0.02</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>YPD(d)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reference pH 5.0</td>
<td>0.32 ± 0.0</td>
<td>3.5 ± 0.3</td>
<td>1.0 ± 0.3</td>
<td>1.0 ± 0.1</td>
<td>0.54 ± 0.02</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>BapA(_{\text{low}})</td>
<td>0.30 ± 0.0</td>
<td>3.0 ± 0.2</td>
<td>0.9 ± 0.3</td>
<td>0.9 ± 0.2</td>
<td>0.56 ± 0.02</td>
<td>—</td>
<td>42 ± 3</td>
</tr>
<tr>
<td>BapA(_{\text{med}})</td>
<td>0.27 ± 0.0</td>
<td>2.6 ± 0.2</td>
<td>0.8 ± 0.1</td>
<td>0.8 ± 0.2</td>
<td>0.60 ± 0.02</td>
<td>—</td>
<td>51 ± 4</td>
</tr>
<tr>
<td>BapA(_{\text{high}})</td>
<td>0.24 ± 0.0</td>
<td>2.1 ± 0.1</td>
<td>0.6 ± 0.1</td>
<td>0.5 ± 0.1</td>
<td>0.63 ± 0.01</td>
<td>—</td>
<td>71 ± 6</td>
</tr>
<tr>
<td>Reference pH 7.5</td>
<td>0.29 ± 0.0</td>
<td>2.9 ± 0.2</td>
<td>1.0 ± 0.1</td>
<td>0.7 ± 0.2</td>
<td>0.56 ± 0.02</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Reference pH 3.5</td>
<td>0.24 ± 0.0</td>
<td>2.2 ± 0.1</td>
<td>0.4 ± 0.2</td>
<td>0.6 ± 0.1</td>
<td>0.60 ± 0.01</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

n.d., not detected.

\(^a\)The results represent the average and the standard error of two independent experiments.

\(^b\)The values given are only an indication for the impact of amino acid supplementation and explicitly are not a true yield on glucose as other carbon sources were present in the media.

\(^c\)Standard minimal media.

\(^d\)Results published in a previous study (Heyland et al., 2010b).

\(^e\)Supplemented with amino acids.

\(^f\)Complex media as described in the Materials and Methods Section.
note, that the metabolic response to different environmental stresses (i.e., pH, osmolarity, stress) is highly similar irrespective of the used stress (Blank et al., 2005b). Applying pH values of 7.5 and 3.5 caused a significant decrease in the growth rate to 0.25 and 0.18 h⁻¹, respectively. Decreased formation of ethanol was also observed under these conditions. In contrast, acetate production seems to be less dependent on the growth and/or glucose uptake rate, but might be influenced more by the pH of the medium as suggested recently for S. cerevisiae under the same conditions (Heyland et al., 2009). Importantly, an increase in biomass yield was also observed under these conditions indicating that the biomass yield is most likely a function of the glucose uptake rate and/or the growth rate (Fig. 2b) and not of recombinant protein production. The data suggest that the biomass yield is asymptotically converging to a maximal value of about 0.35–0.36 gCDW g⁻¹ glucose at zero growth under the here tested conditions.

NADPH is used as an electron donor during biomass synthesis and is derived mainly from the pentose phosphate pathway (PPP) (glucose-6-P-dehydrogenase and 6-P-gluconate dehydrogenase) and the cytosolic NADP⁺ dependent acetaldehyde dehydrogenase. Indeed, a correlation between PPP flux and biomass yield was observed (Fig. 2c), as reported earlier for 14 hemiascomycetous yeast (Blank et al., 2005b). The NADPH formation in the PPP was likely driven by the demand from biomass synthesis, as a good correlation between PPP and biomass yield exists (Fig. 2c).
Our assumption that the glucose uptake rate (resulting in different biomass yields) rather than protein production causes the response of the PPP activity was supported by the low correlation between protein production and PPP activity (Fig. 2d).

The data highlight that recombinant protein production has a substantial impact on cell fitness and physiology. Thus, the question arises how recombinant protein production interferes with cellular metabolism. Since recombinant protein synthesis increases the demand for energy, one can assume that the operation of the TCA cycle is important for the additional task, which will be discussed next.

Impact of Protein Production on Energy Metabolism

Is protein production limited by precursor delivery, that is, by the operation of central carbon metabolism? The specific growth rate decreased with increasing BapA selectivity (or yield on total protein) from 0.28 h⁻¹ for the reference strain to 0.26 h⁻¹ for BapAlow (22 mgBapA g<sub>CDW</sub>⁻¹), 0.23 h⁻¹ for BapAmed (27 mgBapA g<sub>CDW</sub>⁻¹) to 0.16 h⁻¹ for BapAhigh (37 mgBapA g<sub>CDW</sub>⁻¹) (Table I, data using Verduyn). The amounts correspond to yields of product on total protein) (Table I, data using Verduyn). The amounts correspond to yields of product on total protein. The amounts correspond to yields of product on total protein. The amounts correspond to yields of product on total protein. The amounts correspond to yields of product on total protein. The amounts correspond to yields of product on total protein.

The production yields were increasing, the production rate was constant with 3.0 ± 0.1 mgBapA g<sub>CDW</sub>⁻¹ h⁻¹ for all strains tested, due to the decrease in the growth rate. Although, a constant product formation rate was determined, BapAhigh would be preferred for establishing a production process, which is underlined by the product yield on glucose, rising from 0.6 mgBapA mmol<sub>glucose</sub>⁻¹ for BapAlow and 0.8 mgBapA mmol<sub>glucose</sub>⁻¹ for BapAmed to finally 1.2 mgBapA mmol<sub>glucose</sub>⁻¹ for BapAhigh. Moreover, the use of a glucose limited cultivation system (e.g., fed-batch) would eliminate the influence of the growth rate and would allow for high productivities using the strain BapAhigh as shown in a previous study (Heyland et al., 2010b). Consequently, using BapAhigh allows recombinant protein production at low substrate cost.

Because the synthesis of proteins is one of the most energy consuming processes in the cell, one might assume that recombinant protein synthesis influences the cellular energy metabolism and causes a metabolic burden (Glick, 1995). In the present study, investigation of central carbon metabolism indeed identified the TCA cycle as the pathway with the highest response to recombinant protein production (Fig. 2d). As a consequence of the increased TCA cycle activity, an increase in the respiration rate occurred, being with 285% of the glucose uptake rate highest in strain BapAhigh. Normalized respiration rates were 181% for the reference strain, 206% for strain BapAlow and 231% for strain BapAmed. These data suggest a redirection of metabolic fluxes towards NADH regeneration and ATP generation. Also other reactions like the acetyl-CoA synthase Acs1p and Acs2p, which convert acetate to acetyl-CoA, most likely increased AS indicated in our experiments by decreased production rates of acetate. The flux through this reaction was shown in previous experiments (Dragosits et al., 2009) to correlate with the TCA cycle activity.

One might assume that the additional energy demand for recombinant protein synthesis can be compensated by increased TCA cycle activity without directly causing a reduced rate of growth. The presented results on increasing relative TCA cycle activities during recombinant protein production might initially support this idea. However, examining the net fluxes through the TCA cycle reveals that the changes of the absolute TCA cycle activity are negligible (Fig. 3). The activity of the TCA cycle was constant with a rate of 2.1 ± 0.1 mmol g<sub>CDW</sub>⁻¹ h⁻¹, resulting in a rather constant ATP generation rate of 19.4 ± 1.9 mmol g<sub>CDW</sub>⁻¹ h⁻¹. Consequently, the biomass yield on ATP is significantly decreasing from 13.2 g<sub>CDW</sub> mol<sub>ATP</sub>⁻¹ for the reference strain, to 12.1 g<sub>CDW</sub> mol<sub>ATP</sub>⁻¹ for BapAlow, to 11.8 g<sub>CDW</sub> mol<sub>ATP</sub>⁻¹ for BapAmed, and finally to 9.9 g<sub>CDW</sub> mol<sub>ATP</sub>⁻¹ for BapAhigh. This decreased yield is supporting the idea that a metabolic burden occurs during recombinant protein production. To overcome this metabolic burden caused by recombinant protein synthesis, the cell adjusts the flux distribution that enables a high yield of ATP on glucose. The yield of ATP on glucose is indeed increasing from 4.2 mol<sub>ATP</sub> mol<sub>glucose</sub>⁻¹ for the reference strain, to 4.8 mol<sub>ATP</sub> mol<sub>glucose</sub>⁻¹ for BapAlow, to 5.3 mol<sub>ATP</sub> mol<sub>glucose</sub>⁻¹ for BapAmed, and finally to 6.5 mol<sub>ATP</sub> mol<sub>glucose</sub>⁻¹ for BapAhigh. The reduced fitness (i.e., low rate of growth) observed, however, suggest that the cell does not have the capacity to catabolize a sufficient amount of carbon in the TCA cycle, to fully compensate the increased energy demand. This data might be evidence for a limited capacity

![Figure 3](image-url)
of the cell’s energy metabolism that subsequently limits recombinant protein production.

De Novo Amino Acid Synthesis and Energy Metabolism

Our findings might be evidence for a limited capacity of the TCA cycle to overcome the metabolic burden. Other limitations for recombinant protein synthesis might occur during transcription and translation. To test the hypothesis that recombinant protein synthesis is limited by (energy) metabolism, protein production, and cell physiology were investigated by varying the growth medium composition.

The growth media employed were either an amino acid supplemented minimal medium (VerduynAA medium) or the complex YPD medium. Amino acids can simultaneously be consumed with the carbon and energy source glucose and can thereby lower the energetic demand of biomass and recombinant protein synthesis. The use of both media resulted in increased growth and importantly in increased BapA production rates (Table I and Fig. 4), indicating strongly that the observed recombinant protein production limitation is not caused by the transcription nor translation machinery, but rather is of metabolic nature, for example, the availability of precursors or energy.

In detail, a slight increase in the rate of growth of the reference strain was measured, rising from 0.28 h⁻¹ on Verduyn medium (standard conditions), to 0.30 h⁻¹ on VerduynAA medium, to 0.32 h⁻¹ on YPD medium. The highest effect on the growth rate was observed for the recombinant strain BapAhigh, rising from 0.16 h⁻¹, to 0.18 h⁻¹, and 0.24 h⁻¹, respectively. The variation of growth media led to a highly efficient production of BapA up to almost doubling the protein yield as compared to standard conditions (Table I). The activities correspond to yields of product on biomass of 56 mgBapA g⁻¹ CDW for BapAlow, 71 mgBapA g⁻¹ CDW for BapAmed, and 95 mgBapA g⁻¹ CDW for BapAhigh using VerduynAA medium, and 84 mgBapA g⁻¹ CDW, 102 mgBapA g⁻¹ CDW, and 142 mgBapA g⁻¹ CDW, respectively, using YPD medium. Consequently, the productivity of strain BapAhigh rose from 3.0 ± 0.1 mgBapA g⁻¹ h⁻¹, to 4.2 ± 0.2 mgBapA g⁻¹ h⁻¹ on VerduynAA medium, and 7.2 ± 1.2 mgBapA g⁻¹ h⁻¹ on YPD medium.

Now the question is how the medium ingredients, mainly amino acids (Sezonov et al., 2007), unburden the energy metabolism. To decipher the contribution of the supplemented amino acids toward biomass synthesis, the relative uptake of 15 amino acids was quantified by ¹³C-labeling experiments. In these experiments de novo synthesized amino acids are ¹³C-labeled, while amino acids originating from...
from the medium are naturally labeled. Under all conditions tested, amino acid de novo synthesis and uptake were observed, while the ratios between the two alternatives depended on the medium composition (Fig. 5).

The total de novo amino acid synthesis—the sum of all de novo synthesized amino acids of each experiment according to the proportionate frequency in the cell, considering the amino acid composition of *P. pastoris* (Carnicer et al., 2009)—was found to be 65–69% on VerduynAA medium and 33–45% on YPD medium. The higher amount of amino acids taken up in YPD medium might be due to di- and oligopeptides that constitute a significant part of the amino acid fraction in yeast extract (Sezonov et al., 2007). Homologs to *S. cerevisiae* di- and oligopeptide transporters encoded by PTR2 and OPT1/2 (Wiles et al., 2006) are present on the *P. pastoris* genome (De Schutter et al., 2009).

It is known that the transport of di- and oligopeptides is less energy consuming per amino acid transported than single amino acid transport (Grenson, 1992). Notably, the highest uptake of extracellular amino acids was observed for the reference strain and decreased with increasing biomass yields. The de novo synthesis of almost all amino acids increased with decreasing growth rates and glucose uptake rates. As the yield of biomass was higher in slow growing, slow glucose utilizing cultures, carbon limitation as possible reason for reduced amino acid de novo synthesis seems unlikely. In summary, for growth on both media, the lowest de novo synthesis was observed for the lowest biomass yielding strain (reference strain) and decreased with increasing biomass yields. The de novo synthesis of almost all amino acids increased with decreasing growth rates and glucose uptake rates. The total de novo amino acid synthesis—the sum of all de novo synthesized amino acids of each experiment according to the proportionate frequency in the cell, considering the amino acid composition of *P. pastoris* (Carnicer et al., 2009)—was found to be 65–69% on VerduynAA medium and 33–45% on YPD medium. The higher amount of amino acids taken up in YPD medium might be due to di- and oligopeptides that constitute a significant part of the amino acid fraction in yeast extract (Sezonov et al., 2007). Homologs to *S. cerevisiae* di- and oligopeptide transporters encoded by PTR2 and OPT1/2 (Wiles et al., 2006) are present on the *P. pastoris* genome (De Schutter et al., 2009).

To decipher the correlation between energy metabolism and de novo amino acid synthesis, we focused on amino acid uptake and the aerobic energetic cost for de novo amino acid synthesis. The production cost for each single amino acid was recently defined by the required number of high energy phosphate bonds (~PO₄) ranging from 9.5 for glutamate to 75.5 for tryptophan (Raiford et al., 2008). Notably, amino acid de novo synthesis correlates inversely with the cost for most amino acids with the exception of phenylalanine, tyrosine and to a lesser extent isoleucine and lysine (Fig. 6). Highly taken up amino acids, such as histidine, methionine, and leucine (Fig. 6) have high energetic costs of 29.0, 36.5, and 37.0, respectively. Amino acid de novo synthesis was high, for example, for glutamine, alanine, glycine, and serine (Fig. 6) that have low energetic costs of 9.5, 14.5, 14.5, and 14.5, respectively. Exceptionally, the aromatic amino acids phenylalanine and tyrosine, although energetically expensive with ~PO₄ values of 61 and 59, respectively, were synthesized up to 65% de novo (Fig. 6). A competition by phenylalanine and tyrosine for the transporter Tat2p might explain the results (Camon et al., 2003). The presence of an additional transporter (encoded by TAT1) for tyrosine might explain the higher uptake of tyrosine.

Consequently, additional experiments using specific amino acid mixtures for supplementation were performed to quantify their contribution on physiology, amino acid uptake, and recombinant protein production (Table II). In order to unburden central carbon metabolism, four different media supplemented with the following amino acids were used: (1) addition of glutamine, which is the main amino donor (ter Schure et al., 2000); (2) addition of TCA cycle (i.e., oxoglutarate) derived amino acids (i.e., glutamine, glutamate, lysine, and proline); (3) addition of energetically expensive amino acids (i.e., histidine, isoleucine, leucine, lysine, methionine, phenylalanine, and tyrosine); (4) addition of highly incorporated amino acids (i.e., leucine, lysine, and methionine).

The addition of glutamine alone had a significant impact on amino acid synthesis and protein production. The recombinant protein yield was increased to 21 mgBapA g⁻¹CDW, causing an increased productivity of 3.5 mgBapA g⁻¹CDW h⁻¹ compared to 17 mgBapA g⁻¹CDW h⁻¹ when grown under standard conditions. The supplementation of all TCA cycle (i.e., oxoglutarate) derived amino acids (i.e., glutamine, glutamate, lysine, and proline) further increased the product yield to 22 mgBapA g⁻¹CDW and the productivity to 3.7 mgBapA g⁻¹CDW h⁻¹ when compared to standard conditions. Supplementation of the energetically expensive amino acids resulted in a product yield of 23 mgBapA g⁻¹CDW and a productivity of 4.1 mgBapA g⁻¹CDW h⁻¹, which were the highest of all four experiments. Remarkably, addition of the highly incorporated amino acids histidine, leucine, and methionine had the lowest impact on product yield (20 mgBapA g⁻¹CDW) and productivity (3.3 mgBapA g⁻¹CDW h⁻¹).

The data suggest that glutamine contributes significantly to the relieve of metabolism from recombinant protein production, allowing enhanced productivity and product titer. Notably, during growth in the presence of glutamine,
glucose–glutamine co-metabolism was high. In detail, besides glutamate/glutamine (uptake of 64%), other amino acids originated partly from the carbon skeleton of glutamine including aspartate/asparagine (29%), isoleucine (23%), lysine (47%), methionine (9%), proline (32%), and threonine (19%). Remarkably, the amounts of aspartate/asparagine and glutamate/glutamine from glutamine uptake were significantly higher than in the previous experiment using all amino acids for supplementation. Thus, glutamine is not solely used as amino donor, but also as additional carbon source.

To identify the amino acid supplementation strategy with the highest impact on recombinant protein production, we determined the production rate of BapA derived solely from the supplemented amino acids. Thereby, the difference between the total protein production rate of 80 mg\textsubscript{BapA} g\textsuperscript{-1} CDW h\textsuperscript{-1} at standard conditions and the total protein production rate using amino acids supplemented media was assumed to be the total protein production rate derived from the supplemented amino acids. The same assumption can be made for the change in product (BapA) yield. Finally, the selectivity of BapA derived from the supplemented amino acids can be calculated. This evaluation highlights identically high product selectivity of 164 mg\textsuperscript{-1} BapA g\textsuperscript{-1} CDW h\textsuperscript{-1} when using glutamine or energetically expensive amino acids for supplementation. The use of TCA cycle derived amino acids allowed for a selectivity of about 137 mg\textsuperscript{-1} BapA g\textsuperscript{-1} CDW h\textsuperscript{-1}. By supplementing highly taken up amino acids a selectivity of only 62 mg\textsuperscript{-1} BapA g\textsuperscript{-1} CDW h\textsuperscript{-1} was achieved. In summary, glutamine as carbon and nitrogen source seems to be a good candidate to partly release the metabolic burden caused by recombinant protein production.

### Discussion

Efficient (high rate, high titer) recombinant protein production has been reported several hundred times using P. pastoris as host (Zhang et al., 2009). In most of the reports, genetic elements including promoters, insertion cassettes, secretion signals, or the DNA sequence of the gene of interest were in focus for optimizing protein production, resulting in a toolbox of high performing genetic elements. Now metabolic limitations that have been described ever since recombinant protein production started (Da Silva and Bailey, 1985; Glick, 1995), move into focus of strain characterization (Heyland et al., 2010b; Mattanovich et al., 2004) and optimization (Graf et al., 2009). The present study provides novel evidences for a direct response of P. pastoris’ metabolic network to recombinant protein production, which cannot only be explained by the direct resources necessary to produce BapA. The evidences are (1) a negative correlation between the rates of recombinant protein production and growth, (2) a constant flux through the TCA cycle irrespectively of the glucose uptake rate, (3) a negative correlation between biomass yield on ATP and protein production, and (4) a partial restoration of the growth rate by amino acid supplementation.

In respect to physiology, it is well-established today that recombinant protein production is connected to different cellular stresses. The stress induced extra energy requirement for recombinant protein production was summarized as metabolic burden (Glick, 1995). This metabolic burden results in inhibition of growth or low level of product accumulation, decreased cell resistance to environmental influences or even cell lysis, plasmid instability, and thus influences the productivity of an expression system. Notably, theoretical considerations have shown that within the generally obtained range of product yields, as also obtained in the present study, only minor effects on growth rate and/or biomass yield would be expected through the energy and precursor demands for recombinant protein production (Da Silva and Bailey, 1985). Experimental data, however, demonstrate that growth is often affected in recombinant protein overproducing cells. One potential mechanism for explaining the discrepancy between theoretical considerations and experimental observations is that
expression and recombinant protein production rates can significantly exceed protein accumulation rates due to mRNA and recombinant protein turnover, respectively (Hoffmann and Rinas, 2004). Also, enhanced maintenance demands can cause an additional energy demand for the cell during heterologous gene expression (Weber et al., 2002). Moreover, resources are required for posttranslational processes and protein degradation (Mattanovich et al., 2004); all summing up to the encountered metabolic burden. Notably, it could be shown that the metabolic burden decreased with temperature (25 and 20°C), despite an up to threefold increased protein production rate. The authors speculated that the energetically expensive refolding of proteins and/or protein degradation of misfolded proteins were reduced at lower temperatures (Dragosits et al., 2009). In another study it was shown that intrinsic stresses due to the unfolded protein response (UPR) and misfolding were significantly reduced at lower temperatures, leading to reduced protein degradation of unfolded or misfolded proteins (Gasser et al., 2007).

Consistent to our results in batch cultures, a previous study using recombinant S. cerevisiae grown on galactose observed a significant increase in the flux through the TCA cycle during the production of β-galactosidase (Jin et al., 1997). Previous studies have shown that in S. cerevisiae the TCA cycle activity correlates with the glucose uptake rate (Blank and Sauer, 2004; Heyland et al., 2009). In P. pastoris the absolute TCA cycle was with a rate of 2.1 mmol g CDW h−1 of proteins and/or protein degradation of misfolded proteins were reduced at lower temperatures (Dragosits et al., 2009). In another study it was shown that intrinsic stresses due to the unfolded protein response (UPR) and misfolding were significantly reduced at lower temperatures, leading to reduced protein degradation of unfolded or misfolded proteins (Gasser et al., 2007).

Whether the here reported upper boundaries of the flux through the TCA cycle are a coincident or result of the highly similar metabolic network structure and enzyme inventory, of which TCA cycle and electron transport chain are located in the mitochondria, should be investigated in the future.

The improved recombinant protein production by amino acid supplementation again indicates strongly the existence of a metabolic burden as increased biomass yields and protein production rates were observed. Productivities are consistent with previous studies in yeasts when using amino acids for supplementation (Gorgens et al., 2005a,b). Previous studies on yeast species have shown that variation in the media compositions, especially the supplementation of certain amino acids or complex extracts (e.g., yeast extract), have positively influenced the production of proteins (Hahn-Hagerdal et al., 2005).

In another study, the increase in the yield of product was obtained by the addition of auxotroph-complementing amino acid to an auxotrophic host, which caused increased strain viability (Paciello et al., 2009). In the present work, glutamine was most effective on productivity and was used as nitrogen and carbon source. We propose glutamine addition as a potential strategy to unburden central carbon metabolism during recombinant protein production. However, the uses of supplemented amino acids have to be considered carefully since it might compromise process economy, especially at larger scale. Furthermore, growth medium additives can interfere with downstream processing.

De novo amino acid synthesis in amino acid supplemented media was strongly selective and correlated inversely with the energetic cost of most amino acids. Hence, the amino acid composition of the recombinant protein might influence the potential of recombinant production. Especially proteins that differ significantly from the average proteome amino acid composition of P. pastoris might require higher fluxes in tightly regulated amino acid pathways such as the pathways for aromatic and branched chain amino acids.

In summary, recombinant protein production in P. pastoris is limited by metabolism, which can be partially overcome by growth medium supplementation. The results indicate that the investigation of the exact metabolic limitations should move into the research focus to develop metabolic engineering strategies for enhanced protein production in inexpensive (minimal) media.

The authors are grateful to Holger Müller (BlueSens Gas Sensor GmbH) for providing the BC prefem gas sensors and for his invaluable insights into off-gas analysis. We would like to thank Anton Glieder and Franz Hartner for their help with codon optimization and fruitful discussions. The Deutsche Bundesstiftung Umwelt (DBU) is gratefully acknowledged for providing financial support.

References


Paciello L, de Alteris E, Mazzoni C, Palermo V, Zuoco J, Parascandola P. 2009. Performance of the autotrophic Saccharomyces cerevisiae BY4741 as host for the production of IL-1b in aerated fed-batch reactor: Role of ACA supplementation, strain viability, and maintenance energy. Microcell Fact 8:70.


