

# Cytosolic NADPH balancing in *Penicillium chrysogenum* cultivated on mixtures of glucose and ethanol

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**Abstract** The in vivo flux through the oxidative branch of the pentose phosphate pathway (oxPPP) in *Penicillium chrysogenum* was determined during growth in glucose/ethanol carbon-limited chemostat cultures, at the same growth rate. Non-stationary  $^{13}\text{C}$  flux analysis was used to measure the oxPPP flux. A nearly constant oxPPP flux was found for all glucose/ethanol ratios studied. This indicates that the cytosolic NADPH supply is independent of the amount of assimilated ethanol. The cofactor assignment in the model of van Gulik et al. (Biotechnol Bioeng 68(6):602–618, 2000) was supported using the published genome annotation of *P. chrysogenum*. Metabolic flux analysis showed that NADPH requirements in the cytosol remain nearly the same in these experiments due to constant biomass growth. Based on the cytosolic NADPH balance, it is known that the cytosolic aldehyde dehydrogenase in *P. chrysogenum* is  $\text{NAD}^+$  dependent. Metabolic modeling shows that changing the  $\text{NAD}^+$ -aldehyde dehydrogenase to  $\text{NADP}^+$ -aldehyde dehydrogenase can increase the penicillin yield on substrate.

**Keywords** Isotopic non-stationary  $^{13}\text{C}$  flux analysis · Metabolic flux ratio analysis · Pentose phosphate pathway · Metabolic engineering · NADPH metabolism

## Introduction

Using classical strain improvement programs, the industrial *Penicillium chrysogenum* strain has been improved for  $\beta$ -lactam production by more than 1,000-fold (Thykaer and Nielsen 2003). Rational metabolic engineering is the next step, allowing further improvements and addressing the relationship between production pathways and the central metabolism (Nasution et al. 2008).

The biosynthesis pathways of the penicillin carbon precursors cysteine and valine require large amounts of NADPH (Kleijn et al. 2006; Lee and Hwang 2003; Blombach et al. 2008). A strong positive correlation between intracellular NADPH demand and the penicillin production rate was calculated by metabolic flux analysis (Henriksen et al. 1996; van Gulik et al. 2000). It has also been known that most of the NADPH demand incurred by penicillin production resides in the cytosol (Evers et al. 2004; Kleijn et al. 2007). Due to its role in NADPH metabolism, the flux through the oxidative branch of the pentose phosphate pathway (oxPPP) was known to be the limiting factor in the production of several amino acids (Wittmann and Heinzle 2002; Georgi et al. 2005; Kabus et al. 2007). Investigation of the cytosolic NADPH sources and sinks can therefore provide further insights into the process and identify potential NADPH-related metabolic engineering targets.

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The oxidative branch of the pentose phosphate pathway is the main cytosolic NADPH source. Study of the oxPPP flux is therefore relevant to rational strain improvement. The source of NADPH has been shown to vary with the carbon source (Minard and McAlister-Henn 2005). An interesting approach used to manipulate the NADPH source is to use mixed substrate, e.g., glucose/ethanol, because aldehyde dehydrogenase (ALD6 of *Saccharomyces cerevisiae*) was shown to be an important cytosolic NADPH source for fungi (Grabowska and Chelstowska 2003; Bro et al. 2004). Similarly, van Gulik et al. (2000) assumed that ethanol oxidation provides NADPH in *P. chrysogenum* based on the results described by Bruinenberg et al. (1983) on *Candida utilis*. Mixing ethanol with glucose can therefore potentially shift the load of NADP<sup>+</sup> reduction from oxPPP to ethanol uptake.

The metabolic model of van Gulik et al. (2000) contained ten cytosolic metabolic reactions that consume cytosolic NADPH for amino acid, fatty acid synthesis, or the reduction of sulfur. Table 1 lists all of the cytosolic NADPH-consuming reactions/pathways in the *P. chrysogenum* metabolic model developed by van Gulik et al. (2000). Based on the published annotation of the genomic sequences (van den Berg et al. 2008), the putative enzymes that catalyzes these redox reactions can be now assigned. This annotation information supports most of the cofactor assignments in the van Gulik et al. (2000) model (compare Tables 1 and 2), except the histidine synthesis pathway r11.32.

This pathway of r11.32 lumped the reaction from  $\alpha$ -5-phosphoribosylpyrophosphate (PRPP) to histidine. Two NAD<sup>+</sup> and one NADPH were required in this lumped pathway (van Gulik et al. 2000). However, the only redox reaction in this pathway is the oxidation from histidinol to histidine (Stepansky and Leustek 2006), which costs two NAD<sup>+</sup> without NADPH consumption. In addition, the pyrroline-5-carboxylate reductase in the proline synthesis pathway (r11.3) also showed activity toward both NADH and NADPH (Rossi et al. 1977).

Recently, the isotopic non-stationary gluconate tracer method (Zhao et al. 2008) was proved to be suitable for the measurement of the oxPPP flux without long-term cultivation (on the order of several residence times). In addition, the method requires neither assumptions on network reversibility nor knowledge of amino acid biosynthesis routes. The latter feature makes the accuracy of this method independent of the accuracy of the biomass composition data. These features made this method appropriate for quantifying the oxPPP flux of *P. chrysogenum* cultures grown on different mixed glucose/ethanol substrates.

To investigate the cytosolic NADPH metabolism of *P. chrysogenum*, the oxPPP flux was measured using the isotopic non-stationary gluconate tracer method. This work reports the results obtained from chemostat cultures grown on different mixed glucose/ethanol substrate mixtures at the same growth rate. This provides further insights for the penicillin production.

**Table 1** Cytosolic NADPH-consuming reactions in the metabolic model of *P. chrysogenum* (van Gulik et al. 2000)

Name	Reaction	Cofactor
r11.1	1 aKG:cyt + 1 H:cyt + 1 NADPH:cyt + 1 NH <sub>4</sub> :cyt $\Rightarrow$ 1 glu:cyt + 1 H <sub>2</sub> O:cyt + 1 NADP:cyt	NADPH
r16.3	9 AcCoA:cyt + 8 ATP:cyt + 8 H:cyt + 16 NADPH:cyt $\Rightarrow$ 8 ADP:cyt + 8 HCoA:cyt + 16 NADP:cyt + 8 Pi:cyt + 1 steaCoA:cyt	NADPH
r11.18	1 asp:cyt + 1 ATP:cyt + 2 H:cyt + 2 NADPH:cyt $\Rightarrow$ 1 ADP:cyt + 1 homser:cyt + 2 NADP:cyt + 1 Pi:cyt	NADPH
r11.12	1 H:cyt + 4 NADPH:cyt + 1 PAPS:cyt $\Rightarrow$ 1 ADP:cyt + 3 H <sub>2</sub> O:cyt + 1 H <sub>2</sub> S:cyt + 4 NADP:cyt	NADPH
r11.3	1 ATP:cyt + 1 glu:cyt + 2 H:cyt + 2 NADPH:cyt $\Rightarrow$ 1 ADP:cyt + 1 H <sub>2</sub> O:cyt + 2 NADP:cyt + 1 Pi:cyt + 1 pro:cyt	NADPH
r11.8	1 aAd:cyt + 2 ATP:cyt + 1 glu:cyt + 1 H <sub>2</sub> O:cyt + 1 NAD:cyt + 2 NADPH:cyt $\Rightarrow$ 2 ADP:cyt + 1 aKG:cyt + 1 H:cyt + 1 lys:cyt + 1 NADH:cyt + 2 NADP:cyt + 2 Pi:cyt	NADPH
r11.27	1 ATP:cyt + 1 E4P:cyt + 1 NADPH:cyt + 2 PEP:cyt $\Rightarrow$ 1 ADP:cyt + 1 chor:cyt + 1 NADP:cyt + 4 Pi:cyt	NADPH
r16.13	3 AcCoA:cyt + 1 H:cyt + 1 H <sub>2</sub> O:cyt + 2 NADPH:cyt $\Rightarrow$ 3 HCoA:cyt + 1 meva:cyt + 2 NADP:cyt	NADPH
r11.32	3 ATP:cyt + 1 CO <sub>2</sub> :cyt + 1 gln:cyt + 3 H <sub>2</sub> O:cyt + 2 NAD:cyt + 1 NADPH:cyt + 1 NH <sub>4</sub> :cyt + 1 PRPP:cyt $\Rightarrow$ 3 ADP:cyt + 1 aKG:cyt + 8 H:cyt + 1 his:cyt + 2 NADH:cyt + 1 NADP:cyt + 6 Pi:cyt	NADPH + NAD <sup>+</sup>
r16.14	18 ATP:cyt + 5 H <sub>2</sub> O:cyt + 6 meva:cyt + 2 NADPH:cyt + 1 O <sub>2</sub> :cyt $\Rightarrow$ 18 ADP:cyt + 6 CO <sub>2</sub> :cyt + 10 H:cyt + 1 lano:cyt + 2 NADP:cyt + 18 Pi:cyt	NADPH

*aAd*  $\alpha$ -amino adipate, *AcCoA* acetyl coenzyme A, *aKG*  $\alpha$ -ketoglutarate, *asp* aspartate, *chor* chorismate, *cyt* cytosol, *E4P* erythrose-4-phosphate, *gln* glutamine, *glu* glutamate, *H* proton, *HCoA* coenzyme A, *his* histidine, *homser* homoserine, *lano* lanosterol, *lys* lysine, *meva* mevalonate, *PAPS* 3-phosphoadenosine-5-phosphosulfate, *PEP* phosphoenolpyruvate, *Pi* orthophosphate, *pro* proline, *PRPP*  $\alpha$ -5-phosphoribosylpyrophosphate, *steaCoA* stearyl coenzyme A

**Table 2** Genes for the cytosolic NADPH-consuming reactions in *P. chrysogenum* based on the genome annotation

Name	Gene name	Annotated function	Cofactor	Reference
r11.1	Pc22g17560	Glutamate dehydrogenase <i>gdhA</i>	NADPH	Díez et al. (1999), Harris et al. (2006), Thykaer et al. (2008)
r16.3	Pc14g00740	Fatty-acyl-CoA synthase beta chain <i>fas1p</i>	NADPH	Wakil et al. (1983)
r11.18	Pc20g02910	Aspartate-semialdehyde dehydrogenase	NADPH	Robichon-Szulmajster et al. (1966)
r11.18	Pc13g08990	Homoserine dehydrogenase	NADPH	Yamaki et al. (1990)
r11.12	Pc12g08580	Sulfite reductase <i>Met10</i>	NADPH	Yoshimoto and Sato (1968)
r11.3	Pc13g05550	Hypothetical protein <i>ncu01412.1<sup>a</sup></i>	NADPH	Baich (1971), Tomenchok and Brandriss (1987)
r11.3	Pc12g13680	Pyrroline-5-carboxylate reductase <i>ProC</i>	NAD(P)H	Rossi et al. (1977)
r11.8	Pc22g06310	L-aminoadipate-semialdehyde dehydrogenase	NADPH	Casqueiro et al. (1998), Zabriskie and Jackson (2000), Hijarrubia et al. (2001)
r11.8	Pc22g05800	Saccharopine reductase <i>Lys7</i>	NADPH	Jones and Broquist (1966), Esmahan et al. (1994)
r11.27	Pc16g12160	Pentafunctional enzyme <i>aroM<sup>b</sup></i>	NADPH	Duncan et al. (1988)
r16.13	Pc18g05230	Hydroxymethylglutaryl-CoA reductase	NADPH	Qureshi et al. (1976)
r11.32	Pc16g14140	Histidinol dehydrogenase <i>his-3</i>	NAD <sup>+</sup>	Creaser and Varela-Torres (1971)
r16.14	Pc21g13930	Squalene synthase <i>ERG9</i>	NADPH	Qureshi et al. (1973)

<sup>a</sup>Homology with Pro2 of *S. cerevisiae* ( $\gamma$ -glutamyl phosphate reductase)

<sup>b</sup>Shikimate dehydrogenase

A potential metabolic engineering target for altering NADPH metabolism based on metabolic flux analysis is discussed.

## Materials and methods

### Strain

A high-yielding *P. chrysogenum* strain (DS17690) was donated by DSM Anti-Infectives (Delft, The Netherlands).

### Media and medium preparation

Three chemically defined chemostat media were used to study the metabolism of *P. chrysogenum* on mixed glucose/ethanol substrate at (C mole ethanol)/(total carbon) fraction of 0, 0.15, and 0.48 Cmol/Cmol (mediums 1a, 1b, 1c) at constant total carbon substrate concentration 0.5 Cmol/L. The composition of medium 1a was 15.68 g/L glucose·H<sub>2</sub>O, 0.74 g/L glucono- $\delta$ -lactone, 0.675 g/L phenylacetic acid (PAA), 0.8 g/L KH<sub>2</sub>PO<sub>4</sub>, 5 g/L (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.5 g/L MgSO<sub>4</sub>·H<sub>2</sub>O, and 2 mL/L trace element solution. The trace element solution contained 75 g/L Na<sub>2</sub>-EDTA·2H<sub>2</sub>O, 2.5 g/L CuSO<sub>4</sub>·5H<sub>2</sub>O, 10 g/L ZnSO<sub>4</sub>·7H<sub>2</sub>O, 10 g/L MnSO<sub>4</sub>·H<sub>2</sub>O, 20 g/L FeSO<sub>4</sub>·7H<sub>2</sub>O, and 2.5 g/L CaCl<sub>2</sub>·2H<sub>2</sub>O. Mediums 1b and 1c were identical to medium 1a except that the ethanol C mole fraction was changed to 0.15 and 0.48 Cmol/Cmol while keeping the molar ratio between glucose and glucono- $\delta$ -lactone at 19:1. The labeled medium equivalent to 1a, 1b, and 1c are called mediums 2a, 2b, and 2c and were chemically identical to their counterparts except that the glucono-

$\delta$ -lactone was replaced by the same molar amount of [U-<sup>13</sup>C<sub>6</sub>]glucono- $\delta$ -lactone (Omicron, South Bend, IN, USA).

### Cultivation conditions and labeling experiment

The chemostats were carried out at a dilution rate of 0.06 h<sup>-1</sup>, 25°C, pH 6.5, and the working volume was controlled at 600 mL by an overflow system. After reaching the metabolic steady state from each batch phase after approximately five residence times (about 3 days), the naturally labeled mediums 1a, 1b, and 1c were replaced by <sup>13</sup>C gluconate tracer labeled mediums 2a, 2b, and 2c, respectively, until the end of the isotopic non-stationary labeling experiments (usually 5 h). The cost of the labeling material is very limited due to the short period of time and the tracer amount needed.

### Sampling and analysis

To calculate the flux through the oxPPP, it was sufficient to quantify the glucose and gluconate uptake rates and the mass isotopomer distributions (MID) of intracellular glucose-6-phosphate (g6p), 6-phosphogluconate (6pg), and gluconate (glnic) (Zhao et al. 2008). Samples for mass isotopomer analysis of intracellular metabolites were taken, processed, and analyzed as described by Zhao et al. (2008). The MID of the intracellular g6p, 6pg, and glnic were measured by LC-MS as described by van Winden et al. (2005), and the MID were calculated as described by Zhao et al. (2008).

The extracellular concentrations of PAA and penicillin-G were analyzed by an isocratic HPLC

method using a Zorbax<sup>®</sup> column at 30°C. Triplicate 5-mL broth samples were taken daily during the fermentation to determine the dry weight of the biomass. Samples were filtered over pre-weighed glass fiber filters (Pall, East-Hills, NY, USA) and dried at 70°C for 24 h and subsequently weighed.

The enzymatic activity of the aldehyde dehydrogenase (ALDH; EC 1.2.1.3, 1.2.1.4, 1.2.1.5) was measured using the same protocol as Harris et al. (2006). The reaction mix contained 100 mM potassium phosphate buffer, pH 8.0, 15 mM pyrazole, 0.5 mM DTT, 10 mM KCl, and 0.4 mM NAD<sup>+</sup> or NADP<sup>+</sup>. The reaction was started with 0.1 mM freshly prepared acetaldehyde. The protein concentration in the cell extract was determined by the method of Lowry et al. (1951). Because the pathway converting ethanol to acetate is linear, the actual in vivo fluxes catalyzed by the ALDH are the same as the ethanol uptake rates (see Table 3). In order to compare the in vivo flux through ALDH with the measured in vitro enzyme activity, unit conversion from flux (millimoles per C mole per hour) to specific activity (micromoles per milligram per minute) is needed. The averaged biomass C mole weight  $M_w$  is  $27.7 \pm 0.5$  g/(Cmol biomass), ranging from glucose-limited biomass composition to ethanol-limited biomass composition. The extracted protein weight fraction in biomass  $f_{pr}$  is a nearly constant 0.25, based on the protein determination results (see the “Results” section). Therefore, the unit conversion factor from millimoles per C mole biomass per hour to micromoles per milligram protein per minute can be calculated as:

$$1 \frac{\text{mmol}}{(\text{Cmol biomass}) \text{ h}} = \frac{1}{M_w f_{pr} \times 60} \frac{\mu\text{mol}}{(\text{mg protein}) \text{ min}} = 0.0024 \frac{\mu\text{mol}}{(\text{mg protein}) \text{ min}}. \quad (1)$$

The off-gas concentrations of O<sub>2</sub> and CO<sub>2</sub> were measured on-line (non-dispersive/paramagnetic in-

frared Rosemount NGA 2000 gas analyzer, Fisher-Rosemount, Hanau, Germany) and the air flow rate was controlled using a mass flow controller.

#### Black-box elemental balancing of fermentation data

The variance weighed optimization procedure developed by van der Heijden et al. (1994) was used to test elemental recovery and subsequently to reconcile the measured primary fermentation data, based on elemental balancing; it was also used to calculate the biomass-specific uptake and production rates. The statistical acceptance of the balanced results were evaluated by the  $\chi^2$ -test.

#### Metabolic flux analysis

The compartmentalized stoichiometric model of *P. chrysogenum* (van Gulik et al. 2000) was used for the metabolic flux analysis. Dedicated software (SPADit, Nijmegen, The Netherlands) was used for the calculations. The biomass compositions of both the glucose-limited culture and the ethanol-limited culture were taken from van Gulik et al. (2000). The difference between these two compositions were insignificant. Moreover, the cytosolic NADPH demand is quite insensitive to the biomass composition. It was therefore considered that linear interpolation is a sufficient approximation to calculate the biomass composition of the mixed substrate cultures.

## Results

### Uptake and production rates

*P. chrysogenum* was cultivated in three different chemostat experiments at the same dilution rate, using

**Table 3** Measured and reconciled steady-state biomass specific  $q$ -rates

$q$ -rates mmol/Cmol/h	0 $\frac{\text{Cmol}}{\text{Cmol}}$ ethanol		0.15 $\pm$ 0.02 $\frac{\text{Cmol}}{\text{Cmol}}$ ethanol		0.48 $\pm$ 0.05 $\frac{\text{Cmol}}{\text{Cmol}}$ ethanol	
	Measured	Reconciled	Measured	Reconciled	Measured	Reconciled
– $q_{\text{ethanol}}$	0.00	0.00	8.79 $\pm$ 0.85	8.36 $\pm$ 0.79	29.9 $\pm$ 4.1	26.7 $\pm$ 2.3
– $q_{\text{glucose}}$	20.8 $\pm$ 2.0	20.1 $\pm$ 1.7	16.6 $\pm$ 1.6	15.3 $\pm$ 1.3	9.36 $\pm$ 0.90	9.29 $\pm$ 0.77
– $q_{\text{gluconate}}$	1.01 $\pm$ 0.09	0.99 $\pm$ 0.08	0.85 $\pm$ 0.08	0.81 $\pm$ 0.08	0.47 $\pm$ 0.05	0.47 $\pm$ 0.04
– $q_{\text{O}_2}$	46.4 $\pm$ 4.7	45.3 $\pm$ 4.4	41.2 $\pm$ 4.0	42.9 $\pm$ 3.9	59.1 $\pm$ 5.5	59.1 $\pm$ 5.1
– $q_{\text{PAA}}^a$	0.37 $\pm$ 0.09	0.34 $\pm$ 0.04	0.50 $\pm$ 0.07	0.46 $\pm$ 0.04	0.24 $\pm$ 0.15	0.24 $\pm$ 0.15
$\mu$	61.0 $\pm$ 4.9	60.5 $\pm$ 4.9	61.8 $\pm$ 4.3	61.1 $\pm$ 4.3	62.0 $\pm$ 4.2	61.6 $\pm$ 3.6
$q_{\text{Pen-G}}$	0.34 $\pm$ 0.04	0.34 $\pm$ 0.04	0.48 $\pm$ 0.04	0.46 $\pm$ 0.04	0.24 $\pm$ 0.14	0.24 $\pm$ 0.10
$q_{\text{CO}_2}^b$	50.0 $\pm$ 4.9	49.6 $\pm$ 4.7	36.9 $\pm$ 3.7	38.8 $\pm$ 3.5	36.4 $\pm$ 3.4	36.2 $\pm$ 3.2
$q_{\text{by-product}}^b$	14.2 $\pm$ 1.4	13.9 $\pm$ 1.2	9.46 $\pm$ 0.87	9.48 $\pm$ 0.87	12.4 $\pm$ 2.1	12.3 $\pm$ 1.1

<sup>a</sup>Phenylacetic acid

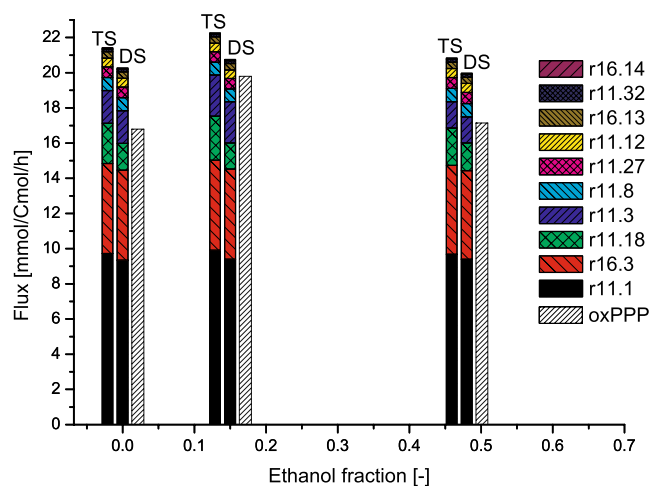
<sup>b</sup>Extracellular peptides and polysaccharides determined by total organic carbon analysis

different glucose/ethanol ratios in the media as described in the “Materials and methods” section. The measured and reconciled uptake and production rates are compared in Table 3. Note that Kleijn et al. (2006) used the residual concentration of the gluconate to show that the added gluconate tracer was completely taken up under carbon limited condition. In all three experiments, good consistency was found between the measured rates and the reconciled rates ( $P$  values<sup>1</sup> are 89%, 54%, and 78%, respectively). It was thus concluded that these data contain no gross measurement errors and that there are no unknown by-products. After the elemental balancing, it was found that the actual ethanol fractions in the three experiments were 0, 0.15, and 0.48 Cmol/Cmol.

In the growth conditions studied, metabolic flux analysis shows that the total cytosolic NADPH demand is nearly constant at a rate of 21 mmol/Cmol/h, irrespective of the ethanol fraction or the cysteine synthesis route (see Fig. 1). This is to be expected given the constant biomass specific growth rate and nearly constant  $q_p$ . Figure 1 also shows that the cytosolic NADPH was consumed mostly for the synthesis of amino acids. The cost for the fatty acid synthesis was only 5.5 mmol/Cmol/h in our experiments. Note that the production of 1 mol penicillin-G cost 5–8 mol cytosolic NADPH (Kleijn et al. 2007). Independent of the cysteine synthesis route, the maximum possible NADPH demand for penicillin-G production is less than 25% of the total. Given that the flux through the histidine synthesis pathway was only 0.16 mmol/Cmol/h at the studied growth rate (Fig. 1), the originally false cofactor assignment in this pathway therefore resulted in a very limited influence on the total cytosolic NADPH demand in our experiments. For the same reason, the uncertain cofactor specificities in r11.3 ( $0.74 \pm 0.02$  mmol/Cmol/h) hardly affect the total cytosolic NADPH requirement. In short, the analysis above showed that the stoichiometric model of van Gulik et al. (2000) can make reasonably well estimations on the cytosolic NADPH requirements.

#### Mass isotopomer distribution dynamics and oxPPP flux

The MID of intracellular g6p, 6pg, and glnic in the three experiments were measured at timed intervals after switching to the labeled medium. The results



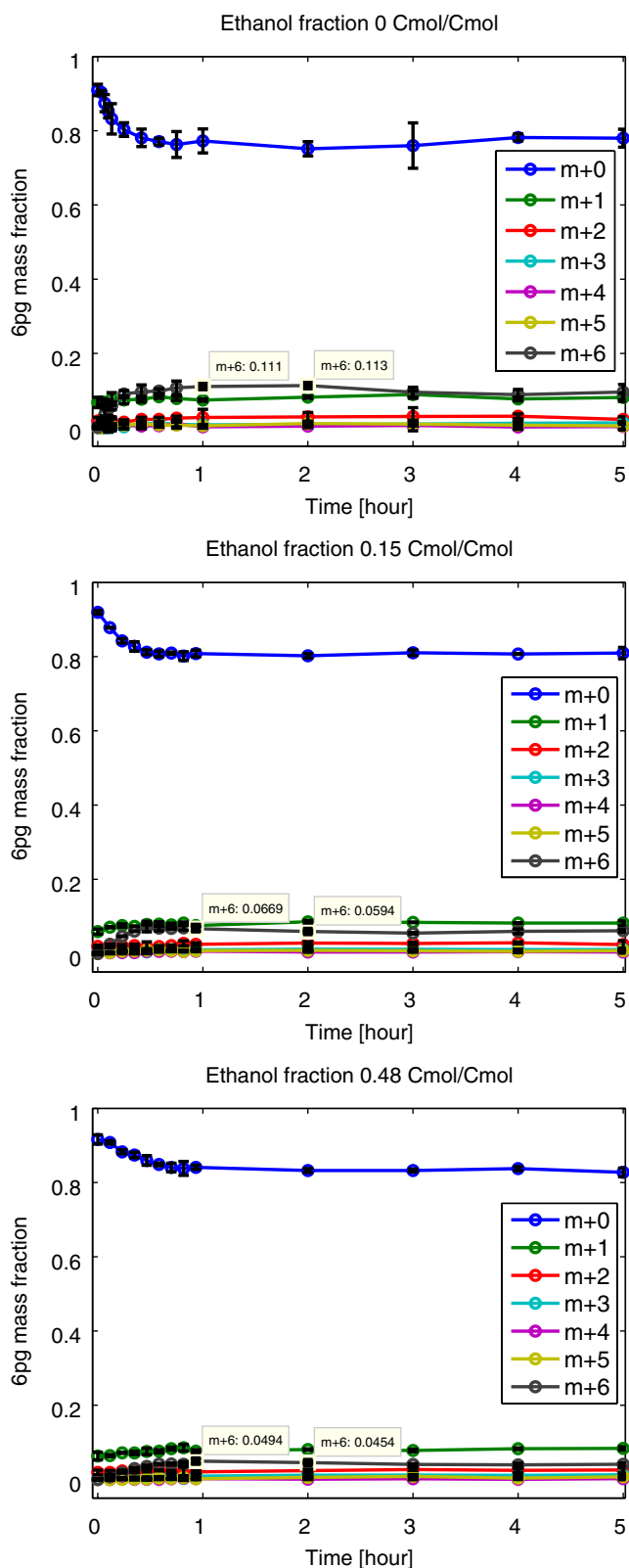
**Fig. 1** Cytosolic NADPH fluxes at different ethanol fractions under different assumptions on cysteine synthesis routes. *TS* The profile of NADPH-consuming reactions under the assumption that cysteine is exclusively synthesized by the transsulfuration pathway. *DS* The profile of NADPH-consuming reactions under the assumption that cysteine is exclusively synthesized by the direct sulfhydrylation pathway. *oxPPP* The NADPH produced by the oxidative branch of PP pathway, based on the flux determined by the <sup>13</sup>C flux analysis result. For the meaning of the reaction names other than oxPPP in the legend, please see Table 1

of 6pg carbon MID in all experiments are shown in Fig. 2. In all experiments, the isotopic steady states were reached within 1 h. The uptake rates of the fully labeled gluconate tracer were much lower than the oxPPP flux originated from unlabeled g6p in all three experiments. As a result, the majority of the 6pg pools were unlabeled (>80%). The isotopic steady-state level of ( $m + 6$ ) mass fraction decreased as the ethanol fraction increased. This reflected a decrease of the ratio between the gluconate tracer uptake rate and the oxPPP flux. Using the method described by Zhao et al. (2008), the oxPPP fluxes were calculated (see Fig. 3) based on the dynamic mass isotopomer data in these experiments (the data obtained in the first hour). The oxPPP fluxes were found to be essentially constant among cultures grown on pure glucose, 15% ethanol and 48% ethanol. This flux satisfied more than 85% of the cytosolic NADPH demand (see Fig. 1).

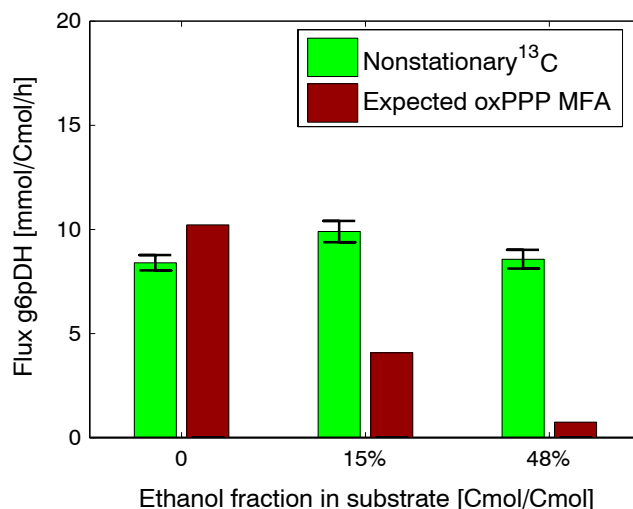
#### Enzymatic activity analysis

The enzyme activity of ALDH in the glucose-limited culture was found to be negligible in *P. chrysogenum* (Harris et al. 2006). However, the ALDH activities with both cofactors ( $\text{NAD}^+$  and  $\text{NADP}^+$ ) were found in the crude cell extracts of the cultures grown on substrates mixed with ethanol (0.15 and 0.48 Cmol/Cmol; see Fig. 4). These activities show that the cells are

<sup>1</sup>At a  $P$  value  $\leq 5\%$ , the validity of the reconciliation is rejected.

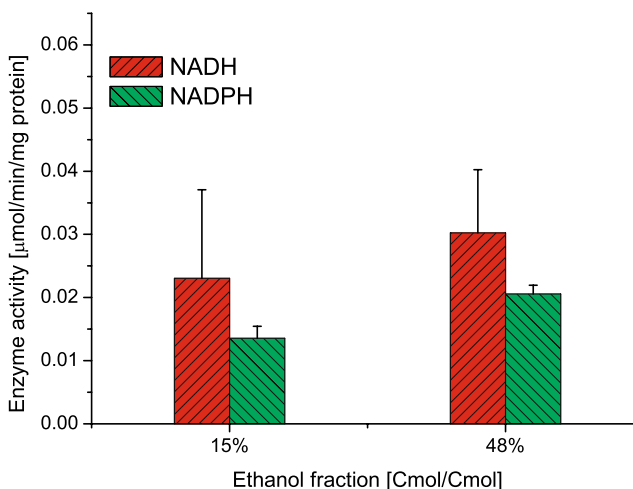


**Fig. 2** Mass distribution of intracellular 6pg during the labeling experiment, *error bar* is the calculated 95% confidence interval



**Fig. 3** Comparison between measured oxPPP flux using  $^{13}\text{C}$  data and the expected oxPPP flux by MFA assuming a fully  $\text{NADP}^+$ -dependent ALDH

already able to oxidize acetaldehyde into acetate at 0.15 Cmol/Cmol ethanol, with the help of either co-factor. Under the applied in vitro conditions,  $\text{NAD}^+$  was found to be slightly preferred over  $\text{NADP}^+$  in both ethanol fractions. However, the preference was not significant. Note that the enzyme activity was expressed per unit amount of extracted protein. In both experiments (15% and 48% ethanol fraction), the extracted protein concentrations in the sample were 3.0 g/L, which means 15 mg of extracted protein per sample because the sample volume was 5 mL. Given that the biomass amount was approximately 60 mg, the extracted protein weight fraction in biomass was 0.25.



**Fig. 4** Measured in vitro aldehyde dehydrogenase activity on different cofactors

## Discussion

### Isotopic non-stationary gluconate tracer method

The isotopic non-stationary gluconate tracer method used is suitable for this study not only because of the short time frame needed and the independence of the assumptions such as reversibility and amino acid synthesis routes but also because it was shown that the precision of the determined oxPPP is higher when compared with methods using only labeled glucose (Kleijn et al. 2006). It is, however, important to be able to accurately quantify the gluconate uptake rate when using this approach, as the labeling data essentially delivered information on the ratio of the oxPPP and the gluconate uptake flux. To minimize the disturbance to the metabolism introduced by gluconate uptake, the ratio of the gluconate uptake rate to the glucose uptake rate was fixed at 1:19. Kleijn et al. (2006) showed that at this ratio, neither the extracellular rates such as growth rate and penicillin production rate nor the intracellular metabolite concentrations were changed. When the ethanol fraction in the substrate mixture increases beyond 48%, accurate quantification of the gluconate uptake rate can become difficult, due to the diminishing glucose uptake rate and the fixed ratio of gluconate/glucose uptake. This method is therefore suitable only for the experiments with high glucose/gluconate uptake rates.

### Cytosolic NADPH balance

The enzyme assays carried out by Harris et al. (2006) showed that cytosolic NADPH can only be produced by the oxPPP pathway and the isocitrate dehydrogenase (IDH), in a glucose-limited culture. In the metabolic model of *P. chrysogenum* (van Gulik et al. 2000), the ALDH reaction was assumed to produce cytosolic NADPH based on the knowledge of other fungi (Grabowska and Chelstowska 2003; Bruinenberg et al. 1983). In this assumption, the oxidation of acetaldehyde to acetate alone can provide sufficient NADPH for the cell when the ethanol uptake rate is greater than 21 mmol/Cmol/h. In order to keep the NADPH balance in the cytosol, the other major cytosolic NADPH producing flux oxPPP would have to be shut down (van Gulik et al. 2000; Kiefer et al. 2004; Kleijn et al. 2007). However, this hypothesis about NADPH production from ALDH (see Fig. 3: calculated oxPPP flux with NADP<sup>+</sup>-ALDH assumption) is clearly contradicted by the oxPPP flux determined based on our present <sup>13</sup>C flux analysis results. This result indicates that the cytosolic ALDH of *P. chrysogenum* is not NADP<sup>+</sup> de-

pendent. Given the fact that both the cytosolic NADPH demand and NADPH production from the oxPPP are nearly constant in the studied ethanol fraction range, the ALDH contribution to the cytosolic NADPH must be negligible (for 15% and 48% ethanol).

It should be noted that the flux through the cytosolic NADP<sup>+</sup>-dependent isocitrate dehydrogenase (NADP<sup>+</sup>-IDH) is not constrained by the cytosolic NADPH balance. The NADP<sup>+</sup>-IDH flux is used to provide the carbon precursor for glutamate, glutamine, proline, and lysine. The production rates of these amino acids are determined by the growth rate and biomass composition. In our experiments, these rates were maintained constant. Therefore, the cytosolic NADP<sup>+</sup>-IDH flux is also fixed. This conclusion is consistent with the results of the experiments conducted by Bruinenberg et al. (1983), in which the oxPPP was then left as the only flux that has the flexibility to balance the cytosolic NADPH pool.

### Additional NADPH source

Besides the cytosolic NADPH sources discussed above, malic enzyme activity is also a potential NADPH source and was found in filamentous fungi (Wynn and Ratledge 1997). In a related work, David et al. (2006) observed that many of the genes of the PP pathway are downregulated for *Aspergillus nidulans* grown on pure ethanol compared with a glucose culture. They suggested that malic enzyme would replace the role for NADPH supply of PP pathway in a pure ethanol culture. It should be noted that the PP pathway resides in cytosol, while the malic enzyme is known to exist either in mitochondria (in *S. cerevisiae*, Boles et al. 1998) or in peroxisome (in *A. nidulans* and *P. chrysogenum*, Kiel et al. 2009). A NADPH shuttle system, such as using IDH would be required. Recent research has shown that IDH can be found in both the cytosol (Harris et al. 2006) and peroxisome (Kiel et al. 2009) of *P. chrysogenum*. These findings suggest that it would be possible that the proposed NADPH shuttle is active. However, according to our <sup>13</sup>C flux analysis results, the oxPPP flux remained largely constant up to 48% ethanol. This indicates that, at least up to this fraction of ethanol, the oxPPP is still the major cytosolic NADPH source in *P. chrysogenum* rather than the malic enzyme. Recently, we analyzed the activity of the malic enzyme for *P. chrysogenum* culture grown in a ethanol-limited chemostat, according to the method described by Harris et al. (2006). No activity could be found beyond the detection limit. This further confirms that the role of malic enzyme in cytosolic NADPH regeneration is

very limited for *P. chrysogenum*, despite the fact that it is closely related to *A. nidulans*.

#### In vitro vs. in vivo aldehyde dehydrogenase activity

When the results of the calculated in vivo (after converting the unit from flux to activity) and the in vitro activities (see Fig. 4) are compared, it can be seen that the in vitro activities of both experiments (15% and 48% ethanol of feed) were comparable or less than the in vivo flux, which were 0.021 and 0.072  $\mu\text{mol}/\text{mg}/\text{min}$ , respectively (for the unit conversion from millimoles per C mole per hour to micromoles per milligram per minute, see the “Materials and methods” section). The main purpose of the in vitro aldehyde dehydrogenase activity assay was to show the cofactor specificity. Even though  $\text{NAD}^+$  was found to be slightly preferred, it is worthwhile to note that the results were only valid qualitatively. Large uncertainties exist in the analysis of the assay with  $\text{NAD}^+$  (see Fig. 4), compared with other enzyme assays for *P. chrysogenum* made by Harris et al. (2006). These uncertainties originated from the enzyme instability during sample storage and the assay. Nevertheless, the results were sufficient to show that the  $\text{NADP}^+$  activity was not significantly higher than that of the  $\text{NAD}^+$ .

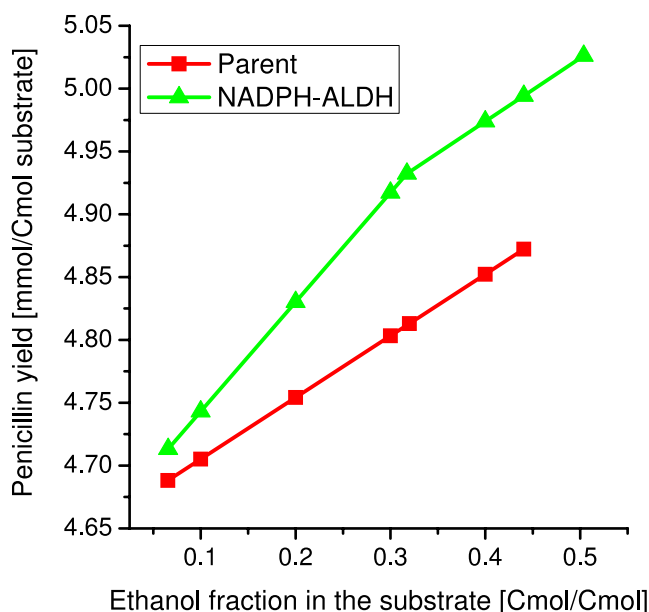
From a thermodynamic point of view, under in vivo condition, the feasibility of the reaction is determined by the ratio of product and substrate, in this case the cytosolic  $\text{NAD}^+/\text{NADH}$  and  $\text{NADP}^+/\text{NADPH}$ . Recently, Canelas et al. (2008) showed that direct measurements of the whole cell average concentrations of these cofactors will result in distorted estimations on their cytosolic ratios. This is mostly due to the presence of the protein-bound  $\text{NADH}$  (Bücher et al. 1972) and unknown distributions of these cofactors over the cytosolic and mitochondrial compartment. Therefore, those cytosolic cofactor ratios cannot be obtained from measurements of their cell averaged concentrations. From various studies using indicator reactions, it was shown that over a wide range of kingdoms, the ratio between cytosolic ( $\text{NAD}^+/\text{NADH}$ ) and ( $\text{NADP}^+/\text{NADPH}$ ) are in the order of 100 (Bautista et al. 1979; Satrustegui et al. 1983; Greenbaum et al. 1971; Canelas et al. 2008). These observations were further reinforced by the analysis results of Henry et al. (2007), who showed the thermodynamic feasible minimal ratio of  $\text{NAD}^+/\text{NADH}$  and maximum ratio  $\text{NADP}^+/\text{NADPH}$ . Consequently, under in vivo conditions, the ALDH reaction proceeding with the  $\text{NAD}^+$  bares much greater thermodynamic driving force than its phosphorylated counterpart. This is in line with our

findings in this study based on the  $^{13}\text{C}$  flux analysis and cytosolic  $\text{NADPH}$  balance.

Some  $\text{NADP}^+$ -dependent reactions can be reversed under certain physiological conditions, so that a normal  $\text{NADPH}$  producing reaction would be changed to  $\text{NADPH}$ -consuming reaction. As demonstrated by Yoo et al. (2008), this can happen to IDH when cofeeding glucose and glutamine. However, as the TCA cycle is running in positive direction in this study, it is unlikely that the IDH reaction can reverse. Therefore, the cytosolic  $\text{NADPH}$  balance discussed above should still hold.

#### Impact of ALDH cofactor change on the penicillin yield

As discussed above, the in vivo cytosolic ALDH in *P. chrysogenum* is found to be  $\text{NAD}^+$  dependent. An  $\text{NADP}^+$ -dependent ALDH would gradually reduce the need for oxPPP flux as the ethanol uptake flux increases. Genetically changing the cofactor dependency of ALDH to exclusive  $\text{NADP}^+$  and cofeeding ethanol to the fermentation could thus result in higher penicillin production. This can be achieved by either protein engineering (Liang et al. 2007) or by the replacement of an ALDH with only  $\text{NADP}^+$  dependency (Estey et al. 2007). As an example, for a defined growth rate  $0.05\text{ h}^{-1}$  and a biomass-specific penicillin production rate of  $0.5\text{ mmol}/\text{Cmol}/\text{h}$ , product yield on substrate



**Fig. 5** Comparing the simulated yields of penicillin-G on the mixed substrate of *P. chrysogenum* with different cofactors of ALDH at different ethanol fractions



can be compared for two different ALDH (NAD<sup>+</sup> or NADP<sup>+</sup>) under different glucose/ethanol ratios (see Fig. 5). It is clear that modifying ALDH cofactor specificity would result in an increase of up to 2% in the penicillin yield on substrates. However, this can only benefit the overall economics of penicillin production when ethanol price drops closer to glucose.

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