PUBLICATION II

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In: Microbiology 2007. Vol. 153, pp. 281–290.

Microbiology (2007), **153**, 281–290 DOI 10.1099/mic.0.29263-0

Metabolic flux profiling of *Pichia pastoris* grown on glycerol/methanol mixtures in chemostat cultures at low and high dilution rates

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Received 29 June 2006
Revised 19 September 2006
Accepted 9 October 2006

The metabolic pathways associated with the tricarboxylic acid cycle intermediates of *Pichia pastoris* were studied using biosynthetically directed fractional ^{13}C labelling. Cells were grown aerobically in a chemostat culture fed at two dilution rates (1.39×10^{-5} s^{-1} and 4.44×10^{-5} s^{-1}) with varying mixtures of glycerol and methanol as sole carbon sources. The results show that, with co-assimilation of methanol, the common amino acids are synthesized as in *P. pastoris* cells grown on glycerol only. During growth at the lower dilution rate, when both substrates are entirely consumed, the incorporation of methanol into the biomass increases as the methanol fraction in the feed is increased. Moreover, the co-assimilation of methanol impacts on how key intermediates of the pentose phosphate pathway (PPP) are synthesized. In contrast, such an impact on the PPP is not observed at the higher dilution rate, where methanol is only partially consumed. This finding possibly indicates that the distribution of methanol carbon into assimilatory and dissimilatory (direct oxidation to CO_2) pathways are different at the two dilution rates. Remarkably, distinct flux ratios were registered at each of the two growth rates, while the dependency of the flux ratios on the varying fraction of methanol in the medium was much less pronounced. This study brings new insights into the complex regulation of *P. pastoris* methanol metabolism in the presence of a second carbon source, revealing important implications for biotechnological applications.

INTRODUCTION

The methylotrophic yeast *Pichia pastoris* has emerged as an important production host for both industrial protein production and basic research, including structural genomics (Lin Cereghino & Cregg, 2000; Lin Cereghino *et al.*, 2002; Yokoyama, 2003; Prinz *et al.*, 2004). However, progress in strain improvement and rational design and optimization of culture conditions for heterologous protein production in *P. pastoris* is currently hampered by the limited number of systematic metabolic and physiological characterization studies under bioprocess-relevant conditions (Sauer *et al.*, 2004; Solà *et al.*, 2004). Information on heterologous gene expression and production of proteins under different physiological states of the cells is scarce. Furthermore, very little information is available on the cellular responses to protein production in *P. pastoris* (Hohenblum *et al.*, 2004). Importantly, the *P. pastoris* genome has been deciphered (see www.integratedgenomics.com), offering innumerable possibilities to pursue coordinated understanding of cellular processes in the framework of systems biology.

P. pastoris has been developed as an expression platform using elements that include strong inducible promoters derived from genes of the methanol utilization pathway, which is compartmentalized in the peroxisomes (Harder & Veenhuis, 1989). During growth on methanol, several key enzymes, e.g. alcohol oxidase, catalase, formaldehyde

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Abbreviations: [^{13}C,^{1}H]-COSY, [^{13}C,^{1}H] correlation NMR spectroscopy; BDF, biosynthetically directed fractional; cyt, cytosolic; GCV, glycine cleavage pathway; mt, mitochondrial; OAA, oxaloacetate; PPP, pentose phosphate pathway; SHMT, serine hydroxymethyltransferase; TCA, tricarboxylic acid.

Tables of *f*-values are available as supplementary data with the online version of this paper.

dehydrogenase and dihydroxyacetone synthase, are present in high amounts and peroxisomes proliferate. The synthesis of these enzymes is regulated at the transcriptional level of the respective genes. Methanol assimilation is subject to a carbon-source-dependent repression/derepression/induction mechanism; it is rather strongly repressed by multicarbon sources such as glucose and glycerol, but highly induced by methanol. Importantly, co-assimilation of a multicarbon source and methanol can be triggered under certain growth conditions (Egli *et al.*, 1982).

Although pathways of methanol metabolism are essentially analogous for all methylotrophic yeasts, important variations do exist with respect to their regulation (Harder & Veenhuis, 1989). It is, for example, well documented that during aerobic growth of different species (e.g. *Hansenula polymorpha*), partial catabolite repression of methanol metabolism specific enzymes may occur, i.e. allowing expression of these enzymes to quite significant levels. Furthermore, control by catabolite repression by different multicarbon compounds in some strains is tighter than in others, while methanol may have a small or a significant inducing effect. In *P. pastoris*, high-level induction of methanol metabolism enzymes is strongly dependent on methanol, i.e. partial catabolite repression of methanol-metabolism-specific enzymes only occurs at a much reduced level. Implications of the regulation of methanol metabolism for central carbon metabolism in *P. pastoris* growing on mixtures of methanol plus a multicarbon source are essentially unexplored. Moreover, most of the comprehensive investigations of methanol mixed carbon metabolism have so far been pursued only for other methylotrophic yeasts (e.g. *H. polymorpha*, *Kloeckera*, *Candida boidinii*; for a review see Harder & Veenhuis, 1989). In this context, the level of protein expression in *P. pastoris* depends critically on the growth conditions, and the attainment of high cell densities has been shown to improve protein yields substantially (Stratton *et al.*, 1998). Although production of recombinant proteins under such culture conditions is typically induced by methanol, which activates the *aox-1* promoter controlling the heterologous gene, feeding mixtures of glycerol (or other multicarbon sources) to the culture has also been successfully used as a means for improving process productivities (for a review see Cos *et al.*, 2006). In view of the outstanding role of *P. pastoris* for biotechnology research, this organism represents an obvious target for studies of its metabolism and physiology.

Stable isotope labelling experiments employed in conjunction with NMR spectroscopy and/or mass spectrometry (Szyperski, 1998) are a powerful tool for metabolic studies. In particular, biosynthetically directed fractional (BDF) ^{13}C labelling of proteinogenic amino acids has been developed into a cost-effective approach to assess the topology of active bioreactions (i.e. active pathways) and to quantify metabolic flux ratios (Szyperski, 1995). BDF labelling has been applied to study central carbon metabolism of eubacteria (Szyperski, 1995; Sauer *et al.*, 1997, 1999) as well as eukaryotic yeast cells (Maaheimo *et al.*, 2001; Fiaux *et al.*, 2003; Solà *et al.*, 2004) growing on glucose or glycerol.

Recently, we have established BDF ^{13}C labelling and metabolic flux ratio formalism (Szyperski, 1995; Maaheimo *et al.*, 2001) as an analytical tool to study intermediary carbon metabolism of *P. pastoris* cells growing on glycerol as sole carbon source in chemostat cultures (Solà *et al.*, 2004). This investigation allowed accurate mapping of the metabolic state of the tricarboxylic acid (TCA) cycle and associated pathways, thus providing a valuable methodological basis for the analysis of *P. pastoris* cells growing on mixtures of glycerol and methanol, which is described in the present study. In addition, here we have applied the metabolic flux ratio formalism for yeast growing on a single carbon source (Maaheimo *et al.*, 2001) to the case of two-carbon-source co-assimilation.

METHODS

Strain and media. A prototrophic *P. pastoris* strain expressing a heterologous protein – a *Rhizopus oryzae* lipase (ROL) – under the transcriptional control of the *aox-1* promoter was chosen for metabolic flux ratio profiling. *P. pastoris* X-33/pPICZαA-ROL (Minning *et al.*, 2001) is the wild-type phenotype X-33 strain (Invitrogen) with the pPICZαA-derived expression vector (Invitrogen) containing the *ROL* gene, pPICZαA-ROL, integrated in its *aox-1* locus. Chemostat cultures were fed with a defined minimal medium containing (per 1×10^{-3} m^3 of deionized water): Yeast Nitrogen Base (YNB; Difco), 0.17×10^{-3} kg; $(NH_4)_2SO_4$, 5×10^{-3} kg; glycerol and methanol (different ratios on w/w basis), 10×10^{-3} kg (total); Antifoam Mazu DF7960 (Mazer Chemicals, PPG Industries), 0.1×10^{-6} m^3. The YNB components and methanol were sterilized separately by microfiltration and then added to the bioreactor. The medium used for starter cultures was YPD medium containing 1 % (w/v) yeast extract, 2 % (w/v) peptone, 2 % (w/v) glucose.

Chemostat cultures. Continuous cultures were carried out at a working volume of 0.8×10^{-3} m^3 in a 1.5×10^{-3} m^3 bench-top bioreactor (BiofloIII; New Brunswick) at 30 °C and with a minimum dissolved oxygen tension of 30 %. Simultaneous cultures using glycerol and methanol in different proportions as carbon source were performed at two different dilution rates, D (defined as volumetric flow rate/working volume) of 1.39×10^{-5} s^{-1} and 4.44×10^{-5} s^{-1}. These values are just below the maximum specific growth rate, μ_{max}, of *P. pastoris* cells growing on an excess of methanol or glycerol, 1.94×10^{-5} and 4.72×10^{-5} s^{-1}, respectively (Solà, 2004). Medium feeding was controlled by a Masterflex pump (Cole-Parmer). The working volume was kept constant by removal of effluent from the centre of the culture volume by use of a peristaltic pump (B. Braun Biotech). The pH of the culture was maintained at 5.5 by addition of 1 M KOH and the airflow was maintained at 0.16×10^{-4} m^3 s^{-1} with filter-sterilized air using a mass flow controller (Brooks Instruments). The agitation speed was set to 500 r.p.m. Starter cultures (1×10^{-4} m^3) were grown in 1 l baffled shake flasks at 200 r.p.m. at 30 °C for 8.64×10^4 s. Cells were harvested by centrifugation and resuspended in fresh medium prior to the inoculation of the bioreactor. The culture was initially run in batch mode to grow cells until the late exponential growth phase and then switched to continuous operational mode.

Analytical procedures. Cell biomass was monitored by measuring OD_{600}. For cellular dry weight, a known volume of culture broth was filtered using pre-weighed filters; these were washed with 2 vols

distilled water and dried to constant weight at 105 °C for 8.64×10^4 s. Samples for extracellular metabolite analyses were centrifuged at 6000 r.p.m. for 120 s in a microcentrifuge to remove the cells. Glycerol, acetic acid and ethanol were analysed by HPLC as described by Solà *et al.* (2004). Methanol was measured by GC as described by Minning *et al.* (2001). The exhaust gas of the bioreactor was cooled in a condenser at 2–4 °C (Frigomix R; B. Braun Biotech) and dried through a silica gel column. Concentrations of oxygen and CO_2 in the exhaust gas of bioreactor cultures were determined on line with a mass spectrometer (Omnistar; Balzers Instruments).

BDF ^{13}C labelling. *P. pastoris* cells were fed with a minimal medium containing 10 kg different glycerol/methanol mixtures m^{-3} (8 : 2, 6 : 4 and 4 : 6, w/w) for five volume changes to reach a metabolic steady-state, as indicated by a constant cell density and constant oxygen and CO_2 concentrations in the bioreactor exhaust gas. BDF ^{13}C labelling of cells growing at steady-state on a single carbon source has been described elsewhere (Sauer *et al.*, 1997; Fiaux *et al.*, 2003; Solà *et al.*, 2004); essentially, it is achieved by feeding the reactor with medium containing about 10 % (w/w) of uniformly ^{13}C-labelled and 90 % unlabelled substrate for one volume change. In this study, where two carbon sources (namely glycerol and methanol) were used simultaneously, the BDF ^{13}C labelling step involved feeding the reactor with medium containing about 10 % (w/w) uniformly ^{13}C-labelled and 90 % unlabelled amounts of each substrate simultaneously for one volume change. Uniformly ^{13}C-labelled glycerol (isotopic enrichment of >98 %) was purchased from Martek Biosciences or Spectra Stable Isotopes. ^{13}C-labelled methanol (isotopic enrichment of 99 %) from Cambridge Isotope Laboratories was purchased from Euriso-top. Cells were then harvested by centrifugation at 4000 ***g*** for 600 s, resuspended in 2×10^{-2} M Tris/HCl (pH 7.6) and centrifuged again. Finally, the washed cell pellets were lyophilized (Benchtop 5L Virtis Sentry), of which 2×10^{-4} kg were resuspended in 3×10^{-6} m^3 of 2×10^{-2} M Tris/HCl (pH 7.6). After addition of 6×10^{-6} m^3 6 M HCl, the biomass was hydrolysed in sealed glass tubes at 110 °C for 8.64×10^4 s, the solutions were filtered using 0.2 μm filters (Millex-GP; Millipore) and lyophilized.

NMR spectroscopy and data analysis. The lyophilized hydrolysates were dissolved in 0.1 M DCl in D_2O and two-dimensional (2D) [^{13}C,^{1}H] correlation NMR spectroscopy (COSY) spectra were acquired for both aliphatic and aromatic resonances as described previously (Szyperski, 1995) at 40 °C on a Varian Inova spectrometer operating at a ^{1}H resonance frequency of 600 MHz. The spectra were processed using standard Varian spectrometer software VNMR (version 6.1, C). The program FCAL (R. W. Glaser; FCAL 2.3.1) (Szyperski *et al.*, 1999) was used for the integration of ^{13}C–^{13}C scalar fine structures in 2D [^{13}C,^{1}H]-COSY, for the calculation of relative abundances, *f*-values, of intact carbon fragments arising from a single carbon source molecule (Szyperski, 1995), and for the calculation of the resulting flux ratios through several key pathways in central metabolism (Szyperski, 1995; Maaheimo *et al.*, 2001). The probabilistic equations relating the ^{13}C fine structures to *f*-values can be readily applied to this case of two simultaneous carbon sources. This is because, as a C_1-compound, methanol does not introduce contiguous multiple-carbon fragments to the metabolism and, therefore, all contiguous $^{13}C_n$ ($n>1$) fragments must originate from glycerol. Since the probabilistic equations for calculating the flux ratios depend on a uniform degree of ^{13}C labelling, both glycerol and methanol were supplied with the same fraction of uniformly ^{13}C-labelled molecules.

As described previously (Szyperski, 1995, 1998; Sauer *et al.*, 1997, 1999; Szyperski *et al.*, 1999; Maaheimo *et al.*, 2001; Fiaux *et al.*, 2003; Solà *et al.*, 2004), the calculation of metabolic flux ratios when using fractional ^{13}C labelling of amino acids is based on assuming both a metabolic (see above) and an isotopomeric steady-state. To establish a cost-effective protocol for a larger number of ^{13}C labelling experiments, we fed a chemostat operating in metabolic steady-state for the duration of one volume change with the medium containing the ^{13}C-labelled substrates (Sauer *et al.*, 1997; Fiaux *et al.*, 2003) before harvesting the biomass. Then, the fraction of unlabelled biomass produced prior to the start of the supply with ^{13}C-labelled medium can be calculated following simple wash-out kinetics (Szyperski, 1998; see also Solà *et al.*, 2004 for additional discussion).

Measurement of the degree of ^{13}C enrichment in CO_2. For the determination of ^{13}C incorporation from ^{13}C-labelled methanol to CO_2, cells were first cultivated with unlabelled medium containing a given glycerol/methanol mixture as carbon source until steady-state was achieved, as described above. During one residence time at steady-state, the CO_2 produced was trapped by bubbling the outlet air through a tube containing 2×10^{-5} m^3 of 10 M KOH. The culture was then fed with medium containing about 50 % (w/w) uniformly ^{13}C-labelled and 50 % unlabelled methanol plus unlabelled glycerol at the same ratio as in the unlabelled medium for one volume change. The $^{13}CO_2$ produced was trapped by bubbling the outlet air through a tube containing 2×10^{-5} m^3 of 10 M KOH for the period of one residence.

The ^{13}C content of carbonate anions in culture off-gas samples was measured by ^{13}C NMR spectroscopy on a Bruker 500 Avance spectrometer using a cryoprobe to improve the signal to noise ratio. Samples were prepared by mixing 0.2×10^{-6} m^3 of the corresponding 10 M KOH solution with 0.2×10^{-6} m^3 of a 1 M solution of dioxane (internal standard for both calibration and integration) in D_2O. ^{13}C NMR spectra were obtained at 125 MHz for each sample under Waltz-16 proton decoupling, using a 31 450 Hz (over 250 p.p.m.) sweep width, with a 30 degree ^{13}C pulse and a relaxation delay of 1.0 s. After accumulation to a good signal to noise ratio, the flame ionization detectors were weighted with a 1.0 Hz line broadening function and Fourier transformed. The resulting spectra showed peaks at 166.6 p.p.m. (carbonate anion) and 66.9 p.p.m. (dioxane), which were integrated. ^{13}C incorporated into CO_2 was estimated by comparing the ^{13}C content in carbonate anions in corresponding unlabelled and labelled samples.

Biochemical reaction network model for *P. pastoris*. The biochemical reaction network model for data interpretation was the one recently identified for *Saccharomyces cerevisiae* (Maaheimo *et al.*, 2001; Fiaux *et al.*, 2003), which was also shown to be suitable for *Pichia stipitis* (Fiaux *et al.*, 2003) and *P. pastoris* (Solà *et al.*, 2004). Considering published data (Harder & Veenhuis, 1989), pathways for methanol metabolism were added (Fig. 1). Briefly, methanol is oxidized by an alcohol oxidase to generate formaldehyde, which is further oxidized to CO_2 or assimilated into carbohydrates. The first step in the formaldehyde assimilation pathway involves a dihydroxyacetone synthase, which catalyses the condensation of formaldehyde with xylulose 5-phosphate to form fructose 6-phosphate. The hydrogen peroxide formed in the initial oxidation of methanol is removed by the action of a catalase. These four enzymes are peroxisomal. Furthermore, methanol assimilation by yeasts is characteristically associated with the biogenesis of peroxisomes.

RESULTS AND DISCUSSION

P. pastoris cultures were performed at two dilution rates, 1.39×10^{-5} s^{-1} and 4.44×10^{-5} s^{-1}, in aerobic chemostats using mixtures of glycerol and methanol at different ratios as sole carbon sources. The lower dilution rate is slightly below the μ_{max} of the organism as observed previously in a batch culture on methanol (1.94×10^{-5} s^{-1}), i.e. where the

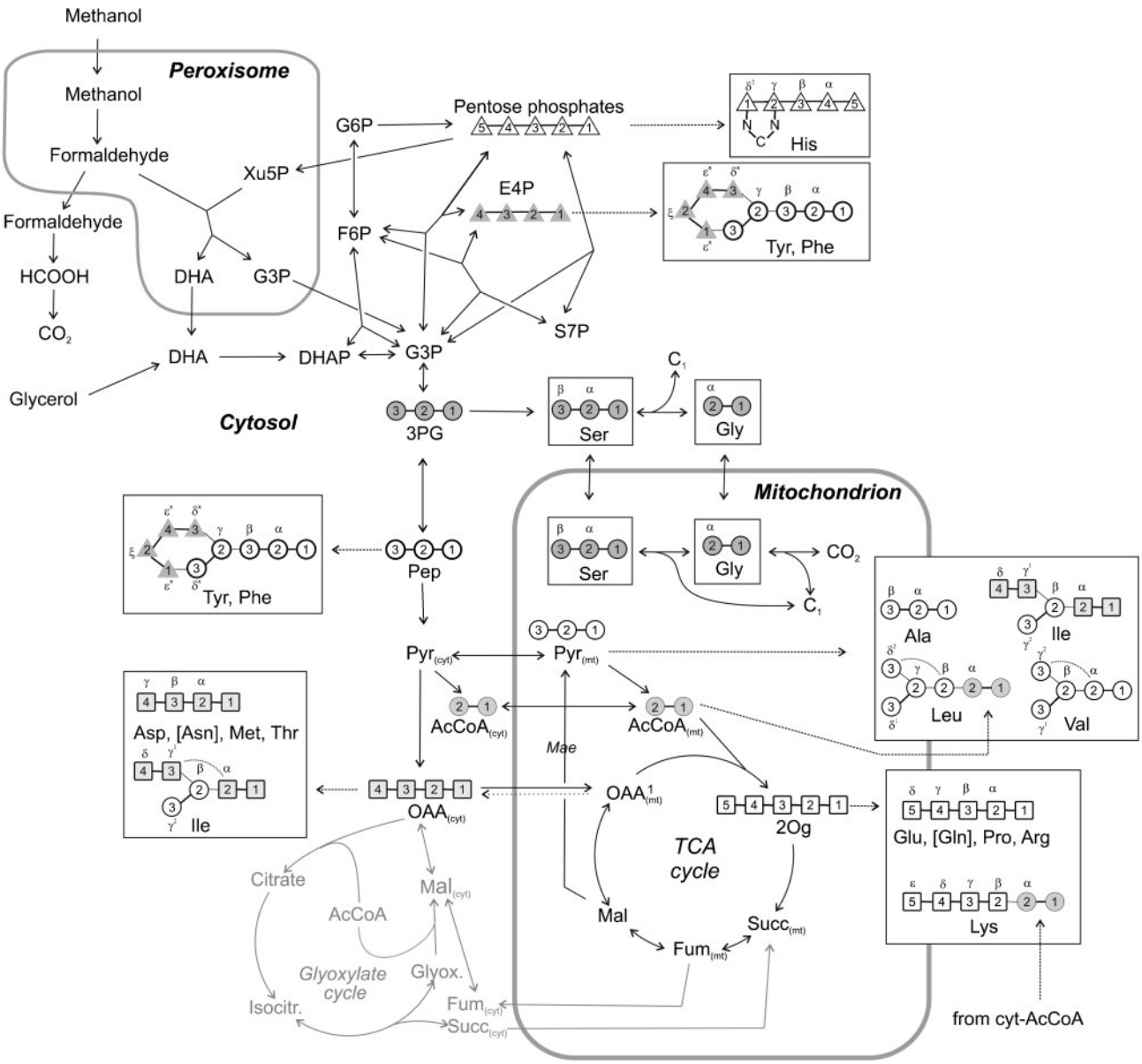

Fig. 1. Network of active biochemical pathways constructed for *P. pastoris* cells grown with glycerol and methanol as mixed carbon source. The network is based on those identified for *P. pastoris* growing on glucose or glycerol (Solà *et al.*, 2004) and on the literature on methanol metabolism of methylotrophic yeasts (Harder & Veenhuis, 1989; see text). The central carbon metabolism of *P. pastoris* is dissected into cytosolic and mitochondrial subnetworks. In addition, the reactions involved in the initial oxidation steps of methanol to formaldehyde (i.e. alcohol oxidase and catalase), the first reaction involved in formaldehyde fixation (i.e dihydroxyacetone synthase), as well as the glyoxylate cycle reactions are supposed to reside in peroxisomes in methylotrophic yeast like *P. pastoris*. Since the reactions of the glyoxylatecycle cannot be identified with the current ^{13}C labelling strategy (see text), its reactions are depicted in grey. Amino acids and carbon fragments originating from a single intermediate of central carbon metabolism are represented in the rectangular boxes. Thin lines between amino acid carbon atoms denote carbon bonds that are formed between fragments originating from different precursor molecules, while thick lines indicate intact carbon connectivities in fragments arising from a single precursor molecule. The carbon skeletons of glycolysis, TCA cycle and PPP intermediates are represented by circles, squares and triangles, respectively. The numbering of the carbon atoms refers to the corresponding atoms in the precursor molecule. Abbreviations: AcCoA, acetyl-Coenzyme A; DHA, dihydroxyacetone; DHAP, dihydroxyacetone phosphate; E4P, erythrose 4-phosphate; F6P, fructose 6-phosphate; Fum, fumarate; G6P, glucose 6-phosphate; Gly, glycine; Glyox., glyoxylate; G3P, glyceraldehyde 3-phosphate; 3PG, 3-phosphoglycerate; Mae, malic enzyme; Mal, malate; OAA, oxaloacetate; 2Og, 2-oxoglutarate; Pyr, pyruvate; Pep, phosphoenolpyruvate; S7P, sedoheptulose-7-phosphate; Ser, serine; Succ, succinate; Xu5P, xylulose 5-phosphate. For AcCoA, Fum, OAA, Pyr and Succ, cytosolic (cyt) and mitochondrial (mt) pools are indicated separately.

glycerol supply is growth-limiting. The higher dilution rate is slightly below the μ_{max} of the organism observed previously in a batch culture on glycerol (4.72×10^{-5} s^{-1}) (Table 1).

All chemostat cultures operated at $D = 1.39 \times 10^{-5}$ s^{-1} simultaneously utilized glycerol and methanol, indicating that glycerol repression of methanol consumption did not occur. In fact, the residual concentrations of glycerol and methanol in the culture media were below the detection limits of the respective assays.

At the higher rate of $D = 4.44 \times 10^{-5}$ s^{-1} with an 80 : 20 glycerol/methanol mixture, some residual glycerol (2.2 kg glycerol m^{-3}) accumulated in the growth medium and, concomitantly, very little methanol was consumed under these conditions. However, residual glycerol concentrations in the chemostat were very close to or below the detection limit when the glycerol/methanol ratio was decreased. Under such conditions, a significant fraction of the methanol was consumed by the cells, though the residual methanol concentration increased as the fraction of methanol increased. These results confirm that cells fed with mixtures of methanol and glycerol are able to utilize methanol at dilution rates considerably higher than μ_{max} in batch cultures grown on methanol as sole carbon source ($D = 1.94 \times 10^{-5}$ s^{-1}) (Zhang *et al.*, 2003). A similar substrate utilization pattern has been observed in *H. polymorpha* growing on different methanol/glucose mixtures (ranging from 0 : 100 to 100 : 0) and growth rates (Egli *et al.*, 1986). At low dilution rates both carbon sources were utilized simultaneously, but at higher dilution rates the cells increasingly accumulated methanol in the culture medium. The dilution rate at which the transition from glucose/methanol growth to glucose growth occurred (D_t) was strictly dependent on the composition of the methanol/glucose mixture in the feed, and D_t increased with decreasing proportions of methanol. Similarly, growth of *P. pastoris* at $D = 4.44 \times 10^{-5}$ s^{-1} is probably close to the upper limit of the specific growth rate at which the regulatory mechanism that determines the onset of repression of methanol-assimilating enzymes in cells growing on glycerol mixtures.

Notably, ethanol and acetate were not detected by HPLC in any of the cultures, and carbon balances closed within 5 %. Hence, *P. pastoris* cells, when growing under the experimental conditions described, used both glycerol and methanol entirely to generate biomass and CO_2. The observed biomass yields ($Y_{x/s}$) in these mixed-substrate cultures gave a reasonable fit with the predicted $Y_{x/s}$ calculated as the weighted mean of the growth yields on the two individual substrates (Table 1). These were calculated from an aerobic chemostat culture at $D = 1.39 \times 10^{-5}$ s^{-1} using methanol as sole carbon source [0.31 kg cell dry wt (kg glycerol)$^{-1}$; Solà, 2004] and from chemostat cultures at $D = 1.39 \times 10^{-5}$ s^{-1} and 4.44×10^{-5} s^{-1} using glycerol as sole carbon source [0.63 kg cell dry wt (kg methanol)$^{-1}$; Solà *et al.*, 2004]. An analogous pattern has been observed in chemostat cultures of *H. polymorpha* growing on different glucose/methanol mixtures (Egli *et al.*, 1986). Also, during growth at $D = 1.39 \times 10^{-5}$ s^{-1} and 4.44×10^{-5} s^{-1} both the specific methanol consumption rate (q_{met}) and specific CO_2 production rate (q_{CO_2}) increased proportionally as the glycerol/methanol ratio decreased. However, this does not necessarily imply that no change in distribution of methanol carbon into assimilatory and dissimilatory pathways took place because of the presence of the second growth substrate, glycerol. Metabolic flux ratio analyses were performed with hydrolysed biomass samples that were harvested from these chemostat cultures in physiological steady-state. 2D [^{13}C,^{1}H]-COSY data were analysed as described by Maaheimo *et al.* (2001), yielding the desired relative abundances (*f*-values) of intact carbon

Table 1. Growth parameters in steady-state chemostat cultures of *P. pastoris*

$Y_{x/s}$ represents the biomass yield, q_{glyc}, q_{gluc} and q_{O_2} are specific utilization rates, q_{CO_2} is the specific production rate, Glyc and Meth indicate glycerol and methanol, respectively, and RQ is the respiratory quotient. ND, Not determined.

Carbon source	Residual substrate concn (Glyc/Meth; kg m^{-3})	$Y_{x/s}$ (kg dry wt kg^{-1}]	q_{glyc}/q_{meth}	q_{CO_2} (mol kg^{-1} per 3600 s)	q_{O_2} (mol kg^{-1} per 3600 s)	RQ
$D = 1.39 \times 10^{-5}$ s^{-1}						
Glycerol	0.0/–	0.63	1.09/–	1.56	2.16	0.72
80 glycerol/20 methanol	0.0/0.09	0.51	0.95/0.63	1.70	2.70	0.63
60 glycerol/40 methanol	0.0/0.17	0.44	0.74/1.48	2.10	3.90	0.54
40 glycerol/60 methanol	0.0/0.09	0.44	0.57/2.33	2.21	4.85	0.46
$D = 4.44 \times 10^{-5}$ s^{-1}						
Glycerol	3.0/–	0.63	2.75/–	2.35	3.62	0.65
80 glycerol/20 methanol	2.2/1.8	0.65	ND	ND	ND	ND
60 glycerol/40 methanol	0.05/2.6	0.51	2.77/1.87	4.18	7.19	0.58
40 glycerol/60 methanol	0.0/3.9	0.53	2.23/2.73	3.60	7.20	0.50

fragments arising from a single source molecule of glycerol (Tables S1 and S2, available with the online version of this paper).

Biosynthesis of proteinogenic amino acids and C1 metabolism in *P. pastoris*

As expected, the *f*-values obtained for the mixed glycerol/methanol cultures (Tables S1 and S2, available with the online version of this paper) show that proteinogenic amino acids are primarily synthesized in *P. pastoris* according to the pathways documented for *S. cerevisiae* (Jones & Fink, 1982; Voet & Voet, 1995; Michal, 1998; Maaheimo *et al.*, 2001), and these have also been validated for *P. pastoris* cells growing on glucose and glycerol (Solà *et al.*, 2004). Remarkably, the fraction of methanol in the feed affects the pool of Ser molecules effected by the reversible cleavage by serine hydroxymethyltransferase (SHMT); about 40–43 % of Ser molecules are cleaved in glucose and glycerol cultures (Solà *et al.*, 2004), but this fraction changes in mixed glycerol/methanol cultures. For instance, it decreased to about 28 % in cells growing at $D = 1.39 \times 10^{-5}$ s^{-1} in the 80 : 20 and 40 : 60 glycerol/methanol mixtures (Table 2). For Gly synthesis, yeasts can cleave either Ser (via SHMT) or Thr (via threonine aldolase). Due to the near degeneracy of *f*-values, however, it is not possible to accurately determine the relative contribution of the two pathways, or to distinguish between cytosolic and mitochondrial SHMT activity (Solà *et al.*, 2004). In contrast to the SHMT pathway, the Thr cleavage reaction via threonine aldolase is, if present,

Table 2. Origins of metabolic intermediates during aerobic growth of *P. pastoris* in glycerol/methanol chemostat cultures

For comparison, corresponding data reported previously for *P. pastoris* growing on glycerol in chemostat aerobic cultures (Solà *et al.*, 2004) are given in the left-most column. Glyc and Meth indicate glycerol and methanol, respectively. PEP, Phosphoenolpyruvate; PYR, pyruvate.

Metabolite	Fraction of total pool (mean % ± SD)			
	Glyc*	80 Glyc/20 Meth	60 Glyc/40 Meth	40 Glyc/60 Meth
$D = 1.39 \times 10^{-5}$ s^{-1}				
Cytosol				
PEP from cyt-OAA (PEP carboxykinase reaction)	<3	<7	9±6	<11
cyt-OAA from cyt-PYR†	32±2	37±4	35±2	33±3
cyt-OAA reversibly converted to fumarate at least once (cytosolic or inter-compartmental exchange)	56±13	50±16	58±19	48±25
Mitochondria				
mt-PYR from malate (upper bound)	<4	<9	<16	<19
mt-PYR from malate (lower bound)	<3	<2	<4	<3
mt-OAA from PEP (anaplerotic supply of TCA cycle)	33±2	32±3	36±3	29±4
mt-OAA reversibly converted to fumarate at least once	65±14	52±17	59±15	50±20
C1 metabolism				
Ser from Gly and C1 unit	43±3	28±2	37±3	28±2
Gly from CO_2 and C1 unit	2±2	4±3	9±3	5±2
cyt-Gly from mt-Gly	<2	<4	<9	<5
$D = 4.44 \times 10^{-5}$ s^{-1}				
Cytosol				
PEP from cyt-OAA (PEP carboxykinase reaction)	<6	<21	<10	<11
cyt-OAA from cyt-PYR†	68±4	89±2	78±2	76±2
cyt-OAA reversibly converted to fumarate at least once (cytosolic or inter-compartmental exchange)	12±6	<5	11±5	<6
Mitochondria				
mt-PYR from malate (upper bound)	<11	<7	<6	0
mt-PYR from malate (lower bound)	<3	<2	<2	0
mt-OAA from PEP (anaplerotic supply of TCA cycle)	48±2	47±2	42±2	43±2
mt-OAA reversibly converted to fumarate at least once	61±14	55±8	52±12	53±12
C1 metabolism				
Ser from Gly and C1 unit	42±2	37±2	33±2	51±2
Gly from CO_2 and C1 unit	5±3	3±3	4±2	7±2
cyt-Gly from mt-Gly	<5	<3	<4	<7

*Data taken from Solà *et al.* (2004).

†Values assuming absence of cytosolic OAA from fumarate conversion.

irreversible in all cultures. This can be readily deduced from the fact that nearly identical *f*-values were obtained from Thr and Asp. Gly may also be synthesized from a C1 unit and CO_2 via the mitochondrial glycine cleavage (GCV) pathway. In contrast to previous studies with *S. cerevisiae* (Maaheimo *et al.*, 2001) and with *P. pastoris* growing on glucose (Solà *et al.*, 2004), we found no evidence for efflux of Gly which had been reversibly cleaved by the GCV into the cytosol in glycerol and glycerol/methanol cultures. Hence, it may be that either the mitochondrial GCV pathway is operating irreversibly, or Gly is not exported into the cytosol when cells are grown on glycerol. Only when cells are grown at $D=1.39\times10^{-5}$ s^{-1} with a 60 : 40 glycerol/methanol mixture do the differences observed in the labelling patterns of Gly Cα and Phe Cα indicate that the mitochondrial GVC is operative. In principle, yeasts can also synthesize Gly from TCA cycle intermediates via isocitrate lyase and the alanine/glyoxylate aminotransferase (Takada & Noguchi, 1985). However, our data suggest that the activity of the glyoxylate cycle is low (see below), so that this route for Gly synthesis is probably of minor importance, if it is active at all.

Comparative flux ratio profiling of *P. pastoris* growing on glycerol- and methanol-limited mixtures in chemostats

The use of the three-carbon source glycerol and methanol for BDF ^{13}C labelling of proteinogenic amino acids enabled the determination of the flux ratios for reactions associated with the TCA cycle (Table 2), while those related to glycolysis and the pentose phosphate pathway (PPP) could not be assessed (Solà *et al.*, 2004). This is because when labelled glycerol is metabolized through gluconeogenesis and oxidative PPP, labelling patterns that are sufficiently distinct from those generated when glycerol is channelled through the non-oxidative PPP are not produced. In fact, the only information that can be derived with respect to the operation of the PPP is obtained from the *f*-values of His Cβ (Tables S1 and S2, available with the online version of this paper). The *f*-values reveal the reversible activity of the transketolase and transaldolase reactions when *P. pastoris* is grown on glycerol. Also, important variations in the observed E4P and R5P labelling patterns can be detected as the methanol fraction in the feed is increased (Table 3), suggesting an increasing activity of the methanol assimilation pathway, which involves PPP intermediates (Fig. 1). The increasing contribution of methanol to biomass constituents is further confirmed by the observation that the fraction of intact PEP molecules (i.e. originating from a single glycerol molecule) sharply decreases as the methanol fraction in the feed is increased, whereas the fraction of PEP molecules with both ^{13}C-^{13}C bonds cleaved (i.e. exclusively originating from methanol) increases (Table 3). Nevertheless, the fraction of methanol carbons assimilated by the cells that enter central carbon metabolism in relation to the methanol carbons that are dissimilated directly into CO_2 cannot be determined.

Ample information for pathways associated with TCA cycle intermediates can be obtained (Fig. 2) and can be summarized as follows. During growth at $D=1.39\times10^{-5}$ s^{-1} one finds that (i) gluconeogenesis from cytosolic oxaloacetate (cyt-OAA) via phosphoenolpyruvate (PEP) carboxykinase is either not detected or active at very low levels; (ii) synthesis of mitochondrial pyruvate (mt-PYR) from malate via malic enzyme is not detected; (iii) the

Table 3. Labelling patterns of erythrose 4-phosphate (E4P) and phosphoenolpyruvate (PEP) metabolic intermediates during aerobic growth of *P. pastoris* in glycerol/methanol chemostat cultures

For comparison, corresponding data reported previously for *P. pastoris* growing on glycerol in chemostat aerobic cultures (Solà *et al.*, 2004) are given in the left-most column. Glyc and Meth indicate glycerol and methanol, respectively. RSP, ribose 5-phosphate.

Metabolite	Fraction of total pool (%)			
	Glyc*	80 Glyc/20 Meth	60 Glyc/40 Meth	40 Glyc/60 Meth
$D=1.39\times10^{-5}$ s^{-1}				
Cytosol				
E4P with intact C_2-C_3-C_4 bonds	44	30	28	19
R5P with intact C_3-C_4-C_5 bonds	96	72	61	46
R5P with cleaved C_1-C_2 bond (TA/TK activity)	55	70	78	90
PEP with intact C_1-C_2-C_3 bonds	96−93	70−67	63	49−48
$D=4.44\times10^{-5}$ s^{-1}				
Cytosol				
E4P with intact C_2-C_3-C_4 bonds	46	43	45	44
R5P with intact C_3-C_4-C_5 bonds	94	86	95	89
R5P with cleaved C_1-C_2 bond (TA/TK activity)	55	65	58	55
PEP with intact C_1-C_2-C_3 bonds	97−93	90−89	97	91

*Data from Solà *et al.* (2004).

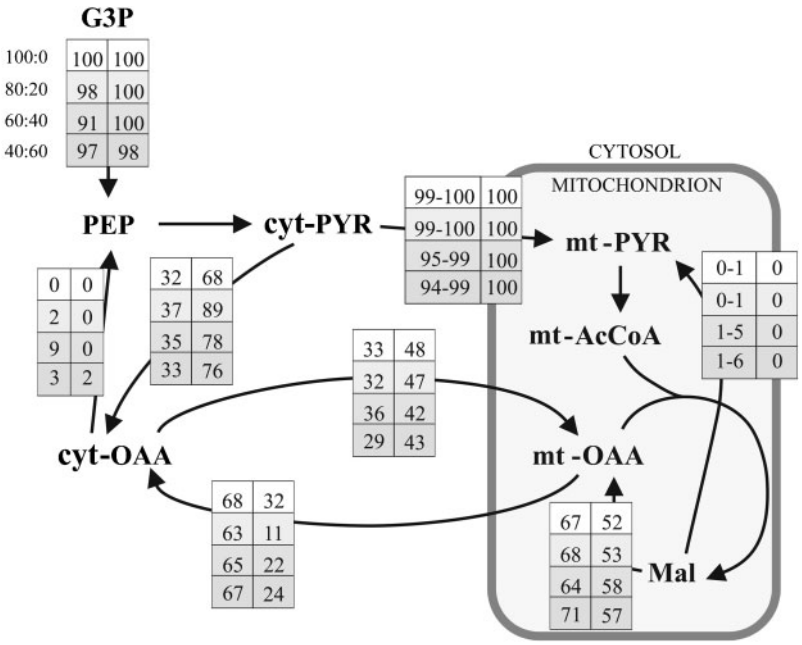

Fig. 2. Summary of flux information involving pools of TCA intermediates when *P. pastoris* cells are grown in a chemostat at $D = 1.39 \times 10^{-5}$ s^{-1} (left-hand values) and at $D = 4.44 \times 10^{-5}$ s^{-1} (right-hand values). The values in the boxes correspond from top to bottom, respectively, to the reference glycerol culture (data from Solà *et al.*, 2004), the 80 : 20, 60 : 40 and 40 : 60 mixed glycerol/methanol cultures. Note that values associated with arrows pointing at the same metabolite pool add up to 100 %. For abbreviations, see the legend to Fig. 1.

fraction of cyt-OAA that stems from the mitochondrial pool of C4 intermediates, e.g. via malate-Asp and/or malate-OAA shuttles (Bakker *et al.*, 2001), or possibly synthesized via the glyoxylate cycle, does not vary significantly as the fraction of methanol in the feed is increased; (iv) the fraction of mt-OAA reversibly interconverted to fumarate and the fraction of cyt-OAA reversibly interconverted to fumarate does not change significantly when the glycerol/methanol ratio is increased; (v) no significant alterations can be identified for the anaplerotic supply of the TCA cycle in cells growing with different glycerol/methanol ratios. The labelling patterns of cyt-OAA and mt-OAA molecules were almost identical, indicating relatively fast exchange between the two pools. Therefore, the anaplerosis was defined here as the fraction of mt-OAA molecules originating from PEP, which can be regarded as the flux of three-carbon molecules from glycolysis to the combined pool of cyt-OAA and mt-OAA.

Comparative flux ratio profiling of *P. pastoris* growing at high growth rates on glycerol/methanol mixtures in chemostats

At $D = 4.44 \times 10^{-5}$ s^{-1}, *P. pastoris* cells grow at about 90 % of the μ_{max} for glycerol provided as sole carbon source. Under these conditions, only part of the methanol being fed is effectively consumed by the cells. In fact, the residual methanol concentration in the culture broth increases with the methanol fraction in the feed, while the residual glycerol concentration is very low or zero (Table 1). In these cultures, the absolute amount of methanol effectively assimilated by the cells increases with the methanol fraction in the feed. Only in the case when glycerol accumulates to significant levels (80 : 20 glycerol/methanol mixture) is methanol virtually not consumed (Table 1). The comparison of flux ratios at this dilution rate revealed that, regardless of the methanol fraction in the feed medium, flux ratios are very similar to those observed in cells growing solely on glycerol as carbon source, e.g. the fraction of intact PEP molecules and the labelling patterns in PPP intermediates is not altered significantly by the assimilated methanol (Tables 2 and 3). *A priori*, this observation might indicate that the methanol that is consumed by the cells has a completely different fate from that in cells growing at the lower growth rate, namely that methanol could be mostly dissimilated directly to CO_2, generating 2 NADH molecules per methanol molecule. However, flux ratios in relation to the anaplerotic supply of the TCA cycle (i.e. the relative TCA cycle activity) are not significantly altered, as one would expect as a result of the extra amount of NADH produced by direct methanol oxidation to CO_2. Also, the observed ratio of the biomass to assimilated substrate ($Y_{x/s}$) still gave a reasonable fit with the predicted $Y_{x/s}$ calculated as the weighted mean of the growth yields on the two individual substrates (i.e. no significant drop in the observed growth yield was observed). Thus, the lower impact of methanol assimilation on the flux ratio distributions in cells growing at $D = 4.44 \times 10^{-5}$ s^{-1} may just reflect the fact that the amount of methanol molecules actually being consumed by the cells is significantly smaller than in cells growing at $D = 1.39 \times 10^{-5}$ s^{-1}, rather than a change in the split flux ratio between methanol assimilatory and dissimilatory pathways. In fact, a replica labelling experiment in which ^{13}C-labelled methanol (isotopic enrichment of 50 %) and unlabelled glycerol were fed at the 60 : 40 glycerol/methanol ratio indicated that the degree of enrichment of CO_2 was about 12.4 % at $D = 1.39 \times 10^{-5}$ s^{-1}, whereas incorporation

of ^{13}C into CO_2 at $D=4.44\times10^{-5}$ s^{-1} was close to negligible, i.e. supporting the latter hypothesis.

Comparison of flux ratio distributions of *P. pastoris* cells growing at $D=1.39\times10^{-5}$ s^{-1} on different glycerol/methanol mixtures with those observed in cells growing at $D=4.44\times10^{-5}$ s^{-1} on the same corresponding substrate mixtures shows the same pattern observed previously when comparing *P. pastoris* cells growing on glycerol as sole carbon source at these growth rates (Solà *et al.*, 2004). In particular, as also observed in cells growing on glycerol as sole carbon source, (i) the values for the fraction of cyt-OAA that stems from the mitochondrial pool of C4 intermediates, e.g. via malate-Asp and/or malate-OAA shuttles (Bakker *et al.*, 2001), or possibly synthesized via the glyoxylate cycle, are about twice as high at the lower dilution rate compared to the corresponding values in cells growing at the higher dilution rate. This indicates that, regardless of the fraction of methanol present in the feed medium, a largely unidirectional flux of OAA from the cytosol to the mitochondria occurs when cells are growing close to their maximal growth rates. (ii) The relative TCA cycle activity is reduced at the higher dilution rate (i.e. the fraction of mt-OAA generated by anaplerosis is a function of the specific growth rate but not of the methanol fraction in the feed medium) – unlike at the lower dilution rate, cyt-OAA and mt-OAA had distinct labelling patterns at the higher dilution rate, indicating slower exchange between the two pools. (iii) The fraction of cyt-OAA reversibly converted to fumarate is very low or zero in cells growing at the higher dilution rate, whereas this value is around 50 % in cells growing at the lower dilution rate. Blank & Sauer (2004) have defined this flux ratio as an upper limit of the fraction of cyt-OAA generated via the glyoxylate cycle, since export of TCA cycle intermediates from the mitochondria can also contribute to the cyt-OAA pool. However, the labelling patterns calculated for mt-OAA (as described by Maaheimo *et al.*, 2001) did not reflect a clear contribution of cytosolic succinate generated by the glyoxylate cycle.

Conclusions

This is the first comprehensive study of central carbon metabolism of the yeast *P. pastoris* growing on glycerol and methanol mixtures. In the framework of this study, we have established the BDF ^{13}C labelling approach of proteinogenic amino acids as an analytical tool to study intermediary metabolism of yeast cells growing on such carbon substrate mixtures. This approach allows the mapping of the metabolic state of the TCA cycle and associated pathways and thus this is an important methodological expansion for investigating the metabolism of eukaryotic cells growing with sole carbon sources. Specifically, we have shown that (i) co-assimilation of methanol as a carbon source does not alter the way the common amino acids are synthesized in *P. pastoris* growing on a sole multicarbon source, and (ii) growth on different glycerol/methanol mixtures at a given growth rate results in rather similar flux ratio profiles in the TCA cycle and related pathways as the fraction of methanol is increased. In contrast, a clear effect of specific growth rate on the relative activity of the TCA cycle and related pathways is observed, regardless of the methanol fraction in the feed, consistent with the observation that TCA cycle activity in *S. cerevisiae* is strongly correlated with the environmentally determined specific growth rate (Blank & Sauer, 2004).

Co-assimilation of methanol as a carbon source has a clear impact with respect to the activity of the PPP, which is consistent with the increasing flux of methanol molecules towards the synthesis of central carbon metabolism intermediates (e.g. PEP), as observed when the methanol fraction in the feed medium is increased. However, this pattern is not observed in cells growing at the higher dilution rate (where methanol is partially accumulated in the medium) suggesting that the distribution of methanol carbon into assimilatory and dissimilatory pathways may be different. Earlier ^{13}C labelling studies of methanol metabolism of the methylotrophic yeast *H. polymorpha* (Jones & Bellion, 1991) showed that the linear methanol oxidation pathway to CO_2 only operates under extreme conditions (e.g. methanol accumulation to toxic levels), suggesting a role in detoxification. Although the data obtained in the present study do not allow directly quantification of the split ratio of formaldehyde between the assimilation pathway and the oxidation pathway (Fig. 1) over the different tested environmental conditions, net fluxes through the metabolic network may be deduced from metabolic flux ratio analysis when combined with metabolic flux balancing (Fischer *et al.*, 2004; Fredlund *et al.*, 2004). Hence, we expect this study will lead to important insights into central carbon metabolism and its regulation in *P. pastoris*.

Overall, our investigation can be expected to become a valuable knowledge base for the optimization of culture processes for the production of recombinant proteins in *P. pastoris*, where parameters such as the residual methanol concentration, specific growth rate, as well as mixed substrate culture strategies have been shown to have a dramatic impact on overall process productivity. In addition, the information derived from our studies may be relevant for the design of isotopic labelling experiments of recombinant proteins (or other cell components, e.g. cell wall glucans) for structural studies. Furthermore, the methodology used in this work can also be applied to study the effect of other bioprocess-relevant parameters such as temperature, oxygen availability, etc., on the metabolic activity of *P. pastoris*.

ACKNOWLEDGEMENTS

This work was supported by the State University of New York at Buffalo, the Spanish Ministry of Science and Education (projects PPQ2001-1908 and CTQ2004-00300) and the Academy of Finland (projects 52311, 202409 and 207435). The authors thank O. Cos for technical assistance with culture off-gas analyses, as well as S. Santos and J. Albiol for physiological data consistency analysis. The

Department of Chemical Engineering of the Universitat Autònoma de Barcelona constitutes the Biochemical Engineering Unit of the Reference Centre in Biotechnology of the Generalitat de Catalunya.

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Edited by: M. Tien