Metabolic flux analysis of recombinant *Pichia pastoris* growing on different glycerol/methanol mixtures by iterative fitting of NMR-derived ^{13}C-labelling data from proteinogenic amino acids

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The yeast *Pichia pastoris* has emerged as one of the most promising yeast cell factories for the production of heterologous proteins. The readily available genetic tools and the ease of high-cell density cultivations using methanol or glycerol/methanol mixtures are among the key factors for this development. Previous studies have shown that the use of mixed feeds of glycerol and methanol seem to alleviate the metabolic burden derived from protein production, allowing for higher specific and volumetric process productivities. However, initial studies of glycerol/methanol co-metabolism in *P. pastoris* by classical metabolic flux analyses using ^{13}C-derived Metabolic Flux Ratio (METAFoR) constraints were hampered by the reduced labelling information obtained when using C3:C1 substrate mixtures in relation to the conventional C6 substrate, that is, glucose. In this study, carbon flux distributions through the central metabolic pathways in glycerol/methanol co-assimilation conditions have been further characterised using biosynthetically directed fractional ^{13}C labelling. In particular, metabolic flux distributions were obtained under 3 different glycerol/methanol ratios and growth rates by iterative fitting of NMR-derived ^{13}C-labelling data from proteinogenic amino acids using the software tool ^{13}CFlux2. Specifically, cells were grown aerobically in chemostat cultures fed with 80:20, 60:40 and 40:60 (w:w) glycerol/methanol mixtures at two dilutions rates (0.05 hour^{-1} and 0.16 hour^{-1}), allowing to obtain additional data (biomass composition and extracellular fluxes) to complement pre-existing datasets. The performed ^{13}C-MFA reveals a significant redistribution of carbon fluxes in the central carbon metabolism as a result of the shift in the dilution rate, while the ratio of carbon sources has a lower impact on carbon flux distribution in cells growing at the same dilution rate. At low growth rate,

Abbreviations: αKG, α-ketoglutarate; *GA3P*, Glyceraldehyde-3-phosphate; *PG2*, 2-phosphoglycerate; *PG3*, 3-phosphoglycerate; $GA3P_{per}$, Glyceraldehyde-3-phosphate peroxisome; *DHA*, Dihydroxyacetone; *DHAP*, Dihydroxyacetone phosphate; *E4P*, Eritrose-4-phosphate; *Rib5P*, Ribose-5-phosphate; *Rul5P*, Ribulose-5-phosphate; *Xul5P*, Xylulose-5-phosphate; *Fru6P*, Fructose-6-phosphate; *Glc6P*, Glucose-6-phosphate; *Sed7P*, Sedoheptulose-7-phosphate; *FBP*, Fructose-1,6-biphosphate; *Pyr*, Pyruvate; *Pep*, Phosphoenolpyruvate; *MAL*, Malate; *SUCC*, Succinate; *CIT*, Citrate; ICIT, Isocitrate; *FUM*, Fumarate; *ACCoA*, Acetyl coenzyme; *Metoh*, Methanol; *MetohL*, labelled Methanol; *MetohN*, non labelled Methanol; FullyGly, Fully labelled Glycerol; CGly, non-labelled Glycerol; X_{bio}, Biomass formation; *Form*, Formaldehyde; *FOR*, formate; *OAA*, Oxaloacetate; *ACALD*, Acetalehyde; *PRPP*, Phosphoribosyl Pirophosphate; *MTHF*, Methylenetetrahydrofolate; *THF*, Tetrahydrofolate; *CHOR*, chorismate; *Pi*, Inorganic phosphate; *PPi*, Inorganic pyrophosphate; *13dpg*, 1,3-Diphophoglycerate; G1P: SUCCoA, Succinyl coenzyme A; *Kval*, Ketovalerate; *DHF*, Dihydrofolate; OUR, Oxigen Uptake Rate; CER, CO_2 Exchange Rate; n.d., not determined; SD, standard deviation; SEM, standard error of the mean; PPP, pentose phosphate pathway; BFD, biosynthetically directed fractional; ROL, *Rhizopus oryzae* lipase; NMR, Nuclear Magnetic Resonance; ETC, electron transfer chain.

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the percentage of methanol directly dissimilated to CO_2 ranges between 50% and 70%. At high growth rate the methanol is completely dissimilated to CO_2 by the direct pathway, in the two conditions of highest methanol content.

Introduction

Over the past few years, systems metabolic engineering has emerged as a powerful tool develop and improve the yeast *Pichia pastoris* as a cell factory platform for heterologous protein production [1,2]. Although most efforts have been focused on the engineering of the protein folding and secretion pathway, recent studies point at the core metabolism as a target for strain improvement [1,3–5]. Also most of the heterologous proteins secreted by this yeast are usually expressed under the control of strong, tightly regulated promoters like the methanol-induced alcohol oxidase promoter AOX1 [6,7]. As the AOX1 promoter is strongly induced in the presence of methanol, protein production on mixed substrates such as glycerol–methanol or sorbitol–methanol strategies have been typically used to improve the amounts of protein secreted [8–13]. More recently, glucose/methanol mixed feed strategies have also been explored [4,14]. To develop improved strategies for optimal production of recombinant proteins in cultures with *P. pastoris* strains, it is necessary to have a better insight into the regulation of methanol metabolic pathways, not only because of their key role in the central carbon and energy metabolism, but also because heterologous gene transcriptional levels are regulated by methanol. For instance, alcohol oxidase levels are strongly influenced by the glucose to methanol ratio and growth rate, in mixed substrate cultures with the methylotrophic yeast *Hansenula polymorpha* [15]. Also, the influence of the methanol–glycerol ratio in the feed medium on the expression of recombinant protein was evaluated in [9]. However, these methanol–glycerol studies did not quantitatively analyse how the carbon assimilated by the cell is distributed through the metabolic network.

In recent years several *P. pastoris* studies were done to evaluate the central carbon metabolic flux distribution in different conditions. Relevant examples include those performed using glucose as a single carbon source [3,16,17] as well as mixed carbon sources such as glucose–methanol [4,18].

In a previous study from our group [19], biosynthetically directed fractional (BDF) ^{13}C-labelling of proteinogenic amino acids was used to investigate the effect of feeding different concentration ratios of the glycerol/methanol at two different growth rates in a recombinant *P. pastoris* strain producing a *Rhyzopus oryzae* lipase (ROL). Although this previous study provided new insights on the impact of methanol co-assimilation on the core metabolism, the use of a C_3:C_1 carbon combination did not allow the straightforward determination of metabolic fluxes using ^{13}C-derived constraints and simplified calculation approaches. That is, determination of the metabolic fluxes, using the traditional metabolic flux ratio (METAFoR) formalism [20,21] or the non-linear minimization approach described in [22], could not be directly used due to the insufficient number of derived flux ratios for the degrees of freedom of the metabolic network studied. Previous studies using these approaches [1] were performed using a C_6 carbon source (glucose) allowing to derive an increased number of flux ratios. This formalism has recently been extended to the case of glucose–methanol mixed carbon sources [4]. However, when using a co-substrate such as glycerol, with a lower number of C atoms, the information derived regarding the metabolic flux ratios is reduced and it is more difficult to derive clear and concise flux ratio equations, useful to solve the metabolic system. In particular, a flux ratio useful to derive information from the contribution of the PPP pathway was not available [19]. Therefore, such methodology was not useful in the present case.

To circumvent this limitation, in the present study we applied an alternative approach based on an iterative global flux calculation methodology [23]. This involves the simulation of isotopomer distributions and labelling measurements combined with the fitting of the calculated values to the labelling and external flux measurements. These calculations were performed using the software tool ^{13}CFlux2 [24], an improved version of the previous ^{13}CFlux program [25] to calculate the carbon flux distribution. The calculations were performed using NMR measurements of labelling patterns from proteinogenic amino acids obtained from stationary labelling experiments [19] to infer the isotopic enrichments of the corresponding metabolite precursors. To obtain complementary data, required to apply the new methodology, new replica experiments were performed. The new experiments allowed determining biomass macromolecular and elemental composition and therefore to derive *P. pastoris* specific data on the amount of biomass precursor metabolites, consumed in these experimental conditions, and not previously available.

Materials and methods

Strain and media

A *P. pastoris* X-33 (Invitrogen) derived strain expressing a heterologous *R. oryzae* lipase (ROL) gene under the transcriptional control of the AOX1 promoter (*P. pastoris* X-33/pPICZαA-ROL) was chosen for this study [26]. Chemostat cultures were fed with a defined minimal medium containing per litre of deionized water: yeast nitrogen base (YNB Difco, Detroit, MI, USA) 0.17 g; $(NH_4)_2SO_4$, 5 g and the corresponding amount of glycerol and methanol up to 10 g, and antifoam Mazu DF7960 (Mazer Chemicals, PPG Industries, Gurnee, IL, USA), 0.1 mL. The different compositions of glycerol and methanol, used in this study were, respectively, 80:20 (w/w), 60:40 (w/w) and 40:60 (w/w). The YNB components were sterilized separately by microfiltration before addition 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) dextrose.

Chemostat cultures

Continuous cultures were carried out as described previously [19]. Briefly the bioreactor working volume was 1 L in a 3 L bench-top bioreactor (Applikon Biotechnology) at 30 °C with a minimum dissolved oxygen tension of 15%. The three different combinations of carbon sources (glycerol/methanol) were performed at two different dilution rates, 0.05 hour^{-1} and 0.16 hour^{-1}. These values