Metabolic Engineering Communications 5 (2017) 34–44

Contents lists available at ScienceDirect

Metabolic Engineering Communications

journal homepage: www.elsevier.com/locate/mec

A comprehensive evaluation of constraining amino acid biosynthesis in compartmented models for metabolic flux analysis

Mathias Lehnen*, Birgitta E. Ebert, Lars M. Blank

iAMB – Institute of Applied Microbiology, ABBt – Aachen Biology and Biotechnology, RWTH Aachen University, Worringer Weg 1, D-52074 Aachen, Germany

ARTICLE INFO

Keywords:
^{13}C-metabolic flux analysis
Eukaryotes
Compartmented metabolism
Non-conventional yeast
S. cerevisiae
H. polymorpha

ABSTRACT

Recent advances in the availability and applicability of genetic tools for non-conventional yeasts have raised high hopes regarding the industrial applications of such yeasts; however, quantitative physiological data on these yeasts, including intracellular flux distributions, are scarce and have rarely aided in the development of novel yeast applications. The compartmentation of eukaryotic cells adds to model complexity. Model constraints are ideally based on biochemical evidence, which is rarely available for non-conventional yeast and eukaryotic cells. A small-scale model for ^{13}C-based metabolic flux analysis of central yeast carbon metabolism was developed that is universally valid and does not depend on localization information regarding amino acid anabolism. The variable compartmental origin of traced metabolites is a feature that allows application of the model to yeasts with uncertain genomic and transcriptional backgrounds. The presented test case includes the baker's yeast *Saccharomyces cerevisiae* and the methylotrophic yeast *Hansenula polymorpha*. Highly similar flux solutions were computed using either a model with undefined pathway localization or a model with constraints based on curated (*S. cerevisiae*) or computationally predicted (*H. polymorpha*) localization information, while false solutions were found with incorrect localization constraints. These results indicate a potentially adverse effect of universally assuming *Saccharomyces*-like constraints on amino acid biosynthesis for non-conventional yeasts and verify the validity of neglecting compartmentation constraints using a small-scale metabolic model. The model was specifically designed to investigate the intracellular metabolism of wild-type yeasts under various growth conditions but is also expected to be useful for computing fluxes of other eukaryotic cells.

1. Introduction

Knowledge of intracellular reaction rates (fluxes) is crucial to understand how cells metabolize nutrients and how they adapt the fluxes in response to environmental or genetic perturbations. The investigation of intracellular reaction rates has become accessible to a wide range of scientists, rather than exclusively experts, and has been applied to expand detailed knowledge on cellular physiology (Blank et al., 2005; Long and Antoniewicz, 2014; Petersen et al., 2000) as well to guide metabolic engineering (Bartek et al., 2011; Stephanopoulos, 1999; Toya and Shimizu, 2013) and biomedical research (Boghigian et al., 2010). The field of fluxomics has not only diversified but has also matured extensively since its inception, and a wide range of biological questions can be addressed through flux balance analysis (Orth et al., 2010), metabolic flux analysis (MFA) (Bonarius et al., 1996; Vallino and Stephanopoulos, 1990), ^{13}C-based MFA (Quek et al., 2009; Weitzel et al., 2013; Wiechert, 2001; Zamboni et al., 2005) or non-stationary MFA (Wiechert and Nöh, 2013). Furthermore, novel methods, such as two-scale (2S)-^{13}C-MFA (Martin et al., 2015), are still emerging. Comprehensive frameworks and guidelines are available to simplify and optimize every step of the analysis, including model generation, experimental design, analytical methods, visualization, and statistical evaluation of simulated results (Crown and Antoniewicz, 2013; Droste et al., 2013; Ebert et al., 2012; Nöh et al., 2014; Wiechert et al., 2001).

MFA computes intracellular fluxes by solving a set of metabolite mass balances for an organism in a metabolic steady state. ^{13}C-MFA is an extension of this type of analysis based on the use of isotopically enriched carbon substrates. As the incorporation of ^{13}C-isotopes into metabolites depends on the flux distribution, tracking the distribution of the ^{13}C-isotopes in metabolic intermediates or end products, e.g., proteinogenic amino acids, allows the generation of additional constraints to the metabolite mass balance equations, either as metabolic

Abbreviations: MFA, metabolic flux analysis; MDV, mass distribution vector; Sf, flux solution from an unconstrained model; Sd, flux solution from a fully constrained model; Sd_{min}, flux solution from a model with minimal constraints; ACCOA, acetyl-CoA; PYR, pyruvate; SER, serine; GLY, glycine; THR, threonine; ILE, isoleucine; LEU, leucine; TP, TargetP 1.1; WP, WoLF PSORT

* Corresponding author.
E-mail addresses: mathias.lehnen@rwth-aachen.de (M. Lehnen), birgitta.ebert@rwth-aachen.de (B.E. Ebert), lars.blank@rwth-aachen.de (L.M. Blank).

http://dx.doi.org/10.1016/j.meteno.2017.07.001
Received 20 March 2017; Received in revised form 29 May 2017; Accepted 5 July 2017
Available online 11 July 2017

flux ratios (Sauer et al., 1999) or in the form of isotopomer (*isotope isomer*) balances (Wiechert and de Graaf, 1996). These additional constraints enhance the potential to identify intracellular fluxes, especially for parallel pathways that result in distinct distributions of the tracer in the metabolites. ^{13}C-MFA has therefore become a standard tool, especially in the context of research on bacterial metabolism, with isotopomer modeling being the most commonly used method at present. More specifically, the metabolite and isotopomer balances are constrained with the experimentally determined substrate uptake, product formation, and biomass production rates as well as labeling information of metabolites in the form of mass distribution vectors (MDVs). An MDV describes the relative abundance of mass isotopomers differing in the number of included ^{13}C-isotopes of a single metabolite or a fragment thereof and are determined by mass spectrometric analyses. The equation system is solved, i.e., the fluxes are computed, by least squares parameter estimation, in which an initial random flux distribution is iteratively adapted to minimize the sum of the squared residuals (SSR) between experimentally and simulated MDVs. A χ^2-cut-off defines the maximum SSR value for a statistically acceptable flux solution. The solution with the smallest SSR in a sufficient number of minimizations is then assumed to be the optimal flux solution. Flux confidence intervals as a result of a sensitivity analysis indicate the resolvability and thus the robustness of the flux solutions (Antoniewicz et al., 2006).

A review of publications on ^{13}C-MFA reveals that few published studies have focused on eukaryotes, with *S. cerevisiae* being the most prominent organism among the investigated eukaryotes. This is partially due to the high scientific and industrial interest in baker's yeast but also to some other extent to the additional modeling challenges arising from the complexity of compartmented organisms. Specifically, the duplication of metabolite pools in distinct compartments and the corresponding parallelization of metabolic pathways increases the solution space and the extent of necessary calculations while decreasing the available constraining information by lumping isotopic labeling data of metabolites from separate compartments in one measurable pool. The issue of modeling compartmentation is usually mentioned in eukaryotic MFA studies but is rarely addressed and resolved in detail (Blank et al., 2005; dos Santos et al., 2003; Förster et al., 2014; Gopalakrishnan and Maranas, 2015). At the core of the pathway parallelization problem is the biosynthesis of proteinogenic amino acids (Förster et al., 2014), whose labeling patterns are usually measured and used as proxy for the labeling of free intracellular metabolites (Szyperski, 1995). The crux in eukaryotic cells is the incomplete knowledge regarding the compartmental origin of precursor metabolites. Even in well-described organisms such as *S. cerevisiae*, these knowledge gaps exist, with alanine biosynthesis being one example (Blank et al., 2005; Buescher et al., 2015). Although defined as catalytically inactive in *S. cerevisiae*, the cytosolic equivalent to the mitochondrial alanine transaminase is genetically available and only transcriptionally regulated. In non-conventional yeasts or other poorly characterized organisms, this problem expands to full uncertainty regarding the existence and activity of enzymes and pathways in any modeled compartment and does not only concern alanine, but theoretically any amino acid that originates from a central metabolite present in more than one compartment. In a modeling context, this indicates that metabolic pathways cannot always be confined to one of multiple compartments, nor is sufficient information available to define a specific ratio between parallel pathways. Thus, we address here the necessity of creating a model with an option of undefined compartmental origin of biosynthetic precursors, especially for eukaryotic systems that are not as well characterized as *S. cerevisiae*. For model validation, we chose the model organism *S. cerevisiae* CEN.PK and the less well-characterized yeast *Hansenula polymorpha*, for which an annotated genome sequence and physiological data were available.

2. Materials and methods

2.1. Strains and culture conditions

S. cerevisiae CEN.PK 113 7D (European *S. cerevisiae* Archive for Functional Analysis, http://www.uni-frankfurt.de/fb15/mikro/euroscarf/) and *H. polymorpha* (*Pichia angusta*) CLIB 421 (Collection de levures d'intérêrt biotechnologique, http://www.inra.fr/Internet/Produits/clib/) were used in all experiments. Growth experiments were conducted in 1.3 L shake flasks filled with 50 mL Verduyn medium containing per liter, 5 g $(NH_4)_2SO_4$, 3 g KH_2PO_4, 0.5 g $MgSO_4{\cdot}7H_2O$, 4.5 mg $ZnSO_4{\cdot}7H_2O$, 0.3 mg $CoCl_2{\cdot}6H_2O$, 1.0 mg $MnCl_2{\cdot}4H_2O$, 0.3 mg $CuSO_4{\cdot}5H_2O$, 4.5 mg $CaCl_2{\cdot}2H_2O$, 3.0 mg $FeSO_4{\cdot}7H_2O$, 0.4 mg $NaMoO_4{\cdot}2H_2O$, 1.0 mg H_3BO_3, 0.1 g KI, 15 mg EDTA, 0.05 mg biotin, 1.0 mg calcium pantothenate, 1.0 mg nicotinic acid, 25 mg inositol, 1.0 mg pyridoxine, 0.2 mg p-aminobenzoic acid, and 1.0 mg thiamine (Verduyn et al., 1992). The medium was supplemented with 5 g/L glucose and buffered with 100 mM potassium hydrogen phthalate. The pH of the medium was adjusted to 5. The shake flasks provided a closed system equipped with O_2- and CO_2-sensors (BlueSens gas sensor GmbH, Herten, Germany) and an air-tight sample port, and were shaken at 130 rpm with an amplitude of 30 mm. ^{13}C-tracer experiments were performed with a mixture of 80% 1-^{13}C-glucose and 20% U-^{13}C-glucose (both purchased from Sigma-Aldrich, Steinheim, Germany, with 99 atom-% purity), reported to have a high potential for resolving the network of central carbon metabolism at a reasonable prize per experiment (Zamboni et al., 2009). The main cultures were inoculated to a starting optical density (OD_{600}) of 0.05 from precultures grown in the same minimal medium, but supplemented with naturally labeled glucose after cells were harvested and washed with 0.9% NaCl solution. Precultures were grown in 100 mL shake flasks at a shaking frequency of 200 rpm at 30 °C and 40 °C for *S. cerevisiae* and *H. polymorpha*, respectively. Samples of the main culture were taken by connecting a syringe to the sample port, opening the sealing clamp, and flushing the sampling line with air before creating a vacuum with the syringe and allowing approximately 1 mL to flow into the sample tube. The sampling line was flushed with air before the sealing clamp was closed to minimize disturbances of the closed headspace and corresponding gas analysis. After the cultures reached stationary phase, the headspace analysis was continued for several hours to verify the tightness of the system by stable gas analysis signals.

2.2. Physiological data acquisition and processing

Immediately after sampling, the OD_{600} was measured, and the samples were centrifuged at 13,000 rpm for 5 min in a tabletop centrifuge. The biomass pellet was separated from the supernatant, and both fractions were stored at −20 °C until further analysis. The supernatant was used to determine the concentrations of glucose and the excreted metabolites ethanol, glycerol, and acetate. The metabolites were separated on an Aminex HPX-87H column (Bio-Rad, Hercules, CA, USA) at 60 °C with a flow rate of 0.8 mL/min of 5 mM H_2SO_4. Glucose, ethanol, and glycerol were detected on a Shodex RI-101 detector, and acetate was detected at a wavelength of 210 nm in a variable wavelength detector of an UltiMate 3000 HPLC system (Dionex, Sunnyvale, CA, USA).

The cell dry weight (CDW) of samples was calculated from the measured OD_{600} and a calibration curve, which was recorded for both yeasts under corresponding experimental conditions. The correlation curves were generated by weighing the biomass from duplicates of 10 mL culture samples with five different OD_{600} values ranging from 0.5 to 6. The samples were cooled on ice before centrifugation in glass tubes at 3500 rpm for 20 min in a Heraeus Megafuge 16 R (Thermo Scientific, Waltham, MA, USA). The supernatant was discarded, and biomass pellets were washed with water and centrifuged again. Finally, the supernatant was discarded again and biomass pellets were dried at