¹³C Metabolic Flux Analysis of acetate conversion to lipids by *Yarrowia lipolytica*

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Abstract

Volatile fatty acids (VFAs) are an inexpensive and renewable carbon source that can be generated from gas fermentation and anaerobic digestion of fermentable wastes. The oleaginous yeast *Yarrowia lipolytica* is a promising biocatalyst that can utilize VFAs and convert them into triacylglycerides (TAGs). However, currently there is limited knowledge on the metabolism of *Y. lipolytica* when cultured on VFAs. To develop a better understanding, we used acetate as the sole carbon source to culture two strains, a control strain and a previously engineered strain for lipid overaccumulation. For both strains, metabolism during the growth phase and lipid production phase were investigated by metabolic flux analysis using two parallel sodium acetate tracers. The resolved flux distributions demonstrate that the glyoxylate shunt pathway is constantly active and the flux through gluconeogenesis varies depending on strain and phase. In particular, by regulating the activities of malate transport and pyruvate kinase, the cells divert only a portion of the glyoxylate shunt flux required to satisfy the needs for anaplerotic reactions and NADPH

production through gluconeogenesis and the oxidative pentose phosphate pathway (PPP). Excess flux flows back to the tricarboxylic acid (TCA) cycle for energy production. As with the case of glucose as the substrate, the primary source for lipogenic NADPH is derived from the oxidative PPP.

Keywords: *Yarrowia lipolytica*, ^{13}C Metabolic Flux Analysis, acetate metabolism, biodiesel, NADPH, gluconeogenesis

1. Introduction

Concerns about global climate change have motivated research in seeking renewable energy sources to replace fossil liquid fuels for transportation. Ethanol generated through the fermentation of sugars from corn and sugarcane has contributed to a reduction of reliance on fossil fuels and is currently produced economically on industrial scales (Nigam & Singh 2011). However, the potential of bioethanol remains limited while demand in other fuels such as diesel continues to increase (Patil et al. 2008; Balat et al. 2008). This trend has increased the attention to alternative renewable biofuels such as the triacylglyceride (TAG)-derived fatty acid methyl esters (FAMES). Various methods have emerged for the production of FAMES with ideal fuel properties from a wide range of cheap and renewable feedstocks (Meng et al. 2009; Li et al. 2008). Along these lines, a two-step process was recently proposed where the first step converts municipal waste into volatile fatty acids (VFAs) using anaerobic fermentation and the second step converts the VFAs into TAGs using an oleaginous organism (Morgan-Sagastume et al. 2011; Fontanille et al. 2012; Fei et al. 2011).

Engineering of microorganisms that exhibit high lipid titer, productivity, and yield from VFAs is key to achieving cost-effective biodiesel production. The model oleaginous yeast *Yarrowia lipolytica* has emerged as a promising biocatalyst to serve such purposes due to its superior capability of TAG overproduction and storage, availability of genome sequencing data and established genetic engineering tools (Dujon et al. 2004; Beopoulos, Cescut, et al. 2009). However, although numerous efforts have been made to understand and engineer lipid accumulation in *Y. lipolytica*, they are almost exclusively focused on using carbohydrates (sugars) as the starting feedstock (Wasylenko et al. 2015; Beopoulos, Chardot, et al. 2009; Qiao et al. 2015; Blazeck et al. 2014; Papanikolaou et al. 2009). Few studies have been conducted to elucidate its metabolism when VFAs are used as carbon source. The metabolism of *Y. lipolytica* on VFAs is significantly different from that of glucose, as it can be illustrated using the model VFA compound—acetate. Initially, acetate is imported into the cytosol and activated to acetyl-CoA (AcCoA) by acetyl-CoA synthetase (ACS, YALI0F05962g) at the expense of two molecules of ATP equivalents (Jogl & Tong 2004). The resulting cytosolic AcCoA has many metabolic destinations as shown in Figure 1. For example, it can be directly incorporated into lipids during de novo fatty acid biosynthesis, or transported into the mitochondria via the carnitine shuttle and enter the tricarboxylic acid (TCA) cycle for energy production. The most prominent distinction between acetate and glucose metabolism is that the former activates the glyoxylate shunt and gluconeogenesis pathways (Eaton 2002; Kornberg 1966; Eschrich et al. 2002). The glyoxylate shunt pathway involves the export of isocitrate from the mitochondria to the cytosol, cleavage of isocitrate into glyoxylate and succinate by isocitrate lyase (ICL, YALI0C16885p and YALI0F31999p), and condensation of glyoxylate with AcCoA to form malate by malate synthase (MES, YALI0D19140p and YALI0E15708g). This process bypasses

the two decarboxylation steps of isocitrate in the TCA cycle and replenishes the metabolic pools of malate and oxaloacetate, thereby conserving carbon atoms for anaplerotic purposes. Cytosolic oxaloacetate also serves as an entry point to gluconeogenesis. Phosphoenolpyruvate carboxykinase (PEPCK, YALI0C16995p) converts the oxaloacetate into phosphoenolpyruvate (PEP), which can then proceed through the gluconeogenic reactions. This process is essentially the reverse of glycolysis and it utilizes all of the glycolytic enzymes except for the step of fructose-1,6-bisphosphate (FBP) to fructose-6-phosphate (F6P) which requires fructose-1,6-bisphosphatase (YALI0A15972p). The significance of this pathway is that it can replenish glycolytic intermediates essential for macromolecule synthesis and that it provides flux through the pentose phosphate pathway (PPP). Apart from the aforementioned metabolic reactions, the source of NADPH is also a major consideration given that lipid biosynthesis requires large amounts of this reducing cofactor. Previous studies on *Y. lipolytica* using glucose as the carbon source suggest that this lipogenic NADPH is supplied primarily from the oxidative pentose phosphate pathway and not from malic enzyme (Wasylenko et al. 2015; Zhang et al. 2013).

These features of acetate metabolism suggest that there must be sufficient flux through gluconeogenesis to support biosynthesis and NADPH generation through oxidative PPP. However, this flux must be tightly regulated as it directly competes with energy (ATP) generation through the TCA cycle, which in turn competes with pathways involved in lipid synthesis for carbon source (Figure 1). Consequently, in order for *Y. lipolytica* to attain high lipid accumulation on acetate, the cells need to distribute its carbon source efficiently among the above pathways as to optimally satisfy both the energy and TAG synthesis requirements.

¹³C Metabolic Flux Analysis (MFA) is an effective tool to determine the intracellular metabolic flux distribution within the cell using experimental data (Wiechert 2001). In this study,

we conducted stationary ^{13}C -MFA on two *Y. lipolytica* strains—a previously engineered strain for lipid overproduction and a control strain, with acetate as the sole carbon source. The goal was to elucidate how the organism partitions the carbon atoms from acetate throughout the lipid synthesis pathway, TCA cycle, glyoxylate shunt, gluconeogenesis, and PPP to achieve lipid overproduction. In addition to the metabolism during normal growth and cell division, a nitrogen limiting condition that triggers lipid accumulation in oleaginous organisms was also investigated, resulting in a total of four cases (Evans & Ratledge 1984; Boulton & Ratledge 1981). Parallel labeling experiments were conducted using two different ^{13}C sodium acetate tracers ($1\text{-}^{13}\text{C}_1$ sodium acetate and $\text{U-}^{13}\text{C}_2$ sodium acetate), metabolic models were constructed for both the growth and lipid production phases, and intracellular flux estimations were obtained by fitting experimental data to the model. Results indicate that malate transport and pyruvate kinase play crucial roles in controlling the flux through gluconeogenesis and that the oxidative PPP is the primary source of NADPH supporting the lipogenesis in *Y. lipolytica*.

2. Materials and methods

2.1 Strain and culture conditions

Two strains of *Y. lipolytica* were used for all ^{13}C -MFA experiments: a control strain MTYL037 and a previously engineered lipid overproducing strain MTYL065 which overexpresses *ACCI* (acetyl-CoA carboxylase 1) and *DGAI* (diacylglycerol acyltransferase 1) (Tai & Stephanopoulos 2013). In the engineered strain, the enzymes encoded by the two overexpressed genes catalyze the first and last step of TAG synthesis respectively, thereby greatly enhancing the flux through this pathway. As a result, this strain has higher demands for cytosolic acetyl-CoA and NADPH. Prior to the experiment, both strains were maintained at 4 °C

on minimal media plates containing 20 g/L glucose, 5 g/L ammonium sulfate, and 1.7 g/L yeast nitrogen base without amino acids and ammonium sulfate (YNB-AA-AS). To prepare for the ^{13}C -MFA experiments, one test tube (14 mL total volume) starter culture was set up for each strain by inoculating from the corresponding plate. The medium contained 2 mL of yeast extract-peptone-dextrose (20 g/L glucose, 20 g/L peptone, and 10 g/L yeast extract) to rapidly accumulate cell density. After 24 hours, 1 mL of the test tube cultures were transferred to 40 mL shake flask (250 mL total volume) cultures containing 50 g/L sodium acetate, 1.34 g/L ammonium sulfate, and 1.7 g/L YNB-AA-AS to adapt the cells and synchronize growth. The carbon to nitrogen (C/N) ratio of the shake flask culture medium was 60:1. All tube and shake flask cultures were incubated at 30 °C and 250 rpm.

After 24 hours, each shake flask culture was used to inoculate three batch mini-bioreactors (Applikon Biotechnology MiniBio 250 mL, Foster City, CA) to an initial OD_{600} of 0.05. The working volume for all bioreactor cultures was 150 mL and the media had the same composition as that of the shake flask. However, the sodium acetate substrate for each of the three bioreactor cultures were different. One contained 100% $1\text{-}^{13}\text{C}_1$ sodium acetate (Cambridge Isotope Laboratories, Tewksbury, MA), another contained 100% sodium acetate labeled to natural abundance, and the last contained 40 mol% $\text{U-}^{13}\text{C}_2$ sodium acetate (Cambridge Isotope Laboratories, Tewksbury, MA). All bioreactor cultures were maintained at a temperature of 30 °C and a pH of 7.0 through the addition of 10 wt% sulfuric acid. The aeration rate was 1 vvm and the dissolved oxygen level (DO) was maintained at 20% through agitation. 100 μL 20 vol% Antifoam 204 (Sigma-Aldrich) was added at 6 and 24 hours after inoculation to prevent foaming. The sampling port was located at the bottom of the bioreactor.

Cells from the shake flask cultures were washed prior to inoculation into the bioreactor. The appropriate culture volume was centrifuged at 18,000 g for 5 min, after which the supernatant discarded and the cell pellet resuspended in 1 mL of the culture medium to be used in the ^{13}C -MFA experiment. A second centrifugation step was carried out and the supernatant was discarded. The cells were then resuspended in 1 mL medium to be used in the ^{13}C -MFA experiment and transferred to the bioreactors.

2.2 Extracellular metabolite quantification

Extracellular acetate and citrate concentrations were quantified using High-Performance Lipid Chromatography (HPLC). 1 mL sample was extracted from each bioreactor culture and centrifuged at 18,000 g for 10 min. The supernatant was filtered through 0.2 μm Nylon syringe filters (Denville Scientific Inc., Holliston, MA) and analyzed on an Agilent 1200 HPLC system coupled to a G1362A Refractive Index Detector. A Bio-Rad HPX-87H column was used for separation with 14 mM sulfuric acid as the mobile phase flowing at a rate of 0.7 mL/min. The injection volume was 10 μL . Extracellular concentration of ammonium sulfate was measured using an Ammonium Assay Kit (Sigma-Aldrich).

2.3 Determination of dry cell weight

The dry cell weight (DCW) was measured by extracting 1 mL sample from the bioreactor culture and vacuum-filtering it on a pre-weighed 0.2 μm nitrocellulose filter paper (Whatman, Pittsburg, PA). After washing with 2 volumes of Milli-Q water, the samples were dried at 60 $^{\circ}\text{C}$ and weighed again after 24 hours. For each time point measurement, a control filter was prepared

by filtering 1 mL natural abundance sodium acetate medium followed by washing. This was used to correct for changes in filter mass during sample preparation.

2.4 Lipid quantification

The fatty acids synthesized by *Y. lipolytica* including palmitate (C16:0), palmitoleate (C16:1), stearate (C18:0), oleate (C18:1) and linoleate (C18:2) were quantified using a Gas Chromatography coupled to a Flame Ionization Detector (GC-FID). 0.1-1 mL cell culture was extracted from each bioreactor such that the sample contained approximately 1 mg biomass. A centrifugation step at 18,000 g for 10 min was performed and the supernatant discarded. Cell pellets were then stored at -20 °C until the analysis of fatty acids.

For the analysis step, 100 µL internal standard containing 2 mg/mL methyl tridecanoate (Sigma-Aldrich) and 2 mg/mL glyceryl triheptadecanoate (Sigma-Aldrich) dissolved in hexane was added to each sample. Methyl tridecanoate was used for volume loss correction during sample preparation and glyceryl triheptadecanoate was used for transesterification efficiency correction. 500 µL 0.5 N sodium methoxide (20 g/L sodium hydroxide in anhydrous methanol) was then added and the samples were vortexed at 1200 rpm for 60 min to allow for the transesterification of lipids to fatty acid methyl esters (FAMES). Afterwards, 40 µL of 98% sulfuric acid was added to neutralize the pH. The FAMES were then extracted through the addition of 500 µL hexane followed by vortexing at 1200 rpm for 30 min. Centrifugation at 6000g for 1 min was performed to remove cellular debris and the top hexane layer was extracted for analysis. Separation of the FAME species was achieved on an Agilent J&W HP-INNOWax capillary column with a Bruker 450-GC system. The injection volume was 1 µL, split ratio was 10, and injection temperature was 260 °C. The column was held at a constant temperature of

200 °C and helium was used as the carrier gas with a flow rate of 1.5 mL/min. The FID was set at a temperature of 260 °C with the flow rates of helium make up gas, hydrogen, and air at 25 mL/min, 30 mL/min, and 300 mL/min respectively.

2.5 Estimation of extracellular fluxes

The extracellular flux for TAG synthesis was decomposed into that of the two constitutive components of TAGs: AcCoA and glycerol-3-phosphate (Glyc3P). This was performed by measuring the fatty acid distribution of the TAGs during each phase to determine the relative amounts of each fatty acid synthesized. Then the total amount of AcCoA and Glyc3P required was calculated by assuming that 1 mol AcCoA was used for every 2 mol carbon incorporated into the fatty acids and 1 mol Glyc3P was used for every 3 mol fatty acids incorporated into the TAGs. The amount of lipogenic NADPH required was also calculated by assuming that 2 NADPH was used for every fatty acid elongation step and 1 NADPH was used for every desaturation step. Note that a total balance on lipogenic NADPH consumption and production was not used in the metabolic flux estimation. As such, a mass balance constraint for NADPH was not included in the model in order to avoid introducing errors due to assumptions on enzyme cofactor preference and unidentified other sources and sinks of NADPH (Schmidt et al. 1998; Ahn & Antoniewicz 2011).

All extracellular fluxes were normalized to an acetate uptake rate of 100. Linear regressions were performed on plots of DCW, citrate, and lipogenic AcCoA, Glyc3P, and NADPH versus acetate and the slope of the best-fit line was determined. Since this slope represents the yield of these metabolites on acetate, its value multiplied by 100 gives the normalized extracellular fluxes based on the arbitrarily fixed acetate consumption rate. The

results from the three bioreactor cultures for each strain were averaged and the standard deviations were viewed as the uncertainties in the obtained extracellular flux values.

2.6 Cell quenching and metabolite extractions

To obtain intracellular metabolites, 7.5 mL cell culture was quenched in 37.5 mL methanol precooled in an ethanol-dry ice bath ($< -70\text{ }^{\circ}\text{C}$). After centrifugation at $-10\text{ }^{\circ}\text{C}$ and 3270g for 5 min, the supernatant was carefully removed through aspiration. The cells were then resuspended in 40 mL cold methanol ($< -70\text{ }^{\circ}\text{C}$) for a wash step and centrifuged again under the same conditions. Following the aspiration of the supernatant, 5 mL 75% ethanol preheated in a water bath ($80\text{ }^{\circ}\text{C}$) was added to the cell pellet for simultaneous lysis of the cell and extraction of intracellular metabolites. Samples were then vortexed for 30 s, incubated in the $80\text{ }^{\circ}\text{C}$ water bath for 3 min, vortexed again for 30 s, briefly cooled in the ethanol-dry ice bath, and centrifuged (same conditions). The supernatant containing the cell extracts was split into two fractions: 3.5 mL was used for LC-MS/MS analysis and 1.5 mL was used for GC-MS analysis. All samples were then dried under airflow using a Pierce Reacti-Therm III Heating/Stirring Module and stored at $-80\text{ }^{\circ}\text{C}$.

2.7 Gas chromatography-mass spectrometry (GC-MS) analysis of intracellular metabolites

Metabolite extracts were resuspended in 20 μL 2% methoxyamine-hydrogen chloride in pyridine (MOX Reagent, Thermo Scientific) and the reaction proceeded for 90 min at $37\text{ }^{\circ}\text{C}$. Subsequently, 25 μL N-tert-butyldimethylsilyl-N-methyltrifluoroacetamide with 1% tert-butyldimethylchlorosilane (TBDMS, Sigma-Aldrich) was added and the samples were incubated for 60 min at $56\text{ }^{\circ}\text{C}$. Following centrifugation to remove cell debris, the supernatant was

analyzed on an Agilent 6890N Network GC System coupled to an Agilent 5975B Inert XL MSD. 3 μ L sample was injected in splitless mode with an inlet temperature of 270 °C. An Agilent J&W DB-35ms column was used with helium as the carrier gas flowing at a rate of 1 mL/min. The temperature of the GC oven was initially set at 100 °C for 1 min, increased to 105 °C at 2.5 °C/min, held at 105 °C for 2 min, increased to 250 °C at 3.5 °C/min, and finally increased to 320 °C at 20 °C/min. The MS operated in electron ionization mode. Electron energy was 69.9 eV and the source and quadrupole temperatures were 230 °C and 150 °C respectively. Mass spectra were obtained using Selective Ion Monitoring (SIM) mode (Ahn & Antoniewicz 2011).

2.8 Liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis of intracellular metabolites

Metabolite extracts were resuspended in 80 μ L Milipore water. An Agilent 1100 Series HPLC system coupled to an API 2000 MS/MS (AB Sciex, Framingham, MA) was used for analysis with an injection volume of 20 μ L. Separation of metabolites was performed on a Waters XBridge C18 Column using an ion pair chromatography method (Luo et al. 2007). The flow rate for the mobile phase (mixture of A and B where A was 10 mM tributylamine + 15 mM acetic acid and B was methanol) was 300 μ L/min with the following solvent profile: 0% B for 8 min; increase to 22.5% B from 8 min to 18 min; increase to 40% B from 18 min to 28 min; increase to 60% B from 28 min to 32 min; increase to 90% B from 32 min to 34 min; held at 90% B from 34 min to 36 min; increase to 100% B from 36 min to 37 min; held at 100% B from 37 min to 42 min. Mass spectra were obtained using multiple reaction monitoring (MRM) mode.

2.9 Metabolic flux estimation

Two separate compartmentalized model bioreaction networks were constructed for intracellular metabolic flux estimation during the growth (G) phase and the lipid production (LP) phase. The G phase model consisted of the enzymatic reactions for the TCA cycle, glyoxylate shunt, gluconeogenesis, PPP, and one-carbon metabolism, as well as the pathways for synthesis of biomass constituents. The reactions for the non-oxidative PPP was modeled using half reactions (Kleijn et al. 2005). The biomass composition for *Y. lipolytica* was adapted from literature (Pan & Hua 2012). To account for variability in lipid content (gram TAGs per gram DCW), the biomass formula was adjusted such that the actual measured lipid content in this study was used. As a result, the biomass equation was slightly different for the MTYL037 versus the MTYL065 strain. Furthermore, synthesis of TAGs was not included in the biomass equation and was represented by the reactions for lipogenic AcCoA and Glyc3P consumption (see section 2.5) in order to better facilitate comparison of TAG production across strains and fermentation phases. Reactions for mitochondrial malic enzyme as well as the gluconeogenic enzymes phosphoenolpyruvate carboxykinase and fructose-1,6-bisphosphatase were also included (Beopoulos et al. 2011; Perea & Gancedo 1982; Jardón et al. 2008). It was shown that in *Y. lipolytica*, the anaplerotic function of pyruvate carboxylase is not essential for growth so long as the glyoxylate shunt pathway is active, which is indeed the case for the conditions used in this study (Flores & Gancedo 2005). Therefore, cytosolic pyruvate carboxylase was omitted from the model to avoid futile cycling mediated by the three enzymes pyruvate carboxylase, pyruvate kinase, and PEP carboxykinase during the modeling process. Separate cytosolic and mitochondrial pools for citrate, succinate, malate, pyruvate, AcCoA and oxaloacetate were constructed. For each of these metabolites, the labeling patterns from both pools were allowed to contribute to the experimentally measured total isotopomer distributions with the relative

contributions of each compartment left as a free parameter for estimation. Reactions for transporting compartmentalized metabolites between the cytosol and the mitochondria were also included. Transportation of cytosolic AcCoA into the mitochondria was assumed to be carried out reversibly by the carnitine shuttle (Eaton 2002). The transport of pyruvate from the cytosol to the mitochondria was assumed to proceed unidirectionally (Maaheimo et al. 2001). Succinate and malate were assumed to be transported through decarboxylate carriers in a unidirectional fashion from the cytosol to the mitochondria (Luévano-Martínez et al. 2010). The carrier for oxaloacetate was omitted since its inclusion did not affect the results for flux estimation significantly (Palmieri et al. 1999). As for the LP phase, all reactions remained the same except for the exclusion of the synthesis reactions for biomass constituents other than TAGs and the inclusion of the reaction for extracellular citrate production. The complete metabolic network models along with carbon atom transitions can be found in Supplementary Tables S2 and S3. Note that reversible fluxes were modeled in terms of a net flux and an exchange flux as opposed to a forward and a reverse flux (Wiechert & de Graaf 1997).

Mass isotopomer distributions (MIDs) were used to describe the labeling patterns of the intracellular metabolites and these data were obtained experimentally from MS measurements using the cell extract samples from cultures with labeled acetate tracers. The cell extracts from natural abundance acetate cultures were also analyzed simultaneously along with the labeled samples. MIDs in the natural abundance samples were compared to that of the theoretical values calculated from the expected effects of naturally occurring heavy isotopes, and the metabolites that have significant discrepancies were excluded during further analysis (Wittmann & Heinzle 1999; Van Winden et al. 2005). This effect of naturally occurring heavy isotopes was also

accounted for in the ^{13}C labeled metabolite samples and the MIDs have been corrected in subsequent analyses.

This study estimates the intracellular metabolic flux distribution using stationary ^{13}C -MFA and all computations were performed using an in-house software that utilizes the concept of elementary metabolite units (Maciek R. Antoniewicz et al. 2007). Under the steady state assumption, a random set of fluxes that satisfies mass balance constraints is first generated and serves as the initial guess to the actual flux distribution. From this initial guess, the expected extracellular fluxes and MIDs can then be simulated and compared to the experimentally determined results. The lack-of-fit between the simulated and experimental values is captured by the weighted sum of squared residuals (WSSR), whose value is minimized through iteratively refining the flux distribution until the minimization algorithm converges. This procedure was repeated 500 times for each strain and phase using different initial guesses and the smallest WSSR was considered as the global minimum. The resulting flux distribution that produced the global minimum is then assumed to be a good estimate to the actual flux distribution within the cell. A Chi-square test was used to evaluate the goodness-of-fit and whether the model for each scenario accurately described the data. 68% and 95% confidence intervals were determined for each flux value using a parameter continuation technique (Antoniewicz et al. 2006). To perform these calculations, the uncertainties in the labeling data for intracellular metabolites were assumed to be 0.4 mol% (Maciek R Antoniewicz et al. 2007; Wasylenko & Stephanopoulos 2013).

3. Results

3.1 Fermentation profiles and establishment of metabolic steady state

The metabolism of *Y. lipolytica* on acetate described in the introduction section is significantly different from that of glucose. Consequently, the culture conditions must also be treated differently. For the case of glucose, the pH of the culture medium gradually decreases. This does not pose a problem when culturing the cells in shake flasks since *Y. lipolytica* can tolerate relatively acidic environments. However, when acetate is used as the carbon substrate, the pH of the culture medium rapidly increases up to 10 after 48 hours, at which the cells can no longer survive (data not shown). The growth rate is also hindered by this pH effect. Consequently, achievable cell density is low and intracellular metabolites cannot be extracted at appreciable amounts, resulting in low signal-to-noise ratios in GC-MS and LC-MS/MS analyses. In order to resolve these issues, 250 mL small scale bioreactors were used for MFA experiments to provide pH control and maintain it at a fixed value of 7.

The performances of the engineered lipid-overproducing strain MTYL065 and the control strain MTYL037 were evaluated and compared when cultured in a low-nitrogen medium (starting C/N = 60) with sodium acetate as the sole carbon source. Each strain was cultured in triplicates with one replicate in 1-¹³C₁ sodium acetate, one in natural abundance sodium acetate, and the other in 40% U-¹³C₂ sodium acetate. Figures 2 and 3 show the time-course fermentation profiles for these six cultures. Addition of the pH control notably extends the fermentation time length beyond 48 hours and the accumulated cell density was sufficient for metabolite extraction. The time course for ammonium consumption (Figure 2b) shows that nitrogen had been depleted from the medium between 44 and 56 hours after inoculation, thereby dividing the entire fermentation period into two phases: the G phase when nitrogen is present allowing for biomass accumulation (24-44 hr), and the LP phase when depletion of nitrogen causes the cell to convert excess carbon into lipids (56-76 hr). During LP phase, the MTYL065 strain consumed acetate

and produced lipids at much faster rates compared to the MTYL037 strain (Figures 2a and 3a). These differences were not as prominent during the G phase. The final lipid contents (g lipid per g dry cell weight) for the three MTYL065 cultures were 53-60%, much higher than the achievable contents for the three MTYL037 cultures (23-28%). Fatty acid distributions shown in Figure 4 were similar between the two strains and the two fermentation phases with oleate being the dominant fatty acid species accounting for ~55% of the total fatty acids. The only byproduct determined through HPLC was citrate. During the G phase, neither strain produced citrate at detectable quantities (Figure 2c). However, citrate began to accumulate during the LP phase with the MTYL037 strain producing nearly four times as much as that of MTYL065 strain.

To perform stationary ^{13}C -MFA, the isotopic labeling patterns of the intracellular metabolites must be at steady state. In order to satisfy this requirement, the cells must be maintained at metabolic steady state during the period of study in which all intra- and extracellular fluxes remain invariant over time. If this condition is maintained for sufficiently long times, the labeling patterns of the intracellular metabolites will eventually reach isotopic steady state, after which the metabolites can be harvested and analyzed (Wiechert 2001). This study uses the labeling patterns of the central carbon metabolites for ^{13}C -MFA. Since these metabolites have very fast turnover rates, they can be expected to reach isotopic steady state relatively quickly if the culture is held in a metabolic steady state (Canelas et al. 2008).

The fermentation profiles can be used to determine whether metabolic steady state has been reached. During G phase, the cells utilize the nitrogen source in the media and actively divide. Under these conditions, it is generally assumed that for batch cultures exponential growth behavior of the cells approximates a metabolic steady state. For the bioreactor batch cultures used in this study, exponential growth is indeed observed as shown in the time courses for dry

cell weight (see Supplementary Figure S1) and therefore metabolic steady state is achieved during the G phase. During the LP phase, the cells no longer have access to nitrogen and can no longer divide, resulting in a constant cell number and loss of exponential growth behavior. Since the total cell number within the culture remains constant, if the cells were to be in a metabolic steady state, the entire culture would be expected to consume acetate and produce citrate and lipids at constant rates. Indeed, the fermentation profiles in Figures 2 and 3 are nearly linear during the LP phase (56-76 hr), implying constant consumption and production rates. Therefore, metabolic steady state is also achieved during this phase. Both the G and LP phases spanned 20-hour timeframes such that the metabolic steady state was maintained sufficiently long for the central carbon metabolites to reach isotopic labeling steady state by the time they were harvested near the end of each fermentation phase.

3.2 Extracellular fluxes

Based on the results shown in Figures 2 and 3, the extracellular fluxes were determined for the two strains MTYL037 and MTYL065. In addition, both fermentation phases were analyzed for each strain. In all four cases, the acetate uptake rate was arbitrarily fixed to a value of 100 in order to compare the intracellular metabolic flux distribution across phases and strains. The extracellular fluxes for dry cell weight, citrate, and lipogenic AcCoA, Glyc3P, and NADPH were normalized based on the acetate uptake rate. Values of these extracellular fluxes along with their uncertainties are listed in Table 1 and they are directly used for metabolic flux analysis. These results show that during the G phase, the control strain MTYL037 utilized the carbon source to generate 20% more biomass compared to the engineered strain MTYL065. On the other hand, the MTYL065 strain generated 50% more TAGs than MTYL037 during the same

phase. As for the LP phase, the MTYL065 strain clearly outperformed the MTYL037 strain by producing nearly twice as much TAGs. In addition, the control strain generated significantly higher amounts of citrate as the byproduct thereby causing its lipid yield to be low.

3.3 Biomass composition

The biomass equation obtained from (Pan & Hua 2012) was modified slightly to reflect the differences in lipid content between the control and engineered strains. The final lipid content achieved by the end of the G phase was 17.2% for the MTYL037 strain and 32.2% for the MTYL065 strain. Amounts of amino acids, carbohydrates, nucleotides, phospholipids, and sterols per gram of dry cell weight are listed in Supplementary Table S1 for both strains. This biomass formula was used in ^{13}C -MFA to estimate the flux for the synthesis of biomass macromolecules other than TAGs.

3.4 Intracellular fluxes

^{13}C -MFA was performed on all four cases. In all scenarios, the measured extracellular fluxes obtained by HPLC and GC-FID as well as the metabolite labeling patterns obtained from GC-MS and LC-MS/MS were used as inputs. The best-fit flux values for central carbon metabolism are shown in Figure 5 and the flux confidence intervals for several important metabolic reactions are shown in Figures 6 and 7. For the G phase, the sum of squared residuals for the MTYL037 and MTYL065 MFA models were 196.1 and 191.0 respectively. Both values fall within the range of 170.0 to 235.6, which is required for the model to accurately describe the data. Similarly, the sum of squared residuals for MTYL037 and MTYL065 LP phase models were 189.3 and 160.2 respectively and they fall within the required range of 134.0 to 199.1

(Antoniewicz et al. 2006). The experimentally measured and model simulated intracellular metabolite MIDs are listed in Supplementary Tables S4 and S5. The complete set of best-fit flux values along with their confidence intervals are listed in Supplementary Tables S6 through S9.

During the G phase, intracellular metabolic flux distributions are largely similar for the two strains despite the flux of AcCoA to TAGs being higher in the MTYL065 strain (Figures 5 and 6a). For instance, both strains exhibit high TCA cycle fluxes to generate ATP for cell growth and maintenance. The glyoxylate shunt pathway and gluconeogenesis are both active to provide fluxes for macromolecule synthesis. The activity of the dicarboxylate carrier, which transports malate from the cytosol to the mitochondria, is present in both strains. This could potentially serve as a way to replenish the TCA cycle metabolite pools for anaplerotic reactions since the high glyoxylate shunt flux constantly draws citrate from the mitochondria to the cytosol. The flux values through major pathways such as the TCA cycle, glyoxylate shunt pathway and gluconeogenesis are nearly the same for the MTYL037 and MTYL065 strains (Figures 5 and 6). The similarities observed between the two strains in terms of intracellular flux distributions during G phase is not surprising. Both strains primarily use the carbon source for biomass accumulation and energy production to support growth and the enzyme activities for these reactions are expected to be comparable in the control vs the engineered strain. Consequently, these similarities outweigh the differences in TAG accumulation, causing the central carbon metabolism flux distribution to have close resemblance between these strains. Nevertheless, the oxidative PPP flux is different with the engineered strain having a higher flux value (17.9 compared to 10.4 for the control strain, Figures 5 and 6b).

During the LP phase, however, several major distinctions emerge between the two strains. When the cells no longer have access to nitrogen, metabolism shifts to allow the conversion of

excess carbon in the medium into TAGs. As a result, the flux of AcCoA to lipids increased significantly in both strains (60% increase in MTYL037 and 100% increase in MTYL065 compared to G phase). Interestingly, the increase in lipid production does not seem to have a significant impact on TCA cycles fluxes for the MTYL037 strain. Although a smaller portion of the cytosolic AcCoA pool is transported into the mitochondria to enter the TCA cycle, the strain uses other metabolic reactions to make up for the loss. Pyruvate kinase, for example, is upregulated by 3-fold compared to the growth phase to divert the majority of the glyoxylate shunt flux back to the TCA cycle (Figures 5 and 7b). The end result is that the MTYL037 strain sacrificed a large portion of the gluconeogenic flux to keep the TCA cycle fluxes high while still synthesizing more lipids during the LP phase. Indeed, the gluconeogenic flux decreased from 11.3 to 7.6 upon entry into this phase (Figure 5). On the other hand, the MTYL065 strain can no longer maintain high fluxes through the TCA cycle. Since the engineered strain produced twice as much TAGs compared to the control strain, the available cytosolic AcCoA that enters the TCA cycle decreased even more. In addition, malate transport activity is nearly shut off and pyruvate kinase activity is much lower compared to the control strain (Figures 5, 7a and 7b). Therefore, even though the engineered strain has considerable glyoxylate shunt flux, the majority of the flux is diverted into gluconeogenesis with only a small portion flowing back into TCA cycle. In the end, this strain has lower TCA cycle fluxes (mitochondrial isocitrate dehydrogenase flux) during LP phase compared to G phase (25.1 versus 33.9, Figures 5 and 6c) but is able to upregulate gluconeogenic enzymes to increase flux through this pathway (13.9 versus 11.9, Figures 5 and 7c).

3.5 Lipogenic NADPH source

The synthesis of amino acids ceases during the LP phase due to the depletion of nitrogen and the cell no longer divides. Therefore, TAG synthesis becomes the primary pathway that requires the reducing cofactor NADPH. In the control strain MTYL037, the estimated consumption of NADPH for fatty acid synthesis is 37.8 mol per 100 mol of acetate (Table 1). In addition, Figure 5 indicates that the flux through oxidative PPP is 20.6. Since 2 molecules of NADPH is generated per reaction through this pathway, the total amount of NADPH generated through oxidative PPP is 41.2 mol per 100 mol acetate, which is enough to fully support lipid synthesis. The minor excess of NADPH (3.4 mol) might be used for other purposes in the cell that are not captured by this truncated model. As for the engineered strain MTYL065, a similar trend can be observed. The NADPH requirement for lipid synthesis in this strain is 69.4 (Table 1) while the amount supplied through oxidative PPP is 75.0 (Figure 5). Once again, NADPH generated is adequate for lipid synthesis with minor excess. In both cases, the amount of lipogenic NADPH required and the amount of NADPH produced through oxidative PPP agree with each other fairly well with less than 8% discrepancy, suggesting that there is a correlation between the two.

To further investigate other possible sources of lipogenic NADPH, the base model for the lipid production phase was revised to include other enzymatic reactions that could potentially generate NADPH. Flux estimation was repeated for the MTYL037 and MTYL065 strains using the modified models. Two cases were analyzed: inclusion of the cytosolic malic enzyme reaction and inclusion of the cytosolic NADP⁺ dependent isocitrate dehydrogenase (IDH). The fluxes through these added reactions and their NADPH producing capacities are listed in Table 2. In the second case, the cytosolic and mitochondrial IDH reactions cannot be differentiated from each other and thus only the upper bound for the flux through cytosolic IDH was listed. The addition

of the cytosolic malic enzyme did not change the flux distribution in either strain (data not shown). Flux through this reaction is near zero despite having large glyoxylate shunt fluxes to generate cytosolic malate as the substrate for the enzyme. Clearly, the lipogenic NADPH cannot come from this reaction. As for the second case, even if the cytosolic IDH flux attains its maximum value, the generated NADPH would only account for 94% and 36% of the required lipogenic NADPH for the MTYL037 and MTYL065 strains respectively. However, this best-case scenario most likely cannot be achieved in the cell since it would require the elimination of all mitochondrial IDH flux thereby significantly lowering energy production due to loss of NADH. Furthermore, similar to the cytosolic malic enzyme case, incorporation of the cytosolic IDH did not alter the fluxes through other pathways (data not shown). Accordingly, as shown in Figure 8, the flux through oxidative PPP remains invariant when either of the two enzymatic reactions are included. Thus, it is largely possible that the required NADPH for TAG biosynthesis is generated through this pathway.

Additional evidence that oxidative PPP is the primary source for lipogenic NADPH comes from the results obtained during the G phase. As mentioned earlier, the metabolic flux map is largely similar for the control versus engineered strain with the exception of the fluxes through oxidative PPP and AcCoA to TAGs. Figure 5 shows that there is a 58% increase in the AcCoA to lipid flux for the MTYL065 strain compared to the MTYL037 strain. Similarly, the oxidative PPP flux is increased by 72% in the engineered strain (Figure 5). The upregulation of oxPPP is likely due to the increase in lipid synthesis flux since most other fluxes are comparable between the two strains. Therefore, correlation between the lipid synthesis flux and the oxidative PPP flux is present in all cases presented in this study, suggesting that oxidative PPP is the primary source of lipogenic NADPH during acetate metabolism.

4. Discussion

We performed ^{13}C -MFA on a control and an engineered strain of *Y. lipolytica* when cultured on acetate as the sole carbon source. For each strain, both the G phase (before nitrogen depletion) and the LP phase (after nitrogen depletion) were analyzed, resulting in a total of four metabolic flux distribution maps. High resolution fluxes for TCA cycle and glyoxylate shunt pathway and good resolution for gluconeogenesis and pentose phosphate pathways fluxes were obtained from parallel labeling experiments using 1- $^{13}\text{C}_1$ sodium acetate and 40% U- $^{13}\text{C}_2$ sodium acetate as separate tracers.

The results demonstrate that the flux through the glyoxylate shunt pathway is high for both strains during both phases and the flux value does not differ significantly among all four cases. As stated previously, in using acetate as the primary carbon source for metabolism, the glyoxylate shunt pathway is active as it provides an avenue for anaplerotic reactions. Gluconeogenic flux is crucial to *Y. lipolytica* during both fermentation phases. In the G phase, gluconeogenesis replenishes the metabolite pools of upper glycolysis and PPP which are constantly syphoned off to generate biomass macromolecules. In the LP phase, the flux through oxidative PPP, which is supported by gluconeogenesis, generates the necessary NADPH for lipid synthesis. However, some steps in gluconeogenesis consume ATP or NADH and thus it is costly for the cells to have a large flux through this pathway. With these considerations, the flux through gluconeogenesis should be tightly regulated.

By observing the differences in how the control and the engineered strains transition from the G phase to the LP phase, this study provides evidence that the point of regulation may occur at two locations, namely, the malate transporter and pyruvate kinase. When the cell needs the

526 gluconeogenic flux for biomass accumulation, as is the case for both strains during the G phase,
527 the malate transporter and pyruvate kinase activities are moderate. In this case, a significant
528 portion of the glyoxylate shunt flux is conserved through gluconeogenesis and only a small
529 portion is diverted back to the TCA cycle. Upon entry into the LP phase, the engineered strain
530 requires an even higher gluconeogenic flux in order to generate sufficient lipogenic NADPH
531 through oxidative PPP. To satisfy this requirement, malate transporter is completely shut off and
532 the activity of pyruvate kinase is relatively low (albeit increased compared to the G phase) to
533 conserve more of the glyoxylate shunt flux for gluconeogenesis. The increase in pyruvate kinase
534 activity could potentially serve as a way to generate more energy compounds through pyruvate
535 dehydrogenase (PDH) and to compensate for the loss of TCA cycle flux. Regardless, the
536 combined effects of malate transporter and pyruvate kinase does indeed allow 63% of the
537 glyoxylate shunt flux to flow through gluconeogenesis which is higher compared to 48% during
538 the G phase. On the other hand, the gluconeogenic flux requirements for the control strain has
539 decreased due to cessation of macromolecule synthesis and low TAG production rates.
540 Consequently, malate transport is active and pyruvate kinase activity is upregulated significantly,
541 thereby recycling the majority of the glyoxylate shunt flux back into the TCA cycle. The effects
542 of these two enzymes are evident in that only 26% of the glyoxylate shunt flux goes into
543 gluconeogenesis, much lower compared to 41% during the G phase. In sum, the changes in
544 activities of the malate transporter and pyruvate kinase along with the conserved high glyoxylate
545 shunt flux can be viewed as a way to dynamically and rapidly alter the flux through
546 gluconeogenesis over a large range. In this way, only the necessary amount of flux for
547 macromolecule and NADPH production flows through gluconeogenesis and the excess

glyoxylate shunt flux is diverted back to the TCA cycle preferentially through pyruvate kinase and PDH in order to produce more ATP.

Another interesting finding comes from the correlation between the amount of lipogenic NADPH required and the amount of NADPH synthesized through the oxidative PPP across two different strains of *Y. lipolytica*. As noted before, the MFA model does not include a cofactor balance and it is entirely possible to have a mismatch of NADPH generated versus consumed in either strain. Therefore, the correlation between the two comes directly from the experimental results and is unbiased by model assumptions. The same conclusion that lipogenic NADPH comes primarily from oxidative PPP was also reached when *Y. lipolytica* was cultured on glucose (Wasylenko et al. 2015). Since the same trend is observed in these two studies where the metabolism of the cell is drastically different, this provides strong evidence that the oxidative PPP might be the only native pathway that can be utilized for lipogenic NADPH production.

For the case of acetate metabolism, it is very costly to divert flux through the oxidative PPP. Overall, a total of 2 AcCoA and 2 ATP molecules are required to generate a maximum of 6 NADPH through this pathway assuming that all the carbons in AcCoA are eventually converted to 4 molecules of CO₂. When the lipid synthesis pathway is active and the demand for lipogenic NADPH is high, the cell needs to use large quantities of AcCoA and ATP. This, along with many other essential reactions such as acetate activation, puts the cell in a state of high energy demand. To make matters worse, constant draining of the available cytosolic AcCoA pool during lipid synthesis and NADPH production decreases the carbons available to enter the TCA cycle and thus the energy producing capacity is reduced. In order to achieve higher lipid titers and yields from acetate, the direct conflict between lipid synthesis and energy production must be resolved through metabolic engineering efforts.

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Figure 1. Overview of the major metabolic pathways involved in triacylglyceride (TAG) synthesis using acetate as the sole carbon source. Metabolites in bold represent the final destinations of the carbon atoms coming from acetate. Abbreviations used: AcCoA, acetyl-CoA; TAGs, triacylglycerides; TCA, tricarboxylic acid; oxPPP, oxidative pentose phosphate pathway; non-oxPPP, non-oxidative pentose phosphate pathway.

Figure 2. Fermentation profiles for extracellular metabolites. Time-course consumption of (a) acetate and (b) ammonium as well as formation of (c) citrate and (d) dry cell weight in 250 mL mini-bioreactors for *Y. lipolytica* strains MTYL037 and MTYL065. Abbreviations used to denote the carbon substrate in the culture medium: 1, 1-¹³C₁ sodium acetate; NA, natural abundance sodium acetate; U, 40% U-¹³C₂ sodium acetate. Other abbreviations used: DCW, dry cell weight.

Figure 3. Fermentation profiles for fatty acids and their biosynthetic precursors acetyl-CoA, Glyc3P and NADPH. Time courses of the total (a) lipid titer as well as lipogenic (b) AcCoA, (c) Glyc3P, and (d) NADPH required for lipid biosynthesis in mini-bioreactors for strains MTYL037 and MTYL065. Abbreviations used to denote the carbon substrate in the culture medium: 1, $1\text{-}^{13}\text{C}_1$ sodium acetate; NA, natural abundance sodium acetate; U, 40% $\text{U-}^{13}\text{C}_2$ sodium acetate. Other abbreviations used: AcCoA, acetyl-CoA; Glyc3P, glycerol-3-phosphate.

Figure 4. Distribution profiles of fatty acids produced by *Y. lipolytica*. The mole fractions of the five principal fatty acids in strains MTYL037 and MTYL065 measured when cells were harvested for ^{13}C -MFA during each phase: (a) growth phase and (b) lipid production phase.

Figure 5. Flux distributions from ^{13}C -Metabolic Flux Analysis. Estimated best-fit values for the MTYL037 strain during growth (WT G) and lipid production phase (WT L) as well as for the MTYL065 strain during growth (AD G) and lipid production phase (AD L). Legend shown on the top right of the figure. For the growth phase, reactions that synthesize biomass macromolecules other than TAGs are not shown. Reversible reactions are described by a net flux and an exchange flux listed in parentheses. Exchange fluxes denoted “nr” could not be resolved.

Figure 6. Flux confidence intervals of selected metabolic reactions. The best-fit flux values and corresponding confidence intervals are shown for reactions that best illustrate differences and similarities across strains (MTYL037 and MTYL065) and fermentation phases (growth and lipid production): (a) AcCoA to lipids; (b) oxidative PPP; (c) mitochondrial IDH (TCA cycle); (d) isocitrate lyase (glyoxylate shunt); (e) PEP carboxykinase. Best-fit values, 68% confidence intervals, and 95% confidence intervals are described by the lines in the middle, the boxes, and the error bars respectively. All flux values are normalized to an acetate uptake rate of 100. Abbreviations: growth, growth phase; lipid, lipid production phase; AcCoA, acetyl-CoA; PPP, pentose phosphate pathway; IDH, isocitrate dehydrogenase; PEP, phosphoenolpyruvate; TCA, tricarboxylic acid.

Figure 7. Flux confidence intervals for metabolic reactions related to glyoxylate shunt pathway and gluconeogenesis. The best-fit flux values and corresponding confidence intervals are shown for reactions that divert the glyoxylate shunt flux back to TCA or into gluconeogenesis: (a) malate transport; (b) pyruvate kinase; (c) enolase. Best-fit values, 68% confidence intervals, and 95% confidence intervals are described by the lines in the middle, the boxes, and the error bars respectively. All flux values are normalized to an acetate uptake rate of 100. Abbreviations: growth, growth phase; lipid, lipid production phase.

Figure 8. Oxidative PPP flux confidence intervals. The best-fit oxidative PPP flux values and corresponding confidence intervals are shown. Four different models of the lipid production phase are used to calculate the oxPPP flux for the two strains (a) MTYL037 and (b) MTYL065. Best-fit values, 68% confidence intervals, and 95% confidence intervals are described by the lines in the middle, the boxes, and the error bars respectively. All flux values are normalized to an acetate uptake rate of 100. Abbreviations: oxPPP, oxidative pentose phosphate pathway; base, base model; malic enz, cytosolic malic enzyme reaction included; cyto IDH, cytosolic IDH reaction included.

752 Table 1. Extracellular fluxes for ¹³C-MFA

	MTYL037 growth	MTYL065 growth	MTYL037 lipid	MTYL065 lipid
Acetate	100	100	100	100
Dry cell weight	2.49 ± 0.19	2.04 ± 0.07	0	0
Citrate	0	0	3.84 ± 0.45	0.693 ± 0.063
AcCoA	12.2 ± 0.9	18.8 ± 0.3	19.4 ± 1.0	37.3 ± 4.1
Glyc3P	0.463 ± 0.036	0.724 ± 0.012	0.750 ± 0.037	1.43 ± 0.15
NADPH	18.8 ± 1.6	33.1 ± 0.6	37.8 ± 1.9	69.4 ± 7.7

753

754 Table 2. Comparison of NADPH generation in modified models

New reaction	Flux		NADPH generated	
	MTYL037	MTYL065	MTYL037	MTYL065
Cytosolic malic enzyme	0.00099	0.00034	0.00099	0.00034
Cytosolic IDH	<35.8	<25.3	<35.8	<25.3

755