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Analysis of *Yarrowia lipolytica* growth, catabolism, and terpenoid biosynthesis during utilization of lipid-derived feedstock

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ABSTRACT

This study employs biomass growth analyses and ^{13}C-isotope tracing to investigate lipid feedstock utilization by *Yarrowia lipolytica*. Compared to glucose, oil-feedstock in the minimal medium increases the yeast's biomass yields and cell sizes, but decreases its protein content (<20% of total biomass) and enzyme abundances for product synthesis. Labeling results indicate a segregated metabolic network (the glycolysis vs. the TCA cycle) during co-catabolism of sugars (glucose or glycerol) with fatty acid substrates, which facilitates resource allocations for biosynthesis without catabolite repressions. This study has also examined the performance of a β-carotene producing strain in different growth mediums. Canola oil-containing yeast-peptone (YP) has resulted in the best β-carotene titer (121 ± 13 mg/L), two-fold higher than the glucose based YP medium. These results highlight the potential of *Y. lipolytica* for the valorization of waste-derived lipid feedstock.

1. Introduction

Yarrowia lipolytica is an attractive industrial yeast for producing natural products due to its well-established genetic tools and beneficial physiological characteristics (Liu et al., 2015; Madzak, 2018; Worland et al., 2020). *Y. lipolytica* can utilize a wide range of carbon substrates, including hydrophobic substances and industrial waste products (Yaguchi et al., 2018). The ability of *Y. lipolytica* to consume lipids and tolerate environmental stresses make this yeast a particularly attractive host for the valorization of waste oils and lipid-based feedstock. In this work, we discuss the biosynthesis of a terpene compound, β-carotene, during growth on lipid and non-lipid feedstocks. Terpenes are naturally occurring compounds that have many diverse and industrially relevant applications, including in pharmaceuticals, food, and fuel, and have been previously been engineered for production in *Y. lipolytica* (Cao et al., 2016; Czajka et al., 2018; Wang et al., 2018; Zhang et al., 2017). Thus, *Y. lipolytica* has the potential to become a lipid-to-terpenoid platform as β-oxidation of fatty acids generates acetyl-CoA, the main precursor of the native mevalonate pathway through which terpenes are synthesized (Du et al., 2016; Marsafari and Xu, 2020). Lipid-based feedstocks have been employed for the production of commodity chemicals by *Y. lipolytica* (Kamzolova & Morgunov, 2013; Poli et al., 2014). During growth on lipid-containing medium, *Y. lipolytica* generates (1) lipases to degrade lipids to its fatty acids and glycerol components (Dulermo and Nicaud, 2011), (2) surfactants to solubilize the hydrophobic components (Gonçalves et al., 2014), and (3) additional cofactors to remove reactive oxygen species generated from β-oxidation of fatty acids (Liu et al., 2019a; Xu et al., 2017). Moreover, *Y. lipolytica* prefers to catabolize medium-chain fatty acids rather than C16:0 and C18:0, while the supplementation of C18:0 leads to accumulation of lipid bodies (Papanikolaou and Aggelis, 2003). Despite the knowledge elucidated by previous work on *Y. lipoylitca* metabolism, the specifics of its lipid catabolism, in particular the partitioning of carbons from lipid-based substrates into central metabolic pathways, is still unclear. Understanding lipid catabolic pathways may inform both fermentation optimization and strain designs.

This work examined the physiology and biosynthesis potential of *Y. lipolytica* wildtype W29 (hereafter referred to as W29) and an engineered strain both cultivated on lipid-derived substrates. W29 is the parent to many industrial *Y. lipolytica* strains (Larroude et al., 2018b;

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Niehus et al., 2018), while the engineered strain has previously been optimized to produce β-carotene (Czajka et al., 2018). β-carotene is both an antioxidant and the precursor for synthesis of diverse carotenoid pharmaceuticals (Grune et al., 2010; Larroude et al., 2018a), serving as the model to study microbial terpenoid synthesis (Wang et al., 2019). In this work, canola oil is used as a representative model lipid feedstock, glycerol as the triglyceride backbone, and oleic acid as the most abundant fatty acid found in canola oil (Ghazani and Marangoni, 2016). Acetate was chosen to represent volatile fatty acids that can be derived from anaerobic digestions of oily feedstock. Glucose was utilized alongside these carbon sources as a control. Nitrogen-limited growth conditions were also evaluated for synergetic effects since nitrogen supply influences yeast lipid metabolism (Zhang et al., 2016). Further, as *Y. lipolytica* often forms hyphae (Timoumi et al., 2018) which is undesirable as it can reduce mass transfer and mixing efficiency in industrial reactors (Bellou et al., 2014), we also explored the effect of carbon sources on the cell morphology. The knowledge gained from examining the effect of carbon sources and nitrogen supply on the biomass yield, metabolic co-utilization, β-carotene production, and morphological changes will facilitate the development of cost-effective bioprocesses for lipid-to-terpenoid production.

2. Materials and methods

2.1. Chemicals, strains, media, and culture conditions

Fully ^{13}C-labeled substrates were purchased from Cambridge Isotopes (Tewksbury, MA, USA). Yeast nitrogen base (YNB) medium without amino acids or carbohydrates (with and without ammonium sulfate) and amino acid supplements were purchased from US Biological Life Sciences (Salem, MA, USA). With amino acid supplements, the medium contained each of 20 amino acids at 0.04 g/L (except for leucine, 0.2 g/L); uracil and inositol at 0.04 g/L; adenine at 0.01 g/L; and p-Aminobenzoic acid at 0.004 g/L. YNB medium which contained no amino acids is referred to as *minimal medium*. YNB medium with amino acid supplements is referred to as *defined medium*. Yeast extract and peptone (YP) were purchased from BD Biosciences (Franklin Lakes, MO, USA). Canola oil was purchased from Schnucks Markets, Inc (St. Louis, MO, USA). All other chemicals were purchased from Sigma Aldrich (St. Louis, MO, USA). To sterilize the growth medium, aqueous substrates were either autoclaved or filtered through 0.22 μm filters. To sterilize the non-aqueous substrates (oleic acid and canola oil), we performed dry heat sterilization at 80 °C for 2 h (Kupiec et al., 2000). Negative controls were conducted alongside experimental conditions to ensure the sterility of the substrates. Two strains were investigated: W29 (ATCC, 20460) and a β-carotene producing strain from Arch Innotek (St. Louis, MO), described in our previous work (Czajka et al., 2018). In brief, β-carotene production was achieved by expressing genes *carB* and *carRP* from the species *Mucor circinelloides*. The seed cultures for both strains were prepared in YP rich medium with 20 g/L peptone, 10 g/L yeast extract, 20 g/L of glucose. 2 g/L of carbon substrates (glucose, glycerol, acetate, oleic acid, canola oil; Supplemental Table S1) in YNB minimal medium (10 mL in shaking tubes) were used for testing yeast growth and biomass yields (in 30 °C at 250 rpm). The low substrate concentration allowed for cell growth under carbon limited aerobic conditions.

2.2. Analysis of amino acid labeling

W29 was cultured with one fully ^{13}C-labeled and one unlabeled carbon substrate, each provided at 1 g/L. A control was conducted with one fully ^{13}C-labeled carbon source, at 2 g/L. The resulting biomass was harvested in the early exponential growth phase (6–8 h after inoculation) by centrifugation (6000 g at 4 °C). The biomass protein was washed then hydrolyzed with 6 M HCl at 100 °C for 20 h. A TBDMS (N-(tert-butyldimethylsilyl)-N-methyltrifluoroacetamide) method was used to analyze the proteinogenic amino acid labeling profiles via GC-MS (You et al., 2012).

2.3. Pulse-trace study of lipid catabolism via inverse ^{13}C-labeling

An inverse labeling technique was applied to track the utilization of lipid carbons. Cultures were first grown in $^{13}C_6$-glucose and then an unlabeled carbon substrate pulsed into the culture at a designated OD_{600} value. Specifically, seed cultures of W29 were prepared in 20 mL YNB with 2 g/L $^{13}C_6$-glucose and were subcultured in the same labeled medium and grown to an OD_{600} of ~2.5. The resulting highly labeled biomass (^{13}C content >90%) was pelleted by centrifugation, washed with 5 mL of 0.9% NaCl solution, and resuspended in YNB media containing no carbon. Unlabeled (1) canola oil, (2) oleic acid, or (3) glycerol was pulsed into the labeled culture. Under conditions (1) and (2), cultures were quenched at 0 s (control), 1 min, 10 min, 1.5 h, 10 h, and 24 h. Due to glycerol being water-soluble and more readily available for consumption, under culture condition (3) the culture was quenched at 20 s instead of at 24 h. The fast-quenching and free metabolite extraction followed our recent protocol (Czajka et al., 2020). Metabolite and isotopomer analyses were performed using LC-MS (a HILIC method) that employed a Shimadzu Prominence-xR UFLC system and a SCIEX hybrid triple quadrupole-linear ion trap MS equipped with Turbo V™ electrospray ionization (ESI) source (Czajka et al., 2020).

2.4. β-carotene production using lipid-derived substrates

The engineered strain was grown in YNB defined medium (with amino acid supplements) or YP rich medium containing glucose or lipid. Cultures were grown for 72 h at 30 °C and 250 rpm. To measure β-carotene content, cultures were pelleted at 6000 g and the supernatant was discarded. The biomass was resuspended in hexane and vortexed in the presence of glass beads until lysed. β-carotene titer was quantified via absorbance at 454 nm (Czajka et al., 2018).

2.5. Cultures under nitrogen-limited conditions

For W29, YNB minimal medium without ammonium sulfate was used with glucose or canola oil as the carbon source. Ammonium sulfate was provided at 0, 0.01, 0.05, 0.2, and 5 g/L. For the engineered strain, YNB defined medium with the same concentrations of ammonium sulfate was tested. Rich YP medium (with and without supplementation of additional 5 g/L of ammonium sulfate) was also tested for the engineered strain. The W29 cultures were grown for 28 h, while the engineered strain cultures were grown for 72 h for β-carotene production.

2.6. Biomass composition and cell imaging analysis

The Molecular Structure Facility in the Genome Core at the University of California at Davis (Davis, CA, USA) performed the compositional analysis of amino acids and total protein contents for *Y. lipolytica* cultures growing in different substrate and nitrogen mediums. All biomass samples were pelleted, washed with sterile water, and lyophilized before sending for compositional analysis. Cell imaging under different carbon conditions was performed at the Air Force Institute of Technology (Dayton, USA). Exponentially growing cells were centrifuged at 4000 rpm for 20 min at 6 °C. Afterward, the cell pellet was washed and then resuspended with 1 mL of sterile water. The sample was then stained with a red-fluorescence lipophilic dye FM 4–64 (5 μg/mL, ThermoFisher Scientific, Waltham, MA, USA) for 5 min in the dark. Afterward, 10 μL of each sample was placed on a glass slide and viewed under an epifluorescence microscope (Zeiss Axioskop). Images were captured with an upright CCD camera attached to the microscope and the exposure time was 2 s for all samples. Five representative regions of interest were randomly selected for each sample. Replicate cultures were processed in the same manner at Washington University, except dye was omitted (Supplemental Fig. S1). The images were captured on an inverted microscope (Zeiss Axio Observer Z1). To identify the OD_{600} correlations with biomass and cell counts, we analyzed dry cell weight (DCW) of

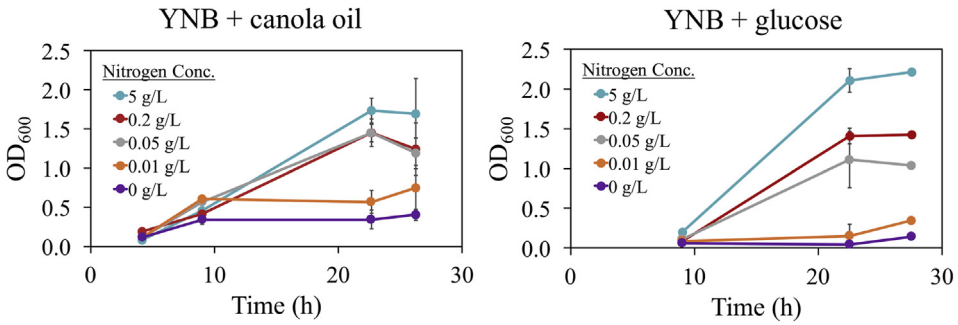

Fig. 1. Nitrogen limitation on W29 growth in YNB medium. Data is presented as averages of biological triplicates (n = 3).

lyophilized biomass pellets and living cell numbers via colony-forming units on agar plates.

3. Results and discussion

3.1. Cell growth on co-carbon substrates and under various nitrogen conditions

Y. lipolytica growth with single carbon substrates has been extensively studied, including with glucose, acetate, glycerol (Gao et al., 2020; Sun et al., 2019; Workman et al., 2013), alkanes (Endoh-Yamagami et al., 2007; Fickers et al., 2005), lipids (Kamzolova et al., 2005; Mlickova et al., 2004), and oleic acid (Michely et al., 2013; Mori et al., 2013). While some studies have characterized growth on co-carbon substrates (Lubuta et al., 2019; Spagnuolo et al., 2018), co-metabolism of mixed substrates with lipid-derived feedstock have not been extensively investigated. Here, we compared the growth of W29 on different carbon substrates under carbon-limited conditions (2 g/L of total carbon, Supplemental Table S1). Our results showed that the fastest growth rate observed for a single carbon source occurred on glycerol-containing medium (0.35 ± 0.05 h^{-1}), consistent with previous reports (Sabra et al., 2017; Workman et al., 2013). As expected, canola oil, the substrate with the highest energy-density, resulted in the highest biomass yield (3.12 ± 0.09 OD_{600}/g substrate). Supplementation of sugars (glucose or glycerol) with acetate, oleic acid, or canola oil promoted cellular growth rates compared to single-substrate cultures. For instance, we found W29 growth rate with canola oil to be 0.28 ± 0.06 h^{-1}, whereas the co-utilization of canola oil with glycerol increased the growth rate to 0.41 ± 0.02 h^{-1}. This increase is likely due to glycerol contributing to the synthesis of surfactants, aiding the digestion of the lipid (Sarubbo et al., 2007). We also found that growth on canola oil led to the most acidified environment (final pH 4.4 ± 0.4), possibly due to the accumulation of overflow organic acids (Kamzolova et al., 2005; Liu et al., 2019b; Morgunov et al., 2013).

The effect of nitrogen conditions on W29 growth was also evaluated. Nitrogen limitation has been shown to increase *de novo* lipid biosynthesis, leading to larger lipid bodies in the cells. In this work, N-limitation was achieved by providing $(NH_4)_2SO_4$ at low concentrations (0–0.2g/L) to the cultures (with 5 g/L as a control). Compared to the nitrogen-limited glucose culture, cells grown on canola oil under N-limitation produced more biomass in YNB minimal medium (Fig. 1) due to canola oil incorporation into intracellular lipid bodies (Beopoulos et al., 2008; Vasiliadou et al., 2018). We also measured protein content to reveal the general abundance of intracellular enzymes and found oil-based feedstocks led to lower protein content than non-oil feedstocks. In rich YP media, canola oil-growing W29 had a protein content of $34.5 \pm 0.2\%$ of biomass, while the protein content in glucose-growing cells was $40.1 \pm 0.6\%$. In YNB minimal media, canola oil cultures had a protein content of $20.5 \pm 0.3\%$, whereas glucose cultures had a protein content of $35.3 \pm 0.2\%$. When cultures were provided 0.01 g/L $(NH_4)_2SO_4$, the resulting protein contents of canola oil and glucose cultures were $10.7 \pm 0.5\%$ and $16.4 \pm 0.8\%$, respectively (Fig. 2a). Despite protein content variations among the tested conditions, the cellular amino acid composition did not significantly differ (Fig. 2b). Therefore, canola oil led to low cellular protein content in minimal mediums, which may limit overall enzyme abundance for terpene biosynthesis (see section 3.3). Next, we aimed to understand the specific metabolic contribution from lipid-derived carbon sources.

3.2. ^{13}C isotopic labeling for determination of carbon contribution to biomass synthesis

^{13}C experiments were performed to elucidate carbon partitioning among pathways from the different carbon sources in minimal media. We provided W29 with a fully ^{13}C-labeled and an unlabeled carbon substrate at a 1:1 ratio by weight and sampled cultures at the early growth phase to determine preferential carbon sources and to evaluate carbon contributions to metabolic nodes. During growth with $^{13}C_6$-glucose and unlabeled glycerol, ^{13}C contributed to 25–35% of the proteinogenic carbons (Fig. 3a), indicating that glycerol is more favorable than glucose (Mori et al., 2013; Workman et al., 2013). When $^{13}C_6$-glucose was provided with either unlabeled acetate or oleic acid, the fatty acids carbons were

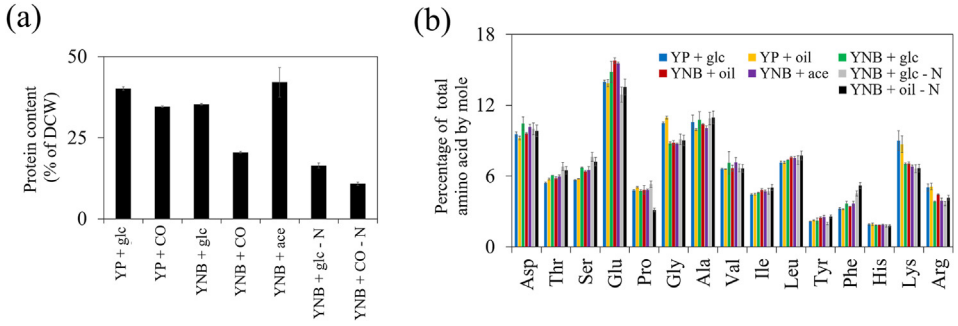

Fig. 2. Total protein content (a) and cell amino acid profiles (b) across different growth medium compositions (n = 2). oil = canola oil and "-N" = nitrogen-limited condition, where nitrogen was supplemented as ammonium sulfate at 0.01 g/L.

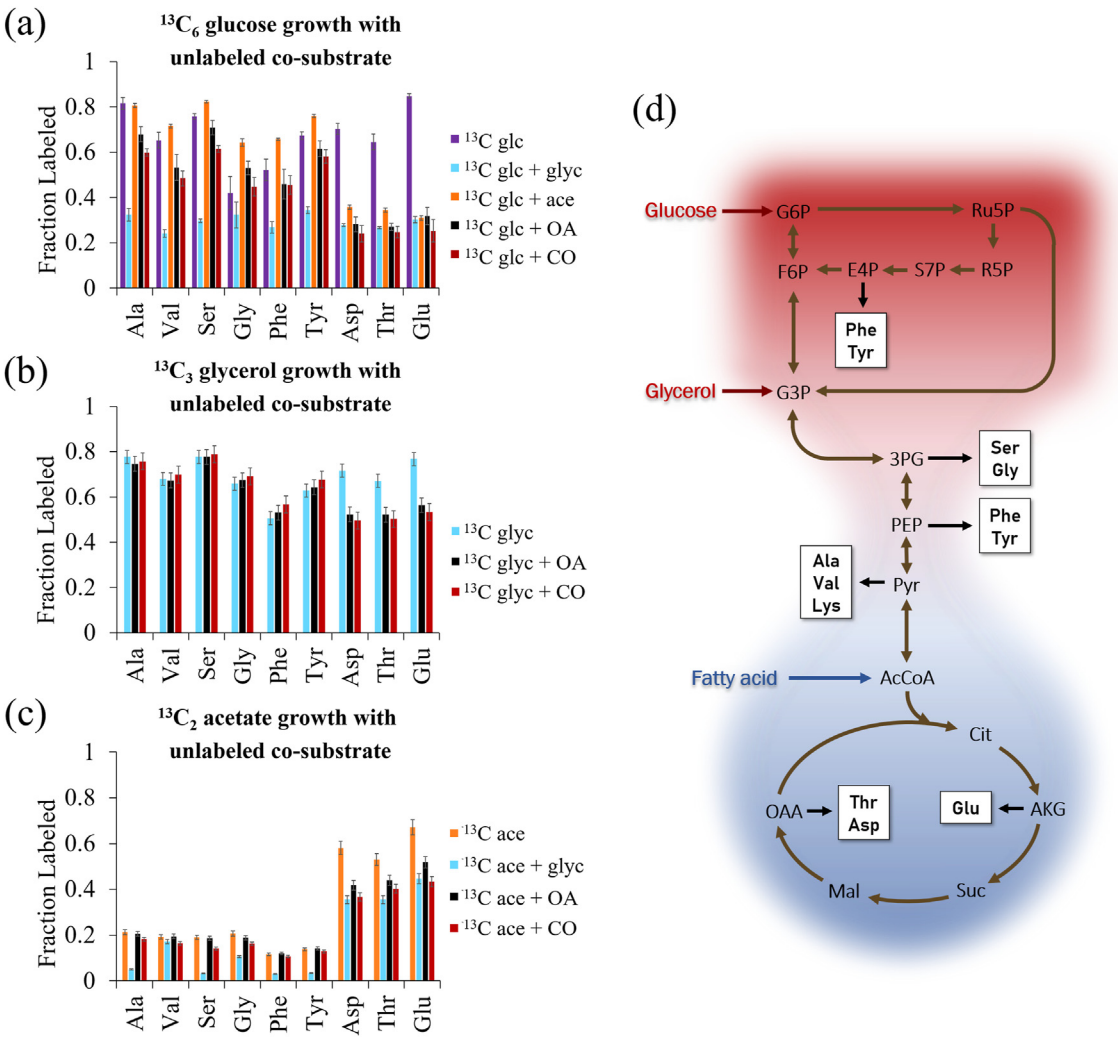

Fig. 3. Amino acid labeling profiles of *Y. lipolytica*. Cultures were grown on single and combinatory carbon sources, where one substrate was fully labeled and the other was unlabeled. The single labeled carbon source was performed as a control, with no additional unlabeled substrate added to the culture. The labeled substrates utilized in this study were: (a) $^{13}C_6$ glucose, (b) $^{13}C_3$ glycerol, and (c) $^{13}C_2$ acetate. Samples were taken in the early exponential phase. Amino acid labeling profiles were measured and the fraction of total labeling is shown on the graphs. Values are averages of biological duplicates and error bars are standard deviations. (d) Metabolic pathway map indicating the contribution of carbon substrates to central carbon pathways and their amino acid production. Fatty acid substrates were seen to contributive to TCA cycle amino acids and metabolites (shaded blue and shaped round) while glucose and glycerol contributed foremost to glycolysis and pentose phosphate amino acids and metabolites (shaded red and shaped square). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

mostly incorporated into the TCA cycle-derived amino acids (aspartate, glutamate, and threonine) without gluconeogenic activity. Cultivation with $^{13}C_6$-glucose and canola oil led to diluted glycolytic derived amino acids labeling compared to the oleic-acid cultures, likely due to the contribution of the unlabeled glycerol backbone of canola oil. The labeling results from the provision of $^{13}C_3$-glycerol (Fig. 3b) with unlabeled canola oil or oleic acid indicated that fatty acid carbons only diluted the labeling of the TCA cycle amino acids (by ~15%). Similarly, $^{13}C_2$-acetate mainly contributed to the labeling of the TCA derived amino acids during its co-utilization with unlabeled glycerol, canola oil or oleic acid (Fig. 3c). In acetate cultures, the yeast could use unlabeled carryover nutrients from the inoculum if the seed was prepared in the YP medium, such that provision of $^{13}C_2$-acetate media to W29 resulted in low labeling in glycolytic derived amino acids (<25%). When a minimal medium seed culture was used, high labeling of all amino acids was observed (>90%). These observations indicate that *Y. lipolytica* requires exogenous nutrients to overcome the low efficiency of gluconeogenesis, and thus fatty acid catabolism is mostly constrained to the peroxisome (β-oxidation) and mitochondria (TCA cycle).

While amino acid labeling information can provide insights into the steady-state growth of the cells on a carbon source, it does not provide a detailed time-dynamic analysis of metabolic activity. Thus, we next employed an inverse pulse-trace approach. Cultures were grown on $^{13}C_6$-glucose to mid-log phase, washed, then pulsed with unlabeled carbon substrates: canola oil (Fig. 4a), oleic acid (Fig. 4b), or glycerol (Fig. 4c). The results for canola oil and oleic acid pulses showed W29 cells required over 10 min before incorporating the carbons from the unlabeled substrate into its metabolism. This lag phase was due to the need for cells to produce surfactants and lipases before utilizing the water-insoluble substrates. In contrast, glycerol utilization was much faster, as shown in the labeling change of glycolytic metabolites (G1P, G6P, and S7P) over time. G6P is an important metabolite node connecting glycolysis and the pentose phosphate pathway. When pulsed with unlabeled glycerol, the three carbon G6P mass isotopologue [m+3] was observed within 20 s, indicating no lag for gluconeogenesis from glycerol. On the other hand, the G6P [m+3] mass isotopologue did not materialize during growth on oleic acid. The canola oil pulse had a similar, albeit delayed, trend as glycerol, providing further evidence that the glycerol backbone of the

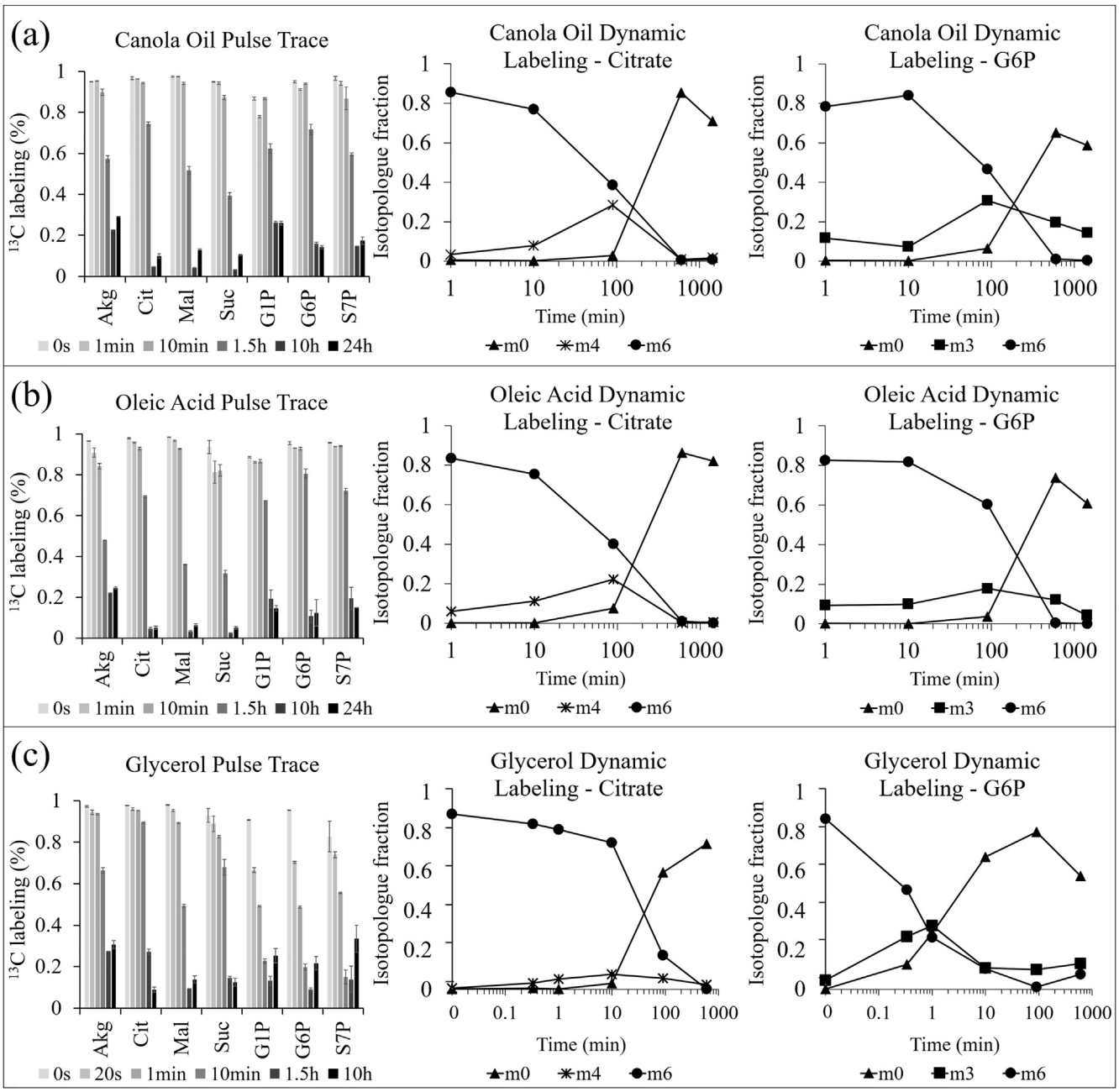

Fig. 4. Dynamic Labeling. W29 cultures were first grown with $^{13}C_6$-glucose, then pulsed with unlabeled canola oil (a), oleic acid (b), or glycerol (c). Left graphs show 7 metabolites' labeling profiles over the course of the experiment. Canola oil and oleic acid cultures were sampled over 24 h, whereas glycerol was sampled up to 10 h, with an additional 20 s sample added in the middle. Middle graphs show the m_0, m_4 and m_6 fragment changes over time for citrate. Right graphs show the m_0, m_3 and m_6 fragment changes over time for G6P. Mass spectrometer peaks m_1, m_2, m_3 means singly, doubly and triply ^{13}C-labeled metabolites. Each data point for all graphs are averages of biological duplicates. The error bars on the far-left graphs of each pane are the standard deviations (n = 2). The error bars on the line graphs are within the markers and therefore are not shown here.

lipid contributed to the glycolytic metabolism while the fatty acid carbons were constrained to the TCA cycle (Fig. 3d). Out of the monitored metabolites, citrate labeling was the slowest during growth on all carbon sources. Citrate is known to have a high intracellular abundance and is in both the cytosol and mitochondria, which can lead to delayed labeling. After pulsing with canola oil or oleic acid, the [m+4] labeling of citrate increased and reached a peak (~22–32%, respectively), consistent with the citrate synthase reaction that condenses an unlabeled acetyl-group from β-oxidation with labeled oxaloacetate.

In summary, pulse-trace labeling indicates that oleic acid is mainly catabolized by the TCA cycle, while glycerol is incorporated into glycolysis and the pentose phosphate pathway before the TCA cycle. Canola oil (a combination of glycerol and oleic acid) showed carbon contribution to all metabolites at equal time frames. During co-utilization of glucose/glycerol and a fatty acid substrate, *Y. lipolytica* lacks catabolite repression and employs a segregated metabolic network. This feature facilitates the metabolic uptake of lipid feedstock with common sugars to promote biosynthesis (Kukurugya et al., 2019).

3.3. *β-carotene producing strain growth and production with lipid-derived substrates*

Y. lipolytica is desirable as a host for production of terpenes due to its strong acetyl-CoA flux and native mevalonate pathway. A β-carotene

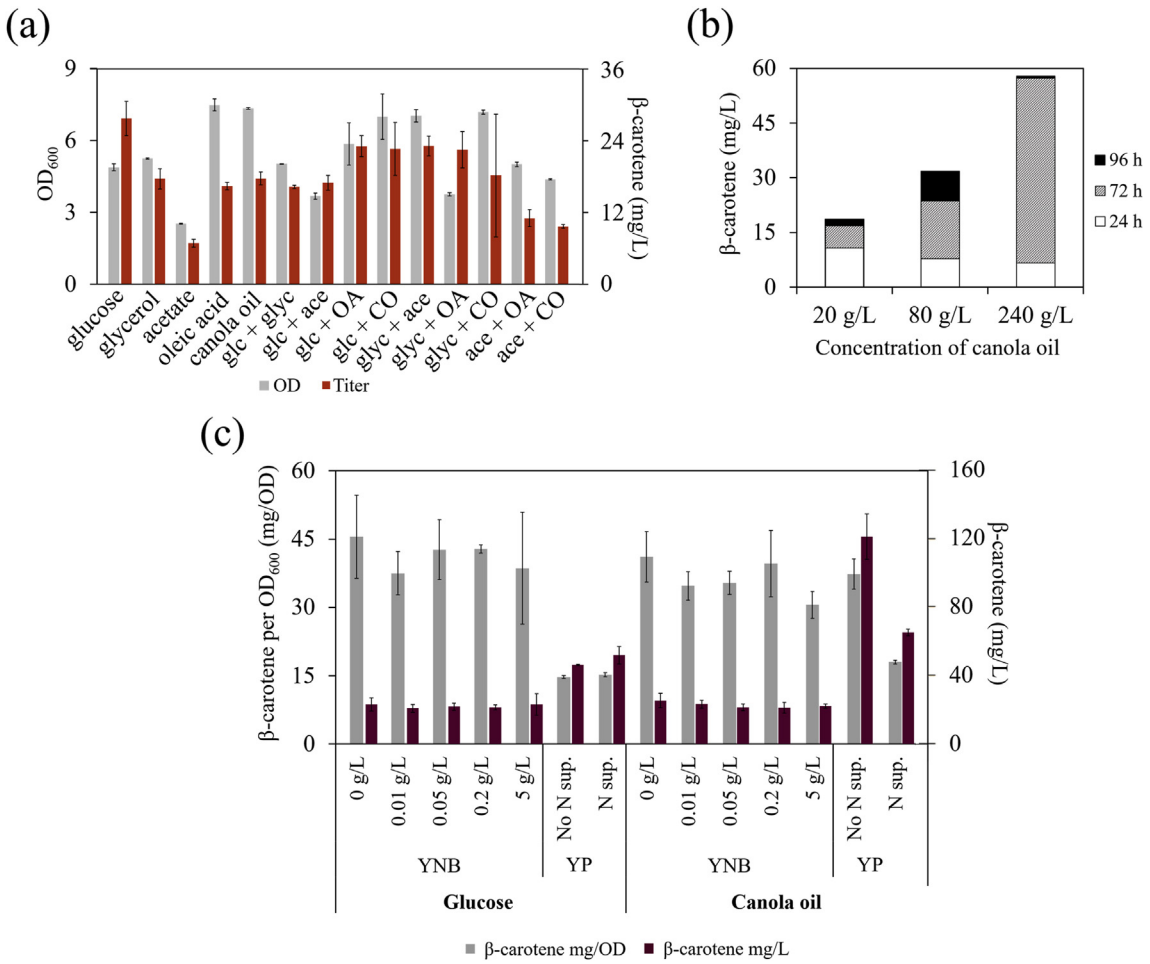

Fig. 5. Effect of carbon substrate on β-carotene production. (A) Biomass and β-carotene production in different single and dual-carbon substrate cultures. The graph indicates 72-h cultivation results with OD_{600} shown in gray and β-carotene content (mg/OD_{600}) in red. Values are an average of biological duplicates and error bars are the standard deviation. (B) High concentrations of lipid were used for β-carotene production. Harvest measurements were taken at 24, 72, and 96 h. Results are presented as β-carotene titer (mg/L). (C) The β-carotene strain was grown in different nitrogen conditions with glucose or canola oil. N sup. = excessive nitrogen supplementation (5 g/L of ammonium sulfate was added in YP medium to further decrease C:N ratio). Data represents the average of biological duplicates of β-carotene content per OD_{600} and β-carotene titer in mg/L. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

producing strain previously described (Czajka et al., 2018) was grown on the different carbon substrates (at a concentration of 2 g/L in YNB defined medium containing amino acids) with the resulting β-carotene and biomass titers examined (Fig. 5a). The glucose-grown engineered strain had the best β-carotene production titer (28 ± 3 mg/L). The cultures grown on canola oil and oleic acid accumulated higher biomass but produced less β-carotene overall (18 ± 1 and 17 ± 1 mg/L, respectively). The decreased β-carotene titers are possibly due to low protein/enzyme content inside of cells (Fig. 2a) and stresses from reactive oxygen species formed during β-oxidation of fatty acids (Xu et al., 2017). The provision of both glucose and canola oil (at 1 g/L each) yielded a β-carotene titer of 23 ± 5 mg/L, because glucose may aid in the cells ability to produce and secrete surfactants to increase the consumption of the lipid substrate (Sarubbo et al., 2007) as well as supporting glycolysis metabolites. Under excessive canola oil (Fig. 5b), an increase in β-carotene titer indicated no substrate inhibitions.

The synergetic effect of carbon and nitrogen supplies on the engineered strain performance were further examined. Although N-limitation has been shown to increase the intracellular supply of acetyl-CoA, we found that limiting $(NH_4)_2SO_4$ supplementation in YNB defined medium showed no effect on β-carotene synthesis (Fig. 5c). The supplied amino acids in the defined medium were a sufficient nitrogen source to promote terpenoid production. We also examined the production of β-carotene in YP mediums: the engineered strain produced up to 53 mg/L of β-carotene with 2 g/L of glucose and 121 ± 13 mg/L β-carotene with 2 g/L of canola oil. Additional $(NH_4)_2SO_4$ supplementation (5 g/L) in the oil YP medium decreased titer to 65 ± 2 mg/L, indicating that the C:N ratio is another factor for optimizing terpene synthesis. Canola oil was more beneficial as a carbon substrate in the YP medium than in defined YNB medium as the extra nutrients could recover cell enzyme/protein abundance (Fig. 2a) and alleviate the pathway bottlenecks due to metabolic segregation (Fig. 3d).

3.4. Effect of culture medium on cell sizes and morphology

One drawback of *Y. lipolytica* systems for industrial fermentations is their potential to transition from ovoid yeast to filamentous forms, causing mixing inefficiencies in bioreactors (Bellou et al., 2014). As reactive oxygen species associated with fatty acid metabolism may induce hypha formation (Xu et al., 2017), we next examined the effects of lipid feedstock on cell morphologies. All experiments were conducted in absence of the dimorphism-inducing compound N-acetylglucosamine (Timoumi et al., 2018). Results showed W29 cells grown on canola oil or acetate in YNB minimal medium appeared more filamentous than other cultures (Fig. 6a). In rich YP medium, W29 showed more elongated cells in canola oil culture than under glucose culture (Supplemental Fig. S1).

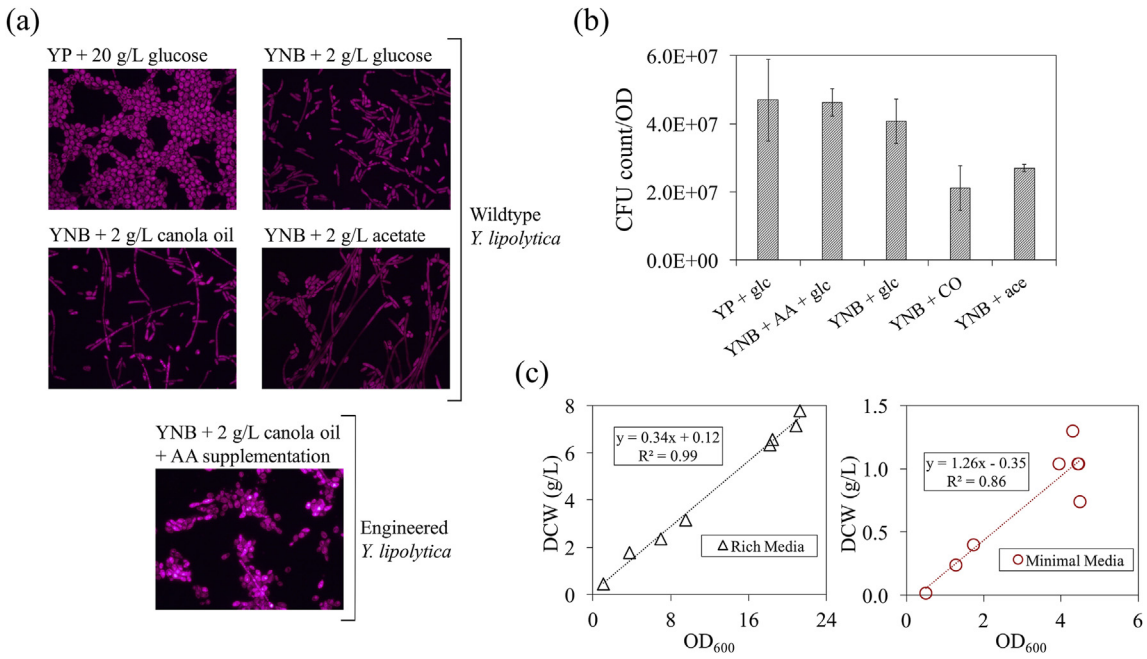

Fig. 6. Cell imaging and colony-forming units. (A) Effect of medium compositions on cell morphology (samples were taken during early growth phase). (B) CFU/OD_{600} ratios, which indicate inconsistent correlation between the number of CFU and OD_{600} due to variability of cell sizes. (C) Cell dry cell weight (DCW) versus OD_{600} measurement for W29 in rich (left) and minimal (right) medium.

The presence of hyphae in the engineered strain cultivated on rich YP medium may be due to the metabolic stress β-carotene imposes on cells (Liu et al., 2016; Verwaal et al., 2010). Interestingly, neither W29 nor the engineered β-carotene strain showed filamentous formation during canola oil growth in YNB defined medium, indicating amino acid supplementation may suppress the transition. Specifically, the presence of leucine has previously been indicated as a key regulator of lipogenesis in *Y. lipolytica* and its high availability in the medium may repress hyphae gene expression (Blazeck et al., 2014; Kerkhoven et al., 2016). Recent research has also shown that the modification of gene *MHY1* is an effective strategy to avoid hyphae formation in *Y. lipolytica* (Konzock and Norbeck, 2020).

The inconsistency of observed cell size and shape across culture conditions strongly implied that OD_{600} measurements do not accurately reflect cell concentration. To test this, we measured colony-forming units (CFU) for different cell conditions and compared them to the OD_{600} measurement. Our results of CFU/OD_{600} ratios confirm that there is not a strong correlation of CFU counts to OD_{600} values across different media conditions (Fig. 6b). However, we found there to be a strong correlation between dry cell weight (DCW) and OD_{600} in rich medium conditions across the growth curve, but a weaker correlation in minimal medium conditions, especially in the stationary phase (Fig. 6c). The decrease in correlation in the minimal media conditions could be due to cell morphological changes skewing the absorbance reading for the OD_{600} measurement. Thus, OD_{600} is a better proxy for total cell mass than it is for cell number, while the correlation between total cell mass and OD_{600} measurements is not consistent across different growth conditions (Stevenson et al., 2016). In summary, *Y. lipolytica* physiologies are sensitive to both cultivation environments and strain backgrounds (Czajka et al., 2020; Egermeier et al., 2017). The interplays of physiochemical (e.g., pH and oxygen), nutritional, and strain background variables on yeast morphological changes are complex and have led to several inconsistent experimental observations (Bellou et al., 2014; Cullen and Sprague, 2012; Timoumi et al., 2018). The prevention of filamentous formation seen during lipid growth needs more in-depth investigation under industrially relevant bioreactor operations.

4. Conclusions

This work explored the potential of *Y. lipolytica* as a platform for the utilization of lipid-based feedstocks for the production of high-value terpene compounds. In previous studies, β-carotene has been shown to partition to the cell membrane, causing stress (Liu et al., 2016; Verwaal et al., 2010), and also shown to sequester to intracellular lipid bodies, alleviating inhibition (Gao et al., 2017). This study also indicates that oil feedstock in minimal medium may decrease enzyme abundances inside cells, leading to lower product synthesis, and thus it is more desirable for co-utilization of lipid feedstock with sugars or nutrient rich sources. The findings of carbon-source contribution to catabolic pathways and metabolic features during substrate co-utilization are valuable for fermentation design and metabolic engineering. Finally, the filamentous morphology of *Y. lipolytica* may be present in lipid-based growth and strategies mentioned could be utilized to suppress hyphae formation. In future research, advanced systems biology tools are needed to reveal the yeasts' regulation of metabolic pathways and morphologies during lipid-based feedstock utilization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Dr. Yechun Wang is the founder of Arch Innotek and company CSO. Dr. Yechun Wang has the following related patent: WANG, Y. 2019. Compositions and methods of biosynthesizing carotenoids and their derivatives. US patent number US20200024607A1.The authors declare no other competing interests.

CRediT authorship contribution statement

Alyssa M. Worland: Writing - original draft, Writing - review & editing, Visualization, Investigation. **Jeffrey J. Czajka:** Writing - review & editing, Investigation. **Yun Xing:** Investigation, Visualization. **Willie F. Harper:** Writing - review & editing. **Aryiana Moore:** Investigation. **Zhengyang Xiao:** Investigation. **Zhenlin Han:** Writing - review &

editing. **Yechun Wang:** Writing - review & editing, Resources. **Wei Wen Su:** Writing - review & editing, Funding acquisition. **Yinjie J. Tang:** Writing - review & editing, Conceptualization, Supervision.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mec.2020.e00130.

Abbreviations

AA	Amino acid supplements
Ace	acetate
AKG	alpha-ketoglutaric acid
CO	canola oil
CFU	colony forming unit
Cit	citrate
DCW	dry cell weight
G1P	glucose 1-phosphate
G6P	glucose 6-phosphate
Glc	glucose
Glyc	glycerol
Mal	malate
OA	oleic acid
S7P	sedoheptulose 7-phosphate
Suc	succinate
TCA	tricarboxylic acid cycle
WT	wildtype
YNB	yeast nitrogen base medium
YP	yeast peptone medium
YPD	yeast peptone dextrose medium

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