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Engineering the push and pull of lipid biosynthesis in oleaginous yeast *Yarrowia lipolytica* for biofuel production

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ABSTRACT

Microbial oil production by heterotrophic organisms is a promising path for the cost-effective production of biofuels from renewable resources provided high conversion yields can be achieved. To this end, we have engineered the oleaginous yeast *Yarrowia lipolytica*. We first established an expression platform for high expression using an intron-containing translation elongation factor-1α (TEF) promoter and showed that this expression system is capable of increasing gene expression 17-fold over the intronless TEF promoter. We then used this platform for the overexpression of diacylglycerol acyltransferase (DGA1), the final step of the triglyceride (TAG) synthesis pathway, which yielded a 4-fold increase in lipid production over control, to a lipid content of 33.8% of dry cell weight (DCW). We also show that the overexpression of acetyl-CoA carboxylase (ACC1), the first committed step of fatty acid synthesis, increased lipid content 2-fold over control, or 17.9% lipid content. Next we combined the two genes in a tandem gene construct for the simultaneous coexpression of ACC1 and DGA1, which further increased lipid content to 41.4%, demonstrating synergistic effects of ACC1+DGA1 coexpression. The lipid production characteristics of the ACC1+DGA1 transformant were explored in a 2-L bioreactor fermentation, achieving 61.7% lipid content after 120 h. The overall yield and productivity were 0.195 g/g and 0.143 g/L/h, respectively, while the maximum yield and productivity were 0.270 g/g and 0.253 g/L/h during the lipid accumulation phase of the fermentation. This work demonstrates the excellent capacity for lipid production by the oleaginous yeast *Y. lipolytica* and the effects of metabolic engineering of two important steps of the lipid synthesis pathway, which acts to divert flux towards the lipid synthesis and creates driving force for TAG synthesis.

1. Introduction

Liquid biofuels are a promising alternative to fossil fuels that can help ease concerns about climate change and smoothen supply uncertainties (Stephanopoulos, 2007). Biodiesel, jet oil and other oil-derived fuels in particular are necessary for aviation and heavy vehicle transport. They are presently produced exclusively from vegetable oils, which is a costly and unsustainable path (Hill et al., 2006). An emerging option is the non-photosynthetic conversion of renewable carbohydrate feedstocks to oil (Li et al., 2008). For biodiesel, a transition from vegetable oil to microbial oil production for the oil feedstock presents numerous additional advantages: adaptability to diverse feedstocks, reduced land requirements, efficient process cycle turnover, and ease of scale-up (Beopoulos et al., 2011). Biological platforms for microbial oil production are also more genetically tractable for further optimization.

Key to a cost-effective microbial technology for the conversion of carbohydrates to oils is high (carbohydrate to oil) conversion yields. Metabolic engineering has emerged as the enabling technology applied to this end (Tai and Stephanopoulos, 2012), building upon experience in successful pathway engineering of microbial biocatalysts for the synthesis of chemical, pharmaceutical and fuel products (Keasling, 2010). Prior efforts at engineering microbes with high lipid synthesis have focused on amplifying presumed rate-controlling steps in the fatty acid synthesis pathway (Courchesne et al., 2009). These efforts, however, have produced mixed results, as fatty acid synthesis tends to be tightly regulated in most organisms (Ohlrogge and Jaworski, 1997). Here we describe an approach that combines the amplification of upstream, metabolite-forming pathways with a similar increase in the flux of downstream, metabolite-consuming pathways. When balanced, this push-and-pull strategy can achieve large flux amplifications with minimal effects due to feedback inhibition.

The oleaginous yeast *Yarrowia lipolytica* is an attractive candidate for microbial oil production, which has also been extensively used in a broad range of other industrial applications: citric acid production, protein production (i.e. proteases and lipases), and

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bioremediation (Beckerich et al., 1998; Papanikolaou et al., 2002b; Scioli and Vollaro, 1997). With a fully sequenced genome and a growing body of tools, engineering of *Y. lipolytica* can be achieved with relative ease (Barth and Gaillardin, 1997). It also has been found to be robust, able to grow on a variety of substrates, and has been used for lipid production on agro-industrial residues, industrial glycerol, and industrial fats (Papanikolaou and Aggelis, 2002, 2003; Papanikolaou et al., 2003). It has excellent lipid accumulation capacity, commonly accumulating up to 36% of its dry cell weight (DCW) in lipids (Beopoulos et al., 2009).

The metabolic pathways for de novo lipid synthesis in *Y. lipolytica* are beginning to be fully mapped out (see Fig. 1). Transport of acetyl-CoA from the mitochondria to the cytosol is carried out by the ATP citrate lyase (ACL)-mediated cleavage of citrate via the citrate shuttle yielding acetyl-CoA and oxaloacetate (OAA). Interestingly, this pathway for cytosolic acetyl-coA generation has been found to be differentially present in oleaginous organisms (Boulton and Ratledge, 1981). Acetyl-CoA carboxylase (ACC) then catalyzes the first committed step towards lipid biosynthesis, converting cytosolic acetyl-CoA into malonyl-CoA, which is the primary precursor for fatty acid elongation. Completed fatty acyl-CoA chains are then transported to the endoplasmic reticulum (ER) or lipid body membranes for the final assembly of triacylglycerol (TAG) via the Kennedy pathway. Over 80% of the storage lipids produced in *Y. lipolytica* are in the form of TAG (Athenstaedt et al., 2006). Cytosolic OAA is converted to malate by malate dehydrogenase (MDH) and transported back into the mitochondria to complete the

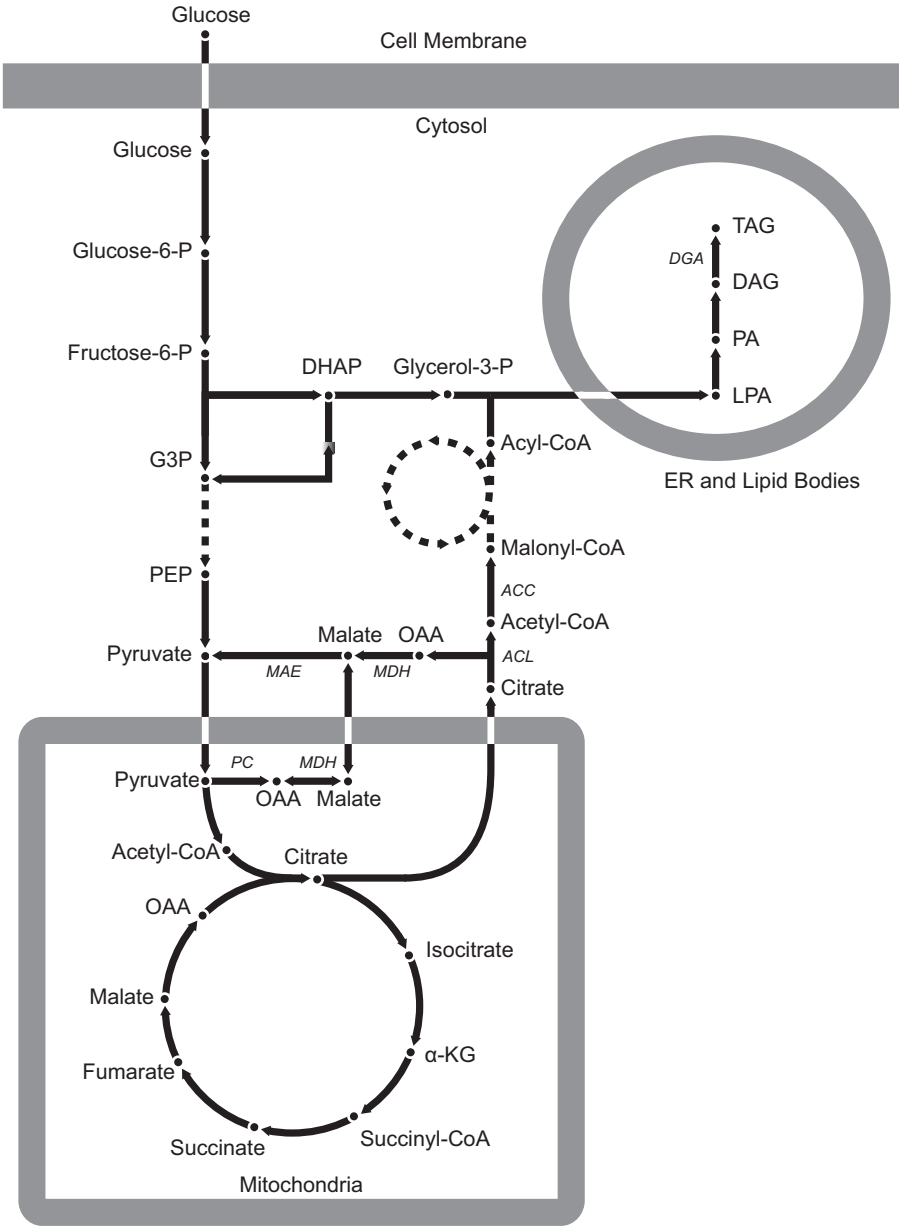

Fig. 1. Overview of the principal metabolic pathways for lipid synthesis in *Y. lipolytica*. Glucose entering glycolysis enters the mitochondria as pyruvate for use in the TCA cycle; however, excess acetyl-coA is transported from the mitochondria to the cytosol via the citrate shuttle. Cytosolic acetyl-CoA is then converted into malonyl-CoA by acetyl-CoA carboxylase (ACC) as the first step of fatty acid synthesis. After fatty acid synthesis, triacylglycerol (TAG) synthesis follows the Kennedy pathway, which occurs in the endoplasmic reticulum (ER) and lipid bodies. Acyl-CoA is the precursor used for acylation to the glycerol-3-phosphate backbone to form lysophosphatidic acid (LPA), which is further acylated to form phosphatidic acid (PA). PA is then dephosphorylated to form diacylglycerol (DAG) and then a final acylation occurs by diacylglycerol acyltransferase (DGA) to produce TAG. OAA oxaloacetate, α-KG alpha-ketoglutarate, PEP phosphoenolpyruvate, G3P Glyceraldehyde 3-phosphate, DHAP dihydroxyacetone phosphate, ACL ATP citrate lyase, PC pyruvate carboxylase, MDH malate dehydrogenase, MAE malic enzyme.

citrate shuttle cycle. Reducing equivalents in the form of NADPH are provided either by the pentose phosphate pathway (PPP) or by malic enzyme (MAE) in the pyruvate/OAA/malate transhydrogenase cycle. In *Y. lipolytica*, high PPP flux and ineffectual MAE overexpression suggest that the former is the primary source for NADPH (Beopoulos et al., 2011; Blank et al., 2005).

Intracellular lipid accumulation can occur via two methods: *de novo* lipid synthesis or *ex novo* incorporation of exogenous fatty acids and lipids. Lipid accumulation most commonly occurs when nutrient supply is exhausted in the presence of excess carbon, typically coinciding with the onset of stationary phase. In practice, the most commonly used limiting-nutrient is nitrogen, as it is easily controllable through media composition (Beopoulos et al., 2009). Ultimately, lipid synthesis pathways are highly regulated in order for the organism to balance cell growth with energy storage. For example, ACC alone is regulated at multiple levels and by multiple factors (Ohlrogge and Jaworski, 1997).

Engineering lipid biosynthesis pathways in *Y. lipolytica* is still relatively unexplored, though initial attempts have shown promise. By eliminating peroxisomal oxidation pathways and engineering glycerol metabolism, *Y. lipolytica* was able to achieve 40%–70% lipids through *ex novo* lipid accumulation (Beopoulos et al., 2008; Dulermo and Nicaud, 2011). Coexpression of Δ6- and Δ12-desaturase genes allowed for significant production of γ-linolenic acid (GLA) (Chuang et al., 2010). Strategies continue to develop for effective engineering of the lipid production pathways to maximize output. By combining the tools for genetic engineering of *Y. lipolytica* with tested strategies developed in the practice of metabolic engineering, we can achieve significant increases of lipid production in this oleaginous yeast host. Here we show how a gene overexpression platform can be established using the intron-enhanced TEF promoter and explore the effects of ACC1 and DGA1 overexpression on lipid accumulation via *de novo* lipid biosynthesis. The coupling of ACC1 and DGA1 allowed effective flux diversion towards lipid synthesis and creation of a driving force by sequestering product formation in lipid bodies.

2. Methods

2.1. Yeast strains, growth, and culture conditions

The *Y. lipolytica* strains used in this study were derived from the wild-type *Y. lipolytica* W29 strain (ATCC20460). The auxotrophic Po1g (Leu^-) used in all transformations was obtained from Yeastern Biotech Company (Taipei, Taiwan). All strains used in this study are listed in Table 3.

Media and growth conditions for *Escherichia coli* have been previously described by Sambrook and Russell (Sambrook and Russell, 2001), and those for *Y. lipolytica* have been described by Barth and Gaillardin (Barth and Gaillardin, 1997). Rich medium (YPD) was prepared with 20 g/L Bacto peptone (Difco Laboratories, Detroit, MI), 10 g/L yeast extract (Difco), 20 g/L glucose (Sigma-Aldrich, St. Louis, MO). YNB medium was made with 1.7 g/L yeast nitrogen base (without amino acids) (Difco), 0.69 g/L CSM-Leu (MP Biomedicals, Solon, OH), and 20 g/L glucose. Selective YNB plates contained 1.7 g/L yeast nitrogen base (without amino acids), 0.69 g/L CSM-Leu, 20 g/L glucose, and 15 g/L Bacto agar (Difco).

Shake flask experiments were carried out using the following medium: 1.7 g/L yeast nitrogen base (without amino acids), 1.5 g/L yeast extract, and 50 g/L glucose. From frozen stocks, precultures were inoculated into YNB medium (5 mL in Falcon tube, 200 rpm, 28 °C, 24 h). Overnight cultures were inoculated into 50 mL of media in 250 mL Erlenmeyer shake flask to an optical density (A_{600}) of 0.05 and allowed to incubate for 100 h (200 rpm, 28 °C), after which biomass, sugar content, and lipid content were taken and analyzed.

Bioreactor scale fermentation was carried out in a 2-liter baffled stirred-tank bioreactor. The medium used contained 1.5 g/L yeast nitrogen base (without amino acids and ammonium sulfate), 2 g/L ammonium sulfate, 1 g/L yeast extract, and 90 g/L glucose. From a selective plate, an initial preculture was inoculated into YPD medium (40 mL in 250 mL Erlenmeyer flask, 200 rpm, 28 °C, 24 h). Exponentially growing cells from the overnight preculture were transferred into the bioreactor to an optical density (A_{600}) of 0.1 in the 2-L reactor (2.5 vvm aeration, pH 6.8, 28 °C, 250 rpm agitation). Time point samples were stored at −20 °C for subsequent lipid analysis. Sugar and organic acid content was determined by HPLC. Biomass was determined by determined gravimetrically from samples dried at 60 °C for two nights.

2.2. Plasmid construction

Standard molecular genetic techniques were used throughout this study (Sambrook and Russell, 2001). Restriction enzymes and Phusion High-Fidelity DNA polymerase used in cloning were obtained from New England Biolabs (Ipswich, MA). Genomic DNA from yeast transformants was prepared using Yeastar Genomic DNA kit (Zymo Research, Irvine, CA). All constructed plasmids were verified by sequencing. PCR products and DNA fragments were purified with PCR Purification Kit or QIAEX II kit (Qiagen, Valencia, CA). Plasmids used are described in Table 3. Primers used are described in Supplementary Table S1.

Table 2
Yield and productivity calculations for 2-L bioreactor fermentation of ACC1+DGA1 transformant. Yield is calculated by grams lipids produced divided by grams glucose consumed. Productivity calculated by concentration of lipids produced per hour. Overall values are calculated from the final values of lipid production over 120 h. Maximum values calculated from the time points between 70 and 100 h, where lipid production was most prominent.

Oil Yield (g/g glucose)	
Overall	0.195
Maximum	0.270
Oil productivity (g/L/h)	
Overall	0.143
Maximum	0.253

Table 1
Total fatty acid content, yield and distribution for *Y. lipolytica* strains. Total fatty acid content is given as means ± S.D. ($n=3$) for a 50 mL culture after 100 h (C/N molar ratio of 20). Fatty acid profiles are given as percent of fatty acid of total fatty acids, with error less than 2.5%.

	Biomass (g DCW)	Lipid content (%)	Lipid yield (g/g glucose)	Fatty acid (fractional abundance %)				
				C16	C16:1	C18:0	C18:1	C18:2
Control (MTYL037)	10.49	8.77 ± 0.37	0.023	20.5	3.9	24.0	43.0	8.7
ACC1 (MTYL040)	6.04	17.9 ± 1.13	0.063	20.3	3.5	23.4	41.7	11.0
DGA1 (MTYL053)	7.53	33.8 ± 0.55	0.094	18.3	2.6	34.1	38.6	6.4
ACC1+DGA1 (MTYL065)	8.60	41.4 ± 1.90	0.114	16.0	2.3	32.8	44.9	4.0

Table 3
Strains and plasmids used in this study.

Strains (host strain)	Genotype or plasmid	Source
E. coli		
DH5α	*fhuA2 Δ(argF-lacZ)U169 phoA glnV44 Φ80 Δ(lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17*	Invitrogen
pINA1269	*JMP62-LEU*	Yeastern
pMT010	*pINA1269 php4d::TEF*	This work
pMT015	*pINA1269 php4d::TEFin*	This work
pMT025	*hp4d-LacZ*	This work
pMT038	*YTEF-LacZ*	This work
pMT037	*YTEFin-LacZ*	This work
pMT013	*YTEF-ACC1*	This work
pMT040	php4d-*ACC1*	This work
pMT053	YTEFin-DGA	This work
pMT065	php4d-*ACC1* + YTEFin-DGA	This work
Y. lipolytica		
Po1g	MATa, leu2-270, ura3-302::URA3, xpr2-332, axp-2	Yeastern
MTYL038	MATa, leu2-270, ura3-302::URA3, xpr2-332, axp-2 TEF-LacZ-LEU2	This work
MTYL037	MATa, leu2-270, ura3-302::URA3, xpr2-332, axp-2 TEFin-LacZ-LEU2	This work
MTYL040	MATa, leu2-270, ura3-302::URA3, xpr2-332, axp-2 hp4d-*ACC1*-LEU2	This work
MTYL053	MATa, leu2-270, ura3-302::URA3, xpr2-332, axp-2 TEFin-*DGA1*-LEU2	This work
MTYL065	MATa, leu2-270, ura3-302::URA3, xpr2-332, axp-2 hp4d-*ACC1* + TEFin-*DGA1*-LEU2	This work

Plasmid pMT010 was constructed by amplifying the translation elongation factor-1α (TEF) promoter region (Accession number: AF054508) from *Y. lipolytica* Po1g genomic DNA using primers MT078 and MT079. The amplicon was inserted between *Sal*I and *Kpn*I sites of the starting vector, pINA1269, also known as pYLEX1, obtained from Yeastern Biotech Company (Taipei, Taiwan). Also included in the reverse primer MT079 were *Mlu*I and *Nsi*I sites to add restriction sites to the multi-cloning site.

Plasmid pMT015 was constructed by amplifying from *Y. lipolytica* Po1g genomic DNA the TEF promoter and the 5′ coding region containing the ATG start codon and 113 bp of the endogenous intron (Accession number: CR382129). Primers MT118 and MT122 were used for this amplification and inserted between *Sal*I and *Mlu*I sites of pMT010. For cloning purposes, some of the intron was omitted so that the *Sna*BI restriction site could be incorporated. Cloning a gene into this plasmid thus requires the omission of the gene's ATG start codon, addition of TAACCGCAG to the beginning of the 5′ primer, and blunt-end ligation at the 5′ end. The plasmid map and sequence of the expression cassette for pMT015 is further detailed in Supplementary Fig. S1.

Plasmid pMT025 was constructed by amplifying the *LacZ* gene, encoding β-galactosidase, from *E. coli* and inserting it into the *Pml*I and *Bam*HI sites of starting vector pINA1269 using primers MT170 and MT171. Plasmid pMT038 was constructed by amplifying the *LacZ* gene and inserting it into the *Mlu*I and *Nsi*I sites of pMT010 using primers MT168 and MT169. Since *LacZ* contains multiple *Mlu*I sites, *Asc*I was used as the 5′ restriction site on MT168 that has a matching overhang. Plasmid pMT037 was constructed by amplifying *LacZ* gene and inserting it into the *Sna*BI and *Nsi*I sites of pMT015. Primers MT172 and MT169 were used, where forward primer MT127 omits the ATG start codon of *LacZ* and instead begins with the sequence TAACCGCAG that completes the intron sequence of pMT015.

Plasmid pMT013 was constructed by amplifying the *ACC1* gene from *Y. lipolytica* Po1g genomic DNA (Accession Number: XM_501721) and inserting it into the *Mlu*I and *Nsi*I sites of pMT010 using primers MT080 and MT081. Plasmid pMT040 was constructed by amplifying the *ACC1* gene and its terminator from pMT013 using primers MT222 and MT137 and inserting this into starting vector pINA1269 digested with *Pml*I and *Cla*I.

Plasmid pMT053 was constructed by amplifying the *DGA1* gene from *Y. lipolytica* Po1g genomic DNA (Accession Number: XM_504700) using primers MT271 and MT272. The amplified gene was digested with *Nsi*I and was inserted into PMT015 in the same manner as in the construction of pMT037.

To produce a single plasmid that could express both *ACC1* and *DGA1*, a promoter-gene-terminator cassette was amplified from pMT053 using primers MT220 and MT265. This was then digested with *Dpn*I and *Ase*I and inserted into pMT040 that was digested with *Nru*I and *Ase*I resulting in tandem gene construct pMT065. The *Ase*I restriction site was selected to facilitate selection, as it resides within the Ampicillin resistance marker. Because *Nru*I is a blunt end restriction site, insertion of the amplicon does not increase the total number of *Nru*I sites to facilitate progressive insertions.

Plasmids were linearized with either *Not*I or *Sac*II and chromosomally integrated into Po1g according to the one-step lithium acetate transformation method described by Chen et al. (Chen et al., 1997). Transformants were plated on selective media and verified by PCR of prepared genomic DNA. Verified transformants were then stored as frozen stocks at −80 °C and on selective YNB plates at 4 °C.

2.3. RNA isolation and transcript quantification

Shake flask cultures grown for 42 h were collected and centrifuged for 5 min at 10,000 g. Each pellet was resuspended in 1.0 mL of Trizol reagent (Invitrogen) and 100 μL of acid-washed glass beads were added (Sigma-Aldrich). Tubes were vortexed for 15 min at 4 °C for cell lysis to occur. The tubes were then centrifuged for 10 min at 12,000 g at 4 °C and the supernatant was collected in a fresh 2-mL tube. 200 μL chloroform was then added and tubes were shaken by hand for 10 s. The tubes were again centrifuged for 10 min at 12,000 g at 4 °C. 400 μL of the upper aqueous phase was transferred to a new tube, and an equal volume of phenol-chloroform-isoamyl alcohol (pH 4.7) (Ambion, Austin, TX) was added. Tubes were again shaken by hand for 10 s and centrifuged for 10 min at 12,000 g at 4 °C. 250 μL of the upper phase was transferred to a new tube with an equal volume of cold ethanol and 1/10th volume sodium acetate (pH 5.2). Tubes were chilled at −20 °C for 30 min to promote precipitation. Tubes were then centrifuged for 5 min at 12,000 g, washed twice with 70% ethanol, dried in a 60 °C oven and finally resuspended in RNAse free water. RNA quantity was analyzed using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE) and samples were stored in −80 °C freezer. qRT-PCR analyses were carried out using iScript One-step RT-PCR Kit with SYBR

Green (Bio-Rad, Hercules, CA) using the Bio-Rad iCycler iQ Real-Time PCR Detection System. Fluorescence results were analyzed using Real-time PCR Miner and relative quantification and statistical analysis was determined with REST 2009 (Qiagen) using actin as the reference gene and MTYL038 as the reference strain (Zhao and Fernald, 2005). Samples were analyzed in quadruplicate.

2.4. β-Galactosidase assay

β-galactosidase enzymatic activity is commonly measured using *ortho*-Nitrophenyl-β-galactoside (ONPG) assay (Juretzek et al., 2001). *LacZ* enzyme activity was measured using the β-gal assay kit from Sigma-Aldrich. Cells were resuspended in PBS buffer and lysed by vortexing with 500 μm glass beads (Sigma-Aldrich) for 2 min. 40 μL of the cell lysate was transferred into 340 μL reaction mix containing 47 mg/mL ONPG, 0.6 M Na_2H-$PO_4 \cdot 7H_2O$, 0.4 M NaH_2PO_4-H_2O, 0.1 M KCl, 0.01 M $MgSO_4 \cdot 7H_2O$. Reaction was incubated at 37 °C for color evolution to occur, and was finally quenched using 500 μL 1 M Sodium Carbonate. Absorbance was then measured with a spectrophotometer at 420 nm. Enzymatic units are calculated based on enzyme activity divided by incubation time and dry cell weight.

2.5. Lipid extraction and quantification

Total lipids were extracted using the procedure by Folch et al. (Folch et al., 1957). A measured quantity of cell biomass (roughly 1 mg) was suspended in 1 mL of chloroform:methanol (2:1) solution and vortexed for 1 h. After centrifugation, 500 μL was transferred to 125 μL saline solution. The upper aqueous layer was removed and the bottom layer was evaporated and resuspend in 100 μL hexane. Samples were then stored at −20 °C until transesterification.

Transesterification of total lipid extracts was performed by adding 1 mL 2% (wt/vol) sulfuric acid in methanol to each sample. Samples were then incubated at 60 °C for 2 h. After that the samples were partially evaporated, and the fatty acid methyl esters (FAME) were extracted by adding 1 mL hexane and vortexing for 10 min. 800 μL of this hexane was then transferred into glass vials for GC analysis.

GC analysis of FAMEs was performed with a Bruker 450-GC instrument equipped with a flame-ionization detector and a capillary column HP-INNOWAX (30 m × 0.25 mm). The GC oven conditions were as follows: 150 °C (1 min), a 10 min ramp to 230 °C, hold at 230 °C for 2 min. The split ratio was 10:1. Fatty acids were identified and quantified by comparison with commercial FAME standards normalized to methyl tridecanoate (C13:0). Total lipid content was calculated as the sum of total fatty acid contents for five FAMEs: methyl palmitate (C16:0), methyl palmitoleate (C16:1), methyl stearate (C18:0), methyl oleate (C18:1), methyl linoleate (C18:2) (Sigma-Aldrich). The addition of tridecanoic acid to the chloroform–methanol extraction fluid was used as the internal standard, which was carried through the entire analysis procedure and transesterified into its methyl ester.

3. Results & discussion

3.1. A high gene expression platform based on the translation elongation factor-1α (TEF) and expression-enhancing intron

In *Y. lipolytica*, several promoters are available for gene expression, including inducible and constitutive ones (Madzak et al., 2004). The TEF promoter was originally identified as being a strong constitutive promoter; however, subsequent cloning, characterization and other alterations resulted in lower expression relative to the inducible XPR2 promoter (Müller et al., 1998). More recently, the hybrid hp4d promoter has been used for its strong quasi-constitutive expression (Madzak et al., 2000), and employed in a number of applications requiring high protein expression (Chuang et al., 2010; Cui et al., 2011; Gasmi et al., 2011).

Analysis of the TEF genomic sequence reveals the presence of a 122-bp spliceosomal intron immediately after the start codon in the 5′ region of the open reading frame. Promoter-proximal spliceosomal introns have often been found to dramatically affect expression of their corresponding genes in a variety of organisms (Le Hir et al., 2003). We hypothesized that the intron impacted strong expression of TEF, and that stronger expression could be achieved by including the intron along with the promoter in the expression vector. Indeed, the initial screening and isolation of the TEF promoter likely relied on the intron-enhanced enrichment in cDNA libraries, a feature that would not have been noticed once the intron was spliced.

To further investigate the effect of introns and compare their impact on relative gene expression driven by three promoters, plasmids pMT025, pMT037 and pMT038 were constructed expressing β-galactosidase (*LacZ*) to compare the relative expression of three promoters (Fig. 2): synthetic hybrid promoter (php4d), TEF promoter without intron (pTEF), and TEF promoter with intron (pTEFintron). Remarkably, the TEFin promoter exhibited a 17-fold increase in expression over the intronless TEF promoter, and a 5-fold increase in expression over the hp4d promoter after 50 h of culture.

The intron enhancement observed in other systems varies wildly: from only 2-fold in human cells and yeast to over 1000-fold in maize (Callis et al., 1987; Furger et al., 2002). Introns are believed to enhance gene expression in a number of ways: by containing regulatory elements, facilitating mRNA export, and increasing transcription initiation rates (Le Hir et al., 2003). Intronic genes, as a group, tend to exhibit higher levels of expression relative to non-intronic genes. For example, in *S. cerevisiae*, intronic genes only represent less than 4% of the total gene count, yet account for 27% of the total RNA generated in the cell (Ares et al., 1999). The genome of *Y. lipolytica* contains introns in 10.6% of its genes (Ivashchenko et al., 2009). Enlisting this endogenous process to enhance expression of our own desired genes represents a simple means for modulating pathway flux, applicable to a broad range of eukaryotic organisms. For example, there is high sequence homology of splice sequences among hemiascomycetous yeast (Bon et al., 2003). While research

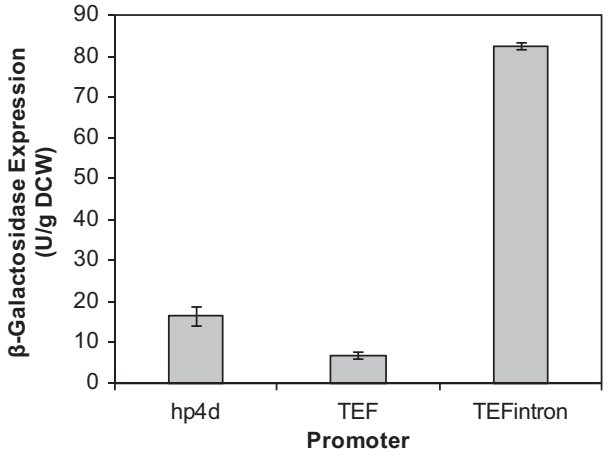

Fig. 2. Enzyme activity of β-galactosidase (LacZ) under the control of different promoters after 50 h of culture. Samples performed in duplicate.

continues to further elucidate the function, evolution, and purpose of introns, the utilization of introns for biotechnological purposes is a relatively untapped opportunity.

3.2. Overexpression of ACC1 and DGA1 leads to significant increases in lipid accumulation

The use of the TEF promoter along with its expression-enhancing intron provides an excellent platform for high gene expression in *Y. lipolytica*. We therefore used this for the overexpression of *DGA1* (pMT053), which has been shown to be important in lipid accumulation in both *Y. lipolytica* and *S. cerevisiae* (Dulermo and Nicaud, 2011; Kamisaka et al., 2007). *ACC1*, already having two endogenous promoter-proximal introns, was not cloned with the *TEFin* promoter. Instead it was cloned with *TEF* and *hp4d* promoters (pMT013 and pMT040, respectively). Growth rates and lipid production were relatively similar between the two (data not shown), so php4d-*ACC1* (pMT040) was used for lipid experiments and for the tandem gene construction of *ACC1*+*DGA1* (pMT065). The hp4d promoter also was selected to minimize the possibility of homologous recombination of the two parallel gene cassettes in the *ACC1*+*DGA1* construct. Simultaneous coexpression of two genes in *Y. lipolytica* using tandem gene construction has been successfully performed elsewhere (Chuang et al., 2010).

Plasmid integration was verified by PCR of prepared genomic DNA of the transformed strains. Overexpression was also measured by RT-PCR of total RNA (Fig. 3A). It was found that the transformants indeed show increased expression of *ACC1* and *DGA1* in the respective strains, with *DGA1* being much more highly expressed than *ACC1*, up to 40-fold increase in expression over the control. *ACC1* was only overexpressed 2 to 3-fold in the transformants, possibly due to the relatively large transcript size of the gene (7000 bp). While gene length is only weakly correlated with mRNA abundance, it has been observed that large genes set upper limits on expression (Akashi, 2001). Slight up-regulation of *ACC1* in the *DGA1* transformant was also observed, suggesting a possible regulatory relationship between the two genes.

The effect of *ACC1* and *DGA1* overexpression on lipid production was first assessed in shake flask experiments (Fig. 3B and Table 1). The control *LacZ* strain only produced 8.77% (g/g DCW) lipids, which is similar to wild-type performance in shake flasks with glucose as sole substrate (Papanikolaou et al., 2006). *ACC1* and *DGA1* transformants both outperformed the control, accumulating 17.9% and 33.8% lipid content, respectively. *DGA1* in particular exhibited almost twice as much lipid accumulation as *ACC1*, almost 4-fold over the control. The biomass generated from the control was significantly higher than the other strains, suggesting that the expression of *ACC1* and *DGA1* impairs somewhat growth of *Y. lipolytica* while diverting cellular resources towards oil synthesis rather than biosynthesis. Overall oil yields were relatively low in comparison to a theoretical maximum yield of 0.32 g/g (Ratledge, 1988). There were also slight shifts in the fatty acid profile, with *ACC1* producing significantly more linoleic acid and *DGA1* maintaining a higher proportion of stearic acid. The proportion of oleic acid stayed relatively even across all transformants.

Improving upon both single gene transformants, *ACC1*+*DGA1* was able to achieve 41.4% lipid content, a 4.7-fold improvement over the control. The biomass production was also improved over the single transformants, but still less than the control. Oil yield improved proportionally, to 0.114 g/g, or 35% of theoretical yield.

Compared to other similar work, the lipid content of the control strain is considered relatively low. However, the media composition in the shake flask experiments had a C/N molar ratio of only 20, and lipid accumulation was derived solely from

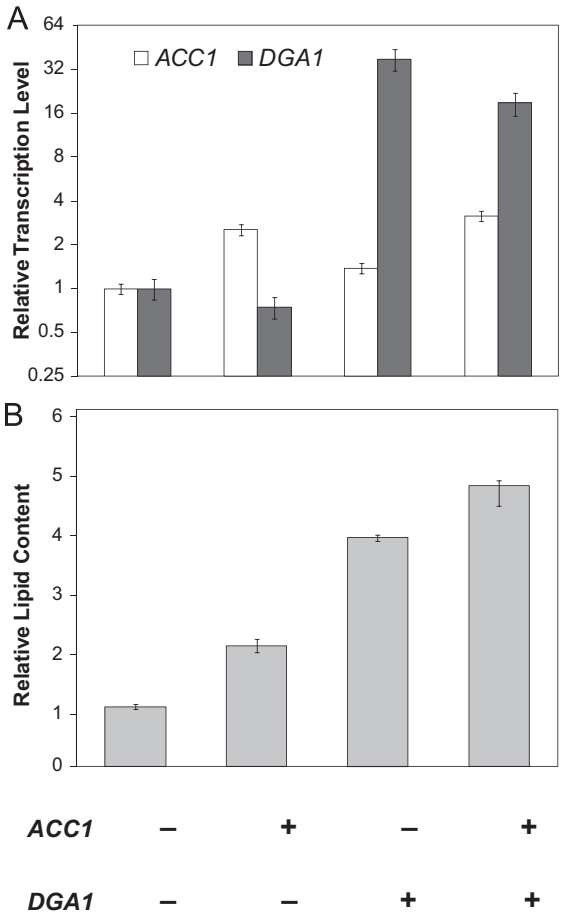

Fig. 3. Combinatorial analysis of strains overexpressing *ACC1* and/or *DGA1*. (A) Relative quantification of RNA transcripts using RT-PCR. Actin was used as the reference gene. Samples performed in quadruplicate, standard error estimated using REST 2009 software. (B) Relative lipid content of strains through total fatty acid analysis after 100 h of culture (C/N Ratio molar ratio of 20). Lipid samples performed in triplicate. MYTL038 was used as the reference strain.

de novo synthesis. Optimal C/N molar ratios for lipid accumulation typically range from 80 to 120 (Beopoulos et al., 2009), suggesting that much higher lipid contents can be achieved under optimized conditions.

In eukaryotic organisms, overexpression of ACC has been met with only limited improvement of lipid production. Heterologous expression of *ACC1* from the oleaginous fungus *Mucor rouxii* in the non-oleaginous yeast *Hansenula polymorpha* was able to achieve only a 40% increase in total fatty acid content, from 3.8% to 5.3% (Ruenwai et al., 2009). In plants, overexpression of *Arabidopsis ACC1* has led to dramatic increases in enzyme activity, but to no more than 30% increase in final lipid content (Klaus et al., 2004; Roesler et al., 1997). It is suspected that improvements in total lipid accumulation have been limited in eukaryotes by the strong metabolic and regulatory control maintained over this enzyme, possibly by free fatty acids. ACC expression and activity is influenced by numerous transcription factors, protein kinases, and metabolites (Brownsey et al., 2006). For example, in *Candida (Yarrowia) lipolytica*, the accumulation of acyl-coA in acetyl-CoA synthetase mutants led to an 8-fold decrease in ACC activity (Kamiryo et al., 1979). Nonetheless, *Y. lipolytica* might represent a regulatory exception in eukaryotic organisms, lending much to its oleaginous nature, as here we achieve a 2-fold increase in lipid content through a commensurate overexpression of endogenous *ACC1*.

The role of DGA has only recently been emphasized to be important for growth and lipid synthesis. In *Y. lipolytica*, three

acyltransferases perform the final step of converting diacylglycerol (DAG) into triacylglycerol (*DGA1/DGA2/PDAT*). A triple knockout of these acyltransferases results in significant growth defect in both lag phase and growth rate, suggesting connections between oil synthesis and normal growth (Zhang et al., 2011). Interestingly, transcriptomic analysis of *Y. lipolytica* identifies only *DGA2* as differentially expressed in lipid accumulation phases, suggesting activation of lipid accumulation may only partially be controlled at the transcription level (Morin et al., 2011). Nonetheless, DGA1p has been identified to predominantly localize to the membrane surface of lipid bodies and is thought to act in concert with triglyceride lipase (*TGL3*) to balance TAG flux in and out of lipid bodies (Athenstaedt et al., 2006). One thus expects the storage of TAGs to rest heavily on the relative activity (and abundance) of DGA1p with respect to its TGL3p counterpart. It has also been hypothesized that DGA diverts flux away from phospholipid synthesis thus creating a driving force for lipid synthesis, as an increased flux is required to produce the still necessary phospholipids (Courchesne et al., 2009). Whether this dynamic and resulting phenotype occurs with overexpression of the other two acyltransferases is unclear, as localization and protein-level interactions may play a role in their regulation. *DGA1* overexpression in other organisms has also led to significant improvements: in an oleaginous Δ*snf2 S. cerevisiae* mutant, *DGA1* overexpression led to accumulation of up to 27% lipid content, a 2.3-fold increase (Kamisaka et al., 2007); in *Arabidopsis, DGAT* overexpression led to a 20-fold increase in lipid content in the leaves, and two-fold overall (Andrianov et al., 2010).

The enhanced lipid accumulation observed in the strains co-expressing *ACC1* and *DGA1* is presumably due to a better balance between the fatty acid and TAG synthesis pathways. acyl-CoA intermediates function as both product and feedback inhibitors in the fatty acid (upstream) pathway and primary precursors in the TAG (downstream) pathway. Up-regulation of the upstream pathway increases the throughput of fatty acid synthesis and, for ACC in particular, diverts flux away from any pathways that would compete for cytosolic acetyl-CoA. Up-regulation of the downstream pathway creates a driving force by depleting acyl-CoA intermediates and increasing the rate of storage of TAG in lipid bodies. However, the two pathways must be well balanced because if modulated individually, they can lead to imbalances that can produce adverse effects on cell metabolism and growth.

Coexpressing *ACC1* and *DGA1* allows one to increase throughput simultaneously in both upstream and downstream pathways without intermediate metabolite accumulation. This leads to increased lipid production as it combines high flux through lipid synthesis from ACC with the driving force provided by the sequestration of TAG into lipid bodies by DGA. The result is a synergistic increase in lipid accumulation, almost 5-fold greater than the control. Indeed, coupling precursor overproduction and driving forces with a metabolic sink to enable a push and pull dynamic has become a very powerful strategy in recent efforts of metabolic engineering, particularly for biofuels (Huo et al., 2011; Liu et al., 2011; Shen et al., 2011).

3.3. Fermentation performance of the ACC1+DGA1 transformant

To further characterize the *ACC1+DGA1* transformant (MTYL065) and explore its lipid accumulation characteristics, large-scale fermentation was conducted using a 2-L stirred-tank bioreactor. Glucose concentration was increased and ammonium sulfate concentration reduced to achieve a C/N molar ratio of 100, which would help promote lipid accumulation.

The strain MTYL037 was used as a control (Fig. 4A), consuming the 90 g/L of glucose within 120 h of fermentation. The culture quickly grew to a biomass concentration of 20 g/L within 48 h, but

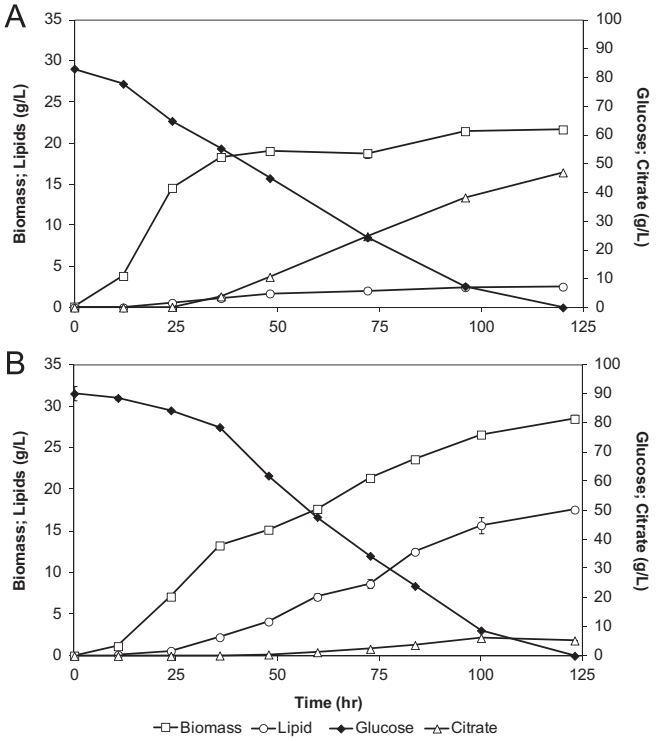

Fig. 4. Batch bioreactor fermentation of control (A) *LacZ* transformant (MTYL037) compared to (B) *ACC1+DGA1* transformant (MTYL065). C/N molar ratio was 100. All sampling was performed in triplicate.

biomass accumulation increased very little afterwards. Over the course of the fermentation, only 2.5 g/L of the 21.6 g/L of biomass was in the form of lipids, resulting in 11.7% total lipid content. Instead, a majority of the carbon was converted into citrate, generating 47 g/L by the end of the fermentation and beginning near the onset of the stationary phase.

For strain MTYL065, glucose was fully consumed over the course of the 120 h fermentation, with final biomass reaching 28.5 g/L (Fig. 4B). Final lipid content was 61.7% of DCW, a 50% increase in lipid accumulation compared to the shake flask experiment, and a 5-fold increase over the control bioreactor, similar to the ratio observed in the shake flasks. Furthermore, citrate production was lower, producing only about 5 g/L despite the same C/N ratio. These results compare favorably with other sugar fermentations (Aggelis and Komaitis, 1999; Cui et al., 2011; Karatay and Dönmez, 2010; Tsigie et al., 2011), as well as values found in *ex novo* lipid accumulation schemes (Dulermo and Nicaud, 2011; Papanikolaou et al., 2002a). Overall yield and productivity was 0.195 g/g and 0.143 g/L/h, respectively; however, during maximum lipid production observed between 70 and 100 h, a yield of 0.270 g/g, and a productivity of 0.253 g/L/h were reached (Table 2). Almost all biomass produced during this phase can be accounted for by the increase in lipid content. The overall and maximum yields achieved are 60.9% and 84.3% of the theoretical yield for TAG synthesis.

While the fatty acid profile is similar between MTYL037 and MTYL065 at the shake flask level, the fatty acid profile changed dramatically during the scale-up (Fig. 5). Relative depletion of stearic acid and enrichment of palmitic acid and oleic acid was observed, with oleic acid ultimately comprising 49.3% of total fatty acids. The ratio between oleic and stearic acids steadily increased throughout the fermentation (data not shown), finally ending in a ratio of 4.6. This is a dramatic change from the ratio of 1.3 seen in shake flask experiments.

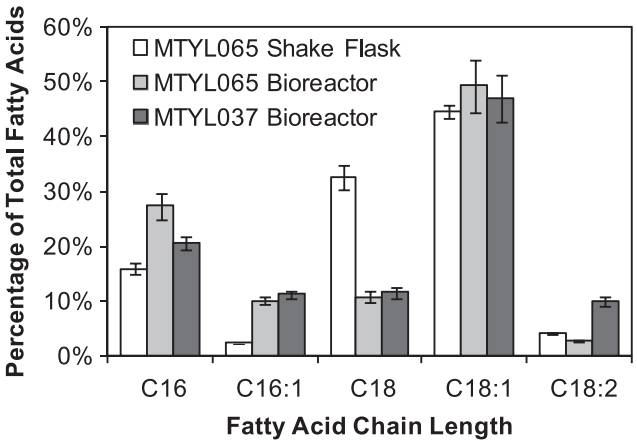

Fig. 5. Fatty acid distribution comparisons for MTYL065 grown in shake flask, 2-L bioreactor fermentation and control strain MTYL037 in 2-L bioreactor fermentation. Samples performed in triplicate. C16 palmitate; C16:1 palmitoleate; C18 stearate; C18:1 oleate; C18:2 linoleate.

High relative oleic acid concentrations (up to 58.5%) have also been observed in other 2-L fermentations (Zhao et al., 2010), and is more similar to profiles of other oleaginous yeasts that accumulate more than 50% lipid content (Beopoulos et al., 2009). In conditions of rapid lipid production, oleic acid might be more rapidly stored and easier to accumulate, as *DGA1*p is known to have varying specificities for different acyl-CoA; in *S. cerevisiae*, C18:1 and C16:0 are the more preferred substrates, having twice the activity of C18:0 (Oelkers et al., 2002). Furthermore, the high oleic acid concentration might also be a response to the higher aeration rate or reactor stresses of the bioreactor and not normally found in shake flask fermentations.

The high lipid content, yield, and productivity seen in the *ACC1*+*DGA1* strain demonstrate the innate capacity of *Y. lipolytica* to accommodate high flux through the lipid synthesis pathway. With further modifications and process optimization, *Y. lipolytica* with engineered lipid synthesis pathways can yield promising breakthroughs in the robust, efficient *de novo* synthesis of lipids.

4. Conclusions

Lipid biosynthesis is a tightly regulated metabolic pathway. For industrially-relevant applications of microbial lipid production, effective engineering of biosynthetic pathways is necessary to maximize yields and productivity. The use of the oleaginous yeast *Y. lipolytica* benefits from its high capacity for lipid accumulation and well-developed tools for engineering the lipid metabolic pathway. Here we show that the intron-enhanced co-overexpression of two important genes in the lipid synthesis pathway, *ACC1* and *DGA1*, provides an enhanced driving force towards the production of lipids, under both moderate and extreme C/N ratios. As the two enzymes carry out the first and last steps of lipid synthesis, the simultaneous push-and-pull of carbon flux towards TAG allows for enhanced production with minimal intermediate accumulation, which can lead to inhibition. The resulting *ACC1*+*DGA1* strain was able to accumulate up to 62% of its DCW as lipids through *de novo* synthesis at an overall volumetric productivity of 0.143 g/L/h.

The concepts of (a) strong overexpression of pathway genes, (b) balance of upstream and downstream pathways, (c) diversion of flux towards desired pathways, and (d) driving forces towards the final product, are prominent strategies in the practice of metabolic engineering, where metabolic networks are engineered and optimized for the generation of desirable products. Implementation of these concepts with respect to lipid accumulation may readily extend to a number of biological platforms, including microalgae. These strategies will be foundational in enabling the technologies of robust, efficient, commodity-scale production of biologically-derived chemicals and fuels. Their application to lipid biosynthesis opens the path for microbial oil overproduction and cost-effective biofuel manufacturing.

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Appendix A. Supplementary information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ymben.2012.08.007.

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