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Metabolic engineering of *Saccharomyces cerevisiae* for the production of triacetic acid lactone

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ABSTRACT

Biobased chemicals have become attractive replacements for their fossil-fuel counterparts. Recent studies have shown triacetic acid lactone (TAL) to be a promising candidate, capable of undergoing chemical conversion to sorbic acid and other valuable intermediates. In this study, *Saccharomyces cerevisiae* was engineered for the high-level production of TAL by overexpression of the *Gerbera hybrida* 2-pyrone synthase (2-PS) and systematic engineering of the yeast metabolic pathways. Pathway analysis and a computational approach were employed to target increases in cofactor and precursor pools to improve TAL synthesis. The pathways engineered include those for energy storage and generation, pentose biosynthesis, gluconeogenesis, lipid biosynthesis and regulation, cofactor transport, and fermentative capacity. Seventeen genes were selected for disruption and independently screened for their effect on TAL production; combinations of knockouts were then evaluated. A combination of the pathway engineering and optimal culture parameters led to a 37-fold increase in titer to 2.2 g/L and a 50-fold increase in yield to 0.13 (g/g glucose). These values are the highest reported in the literature, and provide a 3-fold improvement in yield over previous reports using *S. cerevisiae*. Identification of these metabolic bottlenecks provides a strategy for overproduction of other acetyl-CoA-dependent products in yeast.

1. Introduction

The demand for renewable, biobased products as an alternative to those of petroleum origin is rapidly reshaping the scope of biotechnology. To date, the chemical industry has relied heavily on petroleum resources and chemical catalysis for the production of a wide array of end products (Nikolau et al., 2008). Using microorganisms and enzymes, for example to ferment lignocellulosic biomass (Hamelinck et al., 2005) or to produce novel compounds (Westfall et al., 2012), is an essential component to establishing a biorenewables infrastructure. By pulling together biocatalysis and chemical catalysis, valuable chemical intermediates can similarly be synthesized into numerous end products, with biorenewable feedstocks replacing those from fossil-carbon (Nikolau et al., 2008).

Triacetic acid lactone (TAL), also known as 4-hydroxy-6-methyl-2-pyrone, can be used in the production of polyketide anthraquinones by deriving the tetraphenolic acid from TAL's methyl ether (Evans et al., 1979). Recent efforts have uncovered novel catalysis methods expanding the utility of the TAL moiety. Chia et al. (2012) illustrated that TAL can undergo chemical upgrading through a variety of reactions to generate attractive end products like sorbic acid and 1,3-pentadiene that are precursors to preservatives and plastics, respectively. The ability to readily convert TAL into numerous chemicals that are currently produced using petroleum feedstocks (Chia et al., 2012) makes TAL a relevant precursor candidate for microbial synthesis. While TAL is commercially available, the chemical manufacture of TAL significantly limits its potential for upgrading to low-cost commodity chemicals due to the presence of various byproducts and the requirement of a high-cost multi-stage process (Goel and Ram, 2009; Taeschler, 2010). Therefore developing a biological route for converting sugars directly to TAL will facilitate its use industrially.

TAL is natively produced by the plant *Gerbera hybrida*, where it is further modified to generate anti-pathogen compounds gerberin and parsorboside (Eckermann et al., 1998). The type III polyketide synthase (PKS) 2-pyrone synthase (2-PS), encoded by the *g2ps1* gene, produces TAL via two iterative decarboxylation/condensation reactions using a starter acetyl-CoA and two extender malonyl-CoA molecules (Austin and Noel, 2003), both of which are common metabolites in bacteria and eukaryotes. Recently, Tang et al. (2013) reported the use of directed evolution to identify 2-PS mutants yielding higher expression than the native 2-PS by a

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fluorescence reporter assay. TAL can also be produced from non-native synthases. A closely related type III PKS from *Medicago sativa*, the *chs2*-encoded chalcone synthase (CHS), was shown to produce TAL following three point mutations (T197L/G256L/S338I) resulting in altered specificity for acetyl-CoA and reduced elongations (Jez et al., 2000). The type I PKS 6-methylsalicylic acid synthase (6-MSA) from *Penicillium patulum* was modified to inactivate the ketoreductase domain and produce TAL *in vivo* (Richardson et al., 1999; Xie et al., 2006), and the *Brevibacterium ammoniagenes* fatty acid synthase B (FAS-B) was also rationally designed with an inactive ketoreductase domain (Y2226F) to produce TAL (Zha et al., 2004). A drawback to these latter two systems is that they require a phosphopantetheinyl transferase for activation, while the 2-PS and CHS type III PKSs do not (Austin and Noel, 2003).

Triacetic acid lactone has been reported to have negative effects on microbial cell growth. Specifically, TAL is extremely toxic to *Escherichia coli* with an observed reduction in growth rate of 25% and 90% in 10 mM and 20 mM TAL, respectively, and no growth detected at 50 mM TAL (Laura Jarboe, personal communication). However, there was no effect on the growth rate of *Saccharomyces cerevisiae* at concentrations of up to 200 mM TAL (the higher concentrations exceeded the solubility limit). In the *in vivo* studies on TAL biosynthesis in *E. coli*, concentrations were much lower than 50 mM, and maximal levels were not achieved until after exponential growth (Xie et al., 2006; Tang et al., 2013). Tang et al. (2013) recently reported titers of 2.06 g/L (16.4 mM), corresponding to a yield of 0.102 g/g glycerol (22% of theoretical) using an engineered 2-PS variant (L202G/M259L/L261N) in *E. coli*. This was a significant increase over the previously reported levels achieved in *E. coli* (0.47 g/L, 0.6% of theoretical yield on glucose) using the wildtype 2-PS and under fermenter-controlled conditions (Xie et al., 2006). In this latter study *S. cerevisiae* was also evaluated and produced titers of 0.37 g/L (1.0% of theoretical) using the wild-type 2-PS enzyme and 1.8 g/L (8.9% of theoretical) using an Y1572F variant 6-MSA synthase. In both cases, this was after 168 h of fed-batch fermentation. Yields thus remain relatively low during microbial production of TAL.

The above studies considered the enzyme and culture conditions used for TAL synthesis. The current study focuses on the engineering of *S. cerevisiae* to enhance TAL production via targeted improvements in the pyruvate, acetyl-CoA, and NAD^+ pools. To our knowledge this is the only study to consider metabolic pathway engineering to increase the synthesis of triacetic acid lactone. An emphasis was placed on yeast as the host based on the TAL sensitivities reported in *E. coli*. After establishing an initial expression system, metabolic pathways were engineered for improved TAL biosynthesis. The computational tool OptKnock (Schellenberger et al., 2011) available in the COBRA Toolbox was used to aid the pathway engineering by identifying additional gene deletions for improved flux toward acetyl-CoA and malonyl-CoA. These strain modifications were evaluated independently and in combination to determine their effect on TAL production in *S. cerevisiae*, and final cultivations were done in fed-batch mode to increase titers. The metabolic pathway interventions shown to generate higher levels of TAL should prove directly applicable to the increased synthesis of other polyketide products.

2. Materials and methods

2.1. Strains and plasmids

E. coli strain XL1-Blue (Stratagene, Santa Clara, CA) was used for amplification of plasmids. *S. cerevisiae* strains BY4741 and BY4741Δ*trp1* (Open Biosystems, Huntsville, AL) and BJ5464 (Jones, 1991) were used as the base strains for TAL production, and subsequent engineered strains were derived from these (Table 1). A variety of additional single gene knockouts from the BY4741 knockout library (Open Biosystems) were also utilized for preliminary strain screening. Details on the strain construction can be found in the Supplementary Data.

pXP vectors (Fang et al., 2011) were used to amplify loxP-flanked selectable markers *LEU2-d8, TRP1, MET15,* or *HIS3* for gene disruption. Using the corresponding primer sets (Supplementary Table S1), the markers were amplified with approximately 50 bp homology to the target gene location. Yeast cells were transformed as previously described (Gietz et al., 1992; Hill et al., 1991). Cell colonies were allowed to grow on selective plates for 3–5 days, and gene disruptions were verified following amplification of genomic DNA using Taq polymerase (New England Biolabs, Ipswich, MA), and confirming the presence of the loxP-flanked selection marker.

The pXP218 and pXP842 yeast 2μ-based vectors (Fang et al., 2011; Shen et al., 2012) harbor a *PGK1* or *ADH2* promoter, respectively, the *CYC1* terminator, and a loxP-flanked *URA3* selection marker. The *g2ps1* gene encoding the 2-PS from *G. hybrida* was PCR amplified from the pHIS8 cassette (Jez et al., 2000). Following *SpeI* and *XhoI* digestion, the gene was inserted into pXP218 and pXP842 using the Rapid DNA Ligation Kit (Thermo Scientific, Waltham, MA) to construct pXP218-2PS and pXP842-2PS. Similar methods were used to introduce the altered *chs2* gene encoding the T197L/G256L/S338I mutant of chalcone synthase (CHS) from *M. sativa* (Jez et al., 2000) and a Y1572F mutant of *6-MSAS* from *P. patulum* into pXP842 (Supplementary Data). Plasmid recovery was performed using the GeneJet™ Plasmid Miniprep Kit (Thermo Scientific, Waltham, MA) and DNA sequence analysis confirmed the correct sequence of all PCR-amplified inserts (GeneWiz, South Plainfield, NJ; Eton Biosciences, San Diego, CA).

KOD Hot-start polymerase (EMD Chemicals, San Diego, CA) was used in PCR reactions for plasmid constructs and knockout strains. PCR for site-directed mutagenesis of *6-MSAS* was performed using *Pfu*Ultra II Fusion HS DNA Polymerase (Stratagene). Restriction enzymes, T4 DNA ligase, Taq DNA polymerase, and deoxynucleotides were purchased from New England Biolabs. Oligonucleotide primers were purchased from IDT DNA (San Diego, CA).

2.2. Media and cultivation

Luria-Bertani (LB) media was used for proliferation of XL1-Blue cells with 150 mg/L ampicillin for selection of plasmid-containing strains (Sambrook and Russell, 2001). Complex YPD media (0.5%, 1%, or 2% dextrose; 1% Bacto yeast extract; 2 % Bacto peptone), selective SDC(A) media (1% dextrose, 0.67% yeast nitrogen base, 0.5% Bacto casamino acids, 0.5% ammonium sulfate and 100 mg/L adenine), selective SDC(A,T) media (SDC(A) with 100 mg/L tryptophan), and minimal selective SD media (2% dextrose, 0.67% yeast nitrogen base, 0.5% ammonium sulfate) supplemented with 100 mg/L adenine, 100 mg/L tryptophan, 100 mg/L histidine, and 150 mg/L leucine were used for cultivation of yeast strains. Strains harboring the pXP218-based vectors (*PGK1* promoter) were grown in selective media. Strains harboring the pXP842-based vectors (*ADH2* promoter) were grown in YPD medium.

S. cerevisiae strains were grown for 16 h overnight in 5 mL selective SDC(A) media in an air shaker (New Brunswick Scientific) at 250 rpm and 30 °C, and used to inoculate 5 mL tube or 50 mL flask cultures to an initial cell density (OD_{600}) of 0.3 (Shimadzu UV-2450 UV–vis Spectrophotometer, Columbia, MD). A correlation factor was used to convert OD to dry cell weight per liter (1 OD = 0.43 g DCW/L). During cultivation, samples were taken at 24, 48, and/or 72 h, cell densities were determined, and the samples were centrifuged at 3000 rpm (2600 g) for 5 min at 4 °C (Beckman

Table 1
List of plasmids and strains.

Plasmids	Description	Source
pXP218	2μ vector, *PGK1* promoter, *CYC1* terminator, *URA3* selectable marker	Fang et al., 2011
pXP214	2μ vector, *PGK1* promoter, *CYC1* terminator, *MET15* selectable marker	Fang et al., 2011
pXP216	2μ vector, *PGK1* promoter, *CYC1* terminator, *TRP1* selectable marker	Fang et al., 2011
pXP220	2μ vector, *PGK1* promoter, *CYC1* terminator, *HIS3* selectable marker	Fang et al., 2011
pXP841	2μ vector, *ADH2* promoter, *CYC1* terminator, *LEU2-d8* selectable marker	Shen et al., 2012
pXP842	2μ vector, *ADH2* promoter, *CYC1* terminator, *URA3* selectable marker	Shen et al., 2012
pXP218-2PS	pXP218 harboring the *g2ps1* insertion encoding *G. hybrida* 2-pyrone synthase	This study
pXP842-2PS	pXP842 harboring the *g2ps1* insertion encoding *G. hybrida* 2-pyrone synthase	This study
pXP842-[3X]CHS	pXP842 harboring the T197L/G256L/S338I CHS encoding the *M. sativa* chalcone synthase mutant	This study
pXP842-[1X]6MSAS	pXP842 harboring the Y1572F 6-MSAS encoding the *P. patulum* 6-MSA synthase mutant	This study

Strains	Description	Source
BY4741	*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Open Biosystems
BY4741Δ*trp1*	BY4741 *trp1::KanMX*	Open Biosystems
BJ5464	*MATα ura3-52 trp1 leu2Δ1 his3Δ200 pep4::HIS3 prb1Δ1.6R can1 GAL*	E.W. Jones (1991)
BYAN	BY4741 with integrated copy of *Aspergillus nidulans npgA*, *ADH2* promoter	This study
BY4741Δ*pyc1*	BY4741 *pyc1::KanMX*	Open Biosystems
BY4741Δ*pyc2*	BY4741 *pyc2::KanMX*	Open Biosystems
BY4741Δ*gpd1*	BY4741 *gpd1::KanMX*	Open Biosystems
BY4741Δ*pgm1*	BY4741 *pgm1::KanMX*	Open Biosystems
BY4741Δ*pgm2*	BY4741 *pgm2::KanMX*	Open Biosystems
BY4741Δ*nte1*	BY4741 *nte1::KanMX*	Open Biosystems
BY4741Δ*mls1*	BY4741 *mls1::KanMX*	Open Biosystems
BY4741Δ*pck1*	BY4741 *pck1::KanMX*	Open Biosystems
BY4741Δ*zwf1*	BY4741 *zwf1::KanMX*	Open Biosystems
BY4741Δ*yia6*	BY4741 *yia6::KanMX*	Open Biosystems
BY4741Δ*gsy1*	BY4741 *gsy1::KanMX*	Open Biosystems
BY4741Δ*gsy2*	BY4741 *gsy2::KanMX*	Open Biosystems
BY4741Δ*fbp1*	BY4741 *fbp1::KanMX*	Open Biosystems
BYtΔ*adh1*	BY4741Δ*trp1 adh1::LEU2*	This study
BYtΔ*pep4*	BY4741Δ*trp1 pep4::LEU2*	This study
BYtΔ*prb1*	BY4741Δ*trp1 prb1::MET15*	This study
BYtΔ*pep4*Δ*prb1*	BY4741Δ*trp1 pep4::LEU2 prb1::MET15*	This study
BYtΔ*prb1*Δ*pyc2*	BY4741Δ*trp1 prb1::MET15 pyc2::LEU2*	This study
BYtΔ*prb1*Δ*pyc2*Δ*yia6*	BY4741Δ*trp1 prb1::MET15 pyc2::LEU2 yia6::TRP1*	This study
BYtΔ*prb1*Δ*pyc2*Δ*nte1*	BY4741Δ*trp1 prb1::MET15 pyc2::LEU2 nte1::TRP1*	This study
BYtΔ*prb1*Δ*pyc2*Δ*yia6*Δ*nte1*	BY4741Δ*trp1 prb1::MET15 pyc2::LEU2 yia6::HIS3 nte1::TRP1*	This study
BJΔ*pyc2*	BJ5464 *pyc2::TRP1*	This study
BJΔ*pyc2*Δ*nte1*	BJ5464 *pyc2::TRP1 nte1::LEU2*	This study

GS-6R Centrifuge, Brea, CA). The supernatants were stored at 4 °C for HPLC analysis of TAL levels in the culture broth.

2.3. Plasmid stability

The yeast strain was cultivated in 1% YPD medium for 48 h, diluted with sterile water, and plated onto YPD plates. Approximately 100 colonies were transferred to selective SDC-A plates and non-selective YPD plates (to verify viability). The percent plasmid-containing cells was determined as the number of colonies on the SDC-A plates divided by the number of viable colonies transferred. Three independent experiments were performed.

2.4. Glucose-limited fed-batch fermentations

A New Brunswick BioFlo III system equipped with a 2.5 L working capacity vessel was employed for fed-batch operation. Initial 5 mL overnight cultures were used to inoculate a 50 mL shake flask culture that was used as the seed inoculum. Media was YPD containing 1% glucose. Following inoculation to an OD_{600} of 0.3 (V=1.5 L), the fermentor pH was controlled at pH 6 by automatic supply of either 6 M sodium hydroxide or 6 M hydrochloric acid. Agitation speed was 400 rpm and sparged air was initially supplied at 0.2 vvm. To maintain the dissolved oxygen levels above 20%, the aeration rate was gradually increased to a maximum of 0.8 vvm. Following 12 h of batch cultivation in the fermentor, a glucose feed (3.6 M) was initiated. The speed of the glucose feed pump was varied to maintain DO levels at 20% with a constant aeration rate of 0.8 vvm and a maximum glucose pump speed of 1.5 mL/h. The fermentation was allowed to proceed for a total of 120 h, leading to a final culture volume of approximately 1.7 L.

2.5. HPLC assay

The concentration of triacetic acid lactone was measured by HPLC using a Shimadzu HPLC system: LC-10AT pumps (Shimadzu), UV–vis detector (SPD-10A VP, Shimadzu), Zorbax SB-C18 reversed-phase column (2.1 × 150 mm, Agilent Technologies). Acetonitrile buffered in 1% acetic acid was used as the mobile phase, while HPLC grade water buffered in 1% acetic acid was used as the aqueous phase. A gradient program using a 95–85% Pump B

gradient (H_2O with 1% acetic acid) provided an elution time of approximately 12 min (flow rate 0.25 mL/min, column temperature 25 °C).

2.6. Yield calculations

Actual yield was calculated as the total TAL formed divided by the total glucose supplied to the culture at inoculation. Maximum theoretical yield was calculated based on the metabolic reactions from glucose to TAL assuming all glucose was converted to product (*i.e.*, no growth) and was determined to be 0.47 g/g glucose. (The theoretical maximum on glycerol was also found to be 0.46 g/g glycerol.) Therefore, the percent of the theoretical yield of TAL on glucose was calculated as: [TAL (g/L)/Glucose Fed (g/L)/0.47 (g/g) * 100%]. Although complex YPD medium was used, it does not support significant cell growth in the absence of glucose (< 6% of total growth). Therefore, other carbon sources in YPD should have little effect on the calculated yields.

3. Results and discussion

3.1. Resistance of Saccharomyces cerevisiae to TAL

Previous research has demonstrated the high tolerance of *S. cerevisiae* to TAL relative to *E. coli*. To further explore the sensitivity of this yeast to TAL, we determined the effects of two TAL concentrations on yeast growth at a pH of 5.0 and 6.0. We first supplemented with increasing amounts of solid TAL and determined the solubility limits at 30 °C, approximately 9.0 g/L in water and 6.5 g/L in SDC(A) and YPD media. A significant drop in solubility was observed when temperature was reduced, with a TAL solubility of 2.8 g/L at 4 °C in SDC(A) medium.

To determine the effects on growth, *S. cerevisiae* strain BY4741 was cultivated in SDC(A) or complex YPD medium containing 1% glucose and spiked with TAL at 5 or 10 g/L. Medium pH was then adjusted to 5.0 or 6.0. Insoluble TAL (in the 10 g/L culture) was allowed to settle prior to measuring cell density. Growth rates and final cell densities (48 h) were determined, and are shown in Table 2. Increasing TAL concentration reduced both growth rate and final cell density; this effect was more pronounced at the lower pH and in SDC(A) medium. In the complex YPD medium, minimal decreases were observed, particularly at pH 6.0. YPD has a natural buffering capacity around pH 6.5 relative to SDC(A); in SDC(A), a drop in pH (up to 1 pH unit) occurs during cultivation. With a pKa of 4.9 (Moreno-Mañas et al., 1988), levels of the protonated form of TAL will increase with decreasing culture pH. Protonated forms of fatty acids are incorporated at higher levels in the membrane resulting in membrane leakage in *S. cerevisiae* and reduced growth rate (Liu et al., 2013). Related lactones can also be incorporated into the membrane and detrimentally affect membrane fluidity and integrity (Aguedo et al., 2003). Therefore, at lower pH, the protonated TAL may similarly be detrimental to the membrane and thus cell growth rate.

The results in Table 2 are obtained for high levels of TAL added at the start of batch culture; even so, the effects on growth were relatively minor in *S. cerevisiae*. High TAL concentrations are not expected until late in the cultivation, therefore *S. cerevisiae* is an excellent host for TAL production.

Table 2
Growth rates and final cell densities for strain BY4741 cultured in the presence of varying concentrations of TAL in two media at a pH of 5.0 or 6.0. Values are mean ± standard deviation ($n=3$).

Media	Starting pH	Maximum specific growth rate (h^{-1})			Final cell density at 48 h (g DCW L^{-1})		
		0 g/L TAL	5 g/L TAL	10 g/L TAL[a]	0 g/L TAL	5 g/L TAL	10 g/L TAL[a]
SDC (A,T,U)	5.0	0.42 ± 0.02	0.33 ± 0.01	0.28 ± 0.02	6.6 ± 0.5	4.3 ± 0.3	2.9 ± 0.5
	6.0	0.43 ± 0.01	0.38 ± 0.01	0.33 ± 0.01	6.2 ± 0.4	5.5 ± 0.1	5.1 ± 0.2
YPD	5.0	0.42 ± 0.01	0.37 ± 0.01	0.32 ± 0.01	9.9 ± 0.4	7.9 ± 0.1	6.2 ± 0.1
	6.0	0.42 ± 0.01	0.40 ± 0.01	0.38 ± 0.01	9.9 ± 0.3	9.1 ± 0.1	8.6 ± 0.1

[a] 10 g/L exceeded solubility and TAL precipitated.

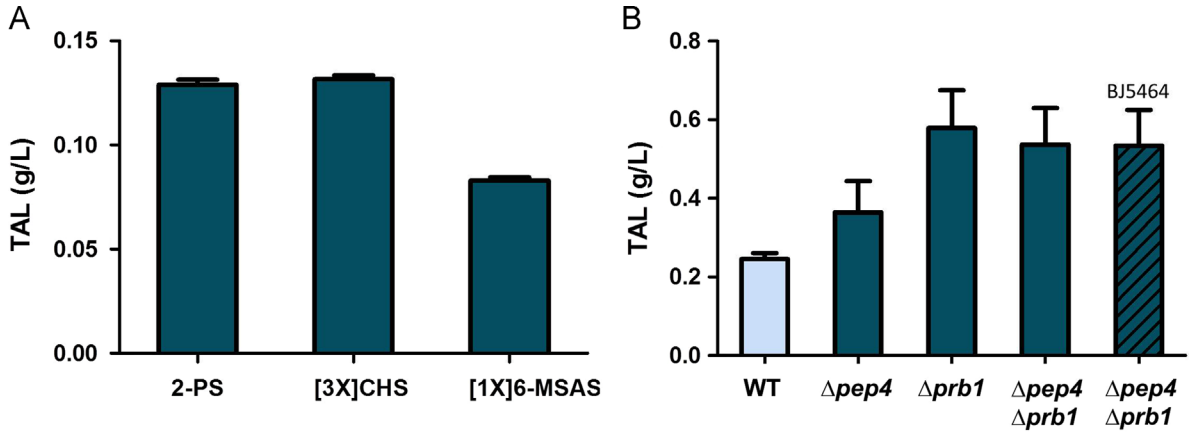

Fig. 1. (A) TAL titers for *S. cerevisiae* strain BY4741 expressing the wildtype *G. hybrida* 2-pyrone synthase (2-PS), the triple mutant (T197L/G256L/S338I) *M. sativa* chalcone synthase ([3X]CHS), and the single mutant (Y1572F) *P. patulum* 6-MSAS ([1X]6-MSAS). The [1X]6-MSAS was expressed in strain BYAN (BY4741 with one integrated *A. nidulans npgA* gene for activation of the synthase). HPLC analysis was performed on samples collected after 48 h of cultivation in YPD (0.5% glucose). (B) Comparison of TAL titers in BY4741 protease knockout strains expressing the 2-PS under the control of the *ADH2* promoter. Expression in protease deficient strain BJ5464 is shown for comparison (hatched bar). Bars represent mean values ± one standard deviation ($n=6$ independent experiments).

3.2. Initial expression system and culture conditions

Our baseline strain BY4741 expressing *G. hybrida* 2-pyrone synthase under the strong glycolytic *PGK1* promoter (plasmid pXP218-2PS) produced only 58 mg/L of TAL (see Supplementary Data) with a yield on glucose of less than 1% (g/g). Therefore, we varied the promoter, synthase, and culture conditions, and introduced protease knockouts to increase TAL levels prior to our pathway engineering.

The late-phase *ADH2* promoter has shown excellent performance for the expression of other polyketides and fatty acid synthases in our laboratory and others (Kealey et al., 1998; Leber and Da Silva, 2013; Lee et al., 2009; Ma et al., 2009; Mutka et al., 2006). This promoter is active when glucose levels fall, and optimal induction occurs in 1% glucose YPD medium (Lee and Da Silva, 2005). Due to the late induction, plasmid stabilities remain high even in complex medium (Shen et al., 2012). Substitution of this promoter (plasmid pXP842-2PS) increased titers 4.5-fold over the *PGK1* promoter to 260 mg/L (see Supplementary Data; Fig. S2). This increase is similar to that observed by Xie et al. (2006); they reported higher levels with the *ADH2* promoter relative to the *GPD1* and *ADH1* promoters. In our batch cultures, only a 10% increase in TAL was observed from 48–72 h (Fig. S2); therefore a 48 h cultivation period was selected for subsequent studies. As expected, a 1% glucose level resulted in the best performance (see Supplementary Data).

The native *G. hybrida* 2-pyrone synthase (2-PS) is responsible for the synthesis of TAL, but mutant PKSs have also been shown to make TAL (Jez et al., 2000; Xie et al., 2006). These two mutants were briefly compared (T197L/G256L/S338I chalcone synthase from *M. sativa*, Y1572F 6-MSA synthase from *P. patulum*) with the native 2-PS using the same promoter and backbone vector (pXP-842). During the 48 h cultivation, the variants produced similar or lower levels of TAL relative to 2-PS (Fig. 1A). The native 2-PS enzyme was selected for use in our strain development studies. An additional advantage of moving forward with the 2-PS relative to the 6-MSAS variant is that overexpression of a phosphopantetheinyl transferase (for activation) is not required.

Yeast proteases have been shown to act on heterologous enzymes produced in *S. cerevisiae*, and can negatively affect PKS levels and thus polyketide production. In our previous work (Lee et al., 2009), disruption of the two vacuolar protease genes (*PEP4* and *PRB1*) encoding the aspartyl protease and proteinase B, respectively, were shown to be critical to detect lovastatin nonaketide synthase (LNKS) produced in yeast. Therefore, BY4741 strains with single and double knockouts of these genes were evaluated, and TAL production was measured following a 48 h cultivation in YPD. The Δ*pep4* strain resulted in an increase in titer of 50% over the base strain, while Δ*prb1* yielded an increase of 135% (Fig. 1B), with no added improvement when combining the deletions (Δ*pep4*Δ*prb1*). An independent strain (BJ5464) that also lacks these two proteases produced similar levels of TAL. Reduction of proteolytic degradation of 2-PS is thus important for TAL production. Titer and yield increased more than 2-fold to 580 mg/L and 12% of theoretical yield (g/g glucose).

3.3. Identification and disruption of competing pathways to improve TAL synthesis

The 2-PS requires one acetyl-CoA starter and two malonyl-CoA extender units to generate one triacetic acid lactone product (Jez et al., 2000). These two precursors are produced naturally by *S. cerevisiae* and are in high demand for energy production, fatty acid biosynthesis, and other cellular activities, with malonyl-CoA pools believed to be a significant limiting factor in these processes (Chen et al., 2012; Wattanachaisaereekul et al., 2008). Synthesis of acetyl-CoA/malonyl-CoA metabolites relies on cytosolic pyruvate,

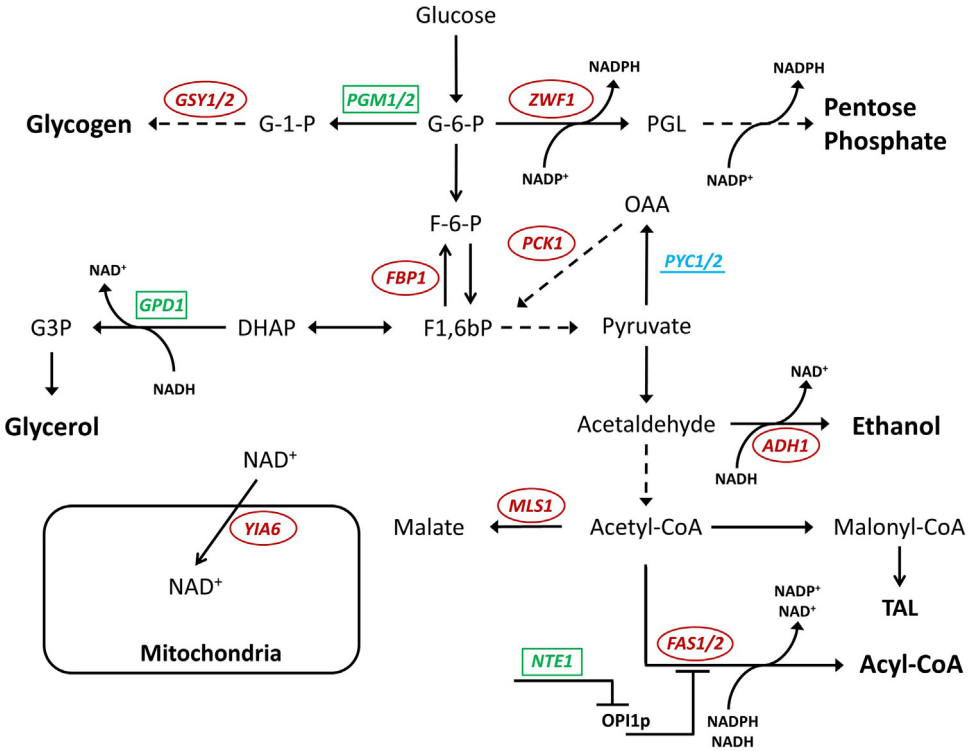

Fig. 2. Metabolic pathways identified for optimizing TAL precursors in *S. cerevisiae*. The highlighted metabolites (bold) identify major by-products generated in central carbon metabolism leading to reduced pyruvate and acetyl-CoA pools. The genes corresponding to enzymes catalyzing the reactions are shown in italics and were determined by pathway analysis (circled), computational predictions (boxed), or both (underlined). In this schematic, reactions not directly used in our analysis have been consolidated as dashed arrows.

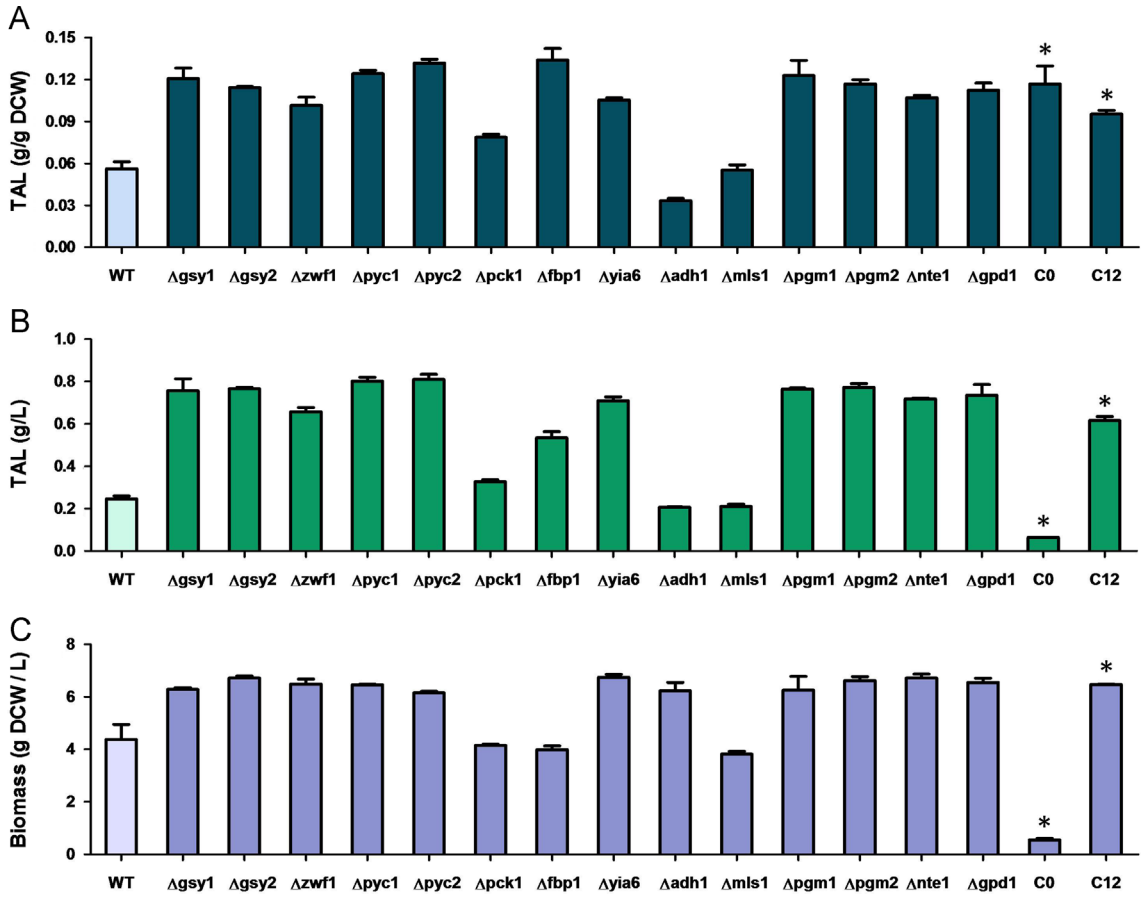

Fig. 3. Comparison of TAL produced in *S. cerevisiae* strains with single gene knockouts after 48 h of cultivation: (A) production on a per cell mass basis [g/g DCW], (B) titers [g/L], and (C) final cell densities (FCD, g DCW/L). WT refers to the base strain BY4741. The asterisk denotes the WT strains supplemented with 10 μM cerulenin at 0 h (bar C0) and 12 h (bar C12). Bars represent mean values ± one standard deviation ($n=6$ independent experiments).

a critical branch point in glycolysis (Pronk et al., 1996). For this reason, engineering *S. cerevisiae* to increase acetyl-CoA and pyruvate levels could alleviate flux limitations toward TAL production.

Enzymes participating in central carbon metabolism that are responsible for producing or consuming pyruvate and acetyl-CoA were analyzed to identify potential gene deletions to increase TAL production. The pathways illustrated in Fig. 2 focus on the utilization of glucose-6-phosphate (G6P) by the cell following glucose uptake from the medium. Significant byproducts (glycogen, pentose sugars, glycerol, and ethanol) that can be produced while generating TAL precursor pools are also shown. Using this pathway analysis, a variety of genes were selected for deletion including *GSY1/2*, *ZWF1*, *PYC1/2*, *PCK1*, *FBP1*, *YIA6*, *ADH1*, and *MLS1* (Fig. 2, circled and underlined genes). The *GSY1/2* and *ZWF1* genes focus on an early branch point in glycolysis; *GSY1* and *GYS2* encode glycogen synthase paralogs that produce glycogen for energy storage in the cell, and *ZWF1* encodes the glucose-6-phosphate dehydrogenase important for driving the pentose phosphate pathway (Ni and LaPorte, 1995; Nogae and Johnston, 1990). The *PYC1/2*, *PCK1*, and *FBP1* genes encode activities important in the gluconeogenic pathway that support increases in biomass and glycogen using non-fermentable carbon sources (Lin et al., 2001). The yeast mitochondrial NAD^+ transporter encoded by *YIA6* was recently identified to perform a unidirectional transport of NAD^+ out of the cytoplasm and into the mitochondria (Todisco et al., 2006). The *ADH1*-encoded alcohol dehydrogenase is known to convert acetaldehyde to ethanol and also plays an important role in redox balance by generating NAD^+ as a byproduct (Denis et al., 1983). Malate synthase is encoded by *MLS1* and is responsible for generating malate from acetyl-CoA in the cytosol (Chen et al., 2012).

To eliminate their native activities and thus improve precursor (acetyl-CoA and malonyl-CoA) pools, strains with single gene disruptions (Table 1) were transformed with pXP842-2PS. This set of single knockout strains was cultivated in YPD medium (1% glucose) for 48 h and TAL production was compared on both a specific (per g cell) basis (Fig. 3A) and a volumetric (titer) basis (Fig. 3B). The final cell densities of the cultures are shown in Fig. 3C (growth curves are in Supplementary Data: Fig. S3). Knockouts of *GSY1/2*, *ZWF1*, *PYC1/2*, or *YIA6* gave greater than 2-fold increases in specific TAL levels and approximately 50% increases in final biomass. In combination, this led to ca. 3-fold increases in TAL with titers of up to 0.81 g/L. Knockouts of *PCK1*, *ADH1*, and *MLS1* did not increase TAL titers or specific production, with levels comparable or lower than the control strain (WT). The knockout of *FBP1* increased titer by 2.2-fold and per cell levels by 2.4-fold, one of the highest specific production levels despite a slight reduction in growth. The significance of this deletion is that Fbp1p is responsible for conversion of fructose 1,6-biphosphate, which is the only reversible step (Pfk1p acts in the forward direction) of glycolysis that uses an independent enzyme (Lin et al., 2001). The results for the best strains (Fig. 3) show that the most important factor was the effect of the gene disruptions on the TAL produced on a per cell basis. However, the highest titers came from strains that were also able to generate increased biomass. By exploiting the well-known metabolic pathways, our analysis suggested ten genes for disruption to redirect the metabolic flux toward TAL precursors. This strategy proved successful as six of the ten knockouts resulted in 3-fold higher TAL titers and >2-fold increases in specific production.

Table 3
List of reactions and gene knockouts identified by the OptKnock algorithm for increased TAL production.

Target	Acetyl-CoA	Pyruvate
Reactions identified	PLBPC_Sce, PGPPAm_Sc, G3PD1ir	PC
Corresponding genes	*NTE1, PGM1/2, GPD1*	*PYC1/2*

3.4. *In silico* prediction of pathway gene knockouts via OptKnock

An *in silico* metabolic engineering approach was also used to identify additional promising single gene disruptions, where identification could be facilitated using a genome-scale model. Using the iMM904 yeast metabolic network, we employed the OptKnock algorithm packaged within the Cobra Toolbox (Mo et al., 2009; Schellenberger et al., 2011). This algorithm has been designed to identify gene knockouts in an organism core model that will increase a target flux value as specified by the user (Burgard et al., 2003), and uses a bilevel optimization framework such that maximization of the target metabolite is subject to maximizing biomass flux. In doing so, solutions should provide gene disruptions free of growth defects, adding a layer of robustness relative to the metabolic engineering approach described above. The procedure has been validated extensively *in vivo* using *E. coli*, and a recent report achieved nearly a 60-fold improvement in 2, 3-butanediol titers using *S. cerevisiae* (Ng et al., 2012). Our optimization was constrained to target the flux of either acetyl-CoA or pyruvate, and limited to up to five deletions in any given solution set. Two solution sets were generated for acetyl-CoA and one unique set for pyruvate, made possible by modulating both biomass and ATP maintenance in the optimization constraints. The reactions and their corresponding genes identified in the optimization problem using these two targets are summarized in Table 3. The metabolic pathways associated with these interventions can be found in Fig. 2 (boxed and underlined genes). For acetyl-CoA flux, three reactions were identified and had genes associated with glycogen biosynthesis (phosphoglucomutase; *PGM1/2*), lipid metabolism (serine esterase; *NTE1*), or glycerol biosynthesis (glycerol-3-phosphate dehydrogenase; *GPD1*). Fig. 2 illustrates how these disruptions benefit TAL production by increasing acetyl-CoA levels. With these deletions there will be a reduction in flux toward significant branch points in glycogen and pentose biosynthesis, as well as fatty acid biosynthesis that is known to consume large quantities of acetyl-CoA and malonyl-CoA. In the case of pyruvate flux as the target, OptKnock selected the PC reaction for disruption, corresponding to pyruvate carboxylase activity encoded by the *PYC1/2* genes, and the first committed step of gluconeogenesis. These two deletions were already evaluated as discussed above. Strains harboring a single deletion in *PGM1*, *PGM2*, *NTE1*, or *GPD1* were transformed with pXP842-2PS, cultivated for 48 h in YPD, and then screened for TAL biosynthesis. All six strains corresponding to interventions identified by the *in silico* method showed robust growth and gave significant improvements with increases that resulted in 3-fold higher TAL titers and >2-fold increases in specific production (Fig. 3).

It was not surprising that interventions selected by OptKnock were in close alignment to those previously identified using a pathway analysis. A clear example is the identification of the *PYC1/2* genes by both strategies. In addition, both strategies sought to manipulate glycogen production by either the *GSY1/2* paralog system or the *PGM1/2* system. The deletion of *GSY1/2* influences the final process in glycogen synthesis (Fig. 2), where *PGM1/2* affects the pathway further upstream, preventing synthesis of unnecessary metabolites. A final feature of the OptKnock strategy is that disruptions not obvious via traditional pathway analysis can be elucidated through the use of genome-scale analysis. Using the core model with OptKnock suggested the regulator of lipid metabolism (*NTE1*) as an avenue for increased TAL precursor pools in *S. cerevisiae*, a logical result that was not readily identified in our previous analysis. As shown in Fig. 2, *NTE1* encodes the serine esterase responsible for, among other things, repression of the *OPI1*-encoded Opi1 repressor protein (Fernández-Murray et al., 2009). The enzyme silences the yeast fatty acid synthase, helping modulate phospholipid biosynthesis in *S. cerevisiae* (Schweizer and Hofmann, 2004). Disrupting *NTE1* potentially relieves the repression of Opi1 and reduces flux of TAL precursors toward competing products like fatty acids. With Nte1 participating in the global regulation of lipid metabolism, further experiments need to be performed to pinpoint exactly how Δ*nte1* is improving TAL production.

3.5. Downregulation of fatty acid synthesis via cerulenin addition

Yeast fatty acid biosynthesis is an obvious choice for downregulation to improve the levels of TAL and other polyketides. The FAS1/FAS2 complex uses acetyl-CoA, malonyl-CoA, and NADPH for the creation of long chain fatty acids, primarily the 16-carbon palmitate (Wakil et al., 1983). An initial experiment was performed to determine the effect of fatty acid downregulation on TAL levels. Strain BY4741 (carrying pXP842-2PS) was cultivated in YPD medium containing 10 μM cerulenin for 48 h. Cerulenin is a known fatty acid synthase inhibitor, and at this concentration the strain would be subject to a significantly repressed FAS complex (Omura, 1976). The cerulenin was added at time zero (C0) or 12 h post-inoculation (C12); the latter time corresponds to glucose depletion in the batch culture. The specific production of TAL for culture C0 was approximately 2-fold higher than the control (Fig. 3A). However, due to the inhibitory effects of this compound, final cell density reached only 10% of the control strain and therefore volumetric (titer) production was very low (Fig. 3B and C). The outcome was very different when the cerulenin was spiked at a later time (C12). By postponing the inhibition of fatty acid biosynthesis, a normal cell density was achieved (Fig. 3B). Specific TAL production was reduced only slightly relative to C0, while titers were 10-fold higher relative to C0. Final titer for the C12 culture was comparable to the other interventions, and 2.5-fold greater than the base strain. This result, and that for the *NTE1* deletion, suggests that modulating fatty acid synthesis will be instrumental for increased production of triacetic acid lactone in *S. cerevisiae*.

3.6. Combining pathway interventions

A total of 17 single gene deletions were identified and tested for improved TAL synthesis in *S. cerevisiae*. To determine whether further increases could be achieved by combining multiple deletions, a subset of the 17 genes was selected that would potentially provide the best gains, with minimal deleterious effects. We selected strong performers from Fig. 3 and introduced the following disruptions: protease activity (*PRB1*), reverse glycolysis (*PYC2*), cofactor transport (*YIA6*), and lipid biosynthesis (*NTE1*). The genes were selected to affect significantly different pathways in order to ensure minimal overlap with the hope of producing substantial increases in TAL production. The strains were constructed first by sequential deletion of genes *PRB1* and *PYC2* in the base strain BY4741Δ*trp1*. *YIA6* or *NTE1* was then knocked out to create strains BYtΔ*prb1*Δ*pyc2*Δ*yia6* and BYtΔ*prb1*Δ*pyc2*Δ*nte1*, respectively. Lastly, *YIA6* was deleted from BYtΔ*prb1*Δ*pyc2*Δ*nte1* to construct the quadruple knockout strain (BYtΔ*prb1*Δ*pyc2*Δ*yia6*Δ*nte1*). The Δ*pyc2* and Δ*pyc2*Δ*nte1* deletions were also introduced into the alternate protease-deficient strain BJ5464 for comparison.

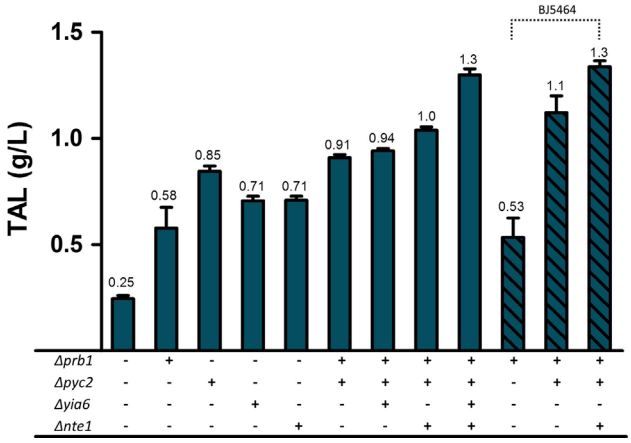

Fig. 4. Improvements in TAL levels following combination of protease and pathway gene knockouts. The open bars represent strain BY4741 and the hatched bars are for strain BJ5464 (this base strain has the *PRB1* gene disrupted). Strains either have (+) or lack (−) the gene deletions shown. Bars represent means ± one standard deviation (n=6 independent experiments).

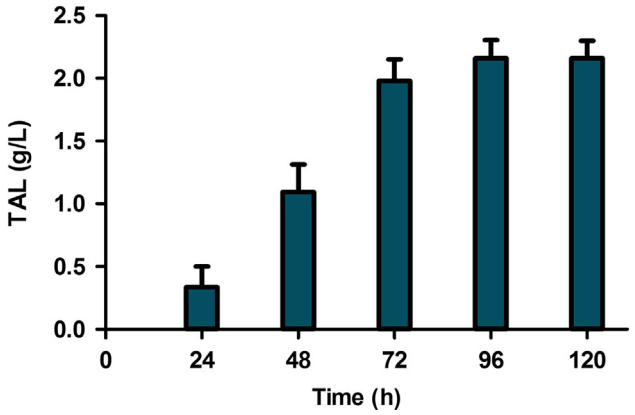

Fig. 5. TAL titers during fermentor-controlled (New Brunswick BioFlo III) glucose fed-batch cultivation using the engineered strain BYtΔ*prb1*Δ*pyc2*Δ*yia6*Δ*nte1*. Following inoculation, the cells were grown in batch culture for 12 h. The glucose feed (3.6 M) was then initiated, and feed rate was controlled (0–1.5 mL/h) to maintain DO at 20% with an aeration rate of 0.8 vvm. Bars represent means ± one standard deviation (n=3 independent experiments).

All strains were then transformed with pXP842-2PS, cultivated for 48 h in YPD (1% glucose), and TAL levels were measured (Fig. 4).

The TAL titer for BYtΔ*prb1*Δ*pyc2* strain was 0.91 g/L, nearly three times higher than that of the base strain and 57% higher than for BYtΔ*prb1*. Subsequent deletion of either *YIA6* or *NTE1* further increased TAL levels, with the latter exceeding 1.0 g/L. The combination of all four deletions in strain BYtΔ*prb1*Δ*pyc2*Δ*yia6*Δ*nte1* resulted in a final titer of 1.3 g/L (29% of theoretical yield) corresponding to an overall 5-fold improvement over the base strain. If the increases for the single gene deletions were cumulative, titer would have been approximately 1.9 g/L, suggesting new bottlenecks might be present. The Δ*pyc2* and Δ*pyc2*Δ*nte1* deletions were also introduced into the alternate strain BJ5464 to see if similar behavior was observed. Sequential gene knockouts increased TAL levels in this strain as well (Fig. 4). Interestingly, the BJ5464-based strains generally showed higher levels of TAL production relative to the comparable BY4741-based strains. BJΔ*pyc2* produced 23% more TAL than BYtΔ*prb1*Δ*pyc2*, and BJΔ*pyc2*Δ*nte1* produced 29% more than BYtΔ*prb1*Δ*pyc2*Δ*nte1*. We have observed similar results with other polyketides in our laboratory.

All strains showed similar growth behaviors and final cell densities. Therefore, specific TAL production (per g cell basis) showed the same trend as that in Fig. 4 (see Supplementary Data: Figure S3). In addition, although the strains were cultivated in YPD medium, plasmid stability was not an issue due to the late-phase induction, and was routinely measured at over 85% for all strains tested (*e.g.*, for strain BYtΔ*prb1*Δ*pyc2*Δ*yia6*Δ*nte1*, 87 ± 2% of the cells retained the 2-PS plasmid after 48 h).

3.7. Glucose fed-batch fermentations

In order to further evaluate the engineered strain BYtΔ*prb1*Δ*pyc2*Δ*yia6*Δ*nte1* for the high-level production of TAL, fed-batch fermentations were performed. The seed and batch cultures were in YPD with 1% glucose. Based on the toxicity data (Section 3.1), we chose to maintain pH at 6.0. After allowing the initial glucose to be consumed for approximately 12 h, pure glucose was continuously fed while ensuring that glucose levels were kept low to maximize expression of the 2-PS under the P_{ADH2} promoter. To do this, glucose feed rate was allowed to vary in order to limit dissolved oxygen levels to 20% throughout the fermentation. In addition, off-line HPLC analysis was performed to quantify glucose levels during the fed-batch stage; glucose concentration never exceeded 0.35 g/L.

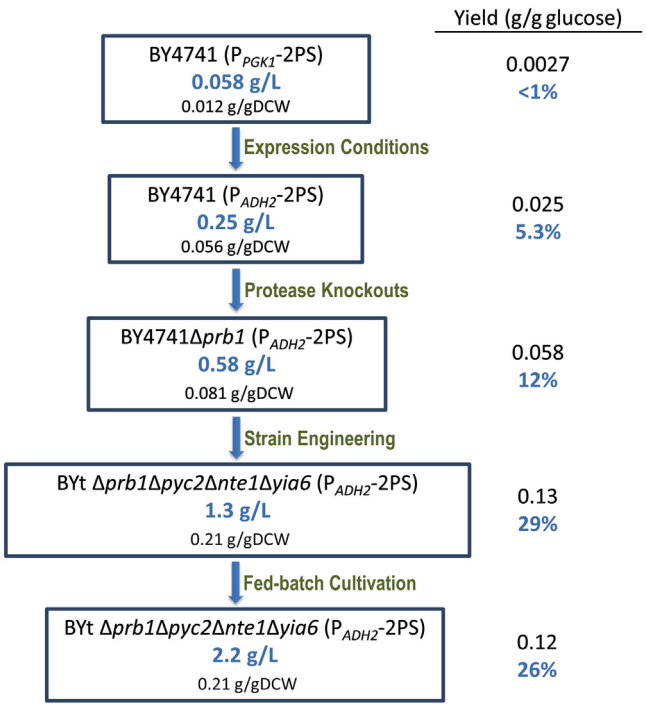

Fig. 6. Final titer and yield summary for TAL production in *S. cerevisiae*. Volumetric titer (g/L), specific production (cell basis; g/g DCW), and corresponding yield on glucose (g/g) with percent theoretical yield are shown.

From 24 to 48 h, triacetic acid lactone levels increased 3-fold to over 1 g/L, and by 72 h began to level off at greater than 2 g/L (Fig. 5). Biomass concentration and TAL production on a per g cell basis are shown in Fig. S5 (Supplementary Data). By 96 h, supplementation of glucose to maintain DO levels was minimal, and culture density was unchanged. This is likely due to other nutrient limitations after 48 h using a glucose-only feed. Using our engineered strain, a final titer of 2.2 g/L was achieved in the fed-batch culture (versus 1.3 g/L in batch). It is noteworthy that a similar yield was obtained (0.12 g/g glucose) as in the small-scale batch cultures (0.13 g/g glucose).

A summary of the substantial improvements in TAL titer and yield obtained with the various interventions is shown in Fig. 6. The highest TAL production was observed with strain

BYtΔ*prb1*Δ*pyc2*Δ*yia6*Δ*nte1* reaching a maximum titer of 2.2 g/L and a maximum yield of 0.12–0.13 g/g glucose (26–29% of theoretical). These are the highest yields and titers reported to date, with the yield 13-fold and 3.0-fold higher than reported by Xie et al. (2006) for *S. cerevisiae* expressing the 2-PS or 6-MSAS variant, respectively.

4. Conclusions

S. cerevisiae is an excellent host for the production of triacetic acid lactone due to the minimal toxicity. Our initial baseline strain produced very low titers (58 mg/L) of TAL with a yield on glucose of less than 1% (g/g). Initial changes in the expression system (*e.g.*, promoter, media) increased TAL titer by approximately 4-fold to 0.25 g/L and increased yield by approximately 10-fold. The introduction of protease knockouts to improve *in vivo* stability of the 2-pyrone synthase produced an additional 2-fold improvement in titer to 0.58 g/L and a further 2-fold increase in yield. Pathway engineering of central carbon metabolism, including the evaluation of computational predictions, identified a total of 15 potential gene knockouts. Up to 3-fold increases in TAL titer were observed for the single gene deletion strains. By combining multiple gene knockouts and performing batch and fed-batch culture, our highest titer was 2.2 g/L and our highest yield was 0.13 (g/g glucose), or 29% of theoretical. To our knowledge, this is the first study implementing metabolic pathway engineering to increase the synthesis of TAL. This study also provides the highest titer and yield of TAL reported to date. Overall, incorporation of optimal culture parameters and combined pathway gene knockouts led to a 37-fold increase in final titer and an approximately 50-fold increase in yield. Additional studies are underway to evaluate the upregulation of specific pathway genes and rational enzyme engineering approaches for improved TAL synthesis in *S. cerevisiae*.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ymben.2014.07.008.

References

Aguedo, M., Beney, L., Waché, Y., Belin, J.-M., 2003. Mechanisms underlying the toxicity of lactone aroma compounds towards the producing yeast cells. J. Appl. Microbiol. 94, 258–265. http://dx.doi.org/10.1046/j.1365-2672.2003.01828.x.

Austin, M.B., Noel, J.P., 2003. The chalcone synthase superfamily of type III polyketide synthases. Nat. Prod. Rep. 20, 79–110. http://dx.doi.org/10.1039/B100917F.

Burgard, A.P., Pharkya, P., Maranas, C.D., 2003. Optknock: a bilevel programming framework for identifying gene knockout strategies for microbial strain optimization. Biotechnol. Bioeng. 84, 647–657. http://dx.doi.org/10.1002/bit.10803.

Chen, Y., Siewers, V., Nielsen, J., 2012. Profiling of cytosolic and peroxisomal acetyl-CoA metabolism in *Saccharomyces cerevisiae*. PLoS One 7, e42475. http://dx.doi.org/10.1371/journal.pone.0042475.

Chia, M., Schwartz, T.J., Shanks, B.H., Dumesic, J.A., 2012. Triacetic acid lactone as a potential biorenewable platform chemical. Green Chem. 14, 1850–1853. http://dx.doi.org/10.1039/C2GC35343A.

Denis, C.L., Ferguson, J., Young, E.T., 1983. mRNA levels for the fermentative alcohol dehydrogenase of *Saccharomyces cerevisiae* decrease upon growth on a non-fermentable carbon source. J. Biol. Chem. 258, 1165–1171.

Eckermann, S., Schröder, G., Schmidt, J., Strack, D., Edrada, R.A., Helariutta, Y., Elomaa, P., Kotilainen, M., Kilpeläinen, I., Proksch, P., Teeri, T.H., Schröder, J., 1998. New pathway to polyketides in plants. Nature 396, 387–390. http://dx.doi.org/10.1038/24652.

Evans, G.E., Leeper, F.J., Murphy, J.A., Staunton, J., 1979. Triacetic acid lactone as a polyketide synthon: synthesis of toralactone and polyketide-type anthracene derivatives. J. Chem. Soc. Chem. Commun., 205–206. http://dx.doi.org/10.1039/C39790000205.

Fang, F., Salmon, K., Shen, M.W.Y., Aeling, K.A., Ito, E., Irwin, B., Tran, U.P.C., Hatfield, G.W., Da Silva, N.A., Sandmeyer, S., 2011. A vector set for systematic metabolic engineering in *Saccharomyces cerevisiae*. Yeast 28, 123–136. http://dx.doi.org/10.1002/yea.1824.

Fernández-Murray, J.P., Gaspard, G.J., Jesch, S.A., McMaster, C.R., 2009. NTE1-encoded phosphatidylcholine phospholipase b regulates transcription of phospholipid biosynthetic genes. J. Biol. Chem. 284, 36034–36046. http://dx.doi.org/10.1074/jbc.M109.063958.

Gietz, D., Jean, A.S., Woods, R.A., Schiestl, R.H., 1992. Improved method for high efficiency transformation of intact yeast cells. Nucl. Acids Res. 20, 1425. http://dx.doi.org/10.1093/nar/20.6.1425 (1425).

Goel, A., Ram, V.J., 2009. Natural and synthetic 2H-pyran-2-ones and their versatility in organic synthesis. Tetrahedron 65, 7865–7913. http://dx.doi.org/10.1016/j.tet.2009.06.031.

Hamelinck, C.N., Hooijdonk, G. van, Faaij, A.P., 2005. Ethanol from lignocellulosic biomass: techno-economic performance in short-, middle- and long-term. Biomass and Bioenergy 28, 384–410. http://dx.doi.org/10.1016/j.biombioe.2004.09.002.

Hill, J., Donald, K.A.I.G., Griffiths, D.E., 1991. DMSO-enhanced whole cell yeast transformation. Nucl. Acids Res. 19, 5791. http://dx.doi.org/10.1093/nar/19.20.5791 (5791).

Jez, J.M., Austin, M.B., Ferrer, J.-L., Bowman, M.E., Schröder, J., Noel, J.P., 2000. Structural control of polyketide formation in plant-specific polyketide synthases. Chem. Biol. 7, 919–930. http://dx.doi.org/10.1016/S1074-5521(00)00041-7.

Jones, E.W., 1991. Tackling the protease problem in Saccharomyces cerevisiae. Methods in Enzymology 194, 428–453.

Kealey, J.T., Liu, L., Santi, D.V., Betlach, M.C., Barr, P.J., 1998. Production of a polyketide natural product in nonpolyketide-producing prokaryotic and eukaryotic hosts. Proc. Natl. Acad. Sci. U. S. A. 95, 505–509.

Leber, C., Da Silva, N.A., 2013. Engineering of *Saccharomyces cerevisiae* for the synthesis of short chain fatty acids. Biotechnol. Bioeng. 111, 347–358. http://dx.doi.org/10.1002/bit.25021.

Lee, K.K.M., Da Silva, N.A., Kealey, J.T., 2009. Determination of the extent of phosphopantetheinylation of polyketide synthases expressed in *Escherichia coli* and *Saccharomyces cerevisiae*. Anal. Biochem. 394, 75–80. http://dx.doi.org/10.1016/j.ab.2009.07.010.

Lee, M.K., Da Silva, N.A., 2005. Evaluation of the *Saccharomyces cerevisiae ADH2* promoter for protein synthesis. Yeast 22, 431–440. http://dx.doi.org/10.1002/yea.1221.

Lin, S.S., Manchester, J.K., Gordon, J.I., 2001. Enhanced gluconeogenesis and increased energy storage as hallmarks of aging in *Saccharomyces cerevisiae*. J. Biol. Chem. 276, 36000–36007. http://dx.doi.org/10.1074/jbc.M103509200.

Liu, P., Chernyshov, A., Najdi, T., Fu, Y., Dickerson, J., Sandmeyer, S., Jarboe, L., 2013. Membrane stress caused by octanoic acid in *Saccharomyces cerevisiae*. Appl. Microbiol. Biotechnol. 97, 3239–3251. http://dx.doi.org/10.1007/s00253-013-4773-5.

Ma, S.M., Li, J.W.-H., Choi, J.W., Zhou, H., Lee, K.K.M., Moorthie, V.A., Xie, X., Kealey, J.T., Da Silva, N.A., Vederas, J.C., Tang, Y., 2009. Complete reconstitution of a highly-reducing iterative polyketide synthase. Science 326, 589–592. http://dx.doi.org/10.1126/science.1175602.

Mo, M.L., Palsson, B.O., Herrgård, M.J., 2009. Connecting extracellular metabolomic measurements to intracellular flux states in yeast. BMC Syst. Biol. 3, 37. http://dx.doi.org/10.1186/1752-0509-3-37.

Moreno-Mañas, M., Prat, M., Ribas, J., Virgili, A., 1988. Palladium catalyzed allylic C-alkylation of highly acidic and enolic heterocyclic substrates: tetronic acid and triacetic acid lactone. Tetrahedron Lett. 29, 581–584. http://dx.doi.org/10.1016/S0040-4039(00)80156-2.

Mutka, S.C., Bondi, S.M., Carney, J.R., Da Silva, N.A., Kealey, J.T., 2006. Metabolic pathway engineering for complex polyketide biosynthesis in *Saccharomyces cerevisiae*. FEMS Yeast Res. 6, 40–47. http://dx.doi.org/10.1111/j.1567-1356.2005.00001.x.

Ng, C.Y., Jung, M., Lee, J., Oh, M.-K., 2012. Production of 2,3-butanediol in *Saccharomyces cerevisiae* by in silico aided metabolic engineering. Microb. Cell Factories 11, 68. http://dx.doi.org/10.1186/1475-2859-11-68.

Ni, H.T., LaPorte, D.C., 1995. Response of a yeast glycogen synthase gene to stress. Mol. Microbiol. 16, 1197–1205.

Nikolau, B.J., Perera, M.A.D.N., Brachova, L., Shanks, B., 2008. Platform biochemicals for a biorenewable chemical industry. Plant J. 54, 536–545. http://dx.doi.org/10.1111/j.1365-313X.2008.03484.x.

Nogae, I., Johnston, M., 1990. Isolation and characterization of the *ZWF1* gene of *Saccharomyces cerevisiae*, encoding glucose-6-phosphate dehydrogenase. Gene 96, 161–169.

Omura, S., 1976. The antibiotic cerulenin, a novel tool for biochemistry as an inhibitor of fatty acid synthesis. Bacteriol. Rev. 40, 681–697.

Pronk, J.T., Yde Steensma, H., Van Dijken, J.P., 1996. Pyruvate metabolism in *Saccharomyces cerevisiae*. Yeast 12, 1607–1633 (10.1002/(SICI)1097-0061(199612)12:16<1607::AID-YEA70>3.0.CO;2-4).

Richardson, M.T., Pohl, N.L., Kealey, J.T., Khosla, C., 1999. Tolerance and specificity of recombinant 6-methylsalicylic acid synthase. Metab. Eng. 1, 180–187. http://dx.doi.org/10.1006/mben.1999.0113.

Sambrook, J.J., Russell, D.D.W., 2001. Molecular Cloning: A Laboratory Manual, Vol. 2. CSHL Press; Cold Spring Harbor, NY, USA.

Schellenberger, J., Que, R., Fleming, R.M.T., Thiele, I., Orth, J., Feist, A.M., Zielinski, D., Bordbar, A., Lewis, N., Rahmanian, S., Kang, J., Hyduke, D.R., Palsson, B., 2011. COBRA toolbox 2.0. Protoc. Exch. 6, 1290–1307. http://dx.doi.org/10.1038/protex.2011.234.

Schweizer, E., Hofmann, J., 2004. Microbial type I fatty acid synthases (FAS): major players in a network of cellular FAS systems. Microbiol. Mol. Biol. Rev. 68, 501–517. http://dx.doi.org/10.1128/MMBR.68.3.501-517.2004.

Shen, M.W.Y., Fang, F., Sandmeyer, S., Da Silva, N.A., 2012. Development and characterization of a vector set with regulated promoters for systematic metabolic engineering in *Saccharomyces cerevisiae*. Yeast 29, 495–503. http://dx.doi.org/10.1002/yea.2930.

Taeschler, C., 2010. Ketenes, ketene dimers, and related substances, Kirk-Othmer Encyclopedia of Chemical Technology. John Wiley & Sons, Inc., Hoboken, NJ, USA.

Tang, S.-Y., Qian, S., Akinterinwa, O., Frei, C.S., Gredell, J.A., Cirino, P.C., 2013. Screening for enhanced triacetic acid lactone production by recombinant *Escherichia coli* expressing a designed triacetic acid lactone reporter. J. Am. Chem. Soc. 135, 10099–10103. http://dx.doi.org/10.1021/ja402654z.

Todisco, S., Agrimi, G., Castegna, A., Palmieri, F., 2006. Identification of the mitochondrial NAD^+ transporter in *Saccharomyces cerevisiae*. J. Biol. Chem. 281, 1524–1531. http://dx.doi.org/10.1074/jbc.M510425200.

Wakil, S.J., Stoops, J.K., Joshi, V.C., 1983. Fatty acid synthesis and its regulation. Ann. Rev. Biochem. 52, 537–579. http://dx.doi.org/10.1146/annurev.bi.52.070183.002541.

Wattanachaisaereekul, S., Lantz, A.E., Nielsen, M.L., Nielsen, J., 2008. Production of the polyketide 6-MSA in yeast engineered for increased malonyl-CoA supply. Metab. Eng. 10, 246–254. http://dx.doi.org/10.1016/j.ymben.2008.04.005.

Westfall, P.J., Pitera, D.J., Lenihan, J.R., Eng, D., Woolard, F.X., Regentin, R., Horning, T., Tsuruta, H., Melis, D.J., Owens, A., Fickes, S., Diola, D., Benjamin, K.R., Keasling, J.D., Leavell, M.D., McPhee, D.J., Renninger, N.S., Newman, J.D., Paddon, C.J., 2012. Production of amorphadiene in yeast, and its conversion to dihydroartemisinic acid, precursor to the antimalarial agent artemisinin. PNAS 109, 111–118. http://dx.doi.org/10.1073/pnas.1110740109.

Xie, D., Shao, Z., Achkar, J., Zha, W., Frost, J.W., Zhao, H., 2006. Microbial synthesis of triacetic acid lactone. Biotechn. Bioeng. 93, 727–736. http://dx.doi.org/10.1002/bit.20759.

Zha, W., Shao, Z., Frost, J.W., Zhao, H., 2004. Rational pathway engineering of type I fatty acid synthase allows the biosynthesis of triacetic acid lactone from D-glucose in vivo. J. Am. Chem. Soc. 126, 4534–4535. http://dx.doi.org/10.1021/ja0317271.