J Ind Microbiol Biotechnol (2015) 42:711–721
DOI 10.1007/s10295-015-1596-7

BIOENERGY/BIOFUELS/BIOCHEMICALS

SIMB
Society for Industrial Microbiology and Biotechnology

Triacetic acid lactone production in industrial *Saccharomyces* yeast strains

Lauren P. Saunders · Michael J. Bowman · Jeffrey A. Mertens · Nancy A. Da Silva · Ronald E. Hector

Received: 22 September 2014 / Accepted: 30 January 2015 / Published online: 15 February 2015

Abstract Triacetic acid lactone (TAL) is a potential platform chemical that can be produced in yeast. To evaluate the potential for industrial yeast strains to produce TAL, the *g2ps1* gene encoding 2-pyrone synthase was transformed into 13 industrial yeast strains of varied genetic background. TAL production varied 63-fold between strains when compared in batch culture with glucose. Ethanol, acetate, and glycerol were also tested as potential carbon sources. Batch cultures with ethanol medium produced the highest titers. Therefore, fed-batch cultivation with ethanol feed was assayed for TAL production in bioreactors, producing our highest TAL titer, 5.2 g/L. Higher feed rates resulted in a loss of TAL and subsequent production of additional TAL side products. Finally, TAL efflux was measured and TAL is actively exported from *S. cerevisiae* cells. Percent yield for all strains was low, indicating that further metabolic engineering of the strains is required.

Mention of trade names or commercial products in this article is solely for the purpose of providing scientific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture. USDA is an equal opportunity provider and employer.

Electronic supplementary material The online version of this article (doi:10.1007/s10295-015-1596-7) contains supplementary material, which is available to authorized users.

L. P. Saunders · M. J. Bowman · J. A. Mertens · R. E. Hector (✉)
Bioenergy Research Unit, National Center for Agricultural Utilization Research, Agricultural Research Service, U.S. Department of Agriculture, 1815 North University Street, Peoria, IL 61604, USA
e-mail: ronald.hector@ars.usda.gov

N. A. Da Silva
Department of Chemical Engineering and Materials Science, University of California, Irvine, CA 92697, USA

Keywords Triacetic acid lactone · Industrial yeast · *Saccharomyces cerevisiae* · Polyketide synthase · 2-Pyrone synthase

Introduction

The current petroleum-based approach for producing transportation fuel and many commodity chemicals is not sustainable. Chemical precursors made from biomass-derived sugars could be used to manufacture a large variety of chemicals without relying on petroleum [29]. Triacetic acid lactone (TAL, or 4-hydroxy-6-methyl-2-pyrone) has been proposed as a platform chemical. TAL is currently produced in a series of chemical steps starting with the pyrolysis of acetic acid [1]. It can also be produced by microbes and subsequently converted into a variety of commercially valuable chemicals via further chemical transformations [10]. Flavoring agents hexenoic acid and γ-caprolactone, as well as commodity chemical 2,4-pentanedione (acetylacetone, used in multiple commercial applications and as a building block for synthesis of heterocyclic compounds), and several other industrially useful chemicals can be synthesized from TAL [2, 10, 21, 50].

TAL is produced by the type III polyketide synthase *g2ps1*, 2-pyrone synthase (2-PS), which was isolated from *Gerbera hybrida* [16]. Like other type III polyketide synthases, 2-PS is a relatively small protein (43.7 kDa) that uses a single active site for decarboxylation and cyclization reactions. 2-PS utilizes acetyl coenzyme A (acetyl-CoA) as an initial substrate and catalyzes the decarboxylation and condensation of two malonyl-CoAs to produce TAL. Several studies have shown that TAL can be produced from both yeast and *Escherichia coli* with the insertion of: *g2ps1*; a genetically modified fatty acid synthase B

gene; or genetically modified ketoreductase mutants of the 6-methylsalycilic acid synthase gene (6-MSAS) [38, 45, 49, 50]. The 2-PS enzyme is significantly smaller and does not require addition of a phosphopantetheinyl transferase gene for activity [49], and thus it requires fewer resources to create active 2-PS than active fatty acid synthase B or 6-MSAS. Reported TAL titers in yeast and bacteria have been low; the highest titer of 2.2 g/L was recently measured in laboratory *Saccharomyces cerevisiae* strain BY4741 [7]. In that study, Cardenas and Da Silva reported a 37-fold increase in TAL concentration compared to the unaltered strain that was achieved through a combination of optimizing culture conditions and metabolic pathway engineering. Additionally, another recent study showed that engineering of the 2-PS enzyme and expression in *E. coli* enabled the production of 2.1 g/L TAL [45].

For TAL to be adopted as a substrate for the production of biorenewable chemicals, an economical process for industrial scale production of TAL using lignocellulosic feedstocks is required. A robust TAL-producing industrial organism will be required for large-scale production. If biomass-derived sugars are eventually to be used as substrates, the strain must also be tolerant to inhibitors that are generated during the deconstruction of these materials into usable monomeric sugars [28, 33]. In this study, inhibitor-tolerant *Saccharomyces* strains of varied genetic (industrial) background were transformed with the *g2ps1* gene to (1) assess the effect of genetic variation on TAL production and (2) to identify candidate strains for further metabolic engineering to produce products derived from acetyl-CoA. The yeast strains were cultured using several different carbon sources and growth conditions to determine which strains and metabolic pathways result in the highest TAL production.

Methods

Strains and media

E. coli strain NEB10β [New England Biolabs (NEB); Ipswich, MA, USA] was used for routine maintenance and preparation of plasmids and was grown in LB medium [40]. Plasmids and microorganisms used in this study are listed in Tables 1 and 2. All yeast strains were grown in YP media and 2 % glucose unless otherwise noted. YP medium (10 g/L yeast extract, 20 g/L bactopeptone) was autoclaved without carbon source. Medium was supplemented with 200 μg/mL G418 (Enzo Life Sciences, Farmingdale, NY, USA) when needed for plasmid maintenance. Sterile carbon sources and G418 were added separately.

Table 1 Plasmids used in this study

Plasmid	Description	Reference
pRS410	pBluescript II SK+, *kanMX4, CEN6, ARSH4*	Addgene #11258
pRS415	pBluescript II SK+, *LEU2, CEN6, ARSH4*	[41]
pRS416	pBluescript II SK+, *URA3, CEN6, ARSH4*	[41]
pUC57	Gene synthesis vector	GenScript
pCR2.1	Cloning vector	Invitrogen
pXP742-2PS	Plasmid containing a his-tagged 2PS gene	Da Silva Lab
pRH499	pUC57+ *Ashbya gossypii TEF* promoter (P_{TEF})	GenScript
pRH503	pRS415+ P_{TEF}−MCS−T_{ADH1}	This work
pRH517	pRS416+ P_{TEF}−MCS−T_{ADH1}	This work
pRH523	pP_{TEF}−MCS−T_{ADH1} (*KanMX marker*)	This work
pRH525	pP_{TEF}−*2PS*−T_{ADH1} (*KanMX* marker)	This work

Cloning of the 2-pyrone synthase gene (*g2ps1*) for expression in *Saccharomyces strains*

The *ADH1* terminator region (T_{ADH1}) from +1,040 to 1,213 was amplified using primers 29 and 30 from *S. cerevisiae* genomic DNA using Phusion® High-Fidelity DNA polymerase (NEB) and cloned into pCR2.1 (Invitrogen, Carlsbad, CA, USA) for sequencing. Oligonucleotides (IDT, Coralville, IA, USA) used for this study are shown in Table 3. The yeast expression vector pRH503 was created by inserting the *TEF* promoter and *ADH1* termination regions into the *Sac*I/*Spe*I and *Sal*I/*Xho*I sites of the shuttle vector pRS415. The high-level, constitutive *Ashbya gossypii TEF* promoter was obtained from pRH499 (GenScript, Piscataway, NJ, USA). The *A. gossypii TEF* promoter region was from −1 to −379 [43]. The [P_{TEF}−MCS−T_{ADH1}] fragment was inserted into the *Sac*I/*Xho*I site of pRS416 to create expression vector pRH517 with the *URA3* marker.

The *URA3* marker was switched to *kanMX4* to generate a vector that can be used with prototrophic strains. Plasmid pRH517 was cut with *Nco*I, which cuts once in the *URA3* open reading frame. *Nco*I-digested pRH517 was transformed into the lab strain CEN.PK2-1C along with a DNA fragment containing the *kanMX4* gene. To generate the *kanMX4* DNA fragment, pRS410 was used as a template for PCR with primers 262 and 263. Flanking homology to direct homologous recombination to replace the *URA3* marker in pRH517 was included in primers 262/263. DNA was transformed into yeast cells using a standard lithium acetate method [19]. Cells from the transformation were resuspended in YPD and allowed to recover for at least 2 h

Table 2 Microorganisms used in this study

Strain	Genotype (description)	Reference
CEN.PK2-1C	*MAT*a *ura3-52 trp1-289 leu2*-3,112 *his3Δ1 MAL2-8^C SUC2*	Euroscarf[a]
BY4727	MATα *his3Δ200 leu2Δ0 lys2Δ0 met15Δ0 trp1Δ63 ura3Δ0*	ATCC[b]
D5A	*S. cerevisiae* isolated from cheese whey	ATCC
Y-7567	*S. cerevisiae* isolated from coconut water sediment	ARS[c]
Y-12633	*S. cerevisiae* isolated from Ivory Coast palm wine	ARS
04-151 Phaff	*S. uvarum* isolated from a banana with *D. melanogaster*	Phaff collection[d]
Y-568	*S. cerevisiae* isolated from a distillery	ARS
Y-634	*S. cerevisiae* (lager production strain)	ARS
Y-863	*S. paradoxus* (lager production strain)	ARS
Y-7408	*S. cerevisiae* (ale pitching yeast)	ARS
Y-11876	*S. cerevisiae* isolated from a British brewery (ale production strain)	ARS
Ethanol Red	*S. cerevisiae* (developed for the industrial alcohol industry)	Lesaffre[e]
Y-630	*S. cerevisiae* isolated from a distillery	ARS
Y-685	*S. cerevisiae* isolated from a distillery	ARS
Y-629	*S. cerevisiae* isolated from distillery	ARS
YRH1152	D5A [pRH525][f]	This work
YRH1174	Y-7567 [pRH525]	This work
YRH1175	Y-12633 [pRH525]	This work
YRH1176	04-151 Phaff [pRH525]	This work
YRH1177	Y-568 [pRH525]	This work
YRH1178	Y-634 [pRH525]	This work
YRH1179	Y-863 [pRH525]	This work
YRH1180	Y-7408 [pRH525]	This work
YRH1181	Y-11876 [pRH525]	This work
YRH1182	Ethanol Red [pRH525]	This work
YRH1183	Y-630 [pRH525]	This work
YRH1184	Y-685 [pRH525]	This work
YRH1185	Y-629 [pRH525]	This work
YRH1186	CEN.PK2-1C [pRH525]	This work
YRH1187	BY4727 [pRH525]	This work

[a] Euroscarf (European *Saccharomyces cerevisiae* archive for functional analysis), Frankfurt, Germany

[b] ATCC (American Type Culture Collection) Manassas, VA

[c] ARS (NRRL) culture collection, National Center for Agricultural Utilization Research, Peoria, IL

[d] Phaff Yeast Culture Collection, University of California, Davis

[e] Lesaffre Yeast Corporation, Milwaukee, WI

[f] Plasmids contained in the strain are shown in brackets

Table 3 DNA oligonucleotides used in this study

Primer #	Sequence
029	5′-G*GTCGAC*TAAATAAGCGAATTTCTTATGAT-3′
030	5′-G*CTCGAG*CGACCTCATGCTATACCTGA-3′
262	5′-TCGGGGCTGGCTTAACTATGCGGCATCAGAGCAGATTGTACTGAGAGTGC-3′
263	5′-TTCCTGATGCGGTATTTTCTCCTTACGCATCTGTGCGGTATTTCACACCG-3′
467	5′-GG*ACTAGT***ATG**GGATCTTACTCATCCGATGATGTGGAGGTG-3′
468	5′-CG*GTCGAC*TCAGTTTCCATTGGCAACCGCAGCAGTAACG-3′

Restriction endonuclease sites are shown italicized and underlined

Start codons are shown in bold

prior to plating to YPD plates containing G418. Plasmid pRH523 (i.e., pRH517 with *URA3* switched to *kanMX4*) was recovered from CEN.PK2-1C.

The open reading frame for the *G. hybrida* 2-PS gene (Accession #Z38097) was PCR amplified from the plasmid pXP742-2PS (Da Silva lab). Primers 467 and 468 were used to generate a DNA fragment containing the *g2ps1* gene (and lacking the 6x-his tag). This DNA fragment was ligated into pCR2.1 for sequencing. Plasmid pRH525 was made by inserting the sequence-verified *g2ps1* gene into the expression vector pRH523 using *Spe*I and *Sal*I restriction endonuclease sites added to the ends of the primers used for amplification. pRH525 was transformed into yeast strains using a standard lithium acetate method [19].

Batch cultivation for TAL production

Strains were grown overnight in YPD plus G418 to an OD_{660} of 3–10. Cells from these overnight cultures were used to inoculate 20 mL cultures in 250-mL Erlenmeyer flasks to an initial cell density (OD_{660}) of 0.1 (GeneSys 10 vis, Thermo Fisher Scientific, Waltham, MA, USA). Cultures were maintained at 30 °C in a shaking incubator. For the time course of TAL production, 1-mL aliquots were taken every 24 h for 120 h. For batch cultivation in medium with varying glucose concentrations (1, 20, 50, 100, 150, 200 g/L glucose), samples were taken at 96 h. OD_{660} values were measured and the cells were removed either by centrifugation or filtering through a 0.45-μm pore PVDF filter (Mini-UniPrep, Whatman, Maidstone, UK). Cell-free supernatant samples were diluted fivefold and 20 μL were analyzed for TAL concentration (see "Analytical methods").

Analytical methods

TAL and modified TAL product (e.g., Compound I and Compound II) concentrations were determined using reverse-phase HPLC (RP-HPLC, Shimadzu Scientific Instruments equipped with: an SIL-20 AHT autosampler, an LC20AD pump, and SPD20A dual wavelength UV detector, under control of EZStart 7.4 SP1 chromatography software). Separation was performed in gradient mode at a flow rate of 0.6 mL/min through a Shim-pack XR-ODS column (3 mm × 75 mm I.D., Shimadzu, Columbia, MD) while maintaining a constant column temperature of 40 °C. The binary gradient of 1.0 % acetic acid in water (A) and 100 % methanol (B) was initially 95 % A and 5 % B followed by a linear gradient to 75 % A and 25 % B over 6.8 min, followed by a washout at 100 % B for 1 min and then re-equilibrated with 95 % A and 5 % B for 10 min. Absorbance was measured at 280 and 254 nm. TAL was identified and quantified based on a five-point standard curve generated with an authentic TAL standard (Sigma-Aldrich, St. Louis, MO, USA). Peak areas were measured with EZStart Software. As the exact identities of Compounds I and II were unknown, extinction coefficients and authentic standards were not available. Since Compounds I and II were derivatives of TAL, the extinction coefficient for TAL was used to estimate their concentrations based on a TAL standard curve.

Residual carbon source remaining in the medium was analyzed by HPLC (Shimadzu Prominence 20, Shimadzu, Columbia, MD), and compounds were separated on an Aminex HPX-87H column (Bio-Rad Laboratories, Hercules, CA, USA) with an isocratic flow rate of 0.6 mL/min (15 mM nitric acid in water) at 60 °C for 65 min. Samples were quantified with an RI detector, using authentic standards. Peak areas were measured using LCSolutions Software v 1.25 (Shimadzu, Columbia, MD), and differences within strains were measured using a Student's *T* test. Differences across multiple strains were analyzed via the Wilcoxon signed rank test.

Compound I analysis

Strain 1185 was resuspended in YP (+G418) media without a carbon source and 0.61 mL of 100 % ethanol was added initially and every 24 h for 72 h. Cultures were harvested at 72 h, cells were collected by centrifugation, and TAL and Compound I peaks were measured by RP-HPLC as described above. The pH of the supernatant was increased in 0.5 pH unit increments by the addition of potassium hydroxide. Aliquots at each 0.5 pH unit were incubated for 20 h and products were analyzed by RP-HPLC.

Yield calculations

Actual yield (g TAL/g carbon source) was calculated as the total TAL formed divided by the total carbon source consumed. Percent yield was determined as the actual yield/theoretical yield × 100. Maximum theoretical yield was determined assuming the entire carbon source was converted to TAL. Theoretical yields for glucose, ethanol, acetate, and glycerol were determined to be 0.47, 0.91, 0.71, and 0.46 g/g, respectively (see Supplementary Data).

TAL production from varied carbon sources

Strains were grown overnight in YPD plus G418 to an OD_{660} of 3–10. Aliquots were collected by centrifugation at 3,000×*g* for 3 min and washed with 1 mL sterile water. Cell pellets were resuspended in 1 mL sterile water. This concentrated cell solution was added to 20 mL of YP (+G418) media containing 2 % w/v glycerol, ethanol, potassium acetate (pH 7), or glucose in a 125 mL Erlenmeyer flask (OD of 1). Cells were gown for 96 h in batch culture at 30 °C. The OD_{660} was measured, and the solution was filtered, diluted fivefold, and analyzed by RP-HPLC and HPLC as described above.

Fed-batch cultivation with ethanol feed

Strain 1185 was grown overnight in YPD plus G418 to an OD_{660} of 3–10. Aliquots were centrifuged at 3,000×*g* for 3 min and cells resuspended in 5 mL water. Cells were added to 60 mL YP (+G418) media in DASGIP bioreactors (300 mL volume bioreactor) (Eppendorf, Hamburg, Germany) for an OD_{660} of 15. Cultures were aerated at 6 L/h and 100 % ethanol was added at rates of 0.03, 0.06, 0.105 and 0.15 g/h. Ethanol did not accumulate during the cultivation. The bioreactor cultures were not subject to pH

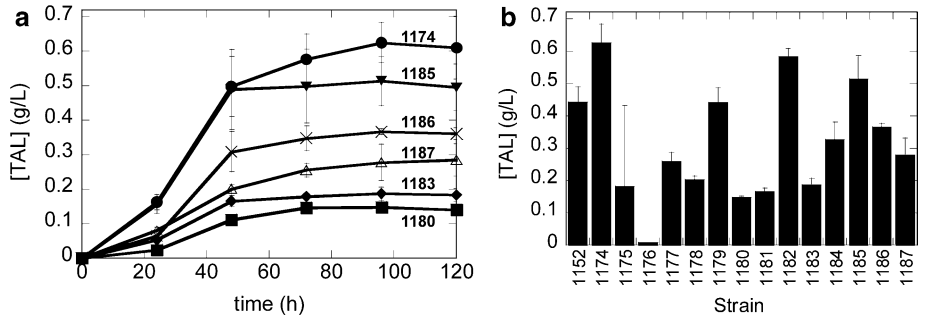

Fig. 1 Screening *Saccharomyces* strains for TAL production from glucose. **a** Representative time courses of TAL production from 6 of the 15 strains engineered to express the *g2ps1* gene. **b** Amount of TAL present at 96 h for all 15 strains evaluated. The average of three independent cultures is shown. *Error bars* represent the standard deviation

control. Samples were taken at 24 h intervals and TAL production was quantified by RP-HPLC as described above.

Efflux measurements

Cells were grown in batch culture in 125-mL flasks using YPD plus G418 medium as described above with a starting OD_{660} of 0.1. Samples (1 mL) were taken at 96 h and quantified by RP-HPLC as described above. The cells were then rinsed twice and resuspended in 5 mL water and incubated at 30 °C. Cells were collected by centrifugation at various time points and the amount of TAL in the cell-free supernatant was quantified by RP-HPLC. Time courses were fit to single exponentials using Kaleidagraph software v 4.1. Intracellular concentrations were calculated from the extracellular concentration at the final time point during the efflux divided by the cellular volume, using the following equation:

$$[\mathrm{TAL_{in}}] = \frac{T_\mathrm{E}}{V_\mathrm{C} \times \mathrm{DW}} \quad (1)$$

in which $[\mathrm{TAL_{in}}]$ is the intracellular TAL concentration (g/L), T_E is the total amount of effluxed TAL (g), V_C is the specific cell volume (L/g DW) of cells, DW is cell dry weight (g DW). The specific cell volume used for calculations ($V_\mathrm{C} = 2.66 \times 10^{-3}$ L/g DW) was based on previous calculations of cell volume and mass and does not change significantly with increasing ploidy [23, 24]. A conversion factor of 0.623 ± 0.0364 g DW/L-OD was determined using g DW and corresponding OD measurements for all seven selected strains. This conversion factor was used to convert OD to dry weight.

Results

Screening *Saccharomyces* strains for TAL production

Fifteen *Saccharomyces* strains of varied genetic background were transformed with the low-copy plasmid pRH525 for expressing the *g2ps1* gene for 2-pyrone synthase. These strains consisted of laboratory and industrial (wine, beer, distillery, and bioethanol production) yeast. The *TEF* promoter was used to induce 2-PS production, as it more consistently produced TAL than the commonly used *ADH2* promoter (Fig. S1). TAL production was initially measured in batch culture with glucose as the carbon source. In all strains, TAL production peaked on day 4 or 5 and did not significantly increase after that (Fig. 1a). The amount of TAL produced varied 63-fold between strains, with the greatest amount of TAL produced by strain 1174 at 0.63 ± 0.058 g/L and the smallest amount produced by strain 1176 at 0.01 ± 0.001 g/L (Fig. 1b). The five highest TAL-producing strains, 1152, 1174, 1179, 1182, and 1185, were analyzed further. Two lab strains, 1186 and 1187 (CEN.PK and BY4727 backgrounds, respectively; Table 2) were also included.

Efflux of TAL

At the concentrations produced in this study, TAL has been shown to cause only a minor decrease in cell growth [7]. When added at elevated concentrations (e.g., 10 g/L TAL), a more significant decrease in growth rate and biomass yield was reported (especially with minimal medium) [7]. High intracellular TAL concentrations could result in toxicity and hinder optimization at the higher TAL production levels required for an industrial process. An efflux experiment was performed to determine if TAL is actively exported from the cell. After TAL production was complete (96 h), yeast cells were removed from media and resuspended in water. TAL efflux values were measured with respect to time (after cell resuspension in water) and fit to a single exponential (Fig. 2a, b). Data that fits a single exponential is consistent with a single efflux event under single turnover conditions [17]. Additional phases were observed in strains 1179 and 1187, and this analysis is beyond the scope of the paper. All other strains fit a single exponential well ($R^2 > 0.87$ for all replicates).

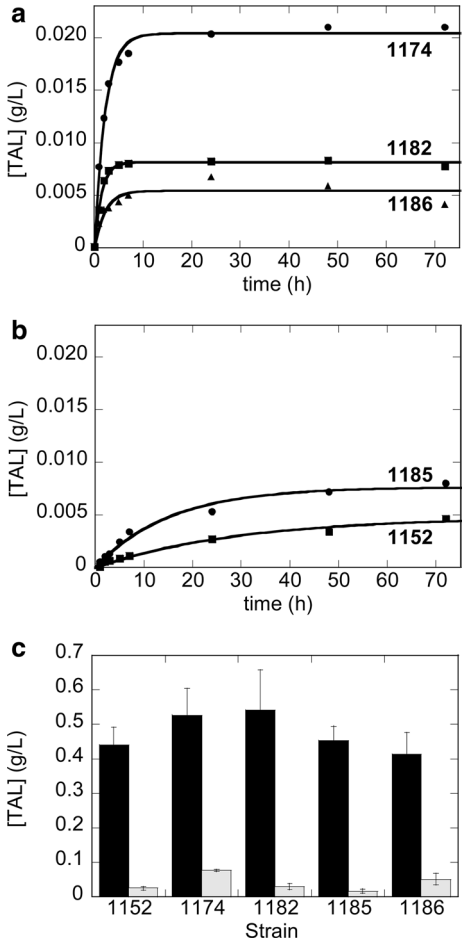

Fig. 2 Efflux of TAL from cells. **a**, **b** Time courses of TAL efflux from cells into water. Efflux experiments were performed in triplicate and efflux data were fit to single exponentials. A representative time course for each strain is shown. *Solid lines* represent the fit to a single exponential. **c** Measured extracellular and calculated intracellular TAL concentrations at 96 h. Extracellular TAL (*black*) and intracellular TAL (*light gray*). Data shown are the averages of three independent cultures. *Error bars* represent the standard deviation

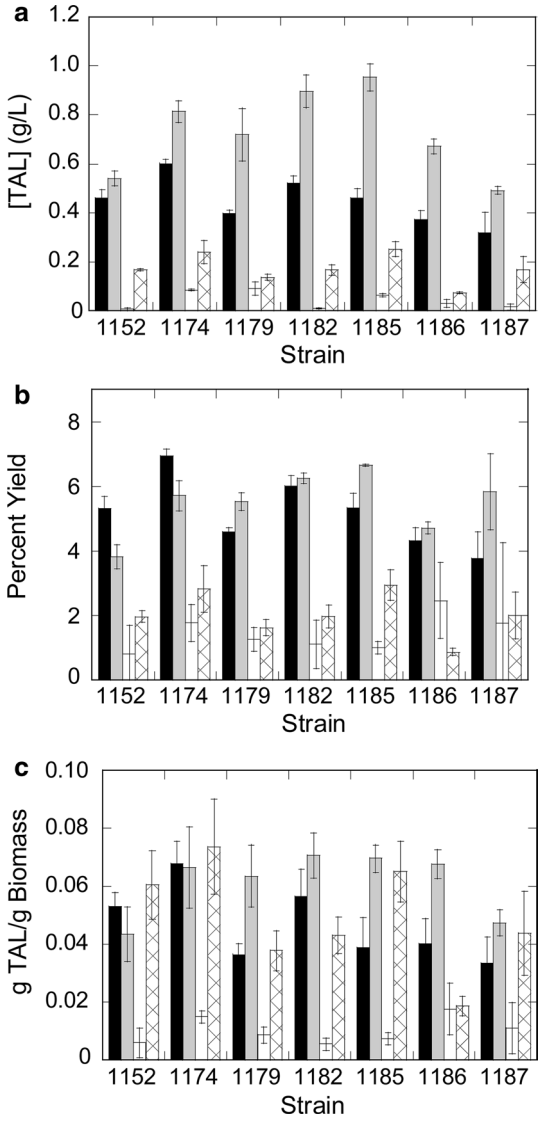

Fig. 3 Analysis of TAL production. **a** TAL production with cells cultured for 96 h in medium containing different carbon sources. **b** Percent yield. **c** Specific TAL production (amount of TAL produced per biomass). Glucose (*black*), ethanol (*gray*), glycerol (*white*), and acetate (*crosshatch*). Data shown are the averages of three independent cultures. *Error bars* represent the standard deviation

The single exponential indicates that one method of efflux predominates, but does not distinguish between active and passive transport. We measured the concentration gradient between intracellular and extracellular TAL to distinguish between diffusion-based and active efflux. Both passive and facilitated diffusion would result in equal intra- and extracellular TAL concentrations at equilibrium. If TAL was actively pumped out of the cell, the extracellular concentration would exceed the intracellular. Intracellular TAL concentrations were determined from the total TAL effluxed into water (Fig. 2c). In all strains, there was a significantly lower (7- to 28-fold less) TAL concentration inside the cells than was present in the medium (Fig. 2c). These data suggest that an active method for TAL efflux is responsible for establishing the concentration gradient.

TAL production using different carbon sources

TAL production was compared for cells grown in medium containing 20 g/L glucose, ethanol, glycerol, or acetate (Fig. 3a). TAL production was highest from cells cultured in medium containing ethanol > glucose > acetate, and lowest in medium containing glycerol regardless of strain (WSR $\alpha < 0.05$ for all pairwise combinations). Cells grown in medium containing ethanol had the highest cell density (OD_{660}), followed by glucose, with acetate and glycerol having similar low cell growth (WSR $\alpha < 0.05$) (Fig. S2a). The highest TAL titer of 0.95 ± 0.056 g/L was achieved

with strain 1185 (Y-629 background) followed by strain 1182 (Ethanol Red background) with 0.90 ± 0.066 g/L. The highest percent yields were approximately 7 % of theoretical yield (Fig. 3b). Specific TAL production (g TAL/g cell dry weight) was compared to identify strains and/or conditions that produce more TAL per cell (Fig. 3c). On average, specific production with glucose was significantly lower than on ethanol for pairwise combinations (Fig. 3c, WSR $\alpha < 0.01$). Using this same test, across all strains, specific production on acetate was not significantly different from production on either ethanol or glucose. Specific TAL production from glycerol was significantly lower than the other three carbon sources (WSR $\alpha < 0.01$).

Comparison of TAL production with varying batch glucose concentration

Several strains produced similar amounts of TAL from glucose and ethanol (Fig. 3a). Strain 1185 (Y-629 with pRH525) was selected for further analysis because it consistently produced high TAL titers, and Y-629 produced TAL when the *ADH2* promoter was used to express 2-PS, whereas the other high-producing strains did not (Fig. S1). Several higher glucose concentrations (50, 100, 150, and 200 g/L) were tested to determine if variation of the initial glucose concentration would lead to an increase in TAL production (Fig. 4a). A low glucose concentration (1 g/L) was also used to determine if respiratory metabolism of glucose, which occurs at low glucose concentrations, would result in a higher percent yield. Increasing the glucose concentration did not result in higher production; final TAL concentration was equivalent for 20 and 50 g/L glucose (0.48 ± 0.068 and 0.55 ± 0.20 g/L) and lower under all other conditions. The highest yield was also observed with 20 g/L glucose (5.2 ± 0.72 percent yield). Respiratory metabolism in the cultures with 1 g/L glucose did not increase TAL yield (Fig. 4b).

TAL production using fed-batch cultivation with ethanol feed

Although respiratory metabolism of glucose (1 g/L glucose in batch culture) did not increase TAL yield compared to glucose fermentation (Fig. 4b), respiratory metabolism of ethanol did result in a higher percent yield compared to cells grown under fermentative conditions (Fig. 3b, batch culture for strain 1185 in medium with ethanol vs. glucose). In an effort to optimize the culture under respiratory conditions and increase cytosolic acetyl-CoA, we evaluated TAL production using strain 1185 cultured in a fed-batch bioreactor with ethanol feed. Dissolved oxygen was maintained at 30 % and the feed rate of ethanol was varied at 0.03, 0.06, 0.105, and 0.15 g/h. All four conditions resulted

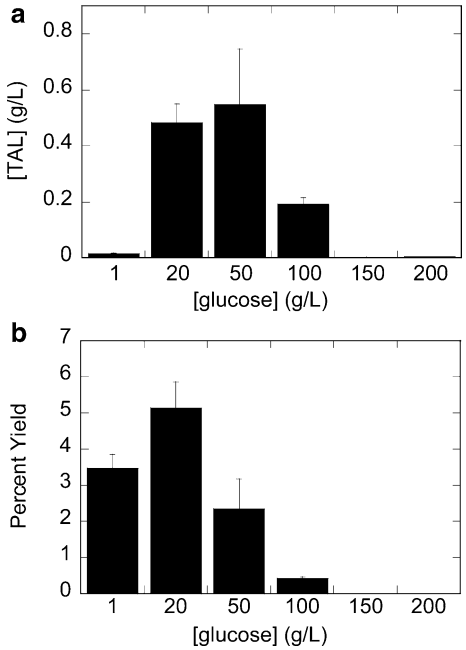

Fig. 4 Batch culture of 1185 with varying glucose concentrations. **a** TAL production by strain 1185. **b** Percent yield with strain 1185. Data shown are the averages of three independent cultures. *Error bars* represent the standard deviation

in significantly higher TAL concentrations than those attained in batch cultivation with the highest titer reaching 5.2 ± 0.46 g/L (Fig. 5a). Lower ethanol feed rates (0.03 and 0.06 g/h) resulted in a steady increase in TAL titer. Initial TAL productivity was increased when the ethanol feed rate was increased to 0.105 and 0.15 g/h. However, rather than further increasing final TAL titer, TAL decreased rapidly after reaching an intermediate concentration (Fig. 5a). This rapid decrease in TAL concentration did not appear to be dependent on the maximum TAL concentration, as the maximum final TAL concentration was higher in cultures using a lower ethanol feed rate than in the 0.15 g/h sample, which had the earliest TAL decrease. Rather, loss of TAL from the medium appeared dependent on the rate of TAL production. Cultures with the highest TAL productivity showed the earliest onset of TAL disappearance, whereas cultures with lower TAL productivity continued to slowly accumulate TAL and were able to reach higher titers.

Modification of TAL

In cell culture with elevated carbon source conditions, such as the fed-batch bioreactors (0.105–0.15 g/h ethanol feed) and batch culture with higher glucose concentrations (50–200 g/L), additional products were observed that eluted at later RP-HPLC retention times. Distinct lower polarity peaks were observed at 12.8 min (Compound II)

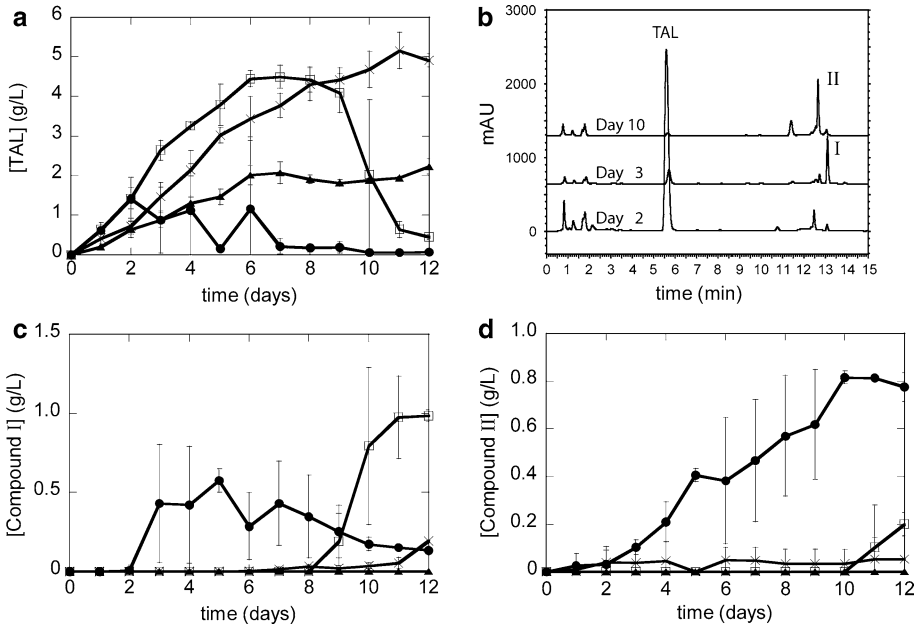

Fig. 5 TAL production using continuous ethanol feed. **a** TAL production (g/L) over time. **b** RP-HPLC chromatograms showing the loss of TAL and subsequent production of Compounds I and II from 0.15 g/h feed. **c** Compound I production (g/L) over time. **d** Compound II production (g/L) over time. Since Compounds I and II were derivatives of TAL, the extinction coefficient for TAL was used to estimate their concentrations based on a TAL standard curve. Data shown are the averages of three independent cultures. *Error bars* represent the standard deviation. Symbols for *panels* **a**, **c**, **d** are: 0.15 g/h (*filled circle*), 0.105 g/h (*square*), 0.06 g/h (*cross*), and 0.03 g/h (*filled triangle*)

and 13.2 min (Compound I) (Fig. 5b). These products had temporal profiles (Fig. 5c, d) that showed a progression in which TAL concentration peaked, followed by appearance of Compound I, then Compound II. These products were not observed in the RP-HPLC chromatograms from strains lacking the *g2ps1* gene (data not shown). Compounds I and II could also be generated by incubating the parent strain with exogenously added TAL at elevated concentrations, indicating that they were TAL derivatives (data not shown).

One potential reactive site on TAL is the 4 position hydroxyl group; we hypothesized that the TAL derivative involved ester formation at this hydroxyl. To test this hypothesis we attempted to convert Compound I back into TAL. To saponify this potential ester linkage, samples containing Compound I were incubated at increasing pH from 6 to 12. Increasing the pH from 6 to 9 resulted in an increased TAL signal and a concomitant lower signal of Compound I; however, higher pH did not increase the amount of Compound I converted to TAL, possibly due to degradation of both TAL and Compound I (Fig. 6). These data indicated that Compound I is a modification of TAL.

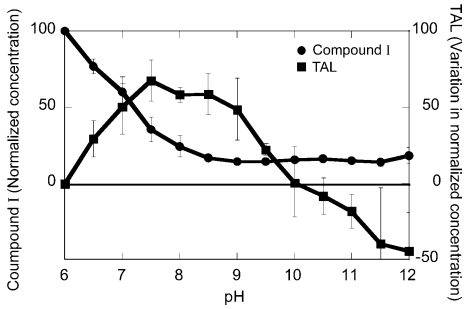

Fig. 6 Base treatment of Compound I. Potassium hydroxide was added to culture supernatant and aliquots were taken at intervals of 0.5 pH. Samples were incubated at RT for 20 h and measured by RP-HPLC. Concentration data were normalized to the amount of Compound I and TAL present at pH 6. Compound I [normalized concentration (*filled circle*)] and TAL [variation in normalized concentration (*filled square*)] are shown on separate axes. Declining values for TAL beyond pH 7.5 indicate that TAL is unstable at elevated pH. Negative values for TAL beyond pH 10 result from degradation of TAL to levels below the amount present at pH 6. *Error bars* represent the standard deviation of three biological replicates

Discussion

Previous studies of TAL production that have utilized *S. cerevisiae* have each investigated a single laboratory strain [7, 49]. This work is the first to increase TAL production through the genetic diversity available in more robust industrial strains. Genome sequencing of industrial strains and comparison to lab strains have identified significant differences at the level of single nucleotide polymorphism and the presence of additional genes and pathways in industrial strains that are lacking in lab strains [4, 5, 42, 48]. Aside from providing the increased stress tolerance [14, 33] required for an industrial process, industrial strains have previously been shown to increase the fermentation of xylose to ethanol [3, 6, 13, 18, 22] compared to lab strains. The strains tested in this study were identified previously in a screen for increased resistance to inhibitors present in corn stover hydrolysate (to be reported elsewhere) and thus should be ideal for further engineering for the production of biorenewable TAL from lignocellulosic biomass feedstocks.

TAL production using 2-PS requires both acetyl and malonyl-CoA. In *S. cerevisiae*, malonyl-CoA is produced from acetyl-CoA by *ACC1*. While some yeast species provide multiple pathways to produce acetyl-CoA, in *S. cerevisiae,* cytosolic acetyl-CoA is only produced by acetyl-CoA synthase (*ACS1* and *ACS2*) [35]. The flux through these pathways is influenced by many factors, and increasing flux to cytosolic acetyl-CoA has been the focus of several metabolic engineering studies [7, 9, 11, 25–27]. Variation due to the genetic differences between yeast strains could also lead to different flux through this pathway, with the potential for certain strains to have an increased capacity to produce TAL. Initial screening of *Saccharomyces* strains of diverse genetic background showed that TAL production varied significantly (63-fold) depending simply on choice of strain used to express *g2ps1*. While industrial strains did produce the most TAL, many of the strains produced less TAL than the intermediate levels produced by laboratory strains.

Cells utilize different metabolic pathways depending on the available carbon source. Changing the carbon source and culture conditions can improve TAL yield and provide useful information for further metabolic engineering strategies and process engineering approaches. This approach (i.e., using fed-batch cultivation with ethanol feed vs. batch culture with glucose) was recently shown in yeast to significantly increase production of amorphadiene, a precursor of the anti-malarial drug artemisinin which also requires acetyl-CoA for its production in yeast [30, 31, 47]. While fermentation is the most efficient pathway for ethanol production, it results in low intracellular acetyl-CoA concentrations, and is thus not ideal for production of products made from acetyl-CoA. Respiratory metabolism provides a sixfold increase in acetyl-CoA flux compared to fermentation [20, 32] and should provide more acetyl-CoA to the TAL production pathway.

Fermentative vs. respiratory production of TAL was investigated by several ways in this study. *S. cerevisiae* uses respiratory metabolism when cultured with ethanol, glycerol, or acetate as the carbon source. Separately, culture with low glucose concentration (<2–5 g/L) also allows respiration. These culture conditions were evaluated for their effect on TAL production and yield. TAL production was highest with ethanol as the carbon source. Polyketide and terpene pathways begin with acetyl and/or malonyl-CoA, and these pathways are glucose-repressed in *S. cerevisiae.* Ethanol is assimilated via conversion to acetyl-CoA and this pathway may supply more acetyl-CoA for conversion to TAL [12, 47] as it has a more direct route to acetyl-CoA than glucose or glycerol. Thus, fewer opportunities exist for intermediates to be siphoned off into other pathways.

Acetate, however, should have a similarly direct pathway to acetyl-CoA, but cells cultured in medium with acetate as the carbon source produced significantly less TAL. While cells cultured with acetate were able to consume all of the acetate, cell density for these cultures was significantly lower compared to cultures with glucose or ethanol (Fig. S2). Acetate flux through acetaldehyde rather than acetyl-CoA may predominate [36], so that cells in acetate media would not have the acetyl-CoA flux of those in ethanol. Weak organic acids like acetic acid are known for their ability to deplete ATP [34]. Acetic acid (p*K*a 4.75) dissociates in the yeast cytosol (pH ~ 7) leading to acidification. Cells counter the acidification by actively transporting the dissociated protons out of the cell, at the expense of 1 ATP:proton. Synthesis of acetyl-CoA requires ATP; ATP limitation due to acidification of the cytosol could explain the low TAL production from acetate.

Biodiesel production generates large amounts of crude glycerol as a by-product. This abundance of glycerol by-product has generated considerable interest in identifying strains capable of using it as a substrate. Most laboratory strains of *S. cerevisiae* grow poorly on glycerol. However, there is intraspecies diversity with respect to glycerol metabolism and strains have been identified that exhibit increased ability to assimilate glycerol [44]. Three of the strains tested were able to metabolize 50–75 % of glycerol (Fig. S2b) and strain 1179 attained a cell density equal to its growth on glucose or ethanol (Fig. S2a). However, TAL production was extremely low for all of the strains cultured with glycerol.

Our work identified a potential bottleneck in TAL production that was not reported in previous studies [7, 49]. At elevated TAL productivity, TAL was rapidly modified by the cells. Since Compound I can be converted back to TAL by increasing pH, we believe that the modified TAL product contains an ester linkage to the 4 position hydroxyl group. Under our conditions, these products did not form after a particular TAL concentration was reached, but instead TAL appears to be modified when a high rate of TAL production is reached. As TAL is slightly toxic to *S. cerevisiae* [7], modification of TAL could be a stress response to a rapid TAL increase. Future work will be required to identify and prevent the formation of these additional products.

Our results are consistent with active TAL export from the cell via a single pathway. However, the identity of the efflux pump is not yet known. Active efflux can keep intracellular TAL concentrations low until the rate of TAL production is faster than the rate of efflux, beyond which we hypothesize that TAL modification occurs. Stress responses can be alleviated with a faster efflux of the toxic chemicals from the cells [37, 39, 46] and transporter engineering has been shown to improve tolerance to alkane biofuels [8, 15]. Identifying the transporter responsible for TAL export and increasing the rate of TAL efflux may be a potential approach to further improve TAL tolerance and limit the conversion of TAL to Compound I.

TAL was produced at concentrations of 5.2 g/L in industrial *Saccharomyces* strains without engineering of either the 2-PS gene or metabolic pathways of the yeast strain. TAL percent yields obtained for these strains were low (4–7 % from glucose), but were comparable to the 1–5.3 % yield reported for an unmodified laboratory strain [7]. Previous studies have shown 20-fold improvement in TAL production from an engineered *g2ps1* gene in *E. coli* [45], and 12-fold improvement through strain engineering in *S. cerevisiae* [7]. The use of either of these methods, combined with the strain identified in our study, will likely increase TAL yield further, indicating that *S. cerevisiae* is an excellent candidate for production of this platform chemical.

Acknowledgments We thank Katherine Card for her excellent technical assistance throughout this study. The authors would also like to thank Dr. Joseph Noel (Salk Institute) for the *g2ps1* gene encoding the 2-pyrone synthase.

Conflict of interest The authors declare no conflict of interest.

References

1. Abaecherli C, Miller RJ (2000) Ketenes, ketene dimers, and related substances. In: Kroschwitz JI, Howe-Grant M (eds) Kirk–Othmer encyclopedia of chemical technology. 4th edn. Wiley. doi :10.1002/0471238961.1105200501020105.a01
2. Achkar J, Xian M, Zhao H, Frost JW (2005) Biosynthesis of phloroglucinol. J Am Chem Soc 127(15):5332–5333. doi:10.1021/ja042340g
3. Bera AK, Ho NW, Khan A, Sedlak M (2011) A genetic overhaul of *Saccharomyces cerevisiae* 424A(LNH-ST) to improve xylose fermentation. J Ind Microbiol Biot 38(5):617–626. doi:10.1007/s10295-010-0806-6
4. Borneman AR, Desany BA, Riches D, Affourtit JP, Forgan AH, Pretorius IS, Egholm M, Chambers PJ (2011) Whole-genome comparison reveals novel genetic elements that characterize the genome of industrial strains of *Saccharomyces cerevisiae*. PLoS Genet 7(2):e1001287. doi:10.1371/journal.pgen.1001287
5. Borneman AR, Forgan AH, Pretorius IS, Chambers PJ (2008) Comparative genome analysis of a *Saccharomyces cerevisiae* wine strain. FEMS Yeast Res 8(7):1185–1195. doi:10.1111/j.1567-1364.2008.00434.x
6. Brat D, Boles E, Wiedemann B (2009) Functional expression of a bacterial xylose isomerase in *Saccharomyces cerevisiae*. Appl Environ Microbiol 75(8):2304–2311. doi:10.1128/AEM.02522-08
7. Cardenas J, Da Silva NA (2014) Metabolic engineering of *Saccharomyces cerevisiae* for the production of triacetic acid lactone. Metab Eng 25:194–203. doi:10.1016/j.ymben.2014.07.008
8. Chen B, Ling H, Chang MW (2013) Transporter engineering for improved tolerance against alkane biofuels in *Saccharomyces cerevisiae*. Biotechnol Biofuels 6(1):21. doi:10.1186/1754-6834-6-21
9. Chen Y, Daviet L, Schalk M, Siewers V, Nielsen J (2013) Establishing a platform cell factory through engineering of yeast acetyl-CoA metabolism. Metab Eng 15:48–54. doi:10.1016/j.ymben.2012.11.002
10. Chia M, Schwartz TJ, Shanks BH, Dumesic JA (2012) Triacetic acid lactone as a potential biorenewable platform chemical. Green Chem 14(7):1850–1853. doi:10.1039/C2gc35343a
11. de Jong-Gubbels P, van den Berg MA, Luttik MA, Steensma HY, van Dijken JP, Pronk JT (1998) Overproduction of acetyl-coenzyme A synthetase isoenzymes in respiring *Saccharomyces cerevisiae* cells does not reduce acetate production after exposure to glucose excess. FEMS Microbiol Lett 165(1):15–20 pii: S0378-1097(98)00249-3
12. de Jong-Gubbels P, Vanrolleghem P, Heijnen S, van Dijken JP, Pronk JT (1995) Regulation of carbon metabolism in chemostat cultures of *Saccharomyces cerevisiae* grown on mixtures of glucose and ethanol. Yeast 11(5):407–418. doi:10.1002/yea.320110503
13. Demeke MM, Dietz H, Li Y, Foulquie-Moreno MR, Mutturi S, Deprez S, Den Abt T, Bonini BM, Liden G, Dumortier F, Verplaetse A, Boles E, Thevelein JM (2013) Development of a D-xylose fermenting and inhibitor tolerant industrial *Saccharomyces cerevisiae* strain with high performance in lignocellulose hydrolysates using metabolic and evolutionary engineering. Biotechnol Biofuels 6(1):89. doi:10.1186/1754-6834-6-89
14. Demeke MM, Dumortier F, Li Y, Broeckx T, Foulquie-Moreno MR, Thevelein JM (2013) Combining inhibitor tolerance and D-xylose fermentation in industrial *Saccharomyces cerevisiae* for efficient lignocellulose-based bioethanol production. Biotechnol Biofuels 6(1):120. doi:10.1186/1754-6834-6-120
15. Dunlop MJ, Dossani ZY, Szmidt HL, Chu HC, Lee TS, Keasling JD, Hadi MZ, Mukhopadhyay A (2011) Engineering microbial biofuel tolerance and export using efflux pumps. Mol Syst Biol 7:487. doi:10.1038/msb.2011.21
16. Eckermann S, Schroder G, Schmidt J, Strack D, Edrada RA, Helariutta Y, Elomaa P, Kotilainen M, Kilpelainen I, Proksch P, Teeri TH, Schroder J (1998) New pathway to polyketides in plants. Nature 396(6709):387–390. doi:10.1038/24652
17. Fersht A (1999) Structure and mechanism in protein science: a guide to enzyme catalysis and protein folding. W.H. Freeman and Company, New York, pp 132–168
18. Garcia Sanchez R, Karhumaa K, Fonseca C, Sanchez Nogue V, Almeida JR, Larsson CU, Bengtsson O, Bettiga M, Hahn-Hägerdal B, Gorwa-Grauslund MF (2010) Improved xylose and arabinose utilization by an industrial recombinant *Saccharomyces cerevisiae* strain using evolutionary engineering. Biotechnol Biofuels 3:13. doi:10.1186/1754-6834-3-13
19. Gietz D, Woods RA (2002) Transformation of yeasts by the lithium acetate/single-stranded carrier/polyethylene glycol method. Methods Enzymol 350:87–96. doi:10.1016/S0076-6879(02)50957-5
20. Gombert AK, Moreira dos Santos M, Christensen B, Nielsen J (2001) Network identification and flux quantification in the central metabolism of *Saccharomyces cerevisiae* under different conditions of glucose repression. J Bacteriol 183(4):1441–1451. doi:10.1128/JB.183.4.1441-1451.2001
21. Hansen CA, Frost JW (2001) Deoxygenation of polyhydroxybenzenes: an alternative strategy for the benzene-free synthesis of aromatic chemicals. J Am Chem Soc 124:5926–5927. doi:10.1021/ja0176346
22. Hector RE, Dien BS, Cotta MA, Qureshi N (2011) Engineering industrial *Saccharomyces cerevisiae* strains for xylose fermentation and comparison for switchgrass conversion. J Ind Microbiol Biotechnol 38(9):1193–1202. doi:10.1007/s10295-010-0896-1
23. Jorgensen P, Nishikawa JL, Breitkreutz BJ, Tyers M (2002) Systematic identification of pathways that couple cell growth and division in yeast. Science 297(5580):395–400. doi:10.1126/science.1070850
24. Klis FM, de Koster CG, Brul S (2014) Cell wall-related bionumbers and bioestimates of *Saccharomyces cerevisiae* and *Candida albicans*. Eukaryot Cell 13(1):2–9. doi:10.1128/EC.00250-13
25. Kozak BU, van Rossum HM, Benjamin KR, Wu L, Daran J-MG, Pronk JT, van Maris AJA (2014) Replacement of the

Saccharomyces cerevisiae acetyl-CoA synthetases by alternative pathways for cytosolic acetyl-CoA synthesis. Metab Eng 21:46–59. doi:10.1016/j.ymben.2013.11.005
26. Krivoruchko A, Serrano-Amatriain C, Chen Y, Siewers V, Nielsen J (2013) Improving biobutanol production in engineered *Saccharomyces cerevisiae* by manipulation of acetyl-CoA metabolism. J Ind Microbiol Biotechnol 40(9):1051–1056. doi:10.1007/s10295-013-1296-0
27. Lian J, Si T, Nair NU, Zhao H (2014) Design and construction of acetyl-CoA overproducing *Saccharomyces cerevisiae* strains. Metab Eng. doi:10.1016/j.ymben.2014.05.010
28. Modig T, Almeida JR, Gorwa-Grauslund MF, Liden G (2008) Variability of the response of *Saccharomyces cerevisiae* strains to lignocellulose hydrolysate. Biotechnol Bioeng 100(3):423–429. doi:10.1002/bit.21789
29. Nikolau BJ, Perera MADN, Brachova L, Shanks B (2008) Platform biochemicals for a biorenewable chemical industry. Plant J 54(4):536–545. doi:10.1111/j.1365-313X.2008.03484.x
30. Paddon CJ, Keasling JD (2014) Semi-synthetic artemisinin: a model for the use of synthetic biology in pharmaceutical development. Nat Rev Microbiol. doi:10.1038/nrmicro3240
31. Paddon CJ, Westfall PJ, Pitera DJ, Benjamin K, Fisher K, McPhee D, Leavell MD, Tai A, Main A, Eng D, Polichuk DR, Teoh KH, Reed DW, Treynor T, Lenihan J, Fleck M, Bajad S, Dang G, Dengrove D, Diola D, Dorin G, Ellens KW, Fickes S, Galazzo J, Gaucher SP, Geistlinger T, Henry R, Hepp M, Horning T, Iqbal T, Jiang H, Kizer L, Lieu B, Melis D, Moss N, Regentin R, Secrest S, Tsuruta H, Vazquez R, Westblade LF, Xu L, Yu M, Zhang Y, Zhao L, Lievense J, Covello PS, Keasling JD, Reiling KK, Renninger NS, Newman JD (2013) High-level semi-synthetic production of the potent antimalarial artemisinin. Nature 496(7446):528–532. doi:10.1038/nature12051
32. Papini M, Nookaew I, Uhlen M, Nielsen J (2012) *Scheffersomyces stipitis*: a comparative systems biology study with the Crabtree positive yeast *Saccharomyces cerevisiae*. Microb Cell Fact 11:136. doi:10.1186/1475-2859-11-136
33. Pereira FB, Romani A, Ruiz HA, Teixeira JA, Domingues L (2014) Industrial robust yeast isolates with great potential for fermentation of lignocellulosic biomass. Bioresour Technol 161C:192–199. doi:10.1016/j.biortech.2014.03.043
34. Piper P, Calderon CO, Hatzixanthis K, Mollapour M (2001) Weak acid adaptation: the stress response that confers yeasts with resistance to organic acid food preservatives. Microbiology 147:2635–2642
35. Pronk JT, Yde Steensma H, Van Dijken JP (1996) Pyruvate metabolism in *Saccharomyces cerevisiae*. Yeast 12(16):1607–1633. doi:10.1002/(SICI)1097-0061(199612)12:16<1607:AID-YEA70>3.0.CO;2-4
36. Remize F, Andrieu E, Dequin S (2000) Engineering of the pyruvate dehydrogenase bypass in *Saccharomyces cerevisiae*: role of the cytosolic Mg(2+) and mitochondrial K(+) acetaldehyde dehydrogenases Ald6p and Ald4p in acetate formation during alcoholic fermentation. Appl Environ Microbiol 66(8):3151–3159
37. Reyes LH, Almario MP, Kao KC (2011) Genomic library screens for genes involved in n-butanol tolerance in *Escherichia coli*. PLoS ONE 6(3):e17678. doi:10.1371/journal.pone.0017678
38. Richardson MT, Pohl NL, Kealey JT, Khosla C (1999) Tolerance and Specificity of recombinant 6-methylsalicylic acid synthase. Metab Eng 1:180–187. doi:10.1006/mben.1999.0113
39. Sa-Correia I, dos Santos SC, Teixeira MC, Cabrito TR, Mira NP (2009) Drug: H+ antiporters in chemical stress response in yeast. Trends Microbiol 17(1):22–31. doi:10.1016/j.tim.2008.09.007
40. Sambrook J, Russell DW (2001) Molecular cloning: a laboratory manual, 3rd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor
41. Sikorski RS, Hieter P (1989) A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. Genetics 122:19–27
42. Stambuk BU, Dunn B, Alves SL Jr, Duval EH, Sherlock G (2009) Industrial fuel ethanol yeasts contain adaptive copy number changes in genes involved in vitamin B1 and B6 biosynthesis. Genome Res 19(12):2271–2278. doi:10.1101/gr.094276.109
43. Steiner S, Philippsen P (1994) Sequence and promoter analysis of the highly expressed *TEF* gene of the filamentous fungus *Ashbya gossypii*. Mol Gen Genet 242(3):263–271
44. Swinnen S, Klein M, Carrillo M, McInnes J, Nguyen HTT, Nevoigt E (2013) Re-evaluation of glycerol utilization in *Saccharomyces cerevisiae*: characterization of an isolate that grows on glycerol without supporting supplements. Biotechnol Biofuels 6:157. doi:10.1186/1754-6834-6-157
45. Tang SY, Qian S, Akinterinwa O, Frei CS, Gredell JA, Cirino PC (2013) Screening for enhanced triacetic acid lactone production by recombinant *Escherichia coli* expressing a designed triacetic acid lactone reporter. J Am Chem Soc 135(27):10099–10103. doi:10.1021/ja402654z
46. Tenreiro S, Nunes PA, Viegas CA, Neves MS, Teixeira MC, Cabral MG, Sa-Correia I (2002) *AQR1* gene (ORF YNL065w) encodes a plasma membrane transporter of the major facilitator superfamily that confers resistance to short-chain monocarboxylic acids and quinidine in *Saccharomyces cerevisiae*. Biochem Biophys Res Commun 292(3):741–748. doi:10.1006/bbrc.2002.6703
47. Westfall PJ, Pitera DJ, Lenihan JR, Eng D, Woolard FX, Regentin R, Horning T, Tsuruta H, Melis DJ, Owens A, Fickes S, Diola D, Benjamin KR, Keasling JD, Leavell MD, McPhee DJ, Renninger NS, Newman JD, Paddon CJ (2012) Production of amorphadiene in yeast, and its conversion to dihydroartemisinic acid, precursor to the antimalarial agent artemisinin. Proc Natl Acad Sci USA 109(3):E111–E118. doi:10.1073/pnas.1110740109
48. Wohlbach DJ, Rovinskiy N, Lewis JA, Sardi M, Schackwitz WS, Martin JA, Deshpande S, Daum CG, Lipzen A, Sato TK, Gasch AP (2014) Comparative genomics of *Saccharomyces cerevisiae* natural isolates for bioenergy production. Genome Biol Evol. doi:10.1093/gbe/evu199
49. Xie D, Shao Z, Achkar J, Zha W, Frost JW, Zhao H (2006) Microbial synthesis of triacetic acid lactone. Biotechnol Bioeng 93(4):727–736. doi:10.1002/bit.20759
50. Zha W, Shao Z, Frost JW, 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(14):4534–4535. doi:10.1021/ja0317271