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Bioengineering triacetic acid lactone production in *Yarrowia lipolytica* for pogostone synthesis

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Abstract

Yarrowia lipolytica is an oleaginous yeast that is recognized for its ability to accumulate high levels of lipids, which can serve as precursors to biobased fuels and chemicals. Polyketides such as triacetic acid lactone (TAL) can also serve as a precursor for diverse commodity chemicals. This study used *Y. lipolytica* as a host organism for the production of TAL via expression of the 2-pyrone synthase gene from *Gerbera hybrida*. Induction of lipid biosynthesis by nitrogen-limited growth conditions increased TAL titers. We also manipulated basal levels of TAL production using a DNA cut-and-paste transposon to mobilize and integrate multiple copies of the 2-pyrone synthase gene. Strain modifications and batch fermentation in nitrogen-limited medium yielded TAL titers of 2.6 g/L. Furthermore, we show that minimal medium allows TAL to be readily concentrated at >94% purity and converted at 96% yield to pogostone, a valuable antibiotic. Modifications of this reaction scheme yielded diverse related compounds. Thus, oleaginous organisms have the potential to be flexible microbial biofactories capable of economical synthesis of platform chemicals and the generation of industrially relevant molecules.

Keywords

Yarrowia lipolytica; triacetic acid lactone; pogostone; polyketide; lipids

The nonconventional yeast *Yarrowia lipolytica* has emerged as a model host organism for biosustainable production of industrial chemicals from renewable carbon sources. It is most notable for its ability to accumulate high levels of lipids under nutrient-limited conditions. Recent studies have shown that it is also a suitable host for efficient production of other industrially relevant metabolites, such as free fatty acids, organic acids, and polyols

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(Beopoulos et al., 2009, Liu et al., 2013; Qiao et al., 2015, Tomaszewska et al., 2012; Zeng et al., 2017).

Another class of natural metabolites with industrial and biomedical applications are polyketides. Triacetic acid lactone (TAL, or 4-hydroxy-6-methyl-2-pyrone) is a polyketide that is synthesized by 2-pyrone synthase (2-PS), originally isolated from *Gerbera hybrida* (Austin and Noel, 2003). TAL synthesis via 2-PS is similar to fatty acid synthesis in that malonyl-CoA molecules are iteratively condensed onto an acetyl-CoA starter molecule. TAL is a platform molecule that can be differentiated into a range of commodity chemicals for use as food additives (sorbic and hexanoic acid), antibiotics (pogostone), and fuel additives (acetylacetone) (Chia et al., 2012; Kraus et al., 2016).

Microbial synthesis of TAL has been conducted in both *Escherichia coli* and *Saccharomyces cerevisiae* (Cardenas and Da Silva, 2014; Saunders et al., 2015; Tang et al., 2013). Previous studies demonstrated that *E. coli* exhibits TAL toxicity (L. Jarboe, Iowa State University, personal communication), thus limiting its feasibility as a host microbe for TAL production. Although recent studies expressing the 2-PS enzyme in industrial strains of *S. cerevisiae* and *Y. lipolytica* produced titers up to 5.1 g/L and 35.9 g/L, respectively (Saunders et al., 2015; Markham et al., 2018), cells were cultured in complex medium with bioactive constituents that complicate the purification workflow (Schwartz et al., 2014).

Y. lipolytica is an ideal host for the production of polyketides like TAL. It is an oleaginous microorganism and differs from *S. cerevisiae* in that it utilizes the ATP:citrate lyase (ACL) pathway to generate *de novo* cytosolic acetyl-CoA, which is in turn used for fatty acid synthesis (Fakas, 2017). This pathway is unique to oleaginous organisms and can be upregulated by nitrogen limitation (Blazeck et al., 2014; Boulton and Ratledge, 1981). This upregulation has the potential to contribute acetyl-CoA not only to fatty acid synthesis, but also to TAL synthesis when 2-PS is expressed. *Y. lipolytica* bears an additional advantage over *S. cerevisiae* in its ability to grow well in minimal medium, potentially avoiding the interference with downstream industrial purifications and reactions caused by constituents of rich media containing yeast extract and peptone.

To investigate TAL production in *Y. lipolytica*, the 2-PS gene was recoded and cloned under the control of the *Y. lipolytica TEF1* intron promoter for expression (Tai and Stephanopoulos, 2013). The 2-PS expression plasmid (pDTN3997) contains three copies of the 2-PS expression cassette with *URA3* and *LEU2* nutritional markers for plasmid retention (Methods; Supporting Information, Table SII). An auxotrophic strain of W29/CLIB89 (yJY2006; Supporting Information, Table SI) was transformed with the 2-PS expression plasmid and cultured in flasks in nitrogen-replete minimal medium (NR) containing 5 g/L ammonium sulfate as a source of nitrogen and 20 g/L glucose. The amount of TAL in the cell-free medium after 96 hrs of culture was 416 ± 21 mg/L as measured by A_{282} (Fig. 1a; Methods; Supporting Information, Fig. S1), which is 4.4% of the maximum theoretical yield.

Previous studies have shown that in nitrogen-limited (NL) conditions, wild type strains of *Y. lipolytica* are capable of accumulating up to 36% lipid and up to 90% lipid with additional

genetic modifications (Beopoulos et al., 2009; Blazeck et al., 2014; Qiao et al., 2015; Xu et al., 2017). Since acetyl-CoA is required for TAL and fatty acid synthesis, we tested whether nitrogen limitation also increased TAL accumulation. When yJY2006 harboring pDTN3997 was grown in NL medium, cultures accumulated 1.4-fold more TAL at 96 hrs compared to NR cultures (566 ± 41 mg/L vs. 416 ± 21 mg/L, respectively, p=0.03) (Fig. 1a), consistent with contributions from the ACL pathway to acetyl-CoA utilized in TAL synthesis. This result suggested that restricting fatty acid synthesis could redirect acetyl-CoA into TAL synthesis.

To investigate whether restriction of fatty acid synthesis can enhance TAL production, we tested the effect of cerulenin, a potent inhibitor of fungal fatty acid synthase, on TAL synthesis (Omura, 1976). yJY2006 + pDTN3997 was cultured in flasks containing NL medium. After 27 hrs of growth, the cultures were split into two sets and cerulenin dissolved in DMSO was brought to a final concentration of 20 μM in test cultures while an equivalent volume of DMSO was added to control cultures. After an additional 73 hrs of growth, cerulenin-treated cultures accumulated 2.7-fold more TAL than in NL medium alone (1872 $\pm$ 88 mg/L vs. 684 ± 247 mg/L, respectively, p=0.03) (Fig. 1b). Taken together, these findings are consistent with the model that acetyl-CoA generated in nitrogen-limiting conditions can be diverted away from fatty acid synthesis and redirected into TAL synthesis.

In addition to the ACL pathway, acetyl-CoA is generated by beta oxidation of fatty acids. In *S. cerevisiae*, beta oxidation occurs exclusively in the peroxisomes (Kunau et al., 1995). To investigate the potential contribution of peroxisomal beta oxidation to acetyl-CoA as a substrate for TAL synthesis, we disrupted the peroxisome biogenesis factor encoded by *PEX10* (YALI0C01023g/ YALI1C01416g) using CRISPR/Cas9 to make the strain yJL2008 (Methods; Supporting Information, Table SI). Pex10p is an integral membrane protein essential for the formation of peroxisomes, thus yJL2008 cannot carry out peroxisomal beta oxidation. The *pex10* strain was transformed with pDTN3997, and TAL production was monitored in NR and NL flask cultures. In NL conditions, disruption of *PEX10* decreased TAL titers at 96 hrs by 65% compared to the control strain (*pex10* NL, 365 ± 45 mg/L; WT NL, 566 ± 41 mg/L, p=0.03) (Fig. 1a), indicating that a basal level of peroxisomal beta oxidation exists within cells even in conditions conducive for lipid accumulation. A similar result was also observed when cultures were grown in NR medium (Fig. 1a). These results are consistent with a model in which acetyl-CoA produced from beta oxidation can be used to synthesize TAL.

In contrast to *S. cerevisiae*, beta oxidation in the mammalian system occurs in both mitochondria and the peroxisomes. Recent studies have demonstrated that some fungi are also capable of mitochondrial beta oxidation (Hynes et al., 2008; Kretschmer et al., 2012; Maggio-Hall and Keller, 2004). A BLAST search of the *Y. lipolytica* genome identified a putative acyl-CoA dehydrogenase (YALI0D15708g/YALI1D19252g) and a putative enoyl-CoA hydratase (YALI0B10406g/YALI1B14044g). Both of these enzymes are required for mitochondrial beta oxidation, providing further suggestive evidence that this pathway exists in this organism. While the *pex10* phenotype indicates that peroxisomal beta oxidation contributes to TAL production (Fig. 1a), residual TAL titers in this mutant could result from non-peroxisomal sources such as the ACL pathway, mitochondrial beta oxidation,

mitochondrial pyruvate dehydrogenase activity and the pyruvate bypass pathway (Markham et al., 2018).

The possibility of an additional source of acetyl-CoA in *Y. lipolytica* warrants further investigation into this question and may have significant industrial implications.

In order to further raise TAL production, we used the DNA cut-and-paste transposon Hermes to increase the chromosomal copy number of the 2-PS gene. Plasmid pJY4089 (Supporting Information, Table SII) was constructed by swapping the transposase coding sequence of a Hermes element with the 2-PS expression cassette and expressing the Hermes transposase in *trans*, enabling an unmarked copy of the 2-PS gene to be mobilized. Sequential transposition of 2-PS from pJY4089 into the yJY1948 genome yielded the strain yJY2039 (Supporting Information, Table SI). Strain yJY2039 + pDTN3997 produced more TAL in both NR and NL medium after 144 hrs culture than the WT strain (yJY2039 NR, 825 ± 46 mg/L; WT NR, 564 ± 43 mg/L, $p = 0.03$; yJY2039 NL, 992 ± 49 mg/L; WT NL, 720 ± 161 mg/L, $p = 0.03$) (Fig. 1c).

To further improve TAL titer and yield, strain yJY2039 + pDTN3997 was cultured in a Multifors 2 bioreactor (INFORS HT, Bottmingen, Switzerland) in batch fermentations (Supporting Information). The strain was first inoculated into a bioreactor containing NL medium with 40 g/L glucose. After 40 hrs of growth, 4.35 g/L ammonium sulfate was added to the medium and the base used to maintain pH was switched from sodium hydroxide to ammonium hydroxide in order to increase the culture's cell density and potentially increase TAL output. Cultures were maintained for an additional 40 hrs before the medium was collected and analyzed for TAL production. These batch fermentations yielded titers of 2.6 g/L TAL, which is 13.8% of the maximum theoretical yield. This level of TAL production was significantly higher than achieved in any of the flask culture conditions.

TAL can serve as a precursor of pogostone and its analogs, important molecules with antifungal and antibacterial activity (Swamy and Sinniah, 2015). Previous syntheses of pogostone reacted dehydroacetic acid with isobutryaldehyde in an aldol reaction followed by hydrogenation to give pogostone. The highest yield obtained using this approach was 58% (Tang et al., 2013). We report a modified synthetic strategy to derive pogostone from TAL in a one-pot procedure using commercially available TAL and 4-methylpentanoic acid in the presence of *N,N´*-dicyclohexylcarbodiimide (DCC) and 4-dimethylaminopyridine (DMAP), which are mild reagents for the preparation of esters and amides (Methods) (Chen et al., 2017; Neise and Steglich, 1978). This reaction proceeds through a Fries-type rearrangement of the O-enol acyl group to the α position of the lactone to get the desired C-acylation product, pogostone (Fig. 2a; Supporting Information). We also tested the suitability of TAL produced in the bioreactor as a substrate for pogostone synthesis. TAL was precipitated from the spent bioreactor medium (94–100% purity) as described (Supporting Information), and subjected to the one-pot synthesis protocol. Pogostone was generated with 96% yield, a substantial improvement over previously reported yields (Chen et al., 2017). A modification of the same reaction protocol (Fig. 2b) was used to produce six different analogs of pogostone, using isobutyric acid, isovaleric acid, 6-methylheptanoic acid, 3-methypentanoic acid, heptanoic acid and 5-methylhexanoic acid. The isolated yields were 58%, 87%, 85%,

99%, 87% and 88% respectively. (Supporting Table SIII). We demonstrate that the renewably sourced TAL produced in minimal medium can be simply extracted and crystalized at high purity with minimal bioactive contaminants to interfere with conversion of TAL into pogostone and related analogs. The TAL concentrated from medium and crystalized is in a form that is conducive to all downstream processes explored here.

Y. lipolytica is widely recognized for its ability to accumulate high levels of lipids. Our work demonstrates that the characteristic high flux into acetyl-CoA that supports lipid accumulation in *Y. lipolytica* can be utilized for the production of polyketides and other acetyl-CoA derived metabolites.

METHODS

Strains and Plasmids

Y. lipolytica strains used in this work are derived from W29/CLIB89 (Supporting Information, Table SI). Strain yJL2006 has deletions of both *URA3* and *LEU2*; yJY2039 contains genomic copies of the 2-PS expression cassette generated by sequential mobilization of the Hermes transposon-flanked 2-PS expression cassette using pJY4089. Strains with disruption of *PEX10* (yJY2008) or *LEU2* (yJY2039) were made by the CRISPR/Cas9 method (Schwartz et al., 2016) using plasmids pJL4074 or pJY4138, respectively. The sgRNA sequence used is in Supporting Information. pDTN3997 is a 2-PS expression plasmid containing three copies of the 2-PS expression cassette. Strain and plasmid construction details and the list of plasmids used in this work are in Supporting Information, Table SII.

Culture conditions

Yeast transformations were performed using a modified lithium acetate transformation protocol (Supporting Information). Nitrogen-limited medium (NL) was composed of 1.6 g/L yeast nitrogen base without amino acids or ammonium sulfate (Difco), 0.625 g/L ammonium sulfate (Fisher), and 5 g/L sodium sulfate (Fisher). Dextrose (20g/L or 40g/L as noted) (Sigma-Aldrich) was provided as the carbon source. Nitrogen–replete medium (NR) was NL medium supplemented with 5g/L ammonium sulfate. Cerulenin (Sigma-Aldrich) was diluted in DMSO (Sigma-Aldrich) to a 50 mM stock solution and supplemented to medium as noted. Cells were grown in flasks or in a bioreactor at 28°C for the times indicated (Supporting Information).

TAL analysis

TAL was quantified from cell-free culture medium by measuring the absorbance at A_{282} using a Nanodrop ND-1000 spectrophotometer (Thermo Scientific). A dilution series of pure TAL (Sigma-Aldrich) dissolved in minimal medium was used to relate A_{282} to titer (Supporting Information, Fig. S1).

Purification of TAL from minimal medium

Spent minimal medium fermentation broth was centrifuged and sterile filtered to remove cells and debris. The clarified broth was acidified using 18.4 M sulfuric acid to pH 2.0 to

facilitate TAL extraction into hexanol. A series of liquid-liquid extractions was performed to concentrate TAL as well as separate TAL from polar molecules. First TAL was extracted from the aqueous broth by three 1:3 (v:v) hexanol:broth extractions in series. The hexanol fractions were pooled and TAL was extracted from the hexanol back into a basic aqueous solution by first using 1:10 (v:v) 3 M ammonium hydroxide:hexanol, followed by two subsequent 1:10 (v:v) 1 M ammonium hydroxide:hexanol extractions. The ammonium hydroxide extracts were pooled and pH was adjusted to < 2.0 with 18.4 M sulfuric acid to precipitate TAL and initiate crystal formation. This solution was placed at 4ºC to further promote precipitation and crystal formation. After reaching equilibrium, the precipitate and crystals were filtered from the mother liquor using a Whatman Grade 50 filter paper, washed with chilled 10 mM sulfuric acid, and dried in a desiccator.

Chemical conversion of TAL into pogostone

In a one-pot procedure, pogostone was synthesized in a reaction containing commercial TAL (Sigma-Aldrich) and 4-methylpentanoic acid in the presence of DCC and DMAP. The reaction, shown in Fig. 2a, was carried out under argon at room temperature for 3 hrs, followed by heating at 100ºC for 5 hrs. Purifying the product by flash column chromatography afforded pogostone in 96% yield. The reaction was first carried out in 250 mg scale, but was later scaled up to 10 g with similar results. TAL purified from spent bioreactor medium (94–100% purity) from culturing of yJY2039+pDTN3997 was subjected to the same protocol. Analogs of pogostone were produced in the same reaction using the relevant acids and heating at 100ºC overnight (Fig. 2b; Supporting Table SIII).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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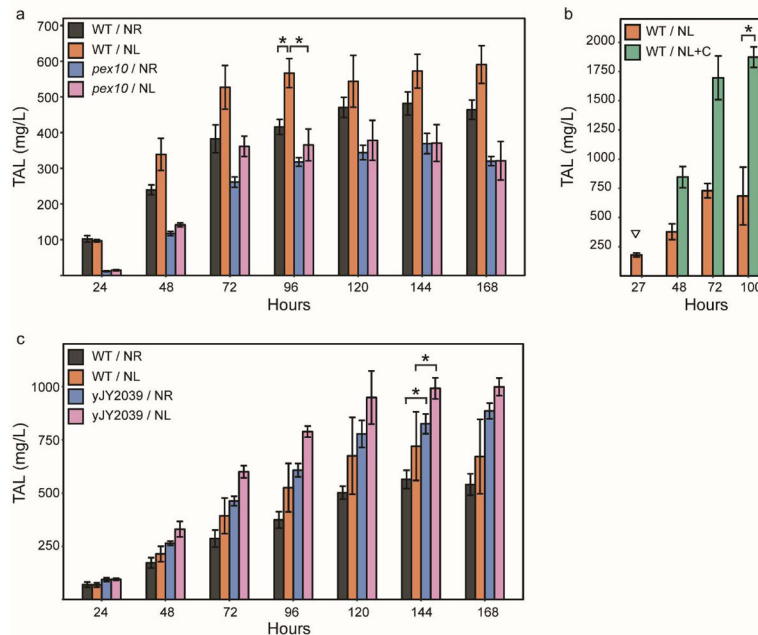
Fig. 1.
TAL production in *Y. lipolytica*. (a) TAL concentration in flask-grown cultures in nitrogen-enriched (NR) and nitrogen-limited (NL) medium. Comparison of TAL production from WT (yJY2006) and *pex10* (yJY2008) strains transformed with pDTN3997. Cells were inoculated at $OD_{600} = 0.05$. (b) Effect of cerulenin on TAL production in WT cultures transformed with pDTN3997 in NL medium. Cerulenin was added at 27 hrs (triangle). (c) Comparison of TAL production from WT (yJY2006) and yJY2039 strains transformed with pDTN3997. Cells were inoculated at $OD_{600} = 0.02$. For statistical analyses, * indicates significant difference as determined by the nonparametric Wilcoxon Rank Sum test. For all comparisons, *p*=0.03 and n=4.
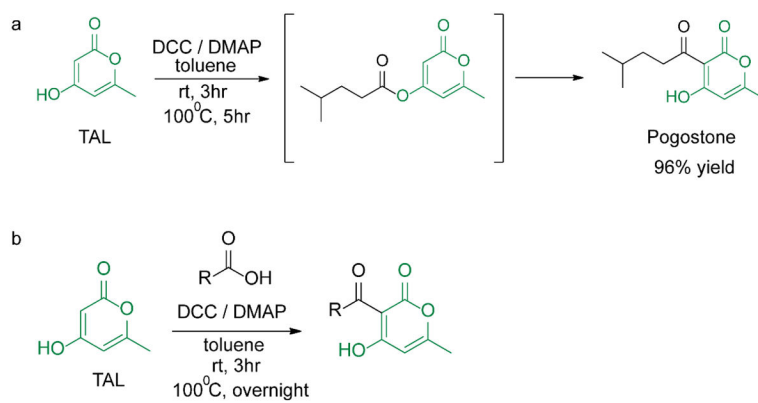
Fig. 2.
One-pot protocol for synthesis of pogostone and pogostone analogs from TAL. (a) Scheme
for synthesis of pogostone. (b) Scheme for synthesis of pogostone analogs from TAL. The
relevant carboxylic acids used to synthesize these analogs were isobutyric acid, isovaleric
acid, 6-methylheptanoic acid, 3-methylpentanoic acid, heptanoic acid and 5-methylhexanoic
acid (Supporting Table SIII).