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4 **Engineering acetyl-CoA metabolic shortcut for eco-friendly**
5 **production of polyketides triacetic acid lactone in *Yarrowia lipolytica***
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Abstract

Acetyl-CoA is the central metabolic node connecting glycolysis, Krebs cycle and fatty acids synthase. Plant-derived polyketides, are assembled from acetyl-CoA and malonyl-CoA, represent a large family of biological compounds with diversified bioactivity. Harnessing microbial bioconversion is considered as a feasible approach to large-scale production of polyketides from renewable feedstocks. Most of the current polyketide production platform relied on the lengthy glycolytic steps to provide acetyl-CoA, which inherently suffers from complex regulation with metabolically-costly cofactor/ATP requirements. Using the simplest polyketide triacetic acid lactone (TAL) as a testbed molecule, we demonstrate that acetate uptake pathway in oleaginous yeast (*Yarrowia lipolytica*) could function as an acetyl-CoA shortcut to achieve metabolic optimality in producing polyketides. We identified the metabolic bottlenecks to rewire acetate utilization for efficient TAL production in *Y. lipolytica*, including generation of the driving force for acetyl-CoA, malonyl-CoA and NADPH. The engineered strain, with the overexpression of endogenous acetyl-CoA carboxylase (ACC1), malic enzyme (MAE1) and a bacteria-derived cytosolic pyruvate dehydrogenase (PDH), affords robust TAL production with titer up to 4.76 g/L from industrial glacier acetic acid in shake flasks, representing 8.5-times improvement over the parental strain. The acetate-to-TAL conversion ratio (0.149 g/g) reaches 31.9% of the theoretical maximum yield. The carbon flux through this acetyl-CoA metabolic shortcut exceeds the carbon flux afforded by the native glycolytic pathways. Potentially, acetic acid could be manufactured in large-quantity at low-cost from Syngas fermentation or heterogenous catalysis (methanol carbonylation). This alternative carbon sources present a metabolic advantage over glucose to unleash intrinsic pathway limitations and achieve high carbon conversion efficiency and cost-efficiency. This work also highlights that low-cost acetic acid could be sustainably upgraded to high-value polyketides by oleaginous yeast species in an eco-friendly and cost-efficient manner.

Keywords: *Yarrowia lipolytica*, acetic acid, metabolic shortcut, cytosolic acetyl-CoA, triacetic acid lactone (TAL), orthogonal pyruvate dehydrogenase complex

1. Introduction

To reduce our dependence on fossil fuels and mitigate climate change concerns, researchers have started seeking alternative routes to produce fuels and commodity chemicals. Motivated by the abundant supply of renewable feedstock and agricultural waste byproducts, biochemical engineers have been able to harness various cellular metabolism to build efficient microbe cell factories with the hope to upgrade the petroleum-based chemical manufacturing process. Our increasing knowledge on microbial physiology, biochemistry and cellular genetics and sophisticated synthetic biology tools have enabled us to explore a variety of host strains with the promise to produce large volume of green and commodity chemicals, including butanol, isobutanol, 1,3- propanediol, 1,4-butanediol, organic acids, amino acids, and fatty acids-based fuels *et al* in the past two decades (Zambanini, Kleineberg et al. 2016, Liu, Hu et al. 2017, Liu, Zhao et al. 2017, Awasthi, Wang et al. 2018).

Triacetic acid lactone (TAL) is an important platform chemical with a broad spectrum of industrial applications ranging from organic synthesis, polymer plasticizers, adhesives to emulsifiers. It is the precursor for the synthesis of fungicides and fine chemicals including 1,3,5-trihydroxybenzene, resorcinol, phloroglucinol *et al* (Hansen 2002). TAL is currently manufactured from chemical catalysis with petroleum-derived dehydroacetic acid followed by acidolysis with concentrated sulfuric acid (H_2SO_4) at high temperature (Weissermel 2008). The use of petroleum feedstock, environmentally-unfriendly catalysts and the generation of toxic byproducts limit its industrial application as green chemicals (Chia, Schwartz et al. 2012). Therefore, there is a pressing need to develop low-cost and eco-friendly platform for sustainable production of TAL from renewable feedstocks.

By manipulating acetyl-CoA and malonyl-CoA metabolism in bacteria and yeast, metabolic engineers have established a number of TAL-producing host strains with *E. coli*, *S. cerevisiae*, *Y. lipolytica* *et al* (Tang, Qian et al. 2013, Cardenas and Da Silva 2014, Li, Qian et al. 2018, Markham, Palmer et al. 2018). Due to cellular toxicity issues, *E. coli*, was found to be a less promising producer to accumulate TAL (Xie, Shao et al. 2006). On the contrary, *S. cerevisiae* and *Y. lipolytica* were insensitive to TAL even at concentrations up to 200 mM (25.7 g/L), opening the opportunity for us to biologically synthesize TAL in large quantity. For example, a recent study has surveyed a large panel of metabolic pathways involving energy storage and generation, pentose biosynthesis, gluconeogenesis and lipid biosynthesis, to improve TAL production in *S. cerevisiae* (Cardenas and Da Silva 2014). In another study, recycling cytosolic NADPH and limiting intracellular acetyl-CoA transportation were also found as efficient strategies to enhance TAL accumulation in *S. cerevisiae* (Cardenas and Da Silva 2016). Compared with *S. cerevisiae*, *Y. lipolytica*, a natural

lipid producer, has recently been identified as an attractive industrial workhorse to produce various compounds due to its broad substrate range, high acetyl-CoA and malonyl-CoA flux as well as the availability of facile genetic manipulation tools (Markham and Alper 2018, Lv, Edwards et al. 2019). These features indicate that *Y. lipolytica* could be engineered as a superior host to produce acetyl-CoA and malonyl-CoA-derived compounds beyond lipid-based chemicals. Indeed, genetically rewired lipogenic pathways have achieved great success to produce lycopene, carotenoids, flavonoids and polyketides in *Y. lipolytica* (Xu, Li et al. 2014, Xu, Qiao et al. 2016, Qiao, Wasylenko et al. 2017, Xu, Qiao et al. 2017, Yu, Landberg et al. 2018). In a recent study, heterologous expression of 2-pyrone synthase along with the cytosolic expression of alternative acetyl-CoA pathways have been implemented to collectively improve TAL production up to 35.9 g/L in a bench-top reactors, with yield up to 43% of the theoretical yield from glucose (Markham, Palmer et al. 2018), highlighting the potential of using this yeast for large-scale production of polyketides.

Acetate presents in high levels in waste streams (municipal organic waste via anaerobic digestion) and lignocellulosic hydrolysates; acetate could also be synthesized from methanol carbonylation at very low cost (about \$340 per metric ton) (Qian, Zhang et al. 2016). And methanol could be abundantly produced by methane oxidation with heterogenous catalysis. These facts indicate that acetic acid will be a promising, sustainable and low-cost carbon source for industrial fermentation. To systematically understand the limiting cofactors associated with polyketide production in oleaginous yeast, we use TAL biosynthetic pathway as a testbed and investigated an arrange of biological factors including cofactor (NADPH) supply, orthogonal acetyl-CoA and malonyl-CoA availability, lipogenesis competition, substrate suitability and pH conditions (**Fig. 1**). Stepwise optimization of these parameters led to an 8.5-times improvement of TAL production (4.76 g/L) from industrial-grade acetic acid (glacial acid), with acetic acid-to-TAL yield at 31.9% of the theoretical yield. The reported acetyl-CoA shortcut pathway delineates a strong case that we could engineer *Y. lipolytica* as an eco-friendly platform to upgrade industrial acetic acids to commodity chemicals. This work also expands our capacity to produce green chemicals from sustainable feedstocks; in particular, acetic acid could be efficiently incorporated as carbon source for cost-efficient production of various polyketides in *Y. lipolytica*.

2. Materials and methods

2.1 Strains and cultivation

Escherichia coli NEB5 α high efficiency competent cells were purchased from NEB for plasmid construction, preparation, propagation and storage. The *Y. lipolytica*

wild type strain W29 was purchased from ATCC (ATCC20460). The auxotrophic Polg (Leu-) was obtained from Eastern Biotech Company (Taipei, Taiwan)(Wong, Engel et al. 2017). All strains and plasmids used in this study are listed in supplementary Table S1.

LB broth or agar plate with 100 µg/mL ampicillin was used to cultivate *E. coli* strains. Yeast rich medium (YPD) was prepared with 20 g/ L Bacto peptone (Difco), 10 g/L yeast extract (Difco), and 20 g/L glucose (Sigma-Aldrich), and supplemented with 15 g/L Bacto agar (Difco) for agar plates. YNB medium with carbon/nitrogen ratio 10:1 for seed culture was made with 1.7 g/L yeast nitrogen base (without amino acids and ammonium sulfate) (Difco), 5 g/L ammonium sulfate (Sigma-Aldrich), 0.69 g/L CSM-Leu (Sunrise Science Products, Inc.) and 20 g/L glucose. Selective YNB plates were made with YNB media with carbon/nitrogen ratio 10:1 supplemented with 15 g/L Bacto agar (Difco). YNB fermentation medium with glucose as substrate and carbon/nitrogen ratio 80:1 was made with 1.7 g/L yeast nitrogen base (without amino acids and ammonium sulfate) (Difco), 1.1 g/L ammonium sulfate (Sigma-Aldrich), 0.69 g/L CSM-Leu (Sunrise Science Products, Inc.) and 40 g/L glucose.

Phosphoric buffer solution (PBS) with pH 6.0 was made with 0.2 M Na₂HPO₄ and 0.2 M Na₂HPO₄, which was used to replace water to make YNB- glucose-PBS fermentation medium. YNB fermentation medium with sodium acetate as substrate and carbon/nitrogen ratio 80:1 was made with 1.7 g/L yeast nitrogen base (without amino acids and ammonium sulfate) (Difco), 0.825 g/L ammonium sulfate (Sigma-Aldrich), 0.69 g/L CSM-Leu (Sunrise Science Products, Inc.), 41 g/L sodium acetate. Glacial acetic acids were purchased from Sigma-Aldrich. Bromocresol purple was a pH-sensitive indicator which could change its color with the pH from 5.2-7.0 (El-Ashgar, El-Basioni et al. 2012) and 40 mg/L bromocresol purple was added into fermentation medium to indicate pH variation. The pH of medium was regulated to 6.0 by 6.0 M HCl in the fermentation process. And 1 mg/L cerulenin solution prepared with dimethylsulfoxide (DMSO) was added into fermentation medium to inhibit precursor competing pathway.

In TAL production process, single *Y. lipolytica* colonies were picked from YNB selective plates and inoculated into YNB seed liquid media, which were grown at 30 °C for 48 h. For tube test, 100 µL seed cultures were inoculated into 5 mL fermentation media in 50 mL tube. 600 µL seed cultures were inoculated into 30 mL fermentation media in 250 mL shake flasks with 250 rpm and 30 °C. Time series samples were taken for analyzing biomass, sugar content, and TAL titer.

2.2 Plasmid construction

All primers are listed in supplementary Table S2. All restriction enzymes were purchased from Fisher FastDigest enzymes. Plasmid miniprep, PCR clean-up, and gel

DNA recoveries were using Zyppy and Zymoclean kits purchased from Zymo research. All the genes, except for *GH2PS*, were PCR-amplified with the primers from genomic DNA of *Y. lipolytica*, *S. cerevisiae*, *E. coli*, *B. subtilis*, *Aspergillus nidulans*, respectively (Supplementary Table S1 and Table S2). *GH2PS* were codon-optimized according to the human codon usage table. All these genes were inserted into downstream of the *Y. lipolytica* TEF-intron promoter in the pYLXP' vector backbone (Wong, Engel et al. 2017) at the *Sna*BI and *Kpn*I site via Gibson assembly (Gibson, Young et al. 2009). Upon sequence verification by Genewiz, the restriction enzyme *Avr*II, *Nhe*I, *Not*I, *Cla*I and *Sal*I (Fermentas, Thermo Fisher Scientific) were used to digest these vectors, and the donor DNA fragments were gel purified and assembled into the recipient vector containing previous pathway components via YaliBricks subcloning protocol (Wong, Holdridge et al. 2019). All assembled plasmids were verified by gel digestion and were subsequently transformed into the *Y. lipolytica* host strain Po1g ΔLeu using the lithium acetate/single-strand carrier DNA/PEG method (Chen, Beckerich et al. 1997). In vector integration process, pYLXP' vector assembled with functional genes was linearized by restriction enzyme *Not*I (Fermentas, Thermo Fisher Scientific). The linear fragment was transformed into the *Y. lipolytica* host strain Po1g ΔLeu for chromosomal gene integration.

2.3 Analytical methods

The cell growth was monitored by measuring the optical density at 600 nm (OD₆₀₀) with a UV-vis spectrophotometer that could also be converted to dry cell weight (DCW) according to the calibration curve DCW : OD₆₀₀ = 0.33:1 (g/L). The fermentation broth was centrifuged at 14,000 rpm for 5 min and the supernatant was used for analyzing the concentration of TAL, glucose, mannitol, acetic acid by HPLC with a SupelcogelTM Carbohydrate column (Sigma). Retention times for each tested chemical were listed in Supplementary Table S3. The quantification of TAL was analyzed by an UV absorbance detector at 280 nm, while glucose, mannitol, acetic acid was examined by a refractive index detector. The column was eluted with 10 mM H₂SO₄ at a column temperature of 50°C, a refractive index detector at temperature of 50°C, and a flow rate of 0.4 mL/min. All chemical standards were purchased from Sigma-Aldrich and standard curve were built to derive the area-concentration relationship. All reported results are three measures with standard deviatoions.

3. Results and discussion

3.1 Boosting precursor malonyl-CoA to improve TAL production in *Y. lipolytica*

It has been well characterized that the plant-derived 2-pyrose synthase (2-PS) is a type III polyketide synthase (Jez, Austin et al. 2000). Specifically, 2-PS from daisy family plant *Gerbera hybrida* was found to efficiently synthesize triacetic acid lactone

(TAL) by joining one acetyl-CoA with two extender malonyl-CoA molecules (Jez, Ferrer et al. 2001, Austin and Noel 2003, Xie, Shao et al. 2006). To test TAL production, the coding sequence of *G. hybrida* 2-pyrone synthase (GH2PS, Uniprot ID: P48391) was codon-optimized and overexpressed in *Y. lipolytica*, yielding 0.5 g/L of TAL at 120 h with chemically-defined complete synthetic media (CSM-leu) in test tube (**Fig. 2A**), demonstrating the potential of using *Y. lipolytica* as a platform to synthesize various polyketides.

It's generally believed that malonyl-CoA is the rate-limiting precursor for polyketide synthase (Zha, Rubin-Pitel et al. 2009, Xu, Ranganathan et al. 2011). We next sought to overexpress the bacteria-derived acetyl-CoA-carboxylase and the endogenous ACC1, to test whether the conversion of acetyl-CoA to malonyl-CoA would facilitate TAL synthesis. *E. coli* intracellular malonyl-CoA could be enhanced by overexpression of the four subunits of acetyl-CoA-carboxylase (accABCD) along with the expression of the biotinylating enzyme (biotin ligase encoded by *birA*), leading to significantly improved production of both flavonoids and fatty acids (Leonard, Lim et al. 2007, Zha, Rubin-Pitel et al. 2009, Xu, Ranganathan et al. 2011, Xu, Gu et al. 2013). With the YaliBrick gene assembly platform (Wong, Engel et al. 2017), we assembled the four catalyzing subunits (accABCD) and the biotinylating subunit (*birA*). Interestingly, a negative effect was observed when these gene clusters were introduced to *Y. lipolytica*, resulting in 40% of TAL production compared to the parental strain. When single bacterial-derived acetyl-CoA-carboxylase was expressed, a similar result was obtained (data was not shown). These results indicate that the bacterial-derived acetyl-CoA carboxylase could not be directly translated to yeast system, presumably due to the depletion of biotins or the protein burden stress of heterologous gene expression, albeit the specific TAL production (mg/gDCW) is about 4.2% higher (Fig. 2A). Bacterial ACC contains four isolated subunits, the non-functionality of bacterial ACC is possibly due to the existence of a lipogenic multienzyme complex (the lipid metabolon) involving malic enzyme, fatty acid synthase, ATP: citrate lyase, acetyl-CoA carboxylase, pyruvate carboxylase and malate dehydrogenase in oleaginous yeast species (Shuib, Ibrahim et al. 2018). Endogenous *ylACC1*, a single polypeptide encoded by YALI0C11407 with two internal introns removed, was also selected to co-express with *Gh2PS*, leading to TAL production increased to 0.66 g/L (Fig. 2A), indicating that overexpression of native ACC1 was beneficial for the TAL production. To test whether endogenous biotin ligase (BPL) was required to improve ACC1 activity (Pendini, Bailey et al. 2008), we co-expressed BPL1 (encoded by YALI0E3059). Similarly, degenerated production titer was observed in our experiment (Fig. 2A), indicating that biotin ligase is likely not the rate-limiting step for ACC1 activity in *Y. lipolytica*.

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257 **3.2 Recycling cytosolic NADPH to improve TAL production in *Y. lipolytica***

258 NADPH, the primary biological reducing equivalent to protect cell from
259 oxidative stress and extend carbon-carbon backbones, has been reported as the major
260 rate-limiting precursor in fatty acids and lipids synthesis in oleaginous species
261 (Ratledge and Wynn 2002, Ratledge 2014, Wasylenko, Ahn et al. 2015, Qiao,
262 Wasylenko et al. 2017). Source of cytosolic NADPH in Baker's yeast was contributed
263 from various alternative routes (Minard, Jennings et al. 1998, Grabowska and
264 Chelstowska 2003, Minard and McAlister-Henn 2005), depending on the carbon
265 source and genetic background of the yeast strain. With glucose as carbon sources,
266 cytosolic NADPH relies on the pentose phosphate pathway. Using TAL as the target
267 molecule, we tested a collection of auxiliary cytosolic NADPH pathways (Liu,
268 Marsafari et al. 2019) and investigated whether these alternative pathways would
269 improve TAL production and cellular fitness (**Fig. 1**). These cytosolic NADPH
270 pathways include malic enzyme (ylMAE), mannitol dehydrogenase (ylMnDH1,
271 ylMnDH2), 6-phosphogluconate dehydrogenase (ylGND2) and succinate
272 semialdehyde dehydrogenase (ylUGA2), which were reported to contribute to
273 NADPH metabolism in various fungal species (Suye and Yokoyama 1985, Minard,
274 Jennings et al. 1998, Wynn, bin Abdul Hamid et al. 1999, Grabowska and
275 Chelstowska 2003, Minard and McAlister-Henn 2005, Rodríguez-Frómata, Gutiérrez
276 et al. 2013, Liu, Marsafari et al. 2019).

277 Among these chosen NADPH source pathway, malic enzyme (MAE, encoded by
278 YALI0E18634) presented the best results to improve TAL production (**Fig. 2B**). MAE
279 was a NADP⁺-dependent enzyme that oxidatively decarboxylates malate to pyruvate
280 and provides NADPH for lipid biosynthesis in oleaginous species (Ratledge 2014).
281 When ylMAE was overexpressed with *GH2PS* and *yLACC1* (strain *HLYali101*), it
282 showed a 60% increase in TAL titer and the volumetric production was increased to
283 0.8 g/L with a specific yield of 75.6 mg/g DCW (**Fig. 2B**). It is generally believed that
284 ylMAE was localized in mitochondria (Zhang, Zhang et al. 2013, Ratledge 2014), we
285 performed bioinformatic studies with TargetP 1.1 (Nielsen, Engelbrecht et al. 1997,
286 Emanuelsson, Nielsen et al. 2000) and predicted that the first 78 nucleotides of
287 ylMAE encodes a 26 AA leader peptide which is responsible for mitochondria
288 targeting. We then overexpressed the truncated ylMAE (t78ylMAE) with the
289 N-terminal MTS removed to compare how the variation of t78ylMAE may affect TAL
290 synthesis. Contrary to our hypothesis, removal of the MTS signal of YALI0E18634
291 exhibits adverse effect on both TAL production and cell growth (**Fig. 2B**). qPCR
292 results confirmed that the ylMAE mRNA abundance was 6.8 times higher compared
293 to the strain without ylMAE overexpression. This result indicates that the 78 nt MTS

sequence is essential to the function of ylMAE. Deletion of the N-terminal 78 nt MTS sequence renders the cell with less MAE catalytic efficiency and leads to degenerated production phenotype.

Protein subcellular location in fungi has been reported to not just depend on the leader peptide sequence. For example, both *S. cerevisiae* acetyl-CoA synthase (ACS) and *Y. lipolytica* malate dehydrogenase (MDH) could move across the mitochondria or peroxisome membrane boundary to function as cytosolic enzymes, depending on the carbon source and environmental conditions (Chen, Siewers et al. 2012, Kabran, Rossignol et al. 2012, Krivoruchko, Zhang et al. 2015). In particular, one of the proven mechanisms is related with alternative splicing of mRNA transcripts in *Y. lipolytica*: where the N-terminal mRNA sequence of malate dehydrogenase could be spliced in different ways to facilitate the mature peptide targeting to distinct cellular compartment (Mekouar, Blanc-Lenfle et al. 2010, Kabran, Rossignol et al. 2012). This mechanism indicates the complicated regulation of malic enzyme in *Y. lipolytica*, highlighting that overcoming metabolite trafficking may have positive effect on TAL accumulation. Increased TAL production could also be linked to the sufficient supply of precursor malonyl-CoA, possibly due to the balanced expression of Gh2PS and ACC1, which presumably creates a metabolic sink and pushes the equilibrium of ylMAE to function in the forward direction.

3.3 Engineering orthogonal pyruvate dehydrogenase complex to boost TAL production in *Y. lipolytica*

Acetyl-CoA, derived from the central carbon metabolism (glycolysis and Krebs cycle), was the starting molecule in TAL synthesis. Acetyl-CoA is also the central hub for lipid synthesis, peroxisomal lipid oxidation and amino acid degradation pathway (Chen, Siewers et al. 2012, Krivoruchko, Zhang et al. 2015). Engineering alternative pathways to enhance cytosolic acetyl-CoA have been proven as efficient strategy to improve fatty acids and oleochemical production in both Bakers' yeast and *Y. lipolytica* (Kozak, van Rossum et al. 2014, van Rossum, Kozak et al. 2016, Xu, Qiao et al. 2016). Here we explored two independent metabolic engineering strategies to improve the oxidation of pyruvate to cytosolic acetyl-CoA. First, we investigated the endogenous pyruvate decarboxylase (PDC), acetaldehyde dehydrogenase (ALD) and acetyl-CoA synthase (ACS) route, which decarboxylates pyruvate to acetaldehyde by PDC, oxidizes acetaldehyde to acetate by ALD, ligates acetate with CoASH to form acetyl-CoA by ACS (Flores, Rodríguez et al. 2000). Previous results have demonstrated that we can increase lipid production when the PDC-ALD genes were overexpressed in *Y. lipolytica* (Xu, Qiao et al. 2016). Comparison of the results by combinatorially overexpressing PDCs and ALDs along with GH2PS and MAE, the

best result was obtained from the strain *HLYali102*, yielding only 0.4 g/L of TAL (**Fig. 3**). We speculated that the synthesis process was possibly limited by the supply of malonyl-CoA. Endogenous *ylACC1* gene was further assembled to the previous pathway, however, no significant TAL improvement was observed in shake flasks (**Fig. 3**). These results indicate the complicated regulation of acetyl-CoA and malonyl-CoA metabolism. In particular, Snf1-mediated AMP kinase may phosphorylate ACC1 to repress ACC1 activity (Shi, Chen et al. 2014), the accumulated acetyl-CoA may lead to histone acetylation and global transcriptional change (Zhang, Galdieri et al. 2013). It could also point out a negative feedback loop of acetyl-CoA regulation: accumulated acetyl-CoA and NADH will undergo oxidative phosphorylation to generate ATP in mitochondria, which instead leads to weaker phosphorylating activity of SNF1 and elevated ACC1 activity that converts acetyl-CoA to malonyl-CoA (Mihaylova and Shaw 2011, Hsu, Liu et al. 2015).

We next attempted a bacterial-derived pyruvate dehydrogenase (PDH) complex pathway, which is orthogonal to *Y. lipolytica* metabolism. Pyruvate dehydrogenase complex (PDH), as a ubiquitous enzyme found in all kingdoms of life, catalyzed the oxidative decarboxylation of pyruvate to acetyl-CoA, forming the connection between glycolysis, the tricarboxylic acid cycle (TCA) and lipid biosynthesis (Kozak, van Rossum et al. 2014). As for all eukaryotic life including *Y. lipolytica*, PDH was localized in the mitochondrial matrix to provide acetyl-CoA for operating Krebs cycle. This mitochondrial acetyl-CoA could not be converted to TAL in the cytosol. To solve this challenge, we investigated whether the expression of the *E. coli* PDH would benefit TAL synthesis in *Y. lipolytica*. Specifically, we assembled the genes encoding the three catalytic subunits of PDH with our YaliBrick vectors (Wong, Engel et al. 2017): including pyruvate dehydrogenase (AceE, E1), dihydrolipoyllysine-residue acetyltransferase (AceF, E2), and lipoamide dehydrogenase (LpdA, E3). Nevertheless, when these three genes were assembled together and overexpressed along with GH2PS, only 0.46 g/L TAL was detected (**Fig. 3**). It has been discovered recently that the functional expression of bacterial PDH in *S. cerevisiae* requires the co-expression of lipoate-protein ligase A (Kozak, van Rossum et al. 2014, Kozak, van Rossum et al. 2014, Nielsen 2014), which plays a pivotal role in lipoylating E2 subunits of PDH. Similar to Baker's yeast, the two annotated lipoyltransferase or lipoate ligase in *Y. lipolytica* (YALI0C03762 and YALI0F27357 respectively) are exclusively localized in mitochondria (Sulo and Martin 1993, Hermes and Cronan 2013, Cronan 2016). To enable cytosolic lipoylation and complement the bacterial PDH function, we next co-expressed the lipoate-protein ligase A (LplA), TAL production was increased to 0.67 g/L with specific yield up to 87.72 mg/gDCW (strain HLYali103) (**Fig. 3** and Table S4), indicating the essential role of EcLplA as an indispensable component for

the *E. coli* PDH functionality. This was the first report to functionally express the bacteria-derived EcPDH with EcLplA in *Y. lipolytica*. Creating this orthogonal acetyl-CoA route may bypass internal pathway regulations, providing a promising path to overcome metabolic limitations and improve metabolite production in *Y. lipolytica*.

Posttranslational modification of apoproteins with lipoyl groups was an important process for many enzymes. There were two complementary systems for protein lipoylation in *E. coli*: one was exogenous route using an ATP-dependent process by lipoate-protein ligase A (LplA); another one required lipoyl-[acyl-carrier protein]-protein-N-lipoyltransferase (LipB) to transfer endogenously synthesized lipoate to apoproteins (Miller, Busby et al. 2000). Both of these two proteins were localized in mitochondria, which presents a critical challenge to functionally express cytosolic PHDs in yeast. The current studies provide a viable option to functionally express bacteria-derived cytosolic PDH steps and leads to significantly improved TAL production. This PDH should be easily transferrable to other metabolite production systems, should there is a need to boost cytosolic acetyl-CoAs.

3.4 Comparison of TAL production from glucose and acetic acid

A unique feature of *Y. lipolytica* is the high acetyl-CoA flux and its broad substrate range, which makes this yeast an attractive host to degrade organic waste (volatile fatty acids, alkanes *et al*). Previous metabolic engineering effort has demonstrated that *Y. lipolytica* possesses strong acetate utilization pathway that is equivalent or even superior to the hexose utilization pathway. For example, triacylglyceride (TAG) production could be improved from ~100 g/L to ~115 g/L in bench-top bioreactors, when the engineered strain was switched from glucose media (Qiao, Wasylenko et al. 2017) to acetate media (Xu, Liu et al. 2017). To investigate how different carbon sources may affect TAL synthesis, both glucose and sodium acetate were tested to compare TAL production efficiency in our engineered strains.

As acetic acid (HAc) has a relatively low pKa (4.75), we used 41 g/L sodium acetate (NaAc), equivalently to 30 g/L acetic acid (HAc) to cultivate the engineered strain. As the cell uptakes acetic acid, the cultivation pH will keep increasing. An *in situ* pH indicator (bromocresol purple) was used to track the pH change and 6 M HCl was used to adjust the pH in the shake flask (**Fig. 4A, 4B and 4C**). Controlling pH (**Fig. 4A**) led to the strain HLYali101 produced 1.05 g/L TAL in glucose-YNB medium, with 98% of glucose depleted and 11.53 g/L biomass produced (**Fig. 4D**). We also detected mannitol as a main byproduct, which increased to 3.7 g/L at 96 h and subsequently was utilized to support cell growth or TAL production (**Fig. 4D**). Strain HLYali101 produced about 1.29 g/L TAL from acetic acid with yield 300.69

mg/g DCW (**Fig. 4E**), this yield represents 230% improvement compared to the yield obtained from glucose medium (91.06 mg/g DCW) (**Fig. 4D** and Table S4). Depletion of acetic acid occurred at 144 h and the DCW reached 4.29 g/L, which is about half of the biomass from glucose.

Our data demonstrate that *Y. lipolytica* could efficiently uptake acetic acid as sole carbon source to produce polyketides. By harnessing the endogenous acetate uptake pathway, this externally provided acetic acid bypassed the pyruvate dehydrogenase steps and created a metabolic “shortcut” to acetyl-CoA with improved carbon conversion efficiency and pathway yield. This acetyl-CoA flux exceeded the capacity of the orthogonal PDH pathway when glucose is used as carbon source.

3.5 pH control and modulating precursor competing pathway to improve TAL production

Despite that glucose is a preferred carbon source for cell growth, we constantly detected moderate or low TAL production in our engineered strains. The high TAL specific yield (300.69 mg/gDCW, Fig. 4E and Table S4) was only obtained in acetate media with pH control. We speculated that the low glucose-to-TAL yield was related with the pH declining of strain *HLYali101* (Fig. 4D): the pH of the fermentation media quickly decreased from 6 to 3.5 due to the accumulation of citric acid when glucose was utilized. This significant pH variation may negatively affect strain physiology, including cell membrane permeability and nutrients transportation process due to the loss of proton driving force. This eventually led to the accumulation of byproduct (mannitol and citric acid *et al*) and suboptimal TAL yield.

To compare how pH control may affect TAL production with the engineered strain fermenting on glucose, we supplemented the minimal YNB media with 0.2 M phosphoric buffer solution (PBS, pH 6.0). Indeed, TAL production was increased to 2.04 g/L at 168 h, which was an increase of 60.1% and 94.3% respectively, compared with the results from pH controlled (supplementary Fig. S1) and uncontrolled (Fig. 4D) process. The major byproduct mannitol reached 4.2 g/L at 72 hour and citric acid reached 6.14 g/L at 96 h, and both were subsequently utilized (**Fig. 5A**). The biomass of strain *HLYali101* reached 8.66 g/L DCW with the TAL specific yield at 235.6 mg/gDCW (**Fig. 5A** and Table S4), which is still 27.6% lower than the specific yield from acetic acid (**Fig. 4E**). The glucose-to-TAL yield was 91.23 mg/g, which was 3.4 times higher than that of the pH uncontrolled fermentation process (**Fig. 4D** and Table S4). Strain *HLYali103* (orthogonal PDH expression) led to similar fermentation profile (**Fig. 5B**) when the glucose YNB media was conditioned with 0.2 M PBS buffer (pH 6.0). This strain led to the accumulation of 7.01 g/L biomass with the glucose-to-TAL yield at 98.44 mg/g glucose, about 7.9% higher than the yield obtained from strain

446 HLYali101. Similarly, only ~50% of glucose was utilized in both strain HLYali101
447 and HLYali103 (**Fig. 5A** and **5B**), indicating the supplementation of PO_4^{3-} buffer may
448 negatively impact the glucose uptake rate.

449 *Y. lipolytica* is a natural lipid producer capable of accumulating 30%~60% cell
450 weight as lipid (Xu, Qiao et al. 2017). The internal fatty acid synthase (FAS) pathway
451 strongly competes with TAL pathway for both acetyl-CoA and malonyl-CoA. In order
452 to mitigate FAS competition, we attempted to use FAS inhibitor (cerulenin) to
453 suppress lipid synthesis in our engineered strain. Cerulenin could irreversibly form a
454 covalent adduct with the active site (cysteine residue) of β -ketoacyl-ACP synthase,
455 inhibiting the incorporation of malonyl-CoA to the extending fatty acid backbone
456 (Vance, Goldberg et al. 1972, Price, Choi et al. 2001). When 1 mg/L cerulenin was
457 supplemented at 48 h, 3.2 g/L TAL was produced by strain *HLYali101* at 168 h (**Fig.**
458 **5C**), a 57% increase compared with the result without cerulenin (**Fig. 5A**).
459 Byproducts mannitol and citric acid was decreased to 0.67 g/L and 3.75 g/L at 168 h,
460 respectively. Glucose was depleted relatively faster compared to the strain without
461 cerulenin (**Fig. 5A**). And the specific TAL production reached to 363.6 mg/gDCW, a
462 54% increase compared to the strain without cerulenin (**Fig. 5A** and Table S4).
463 Similarly, strain *HLYali103* produced about 2.57 g/L TAL (**Fig. 5D**), a 32% increase
464 compared to the strain without cerulenin (**Fig. 5B**). These results indicated that FAS
465 pathway was the major competing pathway and malonyl-CoA flux became the main
466 driver of TAL synthesis in the engineered strain HLYali101 and HLYali103. Taken
467 together, pH control and inhibition of FAS activity were effective strategies for
468 diverting malonyl-CoA flux to TAL synthesis.

470 **3.6 High-yield TAL production from acetic acid with chromosomally-integrated** 471 **strains**

472 The low biomass (**Fig. 5C** and **5D**) and significant cost of cerulenin (10 mg for
473 \$272 from Sigma Aldrich) prohibits the supplementation of cerulenin as chemical
474 inhibitors to modulate malonyl-CoA flux in the engineered strain. On the other hand,
475 ACC1, the key metabolic node of malonyl-CoA source pathway, was primarily
476 controlled through the phosphorylation of serine residues by Snf1-mediated AMP
477 kinase. The overflow of glucose metabolism or nitrogen starvation activates Snf1
478 kinase and slows down ACC1 activity (Hedbacker and Carlson 2008, Seip, Jackson et
479 al. 2013, Zhang, Galdieri et al. 2013, Shi, Chen et al. 2014), which makes glucose a
480 unfavorable carbon source for the production of malonyl-CoA-derived compounds.
481 Since acetic acid could be directly converted to acetyl-CoA, we speculate that this
482 acetyl-CoA shortcut may present little or no activation of the Snf1 kinase. In
483 comparison with glucose, we hypothesize this alternative carbon source (acetic acid)

will not cause inhibitory effect to the malonyl-CoA source pathway (ACC1).

To increase strain stability in acetic acid, we integrated the genes Gh2PS-ylMAE1-EcPDH-EcLplA and Gh2PS-ylMAE1-ylACC1-EcPDH-EcLplA (Fig. S2) into the chromosome of *Y. lipolytica*, leading to strains *LHYali105* and *LHYali106*, respectively. The integrated strain *LHYali105* yielded about 4.18 g/L TAL from YPA complex media (yeast extract, peptone and sodium acetate) in shake flask (Fig. 6A), a 37% increase compared to the strain *LHYali105* grown in glucose-based YPD (yeast extract, peptone and dextrose) media (supplementary Fig. S3). With the complementation of cytosolic PDH pathway, the strain *LHYali106* yielded about 4.76 g/L TAL from YPA media (Fig. 6B), a 56% increase compared to the strain *LHYali105* grown in YPD (supplementary Fig. S3). The biomass of *LHYali106* reached 11.68 g/L at the end of fermentation with little mannitol (3.05 g/L) but no citric acid formation in the fermentation broth (Fig. 6B). The specific TAL yield reaches 407.5 mg/gDCW, which is 12.1% higher than the strain with cerulenin supplementation (Fig. 5C). The acetate-to-TAL conversion yield was increased to 0.149 g/g (Table S4), representing 31.9% of the theoretical maximum yield (0.467 g/g). This result demonstrates that acetic acid could lead to much higher TAL yield, exceeding the capability when glucose was used as carbon sources. Meanwhile, acetate has not been explored widely as sole carbon source in industrial fermentation. This work highlights the potential of engineering *Y. lipolytica* as a promising microbial platform for commercial manufacturing of high-value polyketides from low-cost acetic acids (glacial acetic acids).

4. Conclusion

In this work, triacetic acid lactone (TAL) biosynthetic pathway was constructed and optimized in oleaginous yeast *Y. lipolytica*. In addition to the codon-optimized 2-pyrone synthase gene, we found that both endogenous acetyl-CoA carboxylase (ylACC1) and malic enzyme (ylMAE1) are critical for the efficient conversion of acetyl-CoA and malonyl-CoA to TAL. Acetyl-CoA is the central hub of carbon metabolism, connecting with glycolysis, Krebs cycle and lipid synthesis. To unleash the potential of the heterologous polyketide synthase pathway, we assessed various cytosolic acetyl-CoA pathways to improve TAL synthesis. A bacteria-derived pyruvate dehydrogenase complex (Ec-PDH) along its cognate lipolyating protein (Ec-lplA) were identified as the most efficient cytosolic acetyl-CoA routes to improve TAL production. The engineered strain could efficiently ferment glucose or sodium acetate (NaAc) to TAL. In particular, the TAL titer (g/L) and specific yield (mgTAL/gDCW) from acetic acid media exceeds the titer and the specific yield obtained in glucose media. An *in situ* pH indicator (bromocresol purple) was used

as a real-time pH reporter for process optimization in shake flasks. Suboptimal level of TAL accumulation (~3.2 g/L) was observed in a pH-controlled fermentation when the glucose media was supplemented with expensive FAS inhibitor (cerulenin). When the engineered strain was cultivated with acetate as sole carbon source in the absence of cerulenin, the TAL production was increased to 4.76 g/L in the chromosomally-integrated strain. The acetate-to-TAL yield reaches 0.149 g/g, representing 31.9% of the theoretical maximum yield (0.467 g/g). Since acetate uptake pathway essentially bypasses the mitochondrial PDH steps, the feeding of acetate creates a metabolic shortcut to acetyl-CoA. The carbon flux through this acetyl-CoA metabolic shortcut exceeds the carbon flux afforded by the native acetyl-CoA pathways. This alternative carbon sources present a metabolic advantage over glucose, which may not elicit or activate Snf1-mediated AMP kinase repressing ACC1 activity. Potentially, acetic acid could be a readily-available, low-cost carbon sources from Syngas fermentation, anerobic digestion or heterogenous catalysis (methanol carbonylation).

This report demonstrates that *Y. lipolytica* possesses superior acetate uptake pathway to support both cell growth and metabolite production. Our engineering strategies highlights that acetic acid could be utilized as low-cost and sustainable carbon feedstock for efficient production of plant-derived polyketides in oleaginous yeast species. More importantly, the use of metabolic shortcut may overcome many intrinsic pathway limitations (kinetic or thermodynamic limitations), and facilitate high carbon conversion efficiency and volumetric titer. Since TAL is the simplest polyketide scaffold, the reported work may also be translated to other microbial platforms for upgrading waste acetic acids to high-value compounds in an eco-friendly and cost-efficient manner.

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Author contributions

PX conceived the topic. HL and MM performed genetic engineering and fermentation experiments. HL and PX wrote the manuscript.

Conflicts of interests

The authors declare that they have no competing interests. A provisional patent

has been filed based on the results of this study.

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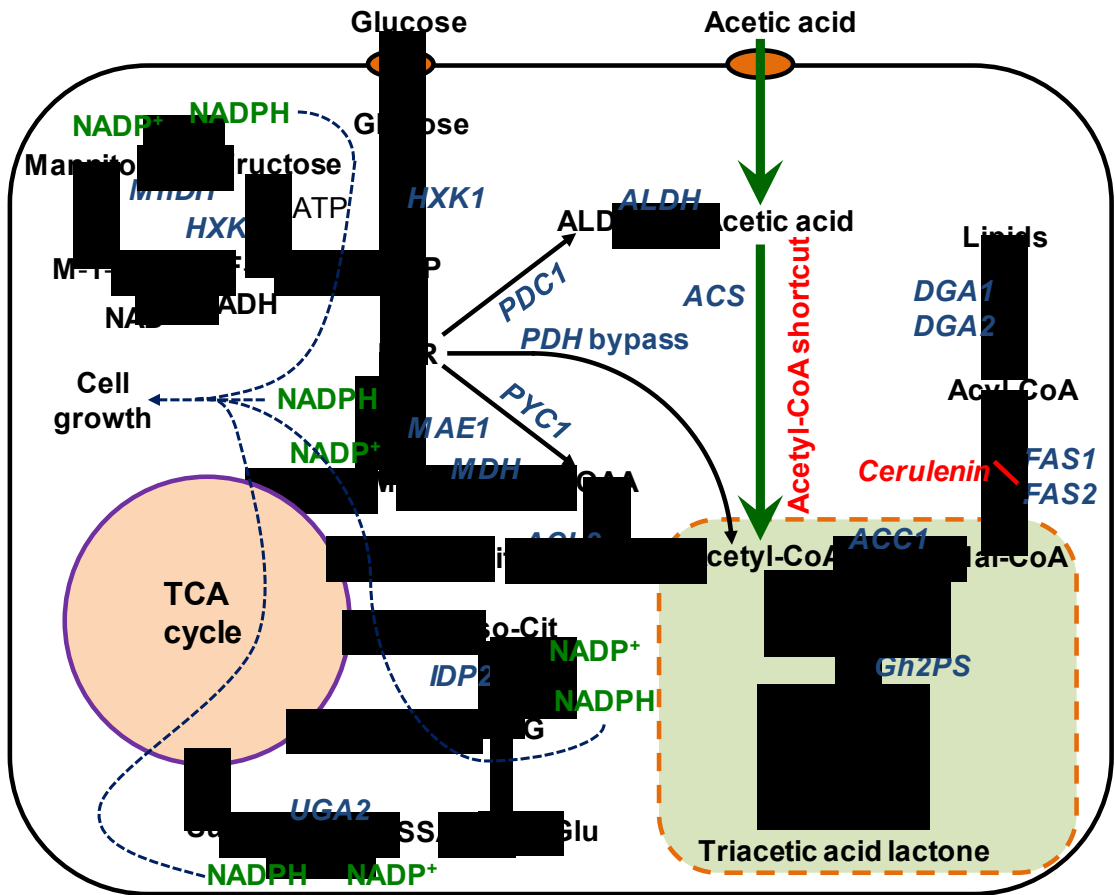
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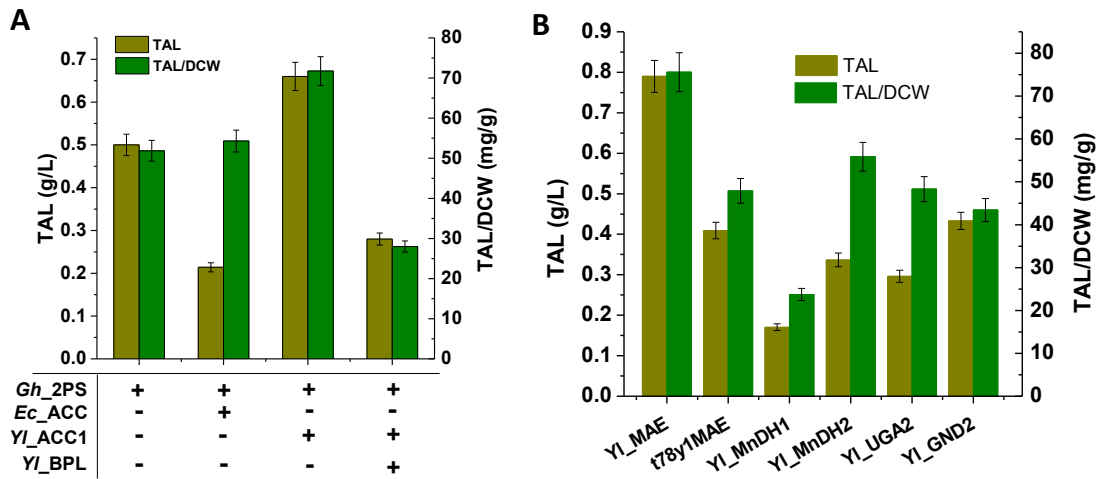
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759 **Fig. 1** Metabolic pathway for TAL synthesis in oleaginous yeast. MnDH, mannitol
760 dehydrogenase; HXK, hexokinase; MAE1, malic enzyme; ACL2, ATP citrate lyase;
761 IDP2, cytosolic NADP-specific isocitrate dehydrogenase; UGA2, succinate
762 semialdehyde dehydrogenase; PYC1, pyruvate carboxylase; PDC1, pyruvate
763 decarboxylase; ALD, aldehyde dehydrogenase; PDH, pyruvate dehydrogenase; ACS,
764 acetic acid synthase; FAS1 and FAS2, fatty acid synthase; ACC1,
765 acetyl-CoA-carboxylase; GH2PS, polyketide synthase.

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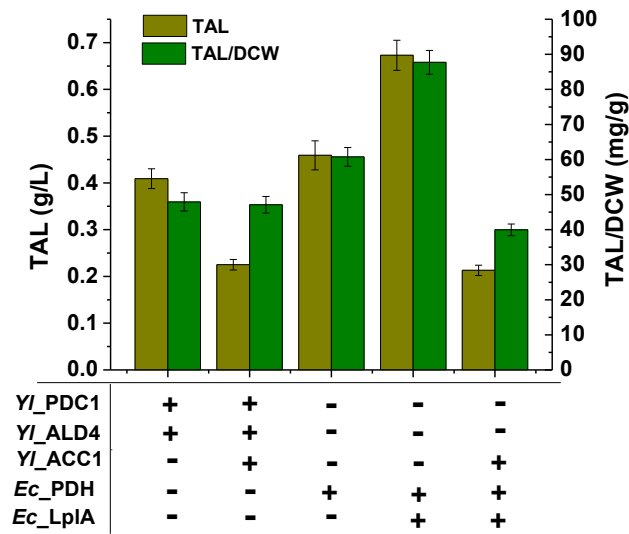


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769 **Fig. 2** Engineering precursor pathway to improve TAL production in *Y. lipolytica*. (A)
770 Comparison of TAL production from native acetyl-CoA carboxylase (ylACC1) and
771 bacteria-derived acetyl-CoA carboxylase (EcACC). (B) Comparison of TAL
772 production from alternative cytosolic NADPH pathways in *Y. lipolytica*.

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774



775

776 **Fig. 3** Engineering cytosolic acetyl-CoA pathway to improve TAL production in *Y.*
777 *lipolytica*. YlPDC1, pyruvate decarboxylase; ylALD4, aldehyde dehydrogenase;
778 EcPDH, pyruvate dehydrogenase complex; EcLplA, lipoate-protein ligase A.

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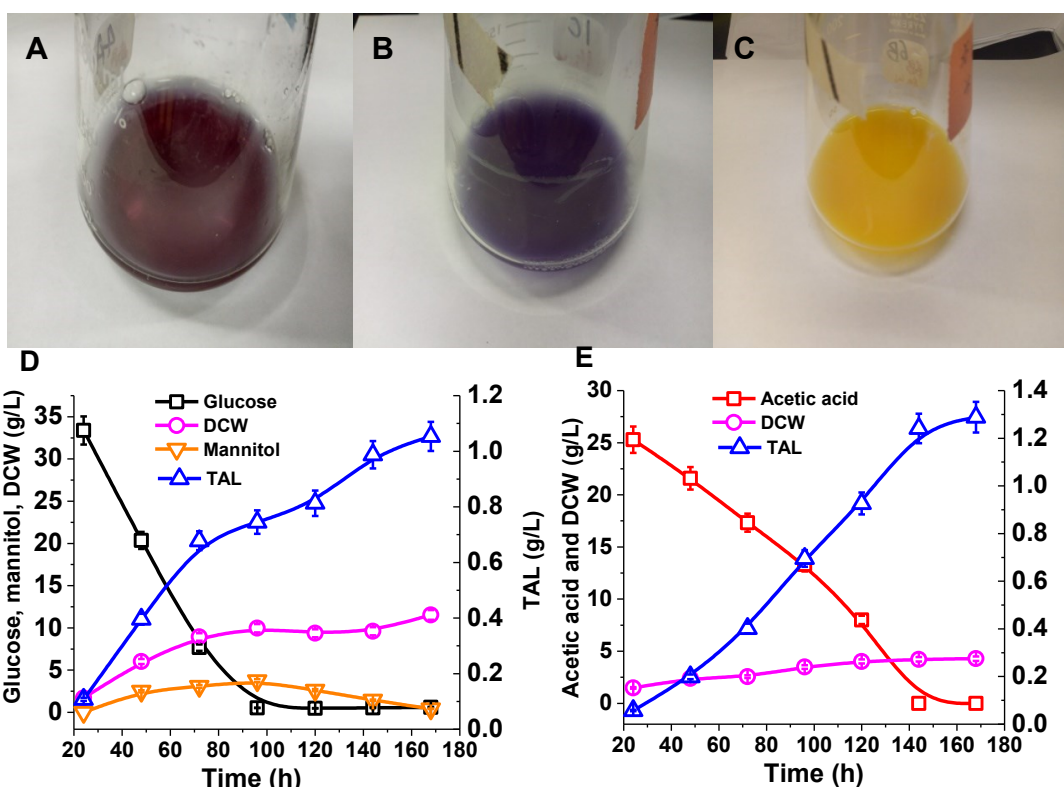


Fig. 4 Eco-friendly production of TAL from glucose and acetic acid. (A~C) Color variation of medium with pH indicator bromocresol purple. (A): Initial sodium acetate (NaAc) medium with pH 6.0; (B): Strain cultured in NaAc medium after 48 h with pH above 8.0; (C): Strain cultured in glucose medium after 48 h with pH below 4.0. (D): TAL production from minimal media supplemented with glucose by *HLYali101*; (E): TAL production from minimal media supplemented with NaAc by *HLYali101*. When NaAc was used as substrate, the medium pH will increase with the consumption of acetate. We adjusted the pH to 6.0 by adding HCl. Bromocresol purple is a pH-sensitive indicator to track the pH variations of fermentation process.

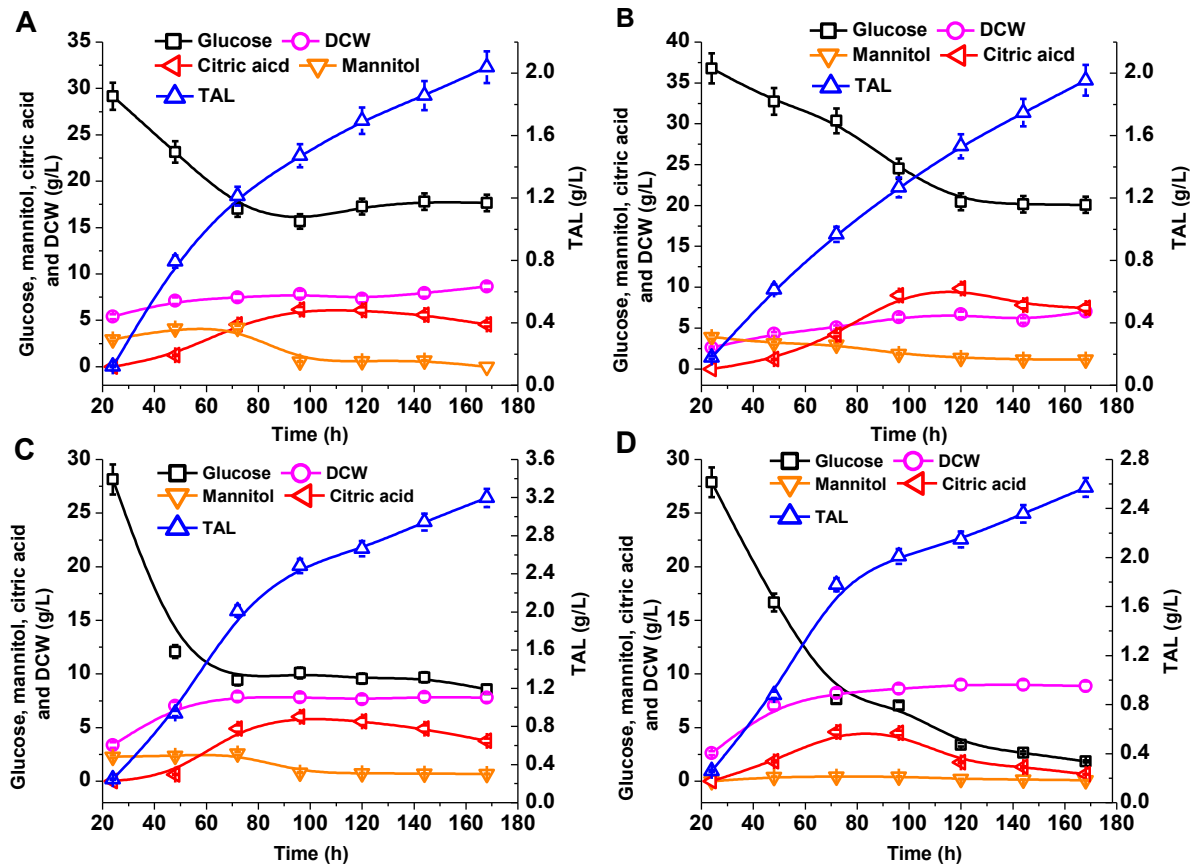
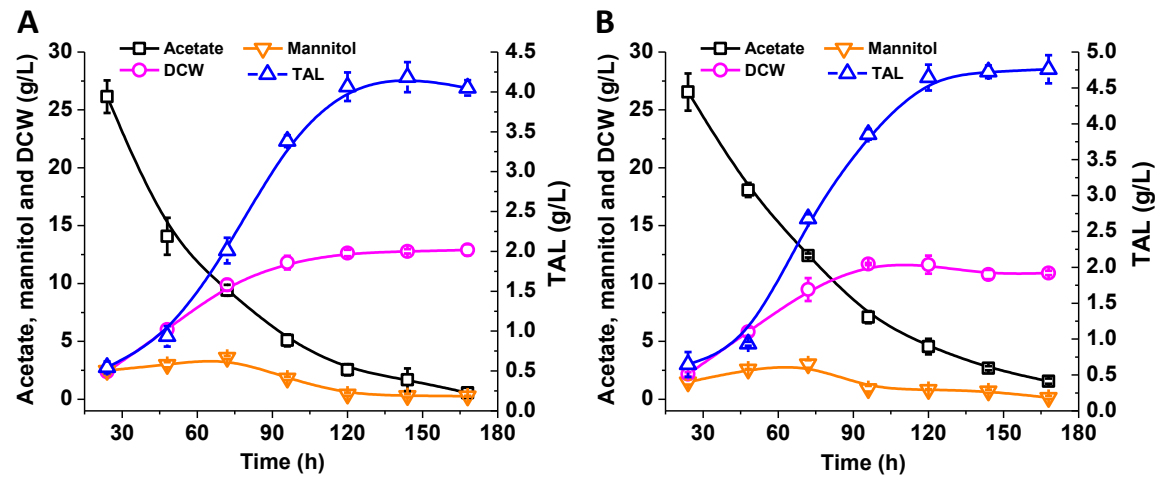


Fig. 5 Improving TAL production by pH control and modulating precursor competing pathway in glucose-minimal media. Fermentation profile of glucose consumption, mannitol, dry cell weight, citric acid and TAL accumulation for strain *HLYali101* cultivated in glucose-minimal media conditioned with PBS buffer (A) or supplemented with 1 mg/L cerulenin (C). Fermentation profile of glucose consumption, mannitol, dry cell weight, citric acid and TAL accumulation for strain *HLYali103* cultivated in glucose-minimal media conditioned with PBS buffer (B) or supplemented with 1 mg/L cerulenin (D).

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803

804 **Fig. 6.** Test of TAL production with chromosomally-integrated pathway. Fermentation
805 profile of NaAc consumption, mannitol, dry cell weight and TAL accumulation in
806 strains *LHYali105* (A) and *LHYali106* (B).