

De novo production of resveratrol from glucose or ethanol by engineered *Saccharomyces cerevisiae*

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ABSTRACT

Resveratrol is a natural antioxidant compound, used as food supplement and cosmetic ingredient. Microbial production of resveratrol has until now been achieved by supplementation of expensive substrates, *p*-coumaric acid or aromatic amino acids. Here we engineered the yeast *Saccharomyces cerevisiae* to produce resveratrol directly from glucose or ethanol via tyrosine intermediate. First we introduced the biosynthetic pathway, consisting of tyrosine ammonia-lyase from *Herpetosiphon aurantiacus*, 4-coumaryl-CoA ligase from *Arabidopsis thaliana* and resveratrol synthase from *Vitis vinifera*, and obtained 2.73 ± 0.05 mg L⁻¹ resveratrol from glucose. Then we over-expressed feedback-insensitive alleles of *ARO4* encoding 3-deoxy-D-arabino-heptulosonate-7-phosphate and *ARO7* encoding chorismate mutase, resulting in production of 4.85 ± 0.31 mg L⁻¹ resveratrol from glucose as the sole carbon source. Next we improved the supply of the precursor malonyl-CoA by over-expressing a post-translational de-regulated version of the acetyl-CoA carboxylase encoding gene *ACC1*; this strategy further increased resveratrol production to 6.39 ± 0.03 mg L⁻¹. Subsequently, we improved the strain by performing multiple-integration of pathway genes resulting in resveratrol production of 235.57 ± 7.00 mg L⁻¹. Finally, fed-batch fermentation of the final strain with glucose or ethanol as carbon source resulted in a resveratrol titer of 415.65 and 531.41 mg L⁻¹, respectively.

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1. Introduction

Resveratrol (trans-3,5,4'-trihydroxystilbene) is a natural polyphenolic compound from the stilbene family. In plants, resveratrol plays a role as a defense compound against pathogens infection and injury. It occurs naturally in several higher plants, e.g. grapes, peanuts, blueberries, and knotweed (Mei et al., 2015). In pre-clinical tests resveratrol has shown a wide range of beneficial properties, i.e., antitumor, anti-inflammatory, antidiabetic, antithrombotic, and antiaging properties (Jeandet et al., 2012; Mei et al., 2015). The evidence of resveratrol effects on human health is however so far inconclusive, due to the limited number of clinical trials and small cohort sizes (Poulsen et al., 2013). Nevertheless, resveratrol has attracted much attention from pharmaceutical,

food and cosmetic industries. Resveratrol is sold as over-the-counter nutritional supplement; it is included in some cosmetics products, energy drinks, and other products. The demand for resveratrol is expected to further increase in the future. The commercial resveratrol on the market is predominantly extracted from the Japanese knotweed *Polygonum cuspidatum* (Mei et al., 2015). The preparations range widely in purity; some can contain as low as 50% of the active ingredient. The unpurified knotweed extracts, however, additionally contain emodin which has a laxative effect (Srinivas et al., 2007). Thus, there is market demand for lower cost and high purity resveratrol.

One promising solution is biotechnological production of resveratrol by fermentation of genetically engineered microbes (Borodina and Nielsen, 2014). A number of studies have been published on microbial production of resveratrol. In all the studies, the production exclusively relied on using complex medium or supplementing the minimal medium with resveratrol precursors, *p*-coumaric acid, tyrosine or phenylalanine, which are expensive for industrial applications. The first study on recombinant resveratrol production described feeding *p*-coumaric acid to *Saccharomyces cerevisiae*, which expressed

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4-coumaroyl-CoA ligase (4CL) and resveratrol synthase (VST1) (Becker et al., 2003). Deamination of tyrosine or phenylalanine, catalyzed by tyrosine/phenylalanine ammonia lyase (TAL/PAL), is the first step of resveratrol biosynthesis pathway in plants. Transcription and enzyme activity of PAL is reduced by its product, *trans*-cinnamic acid (Blount et al., 2000; Bolwell et al., 1988), which means that efficient conversion of *trans*-cinnamic acid into *p*-coumaric acid is required to avoid the accumulation of toxic intermediate. The hydroxylation of *trans*-cinnamic acid into *p*-coumaric acid is catalyzed by cinnamic acid hydroxylase (C4H), a P450 enzyme, which requires cytochrome P450 reductase for efficient electron transfer. Trantas et al. expressed phenylalanine ammonia-lyase (PAL), cinnamate-4-hydroxylase (C4H), and cytochrome P450 reductase (CPR) in addition to 4CL and VST1 to produce resveratrol from phenylalanine (Trantas et al., 2009). In the biosynthesis of *p*-coumaric acid-derived compounds via phenylalanine, C4H was reported as the rate-limiting step (Trantas et al., 2009; Yan et al., 2005). A reduction of the C4H transcriptional level was observed to result in an 8-fold decrease of PAL and 2.5-fold decrease of 4CL activities in tobacco (Kumar et al., 2012). Alternatively, tyrosine ammonia-lyase (TAL) can be used to directly synthesize *p*-coumaric acid from tyrosine (Fig. 1), thus averting the rate-limiting hydroxylation step. Wang et al. have successfully expressed codon-optimized TAL from *Rhodospseudomonas sphaeroides* in yeast to obtain 1.90 mg L⁻¹ resveratrol, when supplemented with 12 mg L⁻¹ tyrosine, and 1.06 mg L⁻¹ resveratrol without supplementation but in complex medium, which contained tyrosine (Wang et al., 2011). In

another case, Shin et al. used a tyrosine/phenylalanine ammonia-lyase from *Rhodospiridium toruloides* to produce 5.8 mg L⁻¹ resveratrol in complex medium supplemented with 2.17 g L⁻¹ tyrosine (Shin et al., 2012). The highest resveratrol titer reported in yeast from the literature so far is 391 mg L⁻¹, obtained by supplementing complex medium with 2.46 g L⁻¹ *p*-coumaric acid and using a genetically engineered industrial Brazilian *S. cerevisiae* strain that overexpressed 4CL1 and STS (stilbene synthase) genes (Sydor et al., 2010).

In the present study, we aimed to achieve *de novo* biosynthesis of resveratrol from cheap carbon sources, glucose or ethanol, without supplementation of aromatic precursors. We chose to apply *S. cerevisiae* as the host, due to its safe use status in pharmaceutical biotechnology and food industry, and due to its high amenability to genetic manipulations. Here we describe step-wise metabolic engineered efforts towards obtaining an efficient cell factory for resveratrol production.

2. Materials and methods

2.1. Strains and growth conditions

The *Escherichia coli* strain DH5α was used for all the cloning work. The *E. coli* transformants were selected and maintained on Luria-Bertani (LB) plates containing 100 μg mL⁻¹ ampicillin.

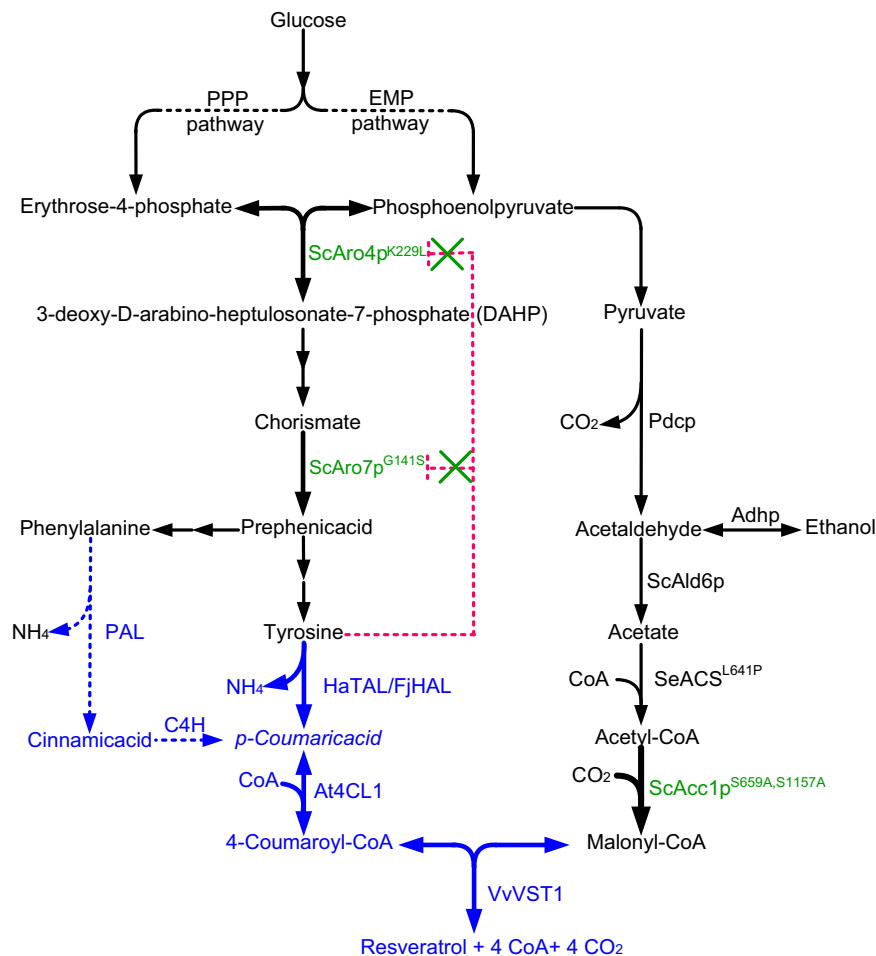


Fig. 1. Biosynthesis pathways towards resveratrol in engineered yeast. ScAro4p: DAHP synthase; ScAro7p: chorismate mutase; PAL: phenylalanine ammonia-lyase; C4H: cinnamate-4-hydroxylase; HaTAL/FjTAL: tyrosine ammonia-lyase from *H. aurantiacus*/Flavobacterium johnsoniae; At4CL1: 4-coumarate-CoA ligase; VST1: resveratrol synthase; PdcP: pyruvate decarboxylase; Adhp: alcohol dehydrogenase; ScAld6p: aldehyde dehydrogenase; SeACS1: acetyl-CoA synthetase; ScAcc1p: acetyl-CoA carboxylase. Native reactions and intermediates are shown in black, heterologous in blue. The enzymes over-expressed in the described resveratrol-producing strain are highlighted in bold. The dash arrows mark the reactions that were not used in this study. Double arrows represent multiple enzymatic steps.

Table 1
List of yeast strains used in the study.

Strains	Parent strains	Integrative plasmids	Genotype	Reference
CEN.PK102-5B			MATa <i>ura3-52 his3Δ1 leu2-3/112 MAL2-8^c SUC2</i>	Entian and Kötter (2000)
ST4120	CEN.PK102-5B	pCfB388, pCfB872	$P_{TEF1}^- > HaTAL$, <i>ura-</i>	This study
ST4121	CEN.PK102-5B	pCfB854, pCfB872	$P_{TEF1}^- > HaTAL$, $P_{PGK1}^- > At4CL1$, $P_{TEF1}^- > VvVST1$, <i>ura-</i>	This study
ST4122	CEN.PK102-5B	pCfB1020, pCfB872	$P_{TEF1}^- > HaTAL$, $P_{TEF1}^- > At4CL1$, $P_{PGK1}^- > VvVST1$, <i>ura-</i>	This study
ST4123	CEN.PK102-5B	pCfB856, pCfB872	$P_{TEF1}^- > HaTAL$, $P_{TEF1}^- > 4CL1::VST1$, <i>ura-</i>	This study
ST4124	CEN.PK102-5B	pCfB855, pCfB872	$P_{TEF1}^- > HaTAL$, $P_{PGK1}^- > At4CL2$, $P_{TEF1}^- > VvVST1$, <i>ura-</i>	This study
ST4125	CEN.PK102-5B	pCfB1021, pCfB872	$P_{TEF1}^- > HaTAL$, $P_{TEF1}^- > At4CL2$, $P_{PGK1}^- > VvVST1$, <i>ura-</i>	This study
ST4126	CEN.PK102-5B	pCfB857, pCfB872	$P_{TEF1}^- > HaTAL$, $P_{TEF1}^- > 4CL2::VST1$, <i>ura-</i>	This study
ST4127	CEN.PK102-5B	pCfB388, pCfB873	$P_{TEF1}^- > FjTAL$, <i>ura-</i>	This study
ST4128	CEN.PK102-5B	pCfB854, pCfB873	$P_{TEF1}^- > FjTAL$, $P_{PGK1}^- > At4CL1$, $P_{TEF1}^- > VvVST1$, <i>ura-</i>	This study
ST4129	CEN.PK102-5B	pCfB1020, pCfB873	$P_{TEF1}^- > FjTAL$, $P_{TEF1}^- > At4CL1$, $P_{PGK1}^- > VvVST1$, <i>ura-</i>	This study
ST4130	CEN.PK102-5B	pCfB856, pCfB873	$P_{TEF1}^- > FjTAL$, $P_{TEF1}^- > 4CL1::VST1$, <i>ura-</i>	This study
ST4131	CEN.PK102-5B	pCfB855, pCfB873	$P_{TEF1}^- > FjTAL$, $P_{PGK1}^- > At4CL2$, $P_{TEF1}^- > VvVST1$, <i>ura-</i>	This study
ST4132	CEN.PK102-5B	pCfB1021, pCfB873	$P_{TEF1}^- > FjTAL$, $P_{TEF1}^- > At4CL2$, $P_{PGK1}^- > VvVST1$, <i>ura-</i>	This study
ST4133	CEN.PK102-5B	pCfB857, pCfB873	$P_{TEF1}^- > FjTAL$, $P_{TEF1}^- > 4CL2::VST1$, <i>ura-</i>	This study
ST4158	ST4121		<i>his-</i> , <i>leu-</i> , <i>ura-</i>	This study
ST4135	ST4158	pCfB255, pCfB257, pCfB258	$P_{TEF1}^- > HaTAL$, $P_{PGK1}^- > At4CL1$, $P_{TEF1}^- > VvVST1$	This study
ST4136	ST4158	pCfB255, pCfB257, pCfB826	$P_{TEF1}^- > HaTAL$, $P_{PGK1}^- > At4CL1$, $P_{TEF1}^- > VvVST1$, $P_{TEF1}^- > ScARO7^{G141S}$, $P_{PGK1}^- > ScARO4^{K229L}$	This study
ST4137	ST4158	pCfB1175, pCfB257, pCfB258	$P_{TEF1}^- > HaTAL$, $P_{PGK1}^- > At4CL1$, $P_{TEF1}^- > VvVST1$, $P_{TEF1}^- > ScACC1^{S659A, S1157A}$	This study
ST4140	ST4158	pCfB1175, pCfB257, pCfB826	$P_{TEF1}^- > HaTAL$, $P_{PGK1}^- > At4CL1$, $P_{TEF1}^- > VvVST1$, $P_{TEF1}^- > ScARO7^{G141S}$, $P_{PGK1}^- > ScARO4^{K229L}$, $P_{TEF1}^- > ScACC1^{S659A, S1157A}$	This study
ST4159	ST4121	pCfB1175	$P_{TEF1}^- > HaTAL$, $P_{PGK1}^- > At4CL1$, $P_{TEF1}^- > VvVST1$, $P_{TEF1}^- > ScACC1^{S659A, S1157A}$	This study
ST4160	ST4159		$P_{TEF1}^- > HaTAL$, $P_{PGK1}^- > At4CL1$, $P_{TEF1}^- > VvVST1$, $P_{TEF1}^- > ScACC1^{S659A, S1157A}$, <i>his-</i> , <i>leu-</i> , <i>ura-</i>	This study
ST4171	ST4160	pCfB257, pCfB826	$P_{TEF1}^- > HaTAL$, $P_{PGK1}^- > At4CL1$, $P_{TEF1}^- > VvVST1$, $P_{TEF1}^- > ScARO7^{G141S}$, $P_{PGK1}^- > ScARO4^{K229L}$, $P_{TEF1}^- > ScACC1^{S659A, S1157A}$, <i>ura-</i>	This study
ST4152	ST4171	pCfB2068	<i>Ty-</i> ($P_{TEF1}^- > HaTAL$, $P_{PGK1}^- > At4CL1$, $P_{TEF1}^- > VvVST1$), $P_{TEF1}^- > ScARO7^{G141S}$, $P_{PGK1}^- > ScARO4^{K229L}$, $P_{TEF1}^- > ScACC1^{S659A, S1157A}$	This study

S. cerevisiae CEN.PK102-5B (MATa *ura3-52 his3Δ1 leu2-3/112 MAL2-8^c SUC2*) (Entian and Kötter, 2007) was used for construction of resveratrol-producing strains. All the yeast strains used in this study are listed in Table 1. Yeast cells transformed with integrative plasmids were selected on synthetic complete (SC) drop-out media.

LB and SC drop-out media were made using pre-mixed powders from Sigma-Aldrich. The defined minimal medium used for fermentation in microtiter plates was described as before (Jensen et al., 2014b).

2.2. Genes and biobricks

Two genes, encoding tyrosine ammonia-lyase from *H. aurantiacus* (*HaTAL*) and from *Flavobacterium johnsoniae* (*FjTAL*), were described in a previous study (Jendresen et al., 2015). The genes, encoding 4-coumarate:CoA ligases from *A. thaliana* (*At4CL1* and *At4CL2*) and stilbene synthase from *V. vinifera* (*VvVST1*) were synthesized by GeneArt (Life Technologies) in codon-optimized versions for *S. cerevisiae*. The feedback-inhibition-insensitive alleles of *ScARO4*^{K229L} (3-deoxy-D-arabino-heptulosonate-7-phosphate (DAHP) synthase) and *ScARO7*^{G141S} (chorismate mutase) were described in the previous study (Rodriguez et al., 2015). Acetyl-CoA synthase from *Salmonella enterica* (*SeACS*^{L641P}), aldehyde dehydrogenase from *S. cerevisiae* (*ScALD6*) were described in (Shiba et al., 2007). Inactivation-resistant acetyl-CoA carboxylase from *S. cerevisiae* *ScACC1*^{S659A, S1157A} was described in Shi et al. (2014). All the biobricks were PCR-amplified using Phu X7 polymerase (Norholm, 2010). The biobricks generated in this study are listed in Table 2 along with the template DNA and primers that were used for PCR amplification.

2.3. Plasmid and strain construction

The primers used in the study are summarized in Table 3 and the plasmids in Table 4. All the biobricks were assembled into integrative EasyClone vectors using USER cloning (Jensen et al., 2014b). The multiple integrative plasmid pCfB2068 was constructed in a different way: plasmid pCfB322 was amplified by PCR using primers Open_fw and Open_rv. Then biobricks BB293 (<-*At4CL1*>), BB302 (<-*P_{PGK1}-P_{TEF1}*>), and BB295 (*VvVST1*->) were cloned into the opened pCfB322 vector following USER protocol, resulting in plasmid pCfB2067. Thereafter the plasmid pCfB2067 was PCR-amplified again by the same primers and biobricks BB429 (*T*<-*NAT5*), BB379 (<-*HaTAL*>), and BB530 (*P*<-*TDH3*) were inserted in the same way to generate plasmid pCfB2068.

Table 2
List of biobricks used in the study.

Biobricks	Description	Templates	Forward primers	Reverse primers
BB008	Promoter, <- <i>P_{TEF1}</i> >	pCfB826	<i>P</i> <- <i>TEF1</i> _fw	<i>P</i> <- <i>TEF1</i> _rv
BB010	Bidirectional promoter, <- <i>P_{TEF1}-P_{PGK1}</i> >	pCfB826	<i>P</i> <- <i>TEF1</i> _fw	<i>P_{PGK1}</i> ->_rv
BB302	Bidirectional promoter, <- <i>P_{PGK1}-P_{TEF1}</i> >	pCfB826	<i>P</i> <- <i>PGK1</i> _fw	<i>P_{TEF1}</i> ->_rv
BB379	<i>HaTAL</i> from <i>Herpetosiphon aurantiacus</i>	pCBJ279	<- <i>HaTAL</i> _fw	<- <i>HaTAL</i> _rv
BB380	<i>FjTAL</i> from <i>Flavobacterium johnsoniae</i>	pCBJ280	<- <i>FjTAL</i> _fw	<- <i>FjTAL</i> _rv
BB293	<i>At4CL1</i> from <i>Arabidopsis thaliana</i>	pCfB757	<- <i>At4CL1</i> _fw	<- <i>At4CL1</i> _rv
BB294	<i>At4CL2</i> from <i>Arabidopsis thaliana</i>	pCfB758	<- <i>At4CL2</i> _fw	<- <i>At4CL2</i> _rv
BB295	<i>VvVST1</i> from <i>Vitis vinifera</i>	pCfB759	<i>VvVST1</i> ->_fw	<i>VvVST1</i> ->_rv
BB304	The front part of <i>4CL1::VST1</i> fusion	pCfB757	<- <i>At4CL1</i> _fw	<- <i>4CL1-VST1</i> _rv
BB305	The latter part of <i>4CL1::VST1</i> fusion	pCfB759	<- <i>4CL1-VST1</i> _fw	<i>VvVST1</i> <-_rv
BB306	The front part of <i>4CL2::VST1</i> fusion	pCfB758	<- <i>At4CL2</i> _fw	<i>4CL2-VST1</i> <-_rv
BB307	The latter part of <i>4CL2::VST1</i> fusion	pCfB759	<- <i>4CL2-VST1</i> _fw	<i>VvVST1</i> <-_rv
BB364	<i>ScARO4</i> ^{K229L} from <i>S. cerevisiae</i>	pCfB826	<i>ScARO4</i> ->_fw	<i>ScARO4</i> ->_rv
BB361	<i>ScARO7</i> ^{G141S} from <i>S. cerevisiae</i>	pCfB826	<- <i>ScARO7</i> _fw	<- <i>ScARO7</i> _rv
BB012	<i>ScACC1</i> ^{S659A, S1157A} from <i>S. cerevisiae</i>	p474	<- <i>ScACC1</i> _fw	<- <i>ScACC1</i> _rv
BB530	Promoter, <- <i>P_{TDH3}</i> >	Genomic DNA of CEN.PK102-5B	<i>P</i> <- <i>TDH3</i> _fw	<i>P</i> <- <i>TDH3</i> _rv
BB429	Terminator, <i>T</i> <- <i>NAT5</i>	Genomic DNA of CEN.PK102-5B	<i>T</i> <- <i>NAT5</i> _fw	<i>T</i> <- <i>NAT5</i> _rv

Colony PCR and sequencing was performed to confirm the correct cloning. Yeast transformations were carried out following lithium acetate protocol (Gietz and Woods, 2002). The correct genomic insertions were verified by yeast colony PCR using the primers listed in Table 3.

ST4158 and ST4160 were generated by removal of the selection markers in ST4122 and ST4159 respectively. It was performed by introducing plasmid pSH65, expressing *creA* gene under control of the *P_{GAL10}* promoter (Gueldener et al., 2002). Strains harboring pSH65 were grown in yeast peptone galactose medium for 12–16 h for induction and then plated on yeast peptone dextrose (YPD) agar plates. The colonies were replica-plated on SC drop-out plates to select for the colonies that have lost the markers. The loss of markers was verified by yeast colony PCR.

2.4. Determination of gene copy number by qPCR

The design of qPCR primers was conducted using the online PrimerQuest[®] Tool at <https://eu.idtdna.com/Primerquest/Home/Index>. All the primers used for qPCR in this study are listed in Supplementary Table 1.

Genomic DNA of ST4140 and ST4152 were extracted using the ZR Fungal/Bacterial DNA MiniPrep[™] kit (Zymo Research) following the manufacturer's manual. The SYBR[®] Green qPCR MasterMix from Life Technologies was used to test for the copy number of *HaTAL*, *At4CL1* and *VvVST1*. The housekeeping gene *ALG9* was used as the reference. Reactions were performed in a 20 µl volume with 10 µl 2 × SYBR Green QPCR master mix, 1 µl of each upstream and downstream primer, 0.3 µl diluted reference dye and 7.7 µl gDNA containing serially diluted gDNA template (62.5–1000 pg). A no-template control reaction (NTC) for each gene was made by replacement of gDNA with nuclease-free PCR-grade water.

qPCR runs were performed in Stratagene Mx3005P instrument using the thermocycler program as follows: 10 min of pre-incubation at 95 °C followed by 40 amplification cycles of denaturation at 95 °C for 20 s, annealing and elongation at 60 °C for 22 s; and a single final cycle of 95 °C for 1 min, 55 °C for 30 s and 95 °C for 30 s.

2.5. Microtiter plate cultivation of yeast strains

Three biological transformants of each strain were inoculated in 0.5 mL SC drop-out liquid medium without histidine and leucine supplemented with 2% glucose in a 96-deep well microtiter plate with air-penetrable lid (EnzyScree, NL). The cultures were incubated at 30 °C with 250 rpm agitation for 24 h. 50 µl of the

seed cultures were inoculated into 0.5 mL minimal medium containing 5 mM tyrosine in a new 96-deep well plate. The minimal medium (pH 6.0) was described in (Jensen et al., 2014b). After 72 h cultivation at 30 °C with 250 rpm agitation, OD₆₀₀ was measured with a 20 times dilution in microplate reader BioTek Synergy MX (BioTek). The remaining cultures were mixed with equal volume of absolute ethanol and centrifuged at 2272 g for 30 min. The supernatants were used to analyze resveratrol concentration by HPLC.

2.6. Batch and fed-batch fermentation of yeast strains in controlled reactors

Inoculum for bioreactors was prepared as following. Yeast strains from –80 °C glycerol stock were inoculated into 5 mL of defined minimal medium with 2% glucose as above, and incubated in 13 mL tubes at 30 °C with shaking at 250 rpm for 18 h. The whole culture volume was consequently transferred into 50 mL of fresh defined minimal medium in a 500 mL baffled shake flask and incubated at 30 °C with shaking at 250 rpm for 12 h. The culture OD₆₀₀ was measured and reactors were inoculated with such a volume of inoculum that an initial OD₆₀₀ of 0.02–0.05 was obtained.

Batch and fed-batch fermentations were performed in 1 L-DasGip stirrerpro[®] bioreactors (DasGip, Julich, Germany) with a starting volume of 0.5 L. Batch medium and medium for batch phase of fed-batch fermentation contained per 1L:30 g (batch fermentation)/40 g (fed-batch fermentation) glucose, 5 g (NH₄)₂SO₄, 3 g KH₂PO₄, 0.5 g MgSO₄·7H₂O, 2 mL trace metals solution, and 1 mL vitamin solution, where the composition of trace metals and vitamin solutions was the same as in (Jensen et al., 2014b). The medium was adjusted to pH 5.0 before autoclaving in reactors at 121 °C for 20 min. Filter-sterilized glucose, trace metal and vitamins solutions were added to the medium after autoclaving. Fed-batch medium contained 10-fold higher concentration of all the nutrients in the batch phase, except for carbon source which was replaced by 200 g L^{–1} glucose or 150 g L^{–1} ethanol in the fed-batch medium, but was otherwise prepared in the same way. The feed was started only after residual ethanol produced from the glucose phase was completely depleted. A volumetric growth rate constant-dependent feed strategy (Villadsen et al., 2011) was adopted as described in (Scalcinati et al., 2012). The fermentations were performed at 30 °C; pH was maintained at 5.0 with automatic addition of 2 M KOH or 2 M HCl. The agitation rate was kept at 800 rpm and the air flow was set to 0.5 L per min. The dissolved oxygen concentration was above 30%

Table 3
List of primers used in the study.

Name	Sequence (5'–3')
P < -TEF1_fw (ID005)	<u>ACCTGCACU</u> TTGTAATTAAACTTAG
P < -TEF1_rv (ID006)	<u>CACGCCAU</u> GCACACACCATAGCTTC
P _{PGK1} - > _rv (ID008)	<u>ATGACAGAU</u> TTGTTTATATTTGTTG
P < -PGK1_fw (ID1562)	<u>ACCTGCACU</u> TTGTTTATATTTGTTG
P _{TEF1} - > _rv (ID1565)	<u>ATGACAGAU</u> TTGTAATTAAACTTAG
< -HaTAL_fw (ID1689)	<u>AGTGCAGGU</u> AAAACAATGAGCACCCTGATTCTG
< -HaTAL_rv (ID1690)	<u>CGTGCGAU</u> TTAGCGAAACAGAAATAT
< -FjTAL_fw (ID1691)	<u>AGTGCAGGU</u> AAAACAATGAACACCATCAACGAATATCTGAGC
< -FjTAL_rv (ID1692)	<u>CGTGCGAU</u> TTAATTGTTAATCAGGTG
< -At4CL1_fw (ID1548)	<u>AGTGCAGGU</u> AAAACAATGGCTCCACAAGAACAAGCTGTTCC
< -At4CL1_rv (ID1549)	<u>CGTGCGAU</u> TCACAAACCGTTAGCCAA
< -At4CL2_fw (ID1550)	<u>AGTGCAGGU</u> AAAACAATGACTACCAAGATGTTA
< -At4CL2_rv (ID1551)	<u>CGTGCGAU</u> TCAGTTCATCAAAACCGTT
VvVST1- > _fw (ID1552)	<u>ATCTGTCAU</u> AAAACAATGGCTTCGTTGAAGAA
VvVST1- > _rv (ID1553)	<u>CACGCCAU</u> TCAATTGGTAACGGTTGG
< -4CL1-VST1_rv (ID1588)	<u>AAACCGTU</u> AGCCAACTTGGCTC
< -4CL1-VST1_fw (ID1589)	<u>AACGGTTU</u> GGTTCGTGGCTTCGGTGAAGAATTCAGAAACGC
< -VvVST1_rv (ID1590)	<u>CGTGCGAU</u> TCAATTGGTAACGGTTGG
< -4CL2-VST1_rv (ID1591)	<u>AGAACCGTU</u> CATCAAAACCGTTAGCC
< -4CL2-VST1_fw (ID1592)	<u>AACGGTTU</u> GGTGCTTCGTTGAAGAATTCAG
ScARO4- > _fw (ID1396)	<u>ATCTGTCAU</u> AAAACA ATGAGTGAATCTCCAATGTTCG
ScARO4- > _rv (ID1397)	<u>CACGCCAU</u> TCATTTCTGTGTAACCTCTCTCTTTG
< -ScARO7_fw (ID1398)	<u>AGTGCAGGU</u> AAAACA ATGGATTTCAAAAACAGAAAC
< -ScARO7_rv (ID1399)	<u>CGTGCGAU</u> TCACTCTTCCAACCTCTTAGCAAG
< -ScACC1_fw (ID053)	<u>CGTGCGAU</u> TCATTTCAAAGTCTTCAACAATTT
< -ScACC1_rv (ID054)	<u>AGTGCAGGU</u> AAAACAATGAGCGAAGAAAGCTTA
< -SeACS_fw (ID1644)	<u>AGTGCAGGU</u> AAAACAATGTCACAAACACAC
< -SeACS_rv (ID1645)	<u>CGTGCGAU</u> TCATGATGGCATAGCAATAG
ScALD6- > _fw (ID738)	<u>ATCTGTCAU</u> AAAACA ATGACTAAGCTACACTTTGACAC
ScALD6- > _rv (ID739)	<u>CACGCCAU</u> TCACAACTTAATCTGACAGCTTTTAC
Open_fw (ID1858)	<u>AGCTGAAGCU</u> TCGTACGCTG
Open_rv (ID2167)	<u>ACGCGATCU</u> TCGAGCGTCC
< -T _{NAT5} _fw (ID2164)	<u>ATCGCACGCU</u> TTCTTAACAGATGGCTG
< -T _{NAT5} _rv (ID2168)	<u>AGATCGCGU</u> TCGGGACCATAAAAAATTC
P < -TDH3_fw (ID2141)	<u>AGCTTCAGCU</u> ATAAAAACACGCTTTTTCAG
P < -TDH3_rv (ID1853)	<u>ACCTGCACU</u> TTGTTTGTATGTGTGTTTATTC
ColPCR_DW_fw (ID2220)	<u>CCTGCAGGACTAGTGTGAG</u>
Loopout_DW_fw (ID400)	<u>ATCGCGTCAGCTGAAGCTTCGTACGC</u>
X-2_DW_rv (ID902)	<u>GAGAACGAGAGGACCCCAACAT</u>
X-3_DW_rv (ID904)	<u>CCGTGCAATACCAAAATCG</u>
X-4_DW_rv (ID906)	<u>GACGGTACGTTGACCAGAG</u>
X1-1_DW_rv (ID908)	<u>GAAGACCCATGGTTCCAAGGA</u>
X1-5_DW_rv (ID2157)	<u>CCCAAAAGCAATCCAGGAAAAACC</u>

Note: Underlined sequences represent overhangs used in USER cloning.

Table 4
List of plasmids used in the study.

Name	Parent plasmids	Properties	Reference
pCBJ279		<i>HaTAL</i> , Amp (ampicillin resistance)	Jendresen et al. (2015)
pCBJ280		<i>FjTAL</i> , Amp (ampicillin resistance)	Jendresen et al. (2015)
pCfB757		<i>At4CL1</i> ^a , Amp (ampicillin resistance)	This study
pCfB758		<i>At4CL2</i> ^a , Amp (ampicillin resistance)	This study
pCfB759		<i>VvVST1</i> ^a , Amp (ampicillin resistance)	This study
p474		<i>ScACC1</i> ^{S659A, S1157A} , <i>CaMCR</i> , <i>KIURA3</i>	Jensen et al. (2014a)
pSH65		<i>P_{GAL1}-CreA</i> ; <i>bleR</i> (phleomycin resistant)	Guedener et al. (2002)
pCfB255		Integrative plasmid, X-2-loxP, <i>KIURA3</i>	Jensen et al. (2014b)
pCfB257		Integrative plasmid, X-3-loxP, <i>KILEU2</i>	Jensen et al. (2014b)
pCfB258		Integrative plasmid, X-4-loxP, <i>SpHIS5</i>	Jensen et al. (2014b)
pCfB388		Integrative plasmid, XI-1-LoxP, <i>KILEU2</i>	Jensen et al. (2014b)
pCfB391		Integrative plasmid, XI-5-LoxP, <i>SpHIS5</i>	Jensen et al. (2014b)
pCfB322		Multiple integrative plasmid, Ty4, <i>KIURA3</i>	(Maury et al., submitted for publication)
pCfB826	pCfB258	Integrative plasmid, X-4-LoxP, <i>SpHIS5</i> , BB0364(< - <i>ScARO7</i> ^{G1415}), BB010(< - <i>P_{TEF1}-P_{PGK1}</i> - >), BB0361(<i>ScARO4</i> ^{K229 L} - >)	(Prado et al., submitted for publication)
pCfB854	pCfB388	Integrative plasmid, XI-1, LoxP, <i>KILEU2</i> , BB293(< - <i>At4CL1</i>), BB302(< - <i>P_{PGK1}-P_{TEF1}</i> - >), BB295(<i>VvVST1</i> - >)	This study
pCfB855	pCfB388	Integrative plasmid, XI-1, LoxP, <i>KILEU2</i> , BB294(< - <i>At4CL2</i>), BB302(< - <i>P_{PGK1}-P_{TEF1}</i> - >), BB295(<i>VvVST1</i> - >)	This study
pCfB856	pCfB388	Integrative plasmid, XI-1, LoxP, <i>KILEU2</i> , BB304 ± BB305(< - <i>4CL1::VST1</i>), BB008(< - <i>P_{TEF1}</i>)	This study
pCfB857	pCfB388	Integrative plasmid, XI-1, LoxP, <i>KILEU2</i> , BB306 ± BB307(< - <i>4CL2::VST1</i>), BB008(< - <i>P_{TEF1}</i>)	This study
pCfB872	pCfB391	Integrative plasmid, XI-5-LoxP, <i>SpHIS5</i> , BB379(< - <i>HaTAL</i>), BB008(< - <i>P_{TEF1}</i>)	This study
pCfB873	pCfB391	Integrative plasmid, XI-5-LoxP, <i>SpHIS5</i> , BB380(< - <i>FjTAL</i>), BB008(< - <i>P_{TEF1}</i>)	This study
pCfB1020	pCfB388	Integrative plasmid, XI-1-LoxP, <i>KILEU2</i> , BB293(< - <i>At4CL1</i>), BB010(< - <i>P_{TEF1}-P_{PGK1}</i> - >), BB295(<i>VvVST1</i> - >)	This study
pCfB1021	pCfB388	Integrative plasmid, XI-1-LoxP, <i>KILEU2</i> , BB294(< - <i>At4CL2</i>), BB010(< - <i>P_{TEF1}-P_{PGK1}</i> - >), BB295(<i>VvVST1</i> - >)	This study
pCfB1175	pCfB255	Integrative plasmid, X-2-LoxP, <i>KILURA3</i> , BB012(< - <i>ScACC1</i> ^{S659A, S1157A}), BB008(< - <i>P_{TEF1}</i>)	This study
p380	pCfB257	Integrative plasmid, X-3-loxP, <i>KILEU2</i> , BB119(< - <i>SeACS</i> ^{L641P}), BB010(< - <i>P_{TEF1}-P_{PGK1}</i> - >), BB158(<i>ScALD6</i> - >)	Jensen et al. (2014a)
pCfB1176	pCfB322	Multiple integrative plasmid, Ty4, <i>KIURA3</i> , BB379(< - <i>HaTAL</i>), BB008(< - <i>P_{TEF1}</i>)	This study
pCfB2067	pCfB1176	Multiple integrative plasmid, Ty4, <i>KIURA3</i> , BB293(< - <i>At4CL1</i>), BB302(< - <i>P_{PGK1}-P_{TEF1}</i> - >), BB295(<i>VvVST1</i> - >)	This study
pCfB2068	pCfB2067	Multiple integrative plasmid, Ty4, <i>KIURA3</i> , BB429(< - <i>NATS</i>), BB379(< - <i>HaTAL</i>), BB530(< - <i>TDH3</i>)	This study

^a Codon optimized for *S.cerevisiae* and synthesized from GeneArt (Life Technologies).

throughout the cultivation. The DASGIP fedbatch pro[®] gas analysis system, equipped with gas analyzer 1 GA4 based on zirconium dioxide and two-beam infrared sensor (DASGIP), was employed for monitoring oxygen and carbon dioxide concentration. Samples were taken at regular intervals to measure OD₆₀₀. Supernatants were stored at -20 °C until HPLC analysis for organic acids, glycerol, ethanol, and residual glucose. Another portion of sample was mixed with an equal volume of absolute ethanol and centrifuged at 12,000 rpm for 2 min. The supernatant was stored at -20 °C until HPLC analysis for resveratrol and *p*-coumaric acid.

2.7. Analytical methods

The OD₆₀₀ was measured on a Genesys 20 Spectrophotometer (Thermo Scientific). The HPLC quantification of glucose, glycerol, ethanol, succinate, and pyruvate was performed as before (Ostergaard et al., 2000). Resveratrol and *p*-coumaric acid were quantified on HPLC (Thermo) equipped with a Discovery HS F5 150 mm × 2.1 mm column (particle size 3 μm). The eluent flow rate was 1.5 mL min⁻¹. Linear gradient from 5% to 60% of solvent A over 0.5–9.5 min was used. Solvent A was 10 mM ammonium formate (pH 3.0, adjusted by formic acid). Solvent B was acetonitrile. Resveratrol was detected by absorbance at 304 nm with a retention time of 6.4 min and *p*-coumaric acid at 277 min with a retention time of 4.7 min. Resveratrol and *p*-coumaric acids concentrations were calculated from the standard curves, and both resveratrol and *p*-coumaric acid standards were purchased from Sigma-Aldrich.

3. Results

3.1. Reconstruction of resveratrol biosynthetic pathway from tyrosine precursor

Our previous study showed that tyrosine ammonia-lyases from *H. aurantiacus* and *Flavobacterium johnsoniae* have high activity in yeast (Jendresen et al., 2015). By over-expressing *HaTAL* (ST4121) and *FjTAL* (ST4128) in *S. cerevisiae*, we obtained respectively 7.04 ± 2.03 and 13.71 ± 2.14 mg L⁻¹ of *p*-coumaric acid in minimal medium supplemented with 5 mM tyrosine (Fig. 2A and B). Two versions of 4-coumarate:CoA ligase from *A. thaliana* (*At4CL1* and *At4CL2*) and stilbene synthase from *V. vinifera* (*VvVST1*) were then introduced under control of strong constitutive promoters in order to convert *p*-coumaric acid into resveratrol. Contrarily to *p*-coumaric acid results, the highest titers were obtained in the strains carrying *HaTAL*. *At4CL1* consistently resulted in slightly higher resveratrol titers than *At4CL2*. The highest titer of 11.66 ± 0.57 mg L⁻¹ resveratrol was obtained in ST4122, over-expressing *HaTAL* and *VvVST1* under *P_{TEF1}* promoters and *At4CL1* under *P_{PGK1}* promoter. When the direction of the double promoter was reversed, so that *At4CL1* was under control of the *P_{TEF1}* promoter and *VvVST1* under control of *P_{PGK1}* promoter, resveratrol titer dropped to 4.48 ± 0.22 to mg L⁻¹ (ST4123). This result underlines the importance of balancing gene expression in the pathway. To reduce loss of pathway intermediates and improve turnover rates due to higher local substrate concentration, fusing related enzymes is a promising strategy (Li and Borodina, 2015). Fusion of two enzymes, *At4CL1* and *VvVST1*, was previously reported to improve resveratrol production due to metabolic channeling

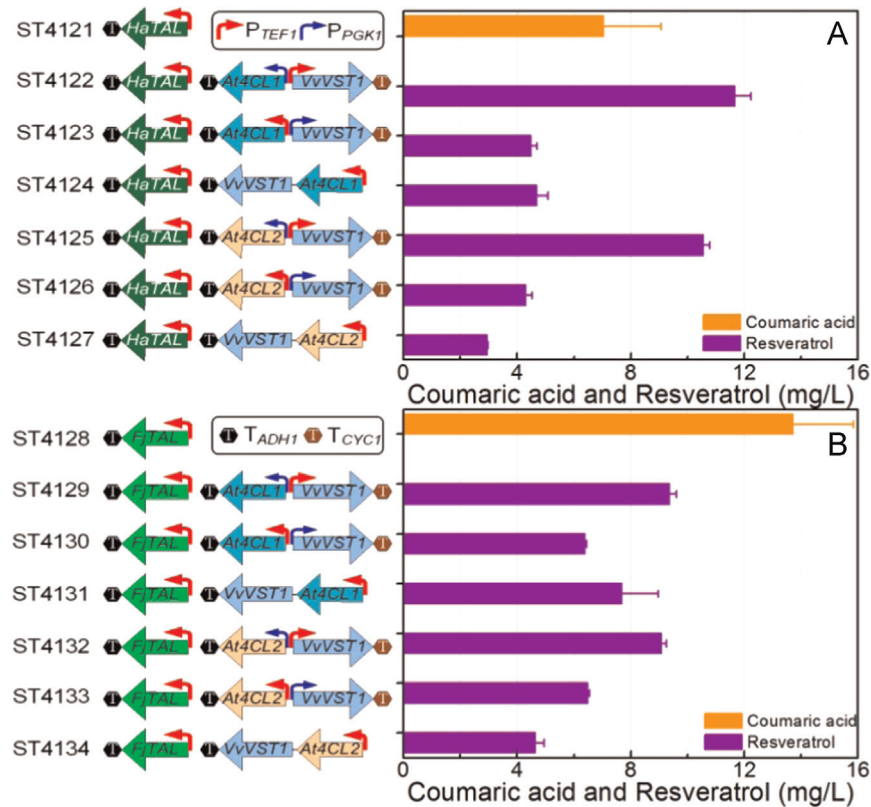


Fig. 2. Microbial production of resveratrol from tyrosine. Strains carrying *HaTAL* from *H. aurantiacus* (A) and *FjTAL* from *F. johnsoniae* (B) together with different combinations of *At4CL1* and *VvVST1* were grown in minimal medium with 20 g L⁻¹ glucose and 5 mM tyrosine and the cultures were sampled at 72 h of cultivation. Strains ST4124, ST4127, ST4131, and ST4134 over-express fusions of *At4CL1* and *VvVST1* genes. The displayed average values \pm standard deviations were calculated from three biological replicates.

(Zhang et al., 2006). We also attempted expressing the fusion of the two proteins, linked by Gly-Ser-Gly linker, in ST4124. This however led to a lower resveratrol titer of 4.69 ± 0.39 mg L⁻¹ so we did not pursue this strategy further.

3.2. De novo biosynthesis of resveratrol from glucose

It was observed that *p*-coumaric acid production continued in batch cultures after the supplemented tyrosine had been consumed, thus indicating that at least a fraction of *p*-coumaric acid was produced *de novo* from glucose or ethanol (Jendresen et al., 2015). To investigate if resveratrol also can be produced directly from glucose and also to test the effect of precursor improvement, we cultivated the strain ST4135 (analogous to ST4122) with the resveratrol pathway (*HaTAL*, *At4CL1* and *VvVST1*) in a minimal medium with glucose as the only carbon source. Fermentation of ST4135 resulted in 2.73 ± 0.05 mg L⁻¹ resveratrol from 30 g L⁻¹ glucose (Fig. 3A). Interestingly, resveratrol was primarily produced during the ethanol consumption phase, which indicates that during growth on glucose the fluxes towards resveratrol precursors, tyrosine and malonyl-CoA, were low.

The aromatic amino acids are the least abundant amino acids in the *S. cerevisiae* cell, with intracellular concentrations of tyrosine and phenylalanine being as low as 0.5 and 0.6 mM, respectively (Braus, 1991). The biosynthesis of aromatic amino acid is strongly regulated, both at the transcriptional and posttranscriptional levels. In order to improve the flux towards tyrosine, we over-expressed feedback-inhibition resistant versions of DAHP synthase *ScAro4p*^{K229L} and chorismate mutase *ScAro7p*^{G141S}. This resulted in a 78%-improvement of resveratrol titer in batch cultures compared with that of the strain ST4135 harboring only the resveratrol pathway, i.e. 4.85 ± 0.31 mg L⁻¹ resveratrol was obtained (Fig. 3B).

Another precursor for resveratrol biosynthesis is malonyl-CoA. Thus, 3 molecules of malonyl-CoA are necessary for biosynthesis of 1 molecule of resveratrol. In *S. cerevisiae*, malonyl-CoA is synthesized in the cytosol by acetyl-CoA carboxylase (*ScAcc1p*). The acetyl-CoA carboxylase can be phosphorylated by the sucrose non-fermenting protein 1 (*Snf1p*) and thus targeted for degradation, which leads to decreased activity of this enzyme in the cell (Shirra et al., 2001). A double mutation of *Acc1p* protein at Ser659 and Ser1157 positions could abolish the phosphorylation (Shi et al., 2014). We over-expressed the inactivation-resistant version of acetyl-CoA carboxylase (*ScAcc1p*^{S659A, S1157A}) in resveratrol-producing yeast, which gave 3.57 ± 0.18 mg L⁻¹ resveratrol, corresponding to a 31% improvement in comparison with ST4135 (Fig. 3C). Finally, we combined over-expression of *ScARO4*^{K229L}, *ScARO7*^{G141S}, and *ScACC1*^{S659A, S1157A} genes in strain ST4140, which improved resveratrol titer by 234% to 6.39 ± 0.03 mg L⁻¹ (Fig. 3D).

In order to further improve the flux towards acetyl-CoA, we also attempted over-expression of acetyl-CoA synthase (*SeACS*^{L641P}) from *Salmonella enterica* and aldehyde dehydrogenase *ScAld6*, in various combinations with *ScAro4p*^{K229L} and *ScAro7p*^{G141S} or *ScAcc1p*^{S659A, S1157A}. Surprisingly no improvement was obtained (Supplementary Fig. S1). Further investigation is needed to determine the reason for the negative effect of this modification (Fig. 4)

3.3. Increasing resveratrol production via integration of multiple copies of resveratrol pathway

We hypothesized that the low activity of the resveratrol biosynthetic pathway may be limiting resveratrol biosynthesis. We therefore introduced multiple copies of the genes *HaTAL*, *At4CL1* and *VvVST1* into a strain, over-expressing *ScARO4*^{K229L}, *ScARO7*^{G141S}, and *ScACC1*^{S659A, S1157A}. The multicopy integration was achieved by using

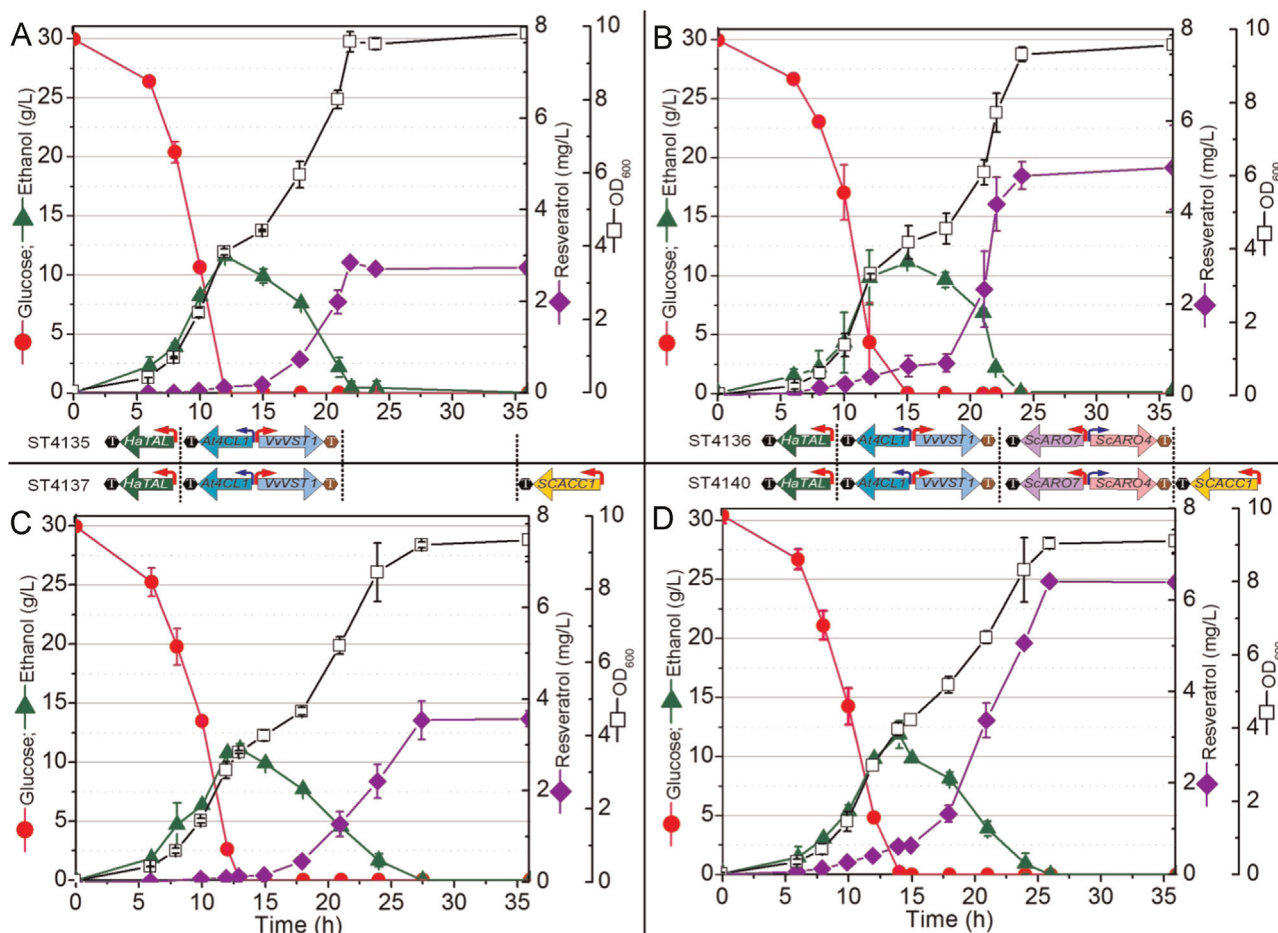


Fig. 3. Microbial production of resveratrol from glucose. The strains were cultivated on minimal medium with 30 g L⁻¹ glucose in bioreactors. (A) ST4135, the reference strain expressing *HaTAL*, *At4CL1* and *VvVST1*; (B) ST4136, over-expressing *ScARO4*^{K229L} and *ScARO7*^{G141S}; (C) ST4137, over-expressing *ScACC1*^{S659A, S1157A}; (D) ST4140, over-expressing *ScARO4*^{K229L}, *ScARO7*^{G141S} and *ScACC1*^{S659A, S1157A}. The displayed average values \pm standard deviations were calculated from two biological replicates.

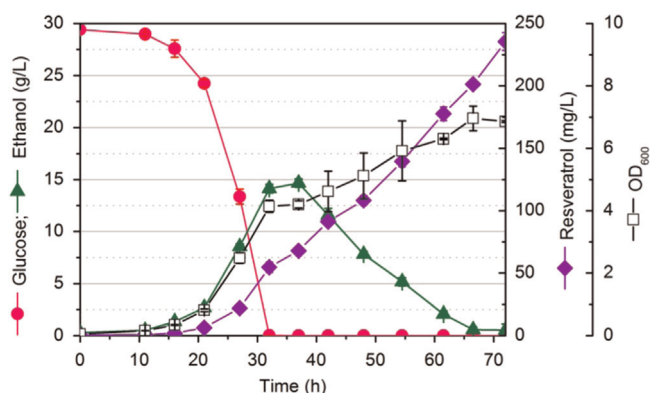


Fig. 4. Optimization of resveratrol pathway by multiple integrations of genes. Multiple integrative plasmid p2068 carrying *HaTAL*, *At4CL1*, and *VvVST1* was integrated onto Ty4 elements of *ScARO4*^{K229L}, *ScARO7*^{G141S} and *ScACC1*^{S659A, S1157A} over-expressing strain. The strain was grown on defined minimal medium with 30 g L⁻¹ glucose in batch bioreactors. The displayed average values \pm standard deviations were calculated from two biological replicates.

an integrative vector that targets Ty4 retrotransposons (Maury et al., submitted for publication). As the transformants were expected to integrate different copy numbers of the expression vector, we randomly screened 8 transformants to select the best producer, named ST4152. The copy number of the genes in the strain ST4152 was

identified to be 8.43 ± 0.85 for *HaTAL*, 8.64 ± 1.60 for *At4CL1*, and 11.16 ± 1.23 for *VvVST1* respectively by qPCR.

The strain ST4152 had a longer lag phase and lower biomass accumulation than the strain ST4140 in batch fermentation. However, the resveratrol titer in both the glucose and ethanol phases was greatly improved (Table 5). The final titer was 235.57 ± 7.00 mg L⁻¹, 36-fold higher than in the parent strain ST4140.

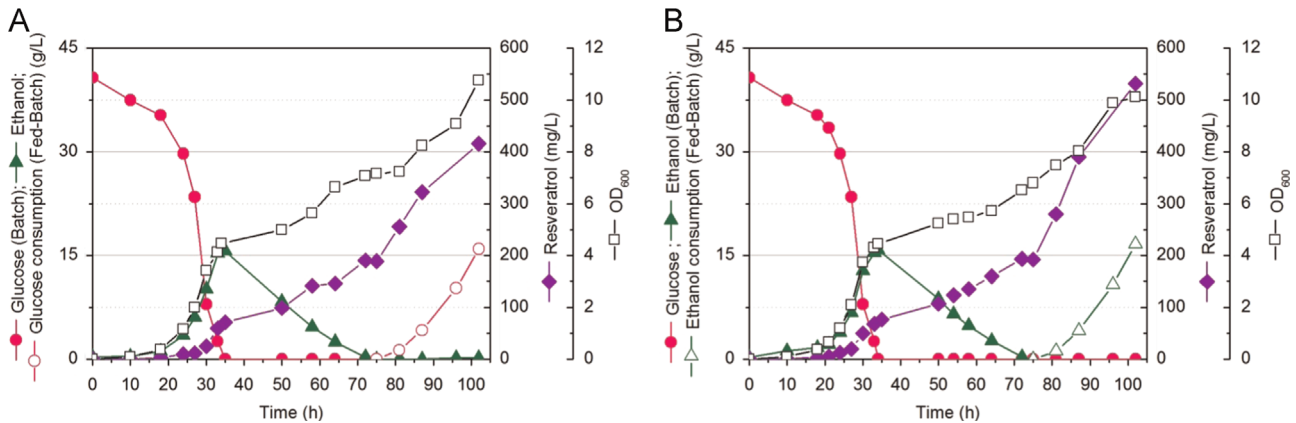
3.4. Resveratrol production in fed-batch fermentation

As resveratrol primarily accumulated after glucose depletion in batch cultivations, we carried out fed-batch cultivations with feeding of glucose or ethanol. The batch phase was on 40 g L⁻¹ glucose, once all carbon sources were consumed, the carbon-limited feeding of glucose or ethanol was initiated. The strain ST4152 produced around 200 mg L⁻¹ in the batch phase. In the glucose feeding phase, 222.08 mg L⁻¹ additional resveratrol was accumulated on the feeding of 15.93 g L⁻¹ glucose (Fig. 5) and resulted in a final titer of 415.65 mg L⁻¹ resveratrol. In the ethanol feeding phase, 333.57 mg L⁻¹ resveratrol was additionally produced from 16.63 g L⁻¹ ethanol resulting in a final titer of 531.41 mg L⁻¹ resveratrol. Small amounts of acetate and glycerol and no other by-products, such as *p*-coumaric acid or succinate were detected during the fed-batch process.

Table 5

Comparison of resveratrol titers and kinetic parameters obtained from different resveratrol producing strains in batch cultivations.

		ST4135	ST4136	ST41222	ST4140	ST4152
μ_{\max} (h ⁻¹)	Glucose phase	0.40 ± 0.01	0.35 ± 0.03	0.36 ± 0.01	0.33 ± 0.04	0.15 ± 0.02
	Ethanol phase	0.10 ± 0.00	0.09 ± 0.01	0.08 ± 0.01	0.07 ± 0.02	0.018 ± 0.00
Production (mg L ⁻¹)	Glucose phase	0.11 ± 0.00	0.64 ± 0.23	0.13 ± 0.02	0.62 ± 0.00	61.69 ± 8.72
	Ethanol phase	2.73 ± 0.11	4.34 ± 0.68	3.41 ± 0.40	6.01 ± 0.44	172.04 ± 13.12
Yield (mg g ⁻¹ glucose)	Glucose phase	0.00 ± 0.00	0.02 ± 0.01	0.00 ± 0.00	0.02 ± 0.00	2.10 ± 0.30
	Ethanol phase	0.25 ± 0.00	0.39 ± 0.06	0.31 ± 0.05	0.51 ± 0.01	12.14 ± 1.02
Productivity (mg (L ⁻¹ h ⁻¹))	Glucose phase	0.01 ± 0.00	0.05 ± 0.01	0.01 ± 0.00	0.04 ± 0.00	1.67 ± 0.24
	Ethanol phase	0.27 ± 0.01	0.39 ± 0.06	0.24 ± 0.03	0.25 ± 0.02	4.92 ± 0.37

**Fig. 5.** Fed-batch fermentation of the optimized resveratrol-producing strain ST4152. Aerobic fed-batch fermentations were carried out by feeding glucose (A) or ethanol (B) respectively following carbon source-limited feeding strategy. The cultivations were performed in duplicates (Supplementary Fig. S2); here representative graphs are shown.

4. Discussion

In the previous studies resveratrol production was achieved by supplementing *p*-coumaric acid (Becker et al., 2003; Beekwilder et al., 2006; Shin et al., 2011; Sydor et al., 2010; Wang and Yu, 2012), which is an expensive precursor. Besides, tyrosine or phenylalanine were also used as the substrates for resveratrol production (Trantas et al., 2009; Zhang et al., 2006). Also several previous studies described resveratrol production without supplementation of precursor, but in SC-dropout (Wang et al., 2011) or Yeast extract-Peptone-Galactose (Shin et al., 2012) media, which contain tyrosine and phenylalanine. This study, for the first time, demonstrates *de novo* microbial production of resveratrol from cheap carbon source (glucose or ethanol) in a minimal medium. We chose *TAL* instead of *PAL* for the first step of resveratrol biosynthesis pathway (deamination from tyrosine or phenylalanine), thus by-passing the *P450*-dependent step. Although previous studies failed in producing resveratrol via the *TAL*-dependent pathway in yeast (Zhang et al., 2006), our study shows that it is possible to construct a high-level resveratrol producing yeast based on the *TAL* route. The resveratrol production of 530 mg L⁻¹ obtained in this study, to our knowledge, is the highest level reported in yeast.

This study adopted a push and pull strategy by increasing precursor supply and multiple integration of resveratrol pathway to improve resveratrol production. Aromatic amino acids were shown to have feedback inhibition on the shikimate pathway (Koopman et al., 2012; Luttik et al., 2008). Deregulation of feedback inhibition is a general way to improve production of aromatic amino acids derivatives. This has been corroborated by overexpression of aromatic amino acids-insensitive *ARO4* and *ARO7* alleles in several studies. Koopman et al. overexpressed feedback-insensitive allele of *ARO4* (*ARO4*^{G226S}), which led to 2-fold increase

of naringenin in *S. cerevisiae* (Koopman et al., 2012). Another study by Curran et al. improved muconic acid production by 50%, when overexpressing tyrosine insensitive *ARO4* allele (*ARO4*^{K229L}) in *S. cerevisiae* (Curran et al., 2013). By combining overexpression of the feedback insensitive alleles of *ARO4* and *ARO7* (*ARO4*^{K229L} and *ARO7*^{G141S}), Luttik et al. obtained 200-fold increase of the extracellular aromatic amino acids concentration (Luttik et al., 2008). Thus, the 78% increase of resveratrol we observed upon overexpression of *ARO4*^{K229L} and *ARO7*^{G141S} is consistent with these studies. Supply of the other precursor, malonyl-CoA, catalyzed by *Acc1p* was a well-known limiting step in the fatty acids biosynthesis pathway due to posttranslational regulation by *Snf1* protein kinase (Tehlivets et al., 2007). Shi et al. found that the phosphorylation of *Acc1p* by *Snf1* could be diminished by site-directed mutagenesis at Ser659 and Ser1157 positions (Shi et al., 2014). Overexpression of *ACC1*^{S659A,S1157A} in engineered *S. cerevisiae* resulted in 3-fold increase of fatty acid ethyl esters production and more than 3.5-fold improvement of 3-hydroxypropionic acid titer (Shi et al., 2014). The 31% improvement was obtained in this study, when we overexpressed *ACC1*^{S659A,S1157A} in the engineered strain. However, it was surprising that further overexpressing *ALD6* and *SeACS*^{L641P}, which was previously shown to enhance acetyl-CoA flux (Shiba et al., 2007), decreased resveratrol production. We suppose that in the strain with a single copy of resveratrol biosynthesis pathway the flux control resided mainly with the downstream of the pathway and therefore the push strategies had only a limited effect. In case of *ALD6* and *SeACS*^{L641P} overexpression, the negative result was possibly due to the effect of acetyl-CoA overproduction, which exhibits genome-wide regulatory effects (Shi and Tu, 2013; Zhang et al., 2013) and may also divert to other products than resveratrol especially in glucose consuming phase. In future studies it would be interesting to

investigate the effect of increased acetyl-CoA supply in a strain carrying multiple copies of resveratrol biosynthesis.

High level-expression of the flux controlling enzymes in the pathway is a common solution to a low enzymatic activity problem. We chose to integrate multiple copies of the resveratrol biosynthetic genes into yeast chromosomes as a pull strategy. In comparison to the high-copy number episomal vectors, e.g., 2 μ -based vectors, integration results in better strain stability. A 36-fold improvement in titer was achieved in this study when three genes, *HaTAL*, *At4CL1* and *VvVST1*, were integrated in multiple copies. This finding highlights that indeed the resveratrol flux was limited by the activity of the biosynthetic enzymes. At the same time, the strain fitness decreased, manifested by a longer lag phase, lower maximum specific growth rate and lower biomass yield on glucose. This could be expected as multiple integration of several genes likely resulted in high metabolic burden for the cell. A solution to the problem could be replacement of constitutive promoters with regulated ones for control of the resveratrol biosynthesis pathway, e.g., one could use a glucose-repressed P_{HXT7} promoter, so the resveratrol biosynthesis is first turned on in the fed-batch phase after sufficient biomass has been accumulated in the batch phase. Moreover, the observation that resveratrol primarily accumulated in the ethanol phase of batch fermentation inspired us to use ethanol feed for fed-batch fermentation. An ethanol pulse feed strategy was also successfully applied earlier for amorphaadiene production (Westfall et al., 2012).

Developing a high-producing microbial strain for industrial production of resveratrol is a serious metabolic engineering challenge. In our study, we implemented the push strategies first, followed by the pull strategy. Retrospectively, it would be better to do it the other way around. Future development of the cell factory may include optimization of activities of the resveratrol biosynthetic genes (via enzyme engineering, protein scaffolding or expression level balancing), further improvement of precursor supply, improvement of reducing co-factor (NADPH) supply, eliminating product degradation (Koopman et al., 2012), decoupling of growth and production phases, and other rational designs. Additionally genome-scale modeling or -omic data can be used to predict non-intuitive metabolic engineering strategies (Curran et al., 2013; Gold et al., 2015).

5. Conclusions

There is an increasing demand for resveratrol in the pharmaceutical, food and cosmetic industries. Production of resveratrol from a cheap carbon source by microbial fermentation is attractive due to short process time, reduction of production costs, reliable supply and high purity, when compared to the extraction from plant materials. In this study we constructed the resveratrol pathway via tyrosine intermediate in *S. cerevisiae* and for the first time demonstrated the possibility of *de novo* resveratrol biosynthesis from glucose. By step-wise metabolic engineering, targeted towards improvement of precursor supply and increased expression of the biosynthetic genes, we obtained an optimized strain, which produced around 0.5 g L⁻¹ resveratrol in fed-batch fermentation on a minimal medium. This strain represents a good basis for development of microbial resveratrol production process.

Author contributions

M.L., I.B. and J.N. conceived the study. M.L., K.R.K. and I.B. designed the experiments. M.L. performed most of the experimental work and A.R. performed parts of the experimental work.

M.L., Y.C., I.B., and J.N. analyzed the results. M.L., I.B., and J.N. wrote the manuscript.

Competing financial interests

The authors declare no competing financial interests.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.ymben.2015.08.007>.

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