Biosensor-Enabled Directed Evolution to Improve Muconic Acid Production in *Saccharomyces cerevisiae*

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Abbreviations: PET, polyethylene terephthalate; **ALE,** adaptive laboratory evolution; **AAA,** aromatic amino acids; **PCA,** protocatechuic acid; **EMS,** ethyl methanesulfonate, **WT,** wild type strain; **ENG,** engineered strain

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Abstract

Muconic acid is a valuable platform chemical with potential applications in the production of polymers such as nylon and polyethylene terephthalate (PET). The conjugate base, muconate, was previously biosynthesized in the bacterial host *Escherichia coli*. Significant pathway engineering led to the first reported instance of rationally engineered production of muconic acid in the yeast *Saccharomyces cerevisiae*. To further increase muconic acid production in this host, we employed an adaptive laboratory evolution (ALE) strategy to complement rational metabolic engineering. To this end, we adapted a biosensor module that responds to the endogenous aromatic amino acid (AAA) as a surrogate for pathway flux. Following two rounds of ALE coupled with an anti-metabolite feeding strategy, we isolate strains with improved AAA pathway flux. Next, we demonstrate that this increased flux can be redirected into the composite muconic acid pathway with a 3-fold increase in the total titer of the composite pathway compared to our previously engineered strain. Finally, we complement a truncation of the penta-functional ARO1 protein and overexpress an endogenous aromatic decarboxylase to establish a final strain capable of producing 0.5 g/L muconic acid in shake flasks and 2.1 g/L in a fed-batch bioreactor with a yield of 12.9 mg muconic acid / g glucose at the rate of 9.0 mg / hour. This value represents the highest titer of muconic acid reported to date in *S. cerevisiae*, in addition to the highest reported titer of a shikimate pathway derivative in this host.

1 Introduction

Metabolic Engineering has enabled the biological production of a wide range of chemicals including fuels, pharmaceuticals, nutraceuticals, polymers, and their precursors [1-3]. In this regard, the unsaturated dicarboxylic acid muconic acid has been a target of particular interest over the past 15 years as an alternative, green platform chemical (i.e. benzene- and NO_2-free) leading to downstream products such as adipic acid and terephthalic acid [4]. These monomers can likewise feed into the over $22 billion per year market associated with nylon and PET [5, 6]. Thus, establishing a high-flux, biosynthetic route to muconic acid can enable high-volume polymer production from renewable feedstocks with reduced environmental impact.

While *Escherichia coli* has served as a high titer host for muconic acid reaching levels of 18 g/L [4], there are challenges associated with using this host to produce similar acid products including the high alkali load required to maintain neutral pH levels [7]. In contrast, cultivations that occur at a pH more closely matching the product pKa value will incur significant savings in downstream separations [8]. To this end, the yeast *Saccharomyces cerevisiae* has been touted as an attractive host for producing muconic acid and similar organic acid products [9]. However, significant efforts involving traditional metabolic engineering, enzyme screening, and metabolic flux analysis have only produced titers of 1.56 mg/L and 141 mg/L [10, 11] in *S. cerevisiae*. More recent work employing protein engineering and bioreactor scale-up have led to a further improvement of titer to nearly 560 mg/L [6]. Yet, these titers clearly do not match the levels seen in *E. coli* and two major issues stymie the production of this molecule in yeast. First, there is a challenge in obtaining highly functional pathway enzymes that fully convert all of the pathway intermediates (esp. protocatechuic acid, or PCA) into muconic acid. Second, the overall pathway flux toward secondary metabolites derived from the shikimate pathway has been a significant challenge due to low precursor availability and a high degree of regulatory control in *S. cerevisiae* [12-14]. These challenges are apparent in other attempts to produce molecules such as naringenin that have only obtained titers of 102 mg/L [15]. The only example of a high-titer shikimate

derivative occurred through the replacement of yeast metabolism with multi-step pathways from *E. coli*. In this case, a titer of 1.93 g/L of p-coumaric acid was obtained [14]. To address these inherent metabolic and regulatory limitations, we sought to employ an adaptive evolution approach to improve muconic acid titers.

Whole-cell adaptive laboratory evolution (ALE) schemes have been effective at increasing growth rates and tolerances [16-20]. However, these approaches are *prima facie* limited to growth-based selection without an alternative mode of screening or selection. Some recent evolutionary strategies exploit a selectable feature of the compound of interest [21, 22] or rely on an anti-metabolite strategy to link the phenotype to growth [23, 24]. As an alternative, the use of biosensors is greatly expanding the potential for ALE applications [25, 26]. Specifically, biosensors can transduce the signal of a small molecule metabolite to either a growth-selectable phenotype (e.g. antibiotic resistance) or a reporter for high-throughput assays (e.g. a fluorescent protein). Recently, ALE coupled with a fluorescent biosensor was used to improve L-Valine production in *C. glutamicum* [27], resulting in 25 % improvement in titers and a 3-4 fold reduction in byproduct formation. Likewise, ALE was coupled with a growth-selectable biosensor (*glmS* riboswitch-based) in *S. cerevisiae* to screen for enzymes capable of synthesizing N-acetyl glucosamine, resulting in its first production in yeast at over 3 mg/g dry cell weight [28]. In another instance, feedback-regulated evolution of phenotype (FREP) was performed by utilizing a sensor to link the concentration of a metabolite to the mutation rate of *E. coli*. This technique resulted in a 5-fold increase in tyrosine and 17-fold increase in lycopene production [29]. Collectively, these selected examples demonstrate the efficacy of a biosensor-enabled ALE approach.

Here, we demonstrate the first coupling of transcription-factor enabled and anti-metabolite-driven ALE in *S. cerevisiae* to improve muconic acid titers. Specifically, we employ our previously established aromatic amino acid (AAA) biosensor that is marked by varying strength and inducibility [30]. Next, this biosensor was coupled with an anti-metabolite selection scheme to screen for improved AAA production phenotypes, as a surrogate to improve muconic acid

production. In doing so, we developed a strain with up to 2-fold higher total AAA production. When complemented with the composite muconic acid pathway, we create a strain that reaches titers of 2.1 g/L muconic acid in bioreactors. This titer represents the highest production of muconic acid in yeast to date, as well as one of the highest production levels for a shikimate pathway derivative.

2 Materials and Methods

2.1 Strains and Media

Yeast expression vectors were propagated in *Escherichia coli* DH10β. *E. coli* strains were cultivated in lysogeny broth (LB, Teknova) at 37°C with 225 RPM orbital shaking. LB was supplemented with 50 µg/mL ampicillin (Sigma) for plasmid maintenance and propagation. Yeast strains were cultivated on a yeast synthetic complete medium containing 6.7 g/L of Yeast Nitrogen Base (YNB, Difco), 20 g/L glucose and a mixture of amino acids, and nucleotides without uracil, leucine, histidine or tryptophan as required to maintain respective plasmids (CSM, MP Biomedicals). All formulations were supplemented with 1.5 % agar for solid media. Yeast and bacterial strains were stored at -80°C in 15 % glycerol.

Saccharomyces cerevisiae BY4741 was obtained from Euroscarf, (*Mat a; his Δ1; leu2Δ0; met15Δ0; ura3Δ0*) while "ENG" refers to the MuA10 engineered strain from Curran *et al* [11]. lacking the Ty2d::PGPD-ECL_01944opt integration (Genotype: *Mat a; his Δ1; leu2Δ0; met15Δ0; ura3Δ0; aro3Δ; aro4Δ::PGPD-aro4k229l; zwf1Δ*).

2.2 Plasmid and Strain Construction

Oligonucleotides were synthesized by Integratd DNA Technologies (**Table S1**). PCR was performed using Phusion or Q5 Hot Start polymerases (NEB). Restriction enzymes were purchased from NEB and reactions performed as per manufacturer's recommendations. PCRs and digestions were cleaned up using the PCR Cleanup Kit (Qiagen). Antarctic phosphatase (NEB) was used to

prepare vectors for ligations. Ligation reactions (Fermentas) were performed from 3-18 hours at 16℃. Gibson assembly [31] was used to construct the PugM vector with ECL_01944opt under control of the UASCLB–UASCIT–UASTEF-GPD hybrid promoter [32] followed by the T-prm9 high capacity terminator [33] with parts amplified with primers listed in **Table S1**. The PugM and p425-TEF-pa5_5120opt/GPD-caHQD2opt vectors were used to amplify parts to construct p426-TEF-pa5_5120opt-Tcyc-GPD-ECL_01944opt-Tprm9 through Gibson Assembly. MoClo assembly was used to construct the *PAD1* integration vector. A "Type 3" entry vector was built for scPAD1 using BsmbI Golden Gate assembly. The *PAD1* integration vector, scPAD1-IV, was assembled from "part vectors" listed in **Table S1** using BsaI Golden Gate assembly [34].

Yeast transformations were performed using both the EZ Yeast II Transformation Kit (Zymo Research) according to manufacturer's recommendations and the Geitz transformation protocol [35], both using 50 µL of chemically competent cells mixed with 500 ng of plasmid DNA, heat shocked and transformants selected on CSM-dropout plates at 30℃ for 2-5 days depending on growth rate. Single colonies were then inoculated into 4ml of CSM-dropout media and glycerol stocked prior to assay. Strains utilized and evaluated in this study are provided in **Table S2**.

2.3 Growth Rate Analysis

Strains of interest were pre-cultured for 3 days in the appropriate selective medium, and 1 µL of this pre-culture was used as an inoculum for a 250 µL culture in the same selective medium. Growth rate measurements were then obtained using a Bioscreen C (Growth Curves USA).

2.4 Flow Cytometry

For the initial biosensor assay, yeast cultures were inoculated in triplicate from glycerol stock in CSM-URA for two days at stationary phase. All cultures were then inoculated at an OD_{600} of 0.01 in fresh media and grown to mid-log phase in 30℃ orbital shaker for 14-16 hours. Induction

was accomplished by subculturing from stationary phase in CSM into media containing 500 mg/L L-Tryptophan, 500 mg/L L-Phenylalanine or 100 mg/L Tyrosine. Fluorescence was analyzed on the Fortessa flow cytometer (BD Biosciences) using the yECitrine fluorophore. 10,000 events were gathered at a flow rate of 1,000 events per second and analyzed using the FlowJo software suite.

For the integrated fluorescent biosensor assay, yeast cultures were inoculated in triplicate from glycerol stock in CSM-HIS in a 96-deep well block, 1ml culture volume and the fluorescence analyzed using the Accuri flow cytometer (BD Biosciences). An average of the fluorescence and standard deviation within biological replicates is provided. All experiments within a single graph were conducted on the same day at the same time.

2.5 EMS Mutagenesis and Subculturing Procedure

The ethyl methanesulfonate (EMS) mutagenesis procedures were performed following the protocol described by Winston [36]. Briefly, an overnight culture was cultivated to OD_{600} of 1.0, cells were then harvested, washed, and suspended with 0.1 M sodium phosphate buffer (pH 7). 30 µL of EMS was added and incubated along with an un-mutagenized control for 1 h at 30℃, with agitation. The cells were then washed twice with 5 % sodium thiosulfate to eliminate residual EMS and the cells were allowed to recover in CSM-URA. This procedure was repeated on a portion of mutagenized cells. Kill fraction was calculated by plating a fraction from mutagenized and control populations.

Following EMS mutagenesis, recovered cells were sequentially subcultured in 30ml of CSM-URA media. After reaching stationary phase, 100 µL of cells were transferred to 30ml of fresh selective media with increasing concentrations of G418 and analog. During this first round of selection, the populations were subcultured six times and cultured for over 750 hours. At this point, the unmutagenized control populations were unable to grow in the selective media conditions whereas the mutagenized populations continued to grow in 1000 mg/L G418 and 2 mM 4-FP.

During the second round of selection, following recovery from EMS, the cultures were inoculated into 1000 mg/L G418 and 1 mM 4-FP. Over the course of selection, the 4FP selective concentration was increased to 7.5 mM which resulted in an observed growth rate difference between the populations. Following selection, colonies were plated and isolated. Colonies were selected and the strain was grown in YPD plus 1 g/L 5-flouroorotic acid to encourage loss of the URA3 containing pSH47 plasmid [37].

2.6 Tyrosine Quantification

Tyrosine quantification was confirmed using nitrosonaphthol derivatization detection, but each with distinct culture conditions. This iteration allowed for us to account for potential changes in growth rate which may affect productivity, with similar controls performed under all condition to allow for inter-assay comparison. The first protocol used to quantify tyrosine production reported in section 3.3 used cultures directly selected from CSM-URA plates following selection. OD_{600} measurements were taken and 100 µL of supernatant were isolated and assayed using nitrosonapthol chemical derivation using nitrosonapthol derivatization which had been previously developed [38] and analyzed using the BioTek Cytation 3 plate reader. Tyrosine concentration was calculated based on standards and production.

The second protocol used to quantify the tyrosine production used cultures directly selected from YPD plates into 4 ml of YPD liquid media. OD_{600} measurements were taken and cells were resuspended to reach 0.5 OD_{600} in 4ml of fresh 2 % glucose CSM. After 48 hour of culturing, 300 µL of supernatant extracted and quantified with the derivatization protocol listed above. The third protocol used to quantify tyrosine production used cultures started directly from glycerol storage and placed into 4 ml of 4 % glucose CSM. OD_{600} measurements were taken and cells were resuspended to reach 0.1 OD_{600} in 30ml of fresh 4 % glucose CSM in 250 ml shake flasks. After 70 hour of culturing, 300 µL of supernatant extracted and quantified with the derivatization protocol listed above.

The fourth and final protocol was used to quantify tyrosine production using 96-deep well blocks inoculated from glycerol stock and grown to stationary phase. OD_{600} was measured with BioTek Cytation 3 plate reader and the block spun down and cells washed with water. The cells were then cultured overnight in minimal media without YNB. After 16 hours of incubation, the cells were pelleted and the supernatant extracted. Tyrosine in the supernatant was quantified using nitrosonaphthol derivatization described above. Tyrosine concentration was calculated based on standards and production normalized per OD unit.

2.7 Flask and Bioreactor Fermentations

For flask scale fermentations, strains were pre-cultured in 4mL aliquots until they reached stationary phase and used to inoculate 30 mL flask cultures at OD_{600} = 0.1. At designated times (72, 120 and 168 h), the OD_{600} was measured and a 1.5 mL sample was taken and pelleted for 5 min. at 3000 x g. The supernatant was filtered using a nylon 0.2 mm syringe filter from Corning Incorporated and components quantified using HPLC.

Duplicate bioreactor fermentations were run at a 1.7 L working volume in a Bioflo 115 system (New Brunswick) using 0.6 g/L CSM-LUHW with 40 g/L glucose and 6.7 g/L YNB (without amino acids) as a fed-batch process with three media spikes/feeds throughout the run. Media feeds contained the same components as the initially charged media, but concentrated 10-fold to enable 0.17 L spikes (i.e. 1.02 g CSM-LUHW, 68 g glucose, 11.39 g YNB in 0.17 L total volume). All fermentations were inoculated to an initial OD_{600} = 0.2 in 1.7 L of media. Dissolved oxygen was maintained above 20 % air saturation through cascaded agitation with a constant air input flow rate of 0.5 SLPM (Standard Liters Per Minute). The pH was maintained at 5.0 with 1M NaOH and temperature was maintained at 30°C. Throughout the 10-day process, samples were removed from the reactor for HPLC determination of glucose, PCA, catechol, and muconic acid concentrations in the supernatant.

2.8 HPLC Analysis

High performance liquid chromatography (HPLC) was used to measure the production of muconic acid and pathway intermediates in *S. cerevisiae* cultures. Supernatant samples were separated using a HPLC Ultimate 3000 from Dionex with dilution as required. Composite pathway products were quantified using the Zorbax SB-Aq column from Agilent Technologies. A 10.0 μL injection volume was used in a mobile phase composed of an 84:16 ratio of ddH2O with 0.1 % Trifluoroacetic Acid to acetonitrile with 0.1 % TFA with a flow rate of 1.0 mL/min. Column temperature was maintained at 30℃ and UV–Vis absorption was measured at 280 nm. Peaks were compared to a standard curve including PCA, catechol, and cis,cis-muconic acid, purchased from Sigma-Aldrich. Cis, trans-muconic acid had been previously provided by Draths Corporation. Reported value represents the highest titer reached during the three timepoints assayed.

Glucose quantification was accomplished using the Aminex HPX-87P Carbohydrate Column from Bio-Rad Laboratories with RI detection performed by Refractomax 520 modular unit. The mobile phase was 100 % ddH2O, with a flow rate of 0.6 mL/min. The column temperature was maintained at 85℃ based on the manufacturer's recommended protocol.

3 Results

3.1 Configuring the Biosensor for Adaptive Laboratory Evolution

Previous approaches for engineering shikimate derivatives in yeast have focused on rational engineering as high-throughput screens are generally lacking. In previous work, we characterized aromatic amino acid (AAA)-responsive hybrid promoters that were constructed through tandem insertions of the UASaro element upstream of a minimal core promoter [30]. Here, we adapt this

biosensor for use in ALE experiments to increase the production of AAAs that could subsequently be re-directed into muconic acid biosynthesis.

While Aro9p-based biosensors are induced through exogenous feeding of AAAs [30, 39, 40], they have not been demonstrated to discern endogenously produced AAAs in the capacity required here. Moreover, ALE applications will require that these biosensors have further inducible capacity in an engineered strain. To evaluate this capacity, we transformed the biosensor plasmid p416-1xUASaro-Leumin-Yecit into BY4741, wild-type (WT) yeast and our previously engineered muconic acid production strain containing *aro3, aro4* and *zwf1* deletions and an integrated feedback resistant $aro4^{k229l}$ (hereafter referred to as ENG [11]). These cells were cultured in Complete Synthetic Media (CSM) with and without additional AAA supplementation and screened via flow cytometry. **Figure 1A** demonstrates that this Aro9p-based biosensor provides an appropriate, holistic output for both intracellular and extracellular AAA concentration in both the WT and ENG strain. Importantly, the biosensor in the ENG strain has a higher basal expression level than in the WT strain, as expected, and this signal can be further induced through exogenous amino acid supplementation. These results demonstrate that this biosensor can be used for ALE of AAA production.

3.2 Enabling a growth-selective ALE of AAA production

After establishing the capacity of the Aro9p-based biosensor, we sought to enable a growth selective ALE scheme using this construct. To do so, we used this AAA inducible hybrid promoter to drive the expression of the KanNeo gene isolated from the piTY vector [41] by replacing the yECitrine for KanNeo to create the biosensor plasmid p416-4xUASaro-Leumin-KanNeo. This gene is known to confer weak resistance to geneticin (G418) in yeast. Moreover, this low-level resistance has been previously used to enable tandem-integrations [42], thus establishing a likely dose-response for this gene. To further enhance selective capacity in our scheme, we sought to

couple this selection with the addition of a known anti-metabolite (specifically, 4-fluorophenylalanine (4FP)) to more reliably direct mutations toward improved AAA production.

To test the efficacy of this scheme, we evaluated growth of the WT strain under distinct G418 and anti-metabolite concentrations (**Figure 1B**). Specifically, we observe that 200 mg/L G418 only slightly reduced growth rate whereas 400 mg/L completely abolished growth in the control and moderately reduces growth in the strain bearing the KanNeo-driven biosensor. Moreover, we demonstrate that this reduction in the KanNeo-driven biosensor strain can be recovered by adding exogenous tryptophan. Interestingly, the presence of exogenous tryptophan appeared to sensitize the control strain to the G418 antibiotic. Likewise, 4FP was evaluated for use in conjunction with biosensor-KanNeo selection. Compounds like 4FP were previously demonstrated to be toxic through competitive inhibition with AAAs [43, 44]. In line with these previous results, the coupling 4FP with 400mg/L G418 appear to be quite selective resulting in near zero growth of the strain. Thus, these results establish a potent selection scheme for improved AAAs.

3.3 ALE for Improved Aromatic Amino Acid Production

ALE schemes have been conducted both with natural drive and forced mutations using checmical mutagenesis [45]. Here, we sought to use both through a sequential subculturing with increasing concentrations of G418 and 4FP. To accomplish this, EMS mutagenesis was conducted as described previously [36]. The ENG strain bearing the p416-4xUASaro-KanNeo plasmid was mutagenized to yield two mutant populations (Single / 1x 99+% lethal, and double / 2x 99+% lethal) and one un-treated control. Performing this treatment in duplicate resulted in six populations which were subcultured at stationary phase into media containing successively increasing concentrations of G418 (antibiotic) and 4FP (anti-metabolite). The overall growth trajectory of these six populations (labeled Pop 1-6) is reported in **Figure 2A**. After 750 hours of subculturing (roughly 6 rounds), the un-mutagenized control populations were unable to grow in the selective media conditions whereas the mutagenized populations continued to grow in 1000 mg/L G418 and

2 mM 4-FP. This growth suggested that at least some fraction of cells in the population produced more AAA than the parental strain.

To confirm improved AAA production, individual colonies were isolated. These colonies were labeled according to the population from which they were isolated (i.e. ALE1.01 denotes the first isolate from Population 1, and ALE 5.02 corresponds to the second isolate from Population 5). Colonies were directly inoculated into liquid CSM and quantified for tyrosine production using a plate-based nitrosonapthol-derivatization quantification [38] (**Figure S1)**. On the basis of these results, ALE-1.01, ALE-2.08 and ALE-5.01 were selected as starter strains for a second round of ALE.

Prior to re-initiating our ALE strain, we cured the biosensor plasmid using counterselection with 5-FOA to reduce the likelihood of cassette integration and/or mutagenesis. Following biosensor re-transformation, EMS mutagenesis was then repeated to generate 9 new populations. The 1x and 2x 99 % kills or no-EMS controls are represented by a 1, 2, or 0 in the final digit of the population name, respectively (i.e. ALE1.01.1 represents a second 1x 99 % kill derived from the ALE1.01 parent, and ALE5.01.2 corresponds to a second 2x 99 % kill derived from ALE 5.01). This second round selection started with 1000 mg/L G418 and 1 mM 4-FP with a gradual ramping of 4FP concentration to 7.5 mM (**Figure 2B)**. These concentrations are quite selective as the first-round mutants failed to grow in 1000 mg/L G418 and 2 mM 4-FP. After 575 hours, individual isolates were once again established for tyrosine using CSM media containing 2 % and 4 % glucose in the media (**Figure S2** and **Figure S3**). The 4 % glucose CSM condition was included as it best matches media conditions used to cultivate the MuA12 strain that previously achieved muconic acid titers of 141 mg/L [11]. The top strains were also characterized on the basis of tyrosine produced per OD600 unit in an overnight culture with media lacking amino acids or nitrogen supplementation (**Figure S4)**.

To complement this tyrosine assay, we employed our Aro9p-biosensor to estimate the total AAA pool. This biosensor comprised the 5xUASaro elements linked with the CORE1 minimal

synthetic core promoter [30, 46]. This promoter was cloned into an EasyClone vector with the yECitrine reporter [47] to form p-XII-5xUASaro-CORE1 and integrated into a neutral, high expression locus on Chromosome XII [47] in the seven selected ALE strains. As shown in **Figure 3**, ALE-2.08 and ALE-5.01 strains did indeed possess increased total AAA production based on this assay which suggests that accrued mutations may be more linked with phenylalanine and/or tryptophan increases instead. ALE-5.01.1.02 appears to be a strong performer from the second round. These results demonstrate successful utility of ALE to improve AAA production, a surrogate target for our desired molecule of muconic acid.

3.4 Muconic Acid Production using Evolved Strains

Following the ALE scheme, we sought to re-direct this improved AAA flux toward the muconic acid biosynthetic pathway. To do so, we transformed the muconic acid pathway into the ALE strains as well as the ENG base strain and analyzed production via HPLC. This pathway consisted of three enzymes: a dehydroshikimate dehydratase from *Podospora anserine* (AROZpodo), protocatechuic acid decarboxylase from *Enterobacter cloacae* (ECL_01944opt), and catechol 1,2-dioxygenase from *Candida albicans* (caHQD2opt) [11]. The interface of this pathway with the *S. cerevisiae* shikimate pathway is schematically represented in **Figure S5**.

Rather than relying on inherent variations from tandem integrations as used in our previous publication [11], we first constructed an integration vector PugM to facilitate high transcriptional strength integration of ECL_01944opt. The resulting cassette was integrated into the *TRP1* locus using the p416-Gal-CAS9 genome editing system [48]. Next, the following plasmids were sequentially transformed and functionally confirmed through HPLC analysis: p425-AROZ-HQD2, p426-AROZ- ECL_01944opt and p413-TKL1 to create strains MuA-1.02.1.04, MuA-1.02.2.01, MuA-5.01.1.02 and MuA-5.01.2.01. The production from this composite pathway in the ALE strains were compared with that of the previously developed ENG strain (MuA13) (**Figure 4A**). These results demonstrate a nearly 3-fold increase in the total titer of the composite pathway relative to the ENG strain, a promising outcome from ALE.

3.5 Expressing an *aro1* Truncation to Further Improve Flux

Next, we sought to obtain further improvements for muconic acid by bypassing AAA production in these ALE strains. The yeast shikimate pathway is largely consolidated into one pentafunctional enzyme, Aro1p, converting DHAP through to shikimate. The muconic acid pathway begins with DHS, one of the key intermediates in this Aro1p catalyzed pathway. Prior efforts to redirect this flux branch have focused on replacing Aro1p with a corresponding heterologous pathway from *E. coli* [49] and through point mutations based on homology modeling [6]. To drive more flux toward the muconic acid pathway, we proposed truncating the pathway to accumulate more DHS. Fortunately, the catalytic step that commits DHS (the precursor for PCA) into the shikimate pathway resides at the C-terminus of Aro1p [50, 51]. However, a cell with a truncated shikimate pathway would be unable to grow without supplementation of AAA (the end product of the shikimate pathway). This specific enzyme is encoded by *aroE* in *E. coli*. Thus, truncating Aro1p and complementing with (a less well-expressed) *aroE* would establish a balance between net pathway flux and flux into the remainder of the shikimate pathway.

In order to test for a successful truncation (i.e. all four of the remaining catalytic activities intact), we performed a complementation assay. Specifically, a complementation with *aroE* would be viable only if the four remaining catalytic functions of the truncated Aro1p could combine with *aroE* to form a complete shikimate pathway. Such a cell would not require supplementation with aromatic amino acids for growth. Initially, we constructed an Aro1p truncation at the reported junction of DHS dehydrogenase function, codon 1305. However, this construct failed to impart growth in an *aro1Δ* strain with *aroE* complementation. Thus we screened additional variations and found that by retaining an additional 40 resides to the C-terminus from Aro1p, we were able to complement *aroE* and form a complete pathway that does not require aromatic amino acid supplementation for growth. In the absence of *aroE*, these cells fail to grow. Therefore, we

confirm that the aro1t-1345 can facilitate growth without aromatic amino acid supplementation only when co-expressed with *aroE*.

The resulting functional *aro1*t gene was then cloned into a high copy p424 vector [52] and transformed into the ALE strains expressing the composite muconic acid pathway. MuA-5.01.1.02+ *aro1*t was capable of producing approximately 1.5 g/L of total pathway intermediates, which represents 7.5 fold relative to the MuA13 strain (**Figure 4A**). Thus, the increased AAA capacity of this ALE strain could be further rewired into muconic acid.

3.6 Composite Pathway Optimization

Despite the high performance of MuA-5.01.1.02+ *aro1*t, most of the pathway capacity was not fully converted to muconic acid. Since our original work in this area, other studies have likewise discussed the poor enzymatic activity of PCA decarboxylase [6, 49]. We sought to increase conversion through the overexpression of an endogenous *S. cerevisiae* gene. Specifically, recent work identified Pad1p as a putative decarboxylase natively used to detoxify cinnamic acid [53]. Thus, to test for Pad1p conversion of PCA into muconic acid, we expressed this gene in the MuA-5.01.1.02+ *aro1*t strain and obtained a final muconic acid titer of over 550 mg/L produced in shake flasks (**Figure 4B**), a collective 4-fold increase over the MuA12 starter strain.

3.7 Bioreactor Fermentation

Finally, we sought to test the production capacity of the MuA-5.01.1.02+ *aro1*t+scPAD1 strain in a bioreactor to achieve further gains using control of pH, oxygenation, and a fed-batch process, which have previously been shown to have great impact on final titer and yield of aromatic compounds [6, 14]. **Figure 5** demonstrates that this strain is capable of producing 2.1 g/L of muconic acid after 10 days, representing the highest titer reported muconic acid from glucose in *S. cerevisiae*. Likewise, this value is the highest reported titer for any product derived from the shikimate pathway in *S. cerevisiae*.

4 Discussion

These results present the highest titer of muconic acid reported in *S. cerevisiae* to date. Certainly, the AAA pathway in *S. cerevisiae* is a highly regulated biosynthetic pathway [51, 54]. This pathway has been of interest for producing many molecules including p-coumaric acid derived from AAA [55] and muconic acid from shikimate intermediates [10, 11]. Previous work focusing on the systematic removal of feedback inhibition and rational pathway rewiring have not been able to fully de-bottleneck this pathway. Here, we hypothesized that an ALE-based approach can allow for whole-cell evolution and subsequent isolation of mutant strains with high AAA production as an effective surrogate for muconic acid. In designing an ALE strategy, it is important to carefully consider the mutation rate and selection mechanism. Here, we employed a multiple selection mechanism simultaneously (using both biosensor-driven and antimetabolite aided selection). In doing so, we reduce the likelihood of isolating only "cheaters" that can either modify the biosensor circuit itself or the general multidrug resistance of the organism. The ALE strains developed here resulted in increases in titer of our desired products. While this work was an engineering proof-of-concept experiment, certainly follow-up experiments sequencing the resulting genomes and tracking down causative mutations can lead to further targets for engineering and a better biochemical understanding. Finally, recent results on the development of other biosensors including one for muconic acid can further refine the approach and strain developed here [56]. Specifically, the use of muconic acid biosensors could facilitate protein engineering of the pathway enzymes and improve muconic acid titers while reducing those of PCA and catechol.

Through ALE and subsequent *aro1*t overexpression, we were able to redirect a significant amount of flux toward the composite pathway. However, muconic acid production is predominantly bottlenecked at PCA due to the poor PCA decarboxylase activity of *AroY* (also referred to as ECL_01944opt in this work). The limitations of this enzyme and other similar decarboxylases have been demonstrated previously [10, 11, 57]. In this work, we established

increased decarboxylation activity through overexpression of the native *S. cerevisiae* Pad1p. Pad1p is homologous to the *E. coli ubiX* enzyme that plays a role in the decarboxylation step in Coenzyme-Q biosynthesis [58]. Previous work has implicated Pad1p in decarboxylation of phenylacrylic acid [59] as well as decarboxylative detoxification of cinnamic acid and hydroxycinnamic acids [53]. Interestingly, we had previously screened Pad1p/Fdc1p as a co-expression module in the ENG strain background and observed minimal activity in that context [11]. However, overexpression of Pad1p in strains developed here led to significantly increased PCA decarboxylase activity and a resulting increase in muconic acid titers. Pad1p has recently been proposed to function as a flavin prenyltransferase capable of synthesizing the flavin cofactor needed for efficient decarboxylation by PCA decarboxylases such as AroY [60, 61]. This mechanism and the variable performance of Pad1p overexpression in different strain backgrounds suggests that cofactor-decarboxylase dynamics may differ in the current ALE strains relative to previously rationally engineered strains. Thus, this avenue represents a unique angle for further increasing titers.

The collective adaptive and rational engineering conducted here led to a strain capable of producing over 550 mg/L muconic acid in shake flasks and 2.1 g/L in duplicate fed-batch bioreactors with pH and dissolved oxygen control with a yield of 12.9 mg muconic acid / g glucose at the rate of 9.0 mg / hour. This represents a 14-fold improvement in titer over our previously reported strain and 4-fold increase relative to the highest previously reported titer of muconic acid in yeast [6, 11]. Significant work still needs to be done in order to reach industrial relevancy, but this approach establishes a promising path forward for shikimate-derived products in *S. cerevisiae*.

Author Information

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The authors declare no commercial or financial conflict of interest.

Table and Figure Legends

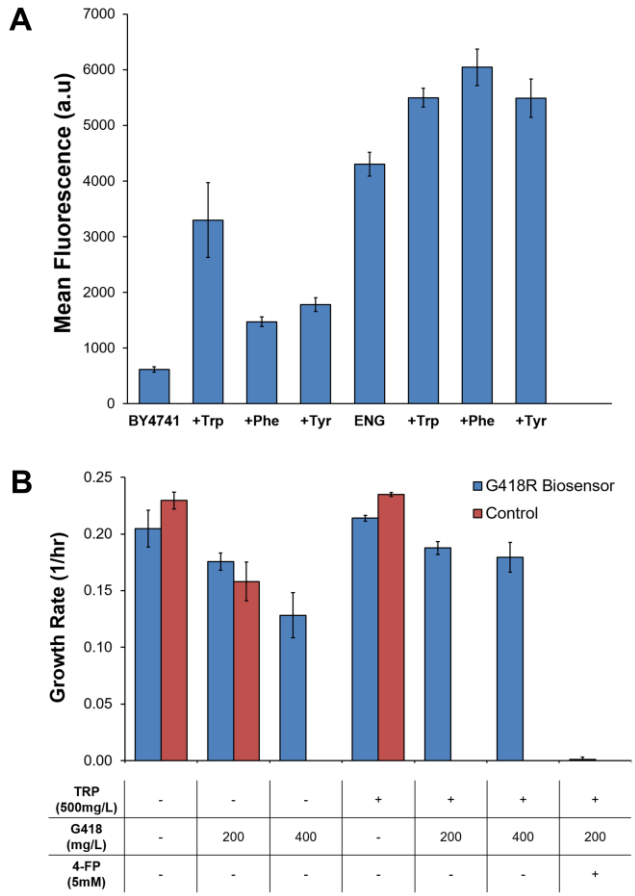

Figure 1 *Establishing an antibiotic and antimetabolite selection scheme for ALE.* **(A)** The ability of the p416-1xUASaro-Leumin biosensor to detect differences in endogenous AAA production as well as further inducible capacity to exogenous AAAs was measured in two strains—BY4741, wild-type (WT) yeast and our previously developed muconic acid strain (ENG). All samples were analyzed by flow cytometry under the same conditions. Error bars represent standard deviations across biological replicates. **(B)** Growth rate is measured for WT yeast strains expressing the 4xUASaro-KanNeo biosensor and control plasmid. The inducible capacity of the 4xUASaro-KanNeo biosensor is demonstrated by recovery of growth rate using exogenous aromatic amino acids as compared to control strain. Further reduction in growth rate is achieved using 4-Fluorophenylalanine (5 mM). Error bars represent standard deviations across technical replicates.

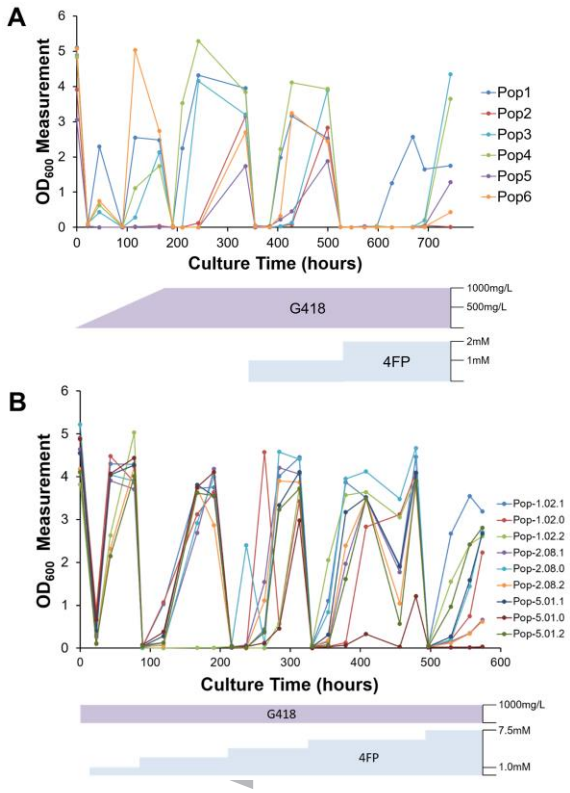

Figure 2 *Adaptive Laboratory Evolution Log.* **(A)** Following EMS mutagenesis, the six populations (1x and 2x 99 % kills and no-EMS control in duplicate) were subcultured in the presence of increasing concentrations of G418 and 4-Fluorophenylalanine. Pop1 and 4 represent 1x 99 % kills, while 2 and 4 represent 2x 99 % kills and 3 and 6 represent no-EMS respectively. T=0 represents populations at stationary phase following recovery from mutagenesis. Following 750 hours of subculturing the populations were plated and isolates screened for aromatic amino acid production. **(B)** Following EMS mutagenesis of the top three strains isolated from the first round of ALE, the 9 populations were subcultured in the presence of 1g/L G418 and increasing concentrations of 4-Fluorophenylalanine. The populations which experienced 1x and 2x 99 % kills or no-EMS controls are represented by 1, 2 and 0 in the final digit of the population name. T=0 represents populations at stationary phase following recovery from mutagenesis. After 575 hours, selection between high performing populations and selection in growth rate was achieved, the AAA production of individual isolates was quantified.

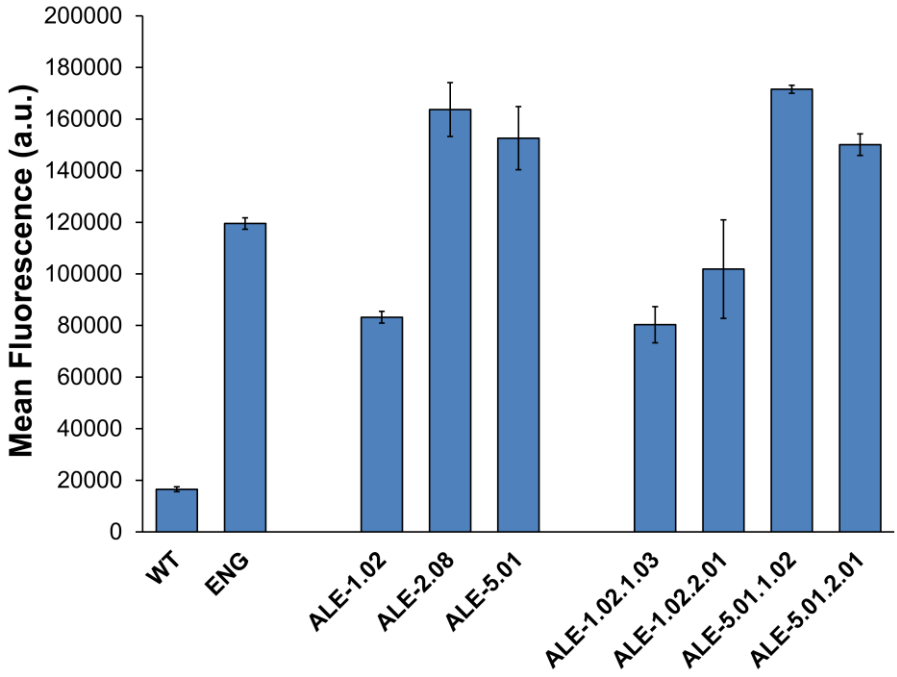

Figure 3 *Fluorescent-based Amino Acid Quantification from Isolated ALE Strains.* The overall AAA production of selected ALE strains and controls was assayed using flow cytometry by aid of an integrated biosensor expressing a fluorescent reporter. All strains were assayed at the same time under the same conditions. Error bars represent standard deviations across biological replicates.

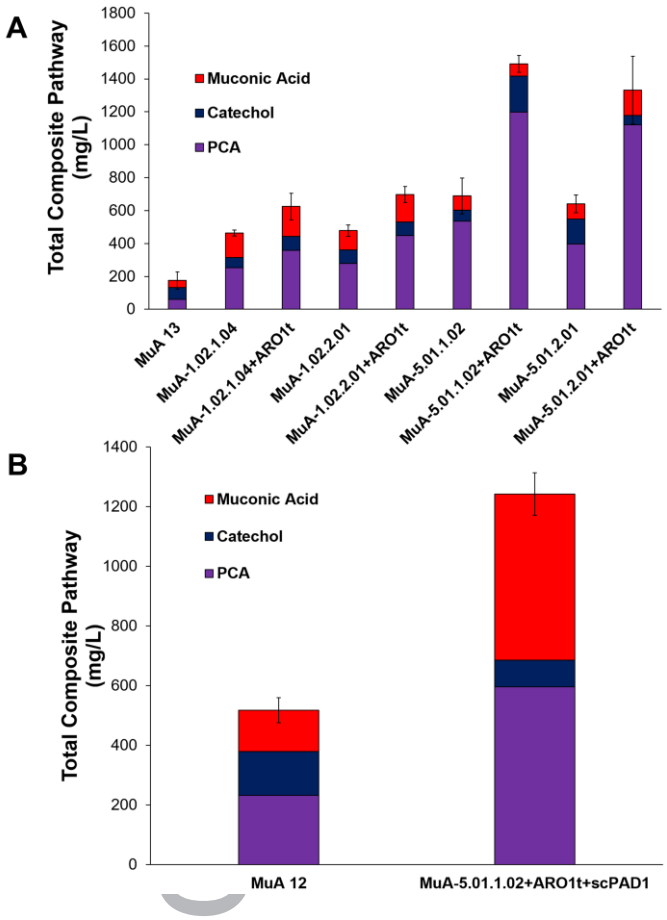

Figure 4 *Composite Pathway Production in ALE Strains.* **(A)** MuA13 represents ENG with similar pathway. Strains were cultured in the flask and total muconic acid pathway production was quantified by HPLC. Strains indicated as "+ARO1t" represent ALE strains with overexpression of the truncated *aro1*t protein rerouting flux into the composite pathway. Individual component titers indicated by color. **(B)** Muconic acid production of the MuA-5.01.1.02+ *aro1*t+scPAD1 strain in flask compared against our previously reported muconic acid producing strain MuA12. The MuA12 and MuA-5.01.1.02+ *aro1*t+scPAD1 fermentations resulted in 138 and 557 mg /L of muconic acid with yields of 3.5 and 13.9 mg muconic acid / g glucose. The MuA-5.01.1.02+ *aro1*t+scPAD1 strain incorporates our initial metabolic engineering work, ALE and final pathway rewiring through *aro1*t and scPAD1 overexpression demonstrating a 4-fold improvement in yield and titer. Individual component titers indicated by color. Error bars represent standard deviations of total composite titers across biological replicates.

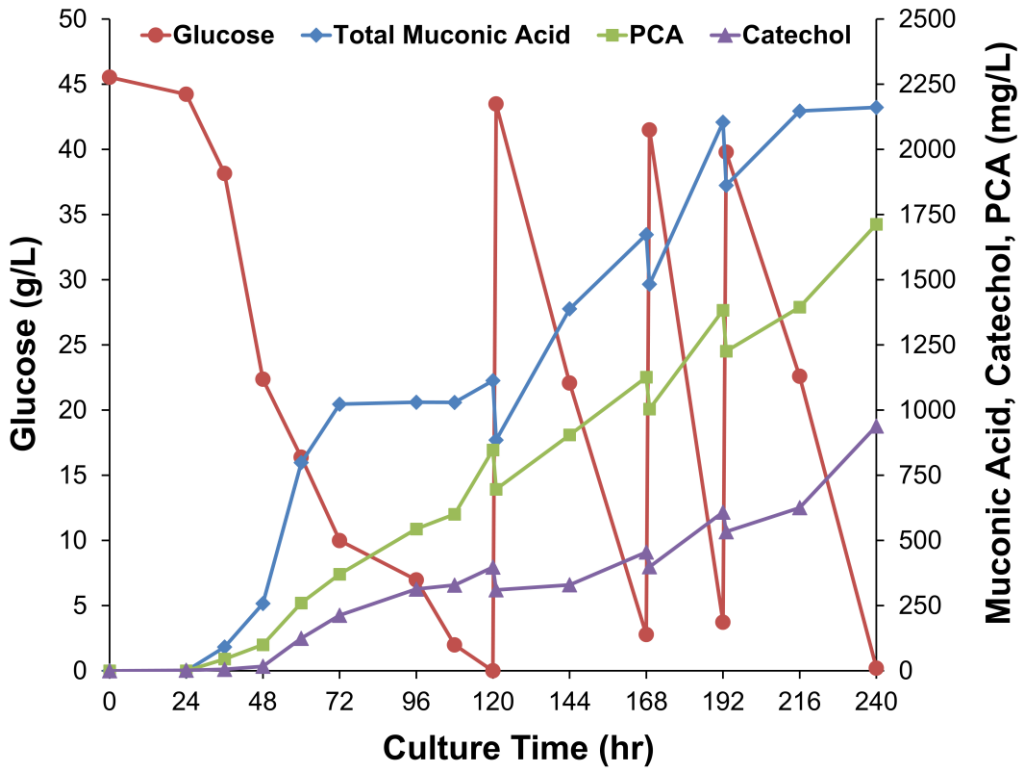

Figure 5 *Bioreactor Fermentation.* Final muconic acid producing strain MuA-5.01.1.02+ *aro1*t+scPAD1 was cultured in duplicate in a bioreactor fed-batch process, and PCA, catechol, total muconic acid, and glucose were quantified by HPLC. Three times throughout the run, as glucose was depleted or nearly depleted, additional media was spiked in to facilitate additional cell growth and production.

5 References

[1] Curran, K. A., Alper, H. S., Expanding the chemical palate of cells by combining systems biology and metabolic engineering. *Metabolic Engineering* 2012, *14*, 289-297.

[2] Lee, J. W., Kim, H. U., Choi, S., Yi, J., Lee, S. Y., Microbial production of building block chemicals and polymers. *Current Opinion in Biotechnology* 2011, *22*, 758-767.

[3] Sun, J., Alper, H. S., Metabolic engineering of strains: from industrial-scale to lab-scale chemical production. *Journal of Industrial Microbiology & Biotechnology* 2015, *42*, 423-436.

[4] Niu, W., Draths, K. M., Frost, J. W., Benzene-free synthesis of adipic acid. *Biotechnology Progress* 2002, *18*, 201-211.

[5] Xie, N.-Z., Liang, H., Huang, R.-B., Xu, P., Biotechnological production of muconic acid: current status and future prospects. *Biotechnology Advances* 2014, *32*, 615-622.

[6] Suastegui, M., Matthiesen, J. E., Carraher, J. M., Hernandez, N., *et al.*, Combining Metabolic Engineering and Electrocatalysis: Application to the Production of Polyamides from Sugar. *Angewandte Chemie International Edition* 2016, *55*, 2368-2373.

[7] Liu, P., Jarboe, L. R., Metabolic engineering of biocatalysts for carboxylic acids production. *Computational and Structural Biotechnology Journal* 2012, *3*, e201210011.

[8] Abbott, D. A., Zelle, R. M., Pronk, J. T., van Maris, A. J., Metabolic engineering of *Saccharomyces cerevisiae* for production of carboxylic acids: current status and challenges. *FEMS Yeast Research* 2009, *9*, 1123-1136.

[9] Liu, L., Redden, H., Alper, H. S., Frontiers of yeast metabolic engineering: diversifying beyond ethanol and *Saccharomyces*. *Current Opinion in Biotechnology* 2013, *24*, 1023-1030. [10] Weber, C., Brückner, C., Weinreb, S., Lehr, C., *et al.*, Biosynthesis of cis,cis-Muconic Acid and Its Aromatic Precursors, Catechol and Protocatechuic Acid, from Renewable Feedstocks by *Saccharomyces cerevisiae*. *Applied and Environmental Microbiology* 2012, *78*, 8421-8430.

[11] Curran, K. A., Leavitt, J. M., Karim, A. S., Alper, H. S., Metabolic engineering of muconic acid production in *Saccharomyces cerevisiae*. *Metabolic Engineering* 2013, *15*, 55-66.

[12] Vargas-Tah, A., Gosset, G., Production of Cinnamic and p-Hydroxycinnamic Acids in Engineered Microbes. *Frontiers in Bioengineering and Biotechnology* 2015, *3*, 116.

[13] Luttik, M. A. H., Vuralhan, Z., Suir, E., Braus, G. H., *et al.*, Alleviation of feedback inhibition in *Saccharomyces cerevisiae* aromatic amino acid biosynthesis: quantification of metabolic impact. *Metabolic Engineering* 2008, *10*, 141-153.

[14] Rodriguez, A., Kildegaard, K. R., Li, M., Borodina, I., Nielsen, J., Establishment of a yeast platform strain for production of p-coumaric acid through metabolic engineering of aromatic amino acid biosynthesis. *Metabolic Engineering* 2015, *31*, 181-188.

[15] Koopman, F., Beekwilder, J., Crimi, B., van Houwelingen, A., *et al.*, *De novo* production of the flavonoid naringenin in engineered *Saccharomyces cerevisiae*. *Microbial Cell Factories* 2012, *11*, 155-155.

[16] Lee, D.-H., Palsson, B. Ø., Adaptive Evolution of *Escherichia coli* K-12 MG1655 during Growth on a Nonnative Carbon Source, L-1,2-Propanediol. *Applied and Environmental Microbiology* 2010, *76*, 4158-4168.

[17] Portnoy, V. a., Bezdan, D., Zengler, K., Adaptive laboratory evolution--harnessing the power of biology for metabolic engineering. *Current Opinion in Biotechnology* 2011, *22*, 590-594.

[18] Oide, S., Gunji, W., Moteki, Y., Yamamoto, S., *et al.*, Thermal and solvent stress cross-tolerance conferred to *Corynebacterium glutamicum* by adaptive laboratory evolution. *Appl Environ Microbiol* 2015, *81*, 2284-2298.

[19] Ai, C., McCarthy, S., Eckrich, V., Rudrappa, D., *et al.*, Increased acid resistance of the archaeon, *Metallosphaera sedula* by adaptive laboratory evolution. *J Ind Microbiol Biotechnol* 2016, *43*, 1455-1465.

[20] Shui, Z. X., Qin, H., Wu, B., Ruan, Z. Y., *et al.*, Adaptive laboratory evolution of ethanologenic *Zymomonas mobilis* strain tolerant to furfural and acetic acid inhibitors. *Appl Microbiol Biotechnol* 2015, *99*, 5739-5748.

[21] Liu, L., Pan, A., Spofford, C., Zhou, N., Alper, H. S., An evolutionary metabolic engineering approach for enhancing lipogenesis in *Yarrowia lipolytica*. *Metabolic Engineering* 2015, *29*, 36-45.

[22] Reyes, L. H., Gomez, J. M., Kao, K. C., Improving carotenoids production in yeast via adaptive laboratory evolution. *Metabolic Engineering* 2014, *21*, 26-33.

[23] Bonomo, J., Lynch, M. D., Warnecke, T., Price, J. V., Gill, R. T., Genome-scale analysis of anti-metabolite directed strain engineering. *Metabolic Engineering* 2008, *10*, 109-120.

[24] Dmytruk, K. V., Yatsyshyn, V. Y., Sybirna, N. O., Fedorovych, D. V., Sibirny, A. A., Metabolic engineering and classic selection of the yeast *Candida famata* (*Candida flareri*) for construction of strains with enhanced riboflavin production. *Metabolic Engineering* 2011, *13*, 82-88.

[25] Rogers, J. K., Taylor, N. D., Church, G. M., Biosensor-based engineering of biosynthetic pathways. *Current Opinion in Biotechnology* 2016, *42*, 84-91.

[26] Mahr, R., Frunzke, J., Transcription factor-based biosensors in biotechnology: current state and future prospects. *Applied Microbiology and Biotechnology* 2016, *100*, 79-90.

[27] Mahr, R., Gätgens, C., Gätgens, J., Polen, T., *et al.*, Biosensor-driven adaptive laboratory evolution of L-valine production in *Corynebacterium glutamicum*. *Metabolic Engineering* 2015, *32*, 184-194.

[28] Lee, S.-W., Oh, M.-K., A synthetic suicide riboswitch for the high-throughput screening of metabolite production in *Saccharomyces cerevisiae*. *Metabolic Engineering* 2015, *28*, 143-150.

[29] Chou, H. H., Keasling, J. D., Programming adaptive control to evolve increased metabolite production. *Nature Communications* 2013, *4*, 2595.

[30] Leavitt, J. M., Tong, A., Tong, J., Pattie, J., Alper, H. S., Coordinated transcription factor and promoter engineering to establish strong expression elements in *Saccharomyces cerevisiae*. *Biotechnology Journal* 2016, *11*, 866-876.

[31] Gibson, D. G., Young, L., Chuang, R.-Y., Venter, J. C., *et al.*, Enzymatic assembly of DNA molecules up to several hundred kilobases. *Nature Methods* 2009, *6*, 343-345.

[32] Blazeck, J., Garg, R., Reed, B., Alper, H. S., Controlling promoter strength and regulation in *Saccharomyces cerevisiae* using synthetic hybrid promoters. *Biotechnol. Bioeng.* 2012, *109*, 2884-2895.

[33] Curran, K. A., Karim, A. S., Gupta, A., Alper, H. S., Use of expression-enhancing terminators in *Saccharomyces cerevisiae* to increase mRNA half-life and improve gene expression control for metabolic engineering applications. *Metabolic Engineering* 2013, *19*, 88-97.

[34] Lee, M. E., DeLoache, W. C., Cervantes, B., Dueber, J. E., A Highly Characterized Yeast Toolkit for Modular, Multipart Assembly. *ACS Synthetic Biology* 2015, *4*, 975-986.

[35] Hegemann, J., Heick, S., Delete and Repeat: A Comprehensive Toolkit for Sequential Gene Knockout in the Budding Yeast *Saccharomyces cerevisiae*, in: Williams, J. A. (Ed.), *Strain Engineering*, Humana Press 2011, pp. 189-206.

[36] Winston, F., EMS and UV Mutagenesis in Yeast, *Current Protocols in Molecular Biology*, John Wiley & Sons, Inc. 2001.

[37] Boeke, J. D., Croute, F., Fink, G. R., A positive selection for mutants lacking orotidine-5′-phosphate decarboxylase activity in yeast: 5-fluoro-orotic acid resistance. *Molecular and General Genetics* 1984, *197*, 345-346.

[38] Lütke-Eversloh, T., Stephanopoulos, G., A semi-quantitative high-throughput screening method for microbial L-tyrosine production in microtiter plates. *Journal of Industrial Microbiology & Biotechnology* 2007, *34*, 807-811.

[39] Iraqui, I., Vissers, S., André, B., Urrestarazu, A., Transcriptional induction by aromatic amino acids in *Saccharomyces cerevisiae*. *Molecular and Cellular Biology* 1999, *19*, 3360-3371.

[40] Williams, T. C., Nielsen, L. K., Vickers, C. E., Engineered Quorum Sensing Using Pheromone-Mediated Cell-to-Cell Communication in *Saccharomyces cerevisiae*. *ACS Synthetic Biology* 2013, *2*, 136-149.

[41] Parekh, R. N., Shaw, M. R., Wittrup, K. D., An Integrating Vector for Tunable, High Copy, Stable Integration into the Dispersed Ty δ Sites of *Saccharomyces cerevisiae*. *Biotechnology Progress* 1996, *12*, 16-21.

[42] Parekh, R. N., Shaw, M. R., Wittrup, K. D., An integrating vector for tunable, high copy, stable integration into the dispersed Ty delta sites of *Saccharomyces cerevisiae*. *Biotechnology Progress* 1996, *12*, 16-21.

[43] Fowden, L., Lewis, D., Tristram, H., Toxic Amino Acids: Their Action as Antimetabolites, *Advances in Enzymology and Related Areas of Molecular Biology*, John Wiley & Sons, Inc. 2006, pp. 89-163.

[44] Shetty, K., Crawford, D. L., Pometto, A. L., Production of L-Phenylalanine from Starch by Analog-Resistant Mutants of *Bacillus polymyxa*. *Applied and Environmental Microbiology* 1986, *52*, 637-643.

[45] Lee, D.-H., Feist, A. M., Barrett, C. L., Palsson, B. Ø., Cumulative Number of Cell Divisions as a Meaningful Timescale for Adaptive Laboratory Evolution of *Escherichia coli*. *PloS One* 2011, *6*, e26172.

[46] Redden, H., Alper, H. S., The development and characterization of synthetic minimal yeast promoters. *Nature Communications* 2015, *6:7810*.

[47] Jensen, N. B., Strucko, T., Kildegaard, K. R., David, F., *et al.*, EasyClone: method for iterative chromosomal integration of multiple genes in *Saccharomyces cerevisiae*. *FEMS Yeast Research* 2014, *14*, 238-248.

[48] DiCarlo, J. E., Norville, J. E., Mali, P., Rios, X., *et al*., Genome engineering in *Saccharomyces cerevisiae* using CRISPR-Cas systems. *Nucleic Acids Research* 2013, 41, 4336-4343.

[49] Horwitz, Andrew A., Walter, Jessica M., Schubert, Max G., Kung, Stephanie H., *et al*., Efficient Multiplexed Integration of Synergistic Alleles and Metabolic Pathways in Yeasts via CRISPR-Cas. *Cell Systems* 2015, 1, 88-96.

[50] Duncan, K., Edwards, R. M., Coggins, J. R., The *Saccharomyces cerevisiae* ARO1 gene An example of the co-ordinate regulation of five enzymes on a single biosynthetic pathway. *FEBS Letters* 1988, 241, 83-88.

[51] Braus, G. H., Aromatic amino acid biosynthesis in the yeast *Saccharomyces cerevisiae*: a model system for the regulation of a eukaryotic biosynthetic pathway. *Microbiological Reviews* 1991, 55, 349-370.

[52] Mumberg, D., Muller, R., Funk, M., Yeast vectors for the controlled expression of heterologous proteins in different genetic backgrounds. *Gene* 1995, 156, 119-122.

[53] Richard, P., Viljanen, K., Penttila, M., Overexpression of PAD1 and FDC1 results in significant cinnamic acid decarboxylase activity in *Saccharomyces cerevisiae*. *AMB Express* 2015, 5, 12.

[54] Luttik, M. A., Vuralhan, Z., Suir, E., Braus, G. H., *et al*., Alleviation of feedback inhibition in *Saccharomyces cerevisiae* aromatic amino acid biosynthesis: quantification of metabolic impact. *Metabolic Engineering* 2008, 10, 141-153.

[55] Rodriguez, A., Kildegaard, K. R., Li, M., Borodina, I., Nielsen, J., Establishment of a yeast platform strain for production of p-coumaric acid through metabolic engineering of aromatic amino acid biosynthesis. *Metabolic Engineering* 2015, 31, 181-188.

[56] Skjoedt, M. L., Snoek, T., Kildegaard, K. R., Arsovska, D., *et al*., Engineering prokaryotic transcriptional activators as metabolite biosensors in yeast. *Nature Chemical Biology* 2016, 12, 951-958.

[57] Draths, K. M., Frost, J. W., Environmentally compatible synthesis of adipic acid from D-glucose. *J. Am. Chem. Soc.* 1994, 116, 399-400.

[58] Gulmezian, M., Hyman, K. R., Marbois, B. N., Clarke, C. F., Javor, G. T., The role of UbiX in *Escherichia coli* coenzyme Q biosynthesis. *Archives of Biochemistry and Biophysics* 2007, 467, 144-153.

[59] Mukai, N., Masaki, K., Fujii, T., Kawamukai, M., Iefuji, H., PAD1 and FDC1 are essential for the decarboxylation of phenylacrylic acids in *Saccharomyces cerevisiae*. *J Biosci Bioeng* 2010, 109, 564-569.

[60] Payne, K. A., White, M. D., Fisher, K., Khara, B., et al., New cofactor supports alpha,beta-unsaturated acid decarboxylation via 1,3-dipolar cycloaddition. *Nature* 2015, 522, 497-501.

[61] White, M. D., Payne, K. A., Fisher, K., Marshall, S. A., et al., UbiX is a flavin prenyltransferase required for bacterial ubiquinone biosynthesis. *Nature* 2015, 522, 502-506.