ETOP 503689: Pre-optimized cell free lysates for rapid prototyping of genes and pathways

Final Project Report

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This study (Project ID: 100834-116; Subcontract# 7399340) was funded by the US Department of Energy Joint Genome Institute (https://ror.org/04xm1d337; operated under Contract No. DE-AC02-05CH11231).

1. Executive summary

This ETOP project aimed to re-conceive how we engineer complex biological systems by linking pathway design, prospecting, and validation into an integrated framework. Specifically, our vision seeks to advance and interweave high-throughput cell-based systems and rapid cell-free technologies in a way suitable for automation and microdroplet manipulation in a DOE JGI user facility setting. The key technology to be investigated toward this vision is a cell-free platform for combinatorial assembly of pathways by mixing-and-matching crude cell lysates derived from a suite of flux-enhanced background strains, each enriched with pathway enzymes. Through this work, we have established rewired *E. coli* and *S. cerevisiae* cells suitable to generated rewired lysates. We have demonstrated that lysates derived from rewired cells can generate higher fluxes of products and can enable a more robust discovery method going from gene to function annotations. Finally, we demonstrate that these rewired lysates can be stored for up to a year and still retain function. Collectively, these technologies enable a more rapid screening approach for enzyme and pathway variants.

2. Introduction

Microbial bioconversion forms the cornerstone of the biotechnology industry with applications spanning from the food and beverage sector[1] to the chemical, fuel, and nutraceutical industries[2, 3]. Among the choice of microbes, the yeast *Saccharomyces cerevisiae* serves an important role as both a model host organism[4-7] and a suitable industrial biocatalyst[8]. Through metabolic engineering, this host can be tailored for *in vivo* production of numerous heterologous products[8], including the biofuel butanol[9], antioxidant nutraceutical resveratrol[10], versatile monomer 3-hydroxypropionic acid[11], anti-malarial drug precursor artemisinin[12], and diverse bioactive alkaloids[13-16], among others. These production strains are substantially modified through both heterologous pathway complementation and rewiring of metabolic pathways to increase pools of precursor compounds, downregulate or eliminate competing pathways, and/or optimize cofactor regeneration routes[2, 3, 17]. Despite the success of these approaches, *in vivo* biosynthesis of chemical products directly competes with cell growth for carbon and energy while withstanding the constant metabolic burden of producing nonessential metabolites. This creates selective pressure against heterologous or overexpressed pathways[18]. Balancing this pressure to push forward innovative *in vivo* biochemical production platforms is a time- and labor-intensive challenge.

In contrast to *in vivo* biosynthesis, many seminal studies of fundamental biological phenomena have embraced cell-free approaches to elucidate mechanisms behind the genetic code[19], eukaryotic translation[20, 21], and fermentation by yeast[22, 23], devoid of growth constraints. Platforms for cell-free gene expression (CFE)[24-26] and cell-free metabolic engineering (CFME)[27-31] have recently matured as complementary approaches for bio-discovery and design of both enzymes and metabolic pathways. Without the cellular impediments of homeostatic maintenance, cell division, and membrane transport, *in vitro* metabolic systems enable the rapid assembly and testing of large combinations of biosynthetic enzymes. This ultimately provides a biological analogue of combinatorial synthetic chemistry with similar implications for scalability and throughput[32-34]. Such systems have been applied to a growing repertoire of metabolic pathways, including 2,3-butanediol (BDO)[35, 36], butanol and isobutanol[33, 37], 3-hydroxybutyrate[38], styrene[39], the

monoterpenes limonene, bisabolene, and pinene[34, 40], and cannabinoids[41], among others[28, 32]. Cell-free systems also offer the ability to explore biosynthetic capabilities using the maximum catalytic rate of enzymes[27, 29] as well as toxic molecules and chemical conditions outside homeostatic ranges[42], such as untreated substrates[35], cytotoxic products[43], and cytotoxic concentrations of product[39]. In the case of *S. cerevisiae*, extracts have been evaluated for the facile assessment of enzyme activity in fatty acids biosynthesis[44] and propanediol production[45] as well as bio-ethanol production at elevated temperatures[46], but the biosynthetic potential of yeast extract remains mostly unexplored for value-added chemical products.

Through the Emerging Technologies Opportunities Program (ETOP), we have combined the benefits of cellular metabolic engineering for strain optimization with *in vitro* metabolism platforms to accelerate the ability to build, study, and optimize biosynthetic pathways. Specifically, *in vivo* systems offer genetic tractability to manipulate and optimize cellular metabolism, while cell-free systems offer a high degree of flexibility to vary enzyme stoichiometry, study enzyme variants, alter cofactor conditions, and assess substrate specificity[27, 34, 47, 48]. First, we demonstrated that metabolic engineering in cells is reflected in cell extracts from these strains, which enables increased titers and volumetric productivities of diverse metabolites in cell-free reactions. Second, we built on this proof-of-principle study using a toolkit of engineered strains from *S. cerevisiae* and *E. coli* backgrounds in combination with purified enzymes to rapidly explore biochemical space and inform strain design. Finally, we assessed the long-term stability of cell extracts to demonstrate the viability of technology transfer to JGI and the extension of our approach developed through ETOP funding.

3. Integrating cellular and cell-free metabolic engineering techniques

a. Cell extracts from metabolically rewired strains have altered flux

We sought to advance and interweave rapid, cell-free technologies and cell-based systems that have been genetically rewired for high flux toward a particular branch of metabolism. The goal was to increase *in vitro* metabolic potential using value-added chemical products as a model. Before testing the impact of genetic rewiring in cells on cell-free metabolism, we first sought to activate metabolic pathways in yeast cell extracts to convert glucose to both ethanol and 2,3-butanediol (BDO) via native and heterologous enzymes, respectively. Commercial BDO is normally generated from petrochemical processes for applications in flavoring, synthetic rubber, and fuels[49, 50], making it an appealing target for biosynthesis both *in vivo*[51, 52] and *in vitro*[35, 36, 53]. Despite decades of research using yeast cell extracts to elucidate biological principles[20, 22], few studies have assessed small molecule biosynthesis from heterologous pathways with *S. cerevisiae* extracts. As such, a yeast cell-free system for heterologous metabolite biosynthesis had not previously been rigorously tested or optimized. To establish such a platform, we began with an extract preparation protocol optimized for *S. cerevisiae* cell-free gene expression (CFE)[54, 55] (**Fig. 1a**).

Both a BY4741 wildtype yeast strain expected to convert glucose to ethanol[22] and a BY4741 strain producing BDO[52] were prepared into extract to assess metabolic activity *in vitro*. The BDO strain expressed *AlsD* and *AlsS* from *Bacillus subtilis* to convert pyruvate to acetoin and *NoxE* from *Lactococcus lactis* for NAD recycling[52] (**Fig. 1b**). The

resulting cell extracts were combined with glucose, cofactors, salts, and buffer prior to incubation for 20 h at 30°C, producing both ethanol and BDO to validate cell-free synthesis of endogenous and heterologous metabolites. The optimization of CFE systems has incorporated several engineered strains for increased protein synthesis yields[27, 56-58] and for specialized applications, such as producing proteins with disulfide bonds[59] or noncanonical amino acids[60, 61]. In contrast, nearly all cell-free, crude extract-based metabolite biosynthesis to date has relied on strains with wildtype metabolism expressing pathway enzymes[36, 38, 47] with few examples of strain or extract modifications to increase *in vitro* product titers[62, 63]. Therefore, we utilized a modified *S. cerevisiae* strain that was metabolically rewired for increased BDO production using multiplexed CRISPR-dCas9 modulation[64] to simultaneously downregulate *ADH1,3,5* and *GPD1* to reduce byproduct formation while upregulating endogenous *BDH1* to increase flux to BDO (**Fig. 1b**)[52]. Cell-free reactions containing extract from the rewired BDO strain indeed retained altered flux to produce on average 46% more BDO and 32% less ethanol than extracts produced from the unmodified BDO strain (**Fig. 1c**), which we sought to enhance further through reaction and strain optimization.

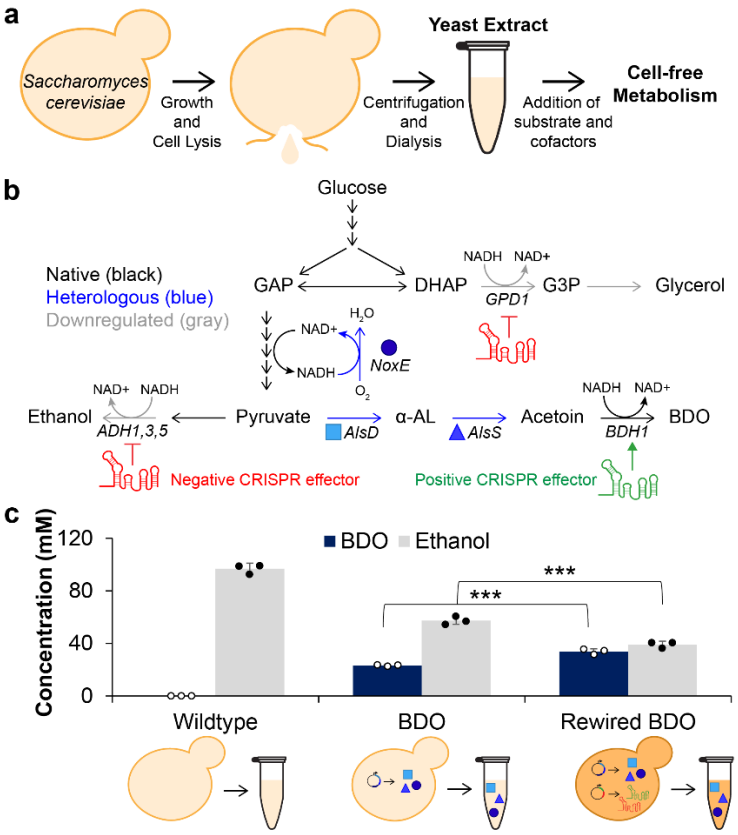

Figure 1. Metabolic rewiring *in vivo* is reflected in cell extracts. **a** Schematic overview of yeast cell extract preparation for cell-free reactions. **b** Metabolic map showing wildtype yeast metabolism in black, heterologous enzymes for BDO strains in blue, and CRISPR effectors expressed by rewired BDO strain. **c** Initial cell-free comparison of wildtype, BDO, and rewired BDO cell extracts converting glucose to native and heterologous metabolites. Extract from the metabolically rewired BDO strain produces more BDO and less ethanol than extract from the unmodified BDO strain (***$p \leq 0.001$).

b. Cell-free metabolic engineering and strain background selection increase product titer

As a first layer of optimization, we applied CFME techniques to increase BDO titers[27-29] by tuning variables that are not as easily altered in fermentations. To do so, extract concentration, reaction pH, cofactor concentrations, and glucose concentration were sequentially optimized (**Fig. 2**). Through this CFME approach, it was possible to increase BDO titer by ~40% with only small differences between initial reaction composition and final parameter selection, thus demonstrating the tunability of CFME systems (compare production in **Fig. 1c** relative to **Fig. 2d**).

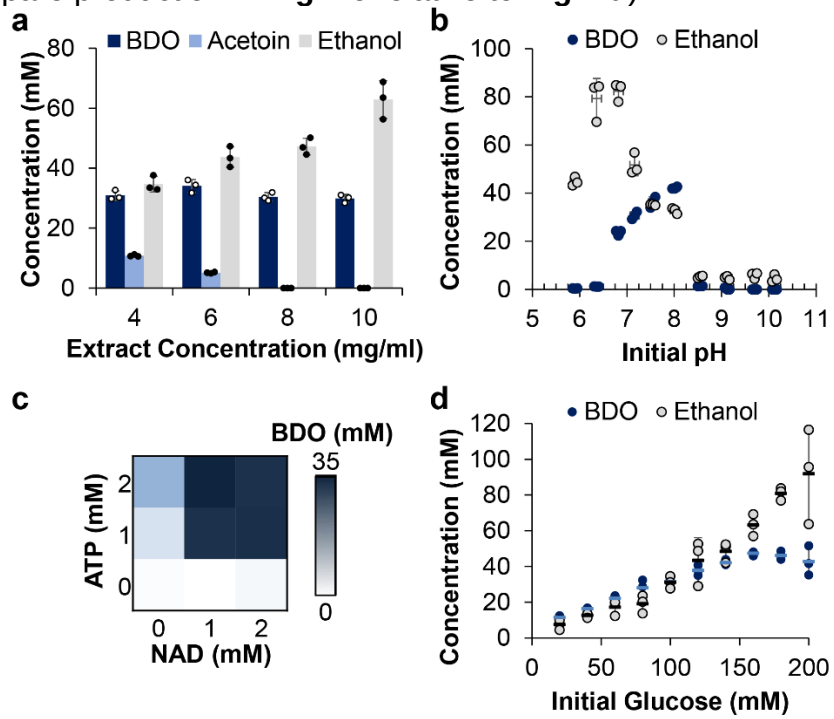

Figure 2. Optimizing the cell-free environment increases BDO titers. **a** The concentration of rewired BDO cell extract was adjusted to maximize conversion of acetoin to BDO while minimizing ethanol production. **b** pH was varied in physiologically compatible ranges that would not be possible *in vivo* to highlight the disparate pH optima for BDO and ethanol biosynthesis *in vitro*. **c** NAD and ATP concentrations were covaried to optimize the cofactor pool. **d** Glucose concentration was varied from 20 to 200 mM to compare the ratio of BDO to ethanol and the final BDO titer.

After optimizing the physiochemical environment of cell-free reactions, we sought to evaluate whether the chassis strain selection was critical for *in vitro* metabolism. To do so, we utilized three strain backgrounds (BY4741, CEN.PK, and Sigma) each rewired using a multiplexed CRISPR-dCas9 approach and applied the optimized reaction conditions shown above (6 mg/mL cell extract, pH 8, 1 mM NAD, 2 mM ATP, and 160 mM glucose). The corresponding extracts for these strains exhibited different behaviors. Notably, extract with the highest BDO titers *in vitro* was derived from the strain that produced the greatest BDO titer *in vivo*[52]. Specifically, reactions driven by extract from the rewired BDO Sigma strain produced 96% more BDO and 63% less ethanol than reactions with extract from the unmodified BDO Sigma background strain. In contrast, glycerol byproduct concentrations remained comparable across all 9 yeast extracts. This effort highlights the potential to compare relative pathway performance between cell and cell-free platforms, as we have observed before[33].

Finally, to better understand the dynamics of yeast cell-free biosynthesis reactions, we conducted a time course using extract derived from the rewired Sigma strain. These reactions exhibited a substantial increase in volumetric productivity compared to the corresponding strain grown *in vivo*[52] with all the glucose consumed and 80% of the BDO produced within 5 h of incubation (**Fig. 3b**). Based on these rapid cell-free reaction rates, we hypothesized that a fed-batch approach could further maximize BDO titer while maintaining relatively short reaction times[36]. To accomplish this, it was necessary to increase NAD and ATP supply to enable full glucose utilization with fed-batch spiking. Ultimately, fed-batch reactions receiving additional glucose, NAD, and ATP (45, 1, and 2 mM, respectively) at 1.5 h intervals displayed a reduced rate of BDO synthesis, but higher overall titer of 99.3 ± 2.3 mM (**Fig. 3c**). The decreased yield observed in this fed-batch system and the requirement of cofactor spiking suggest limitation due to cofactor recycling and/or overflow metabolism, which could potentially be alleviated by increasing *NoxE* expression or reducing the magnitude of glucose spikes through a continuous feed[65].

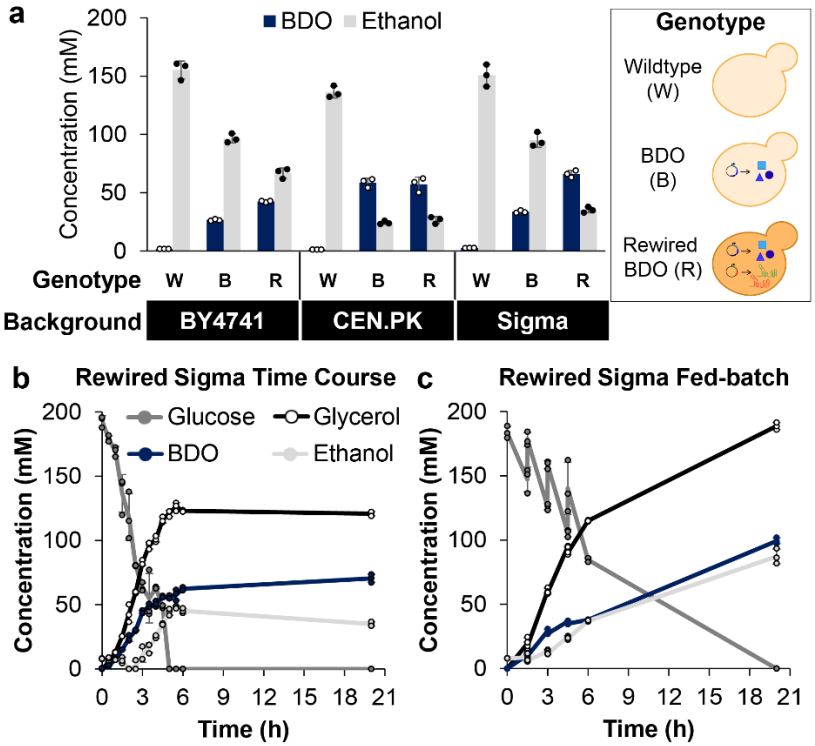

Figure 3. Yeast extracts from different strain backgrounds exhibit similar metabolism and rapidly consume glucose. **a** Panel of cell-free reactions containing yeast extracts from 3 strain backgrounds with the optimized conditions from Figure 2. All extracts retained metabolic activity, consuming glucose and producing ethanol and BDO. Extract from the rewired BDO Sigma strain produced the highest BDO titer and was characterized further. **b** Time course of cell-free reactions containing extract from the rewired BDO Sigma strain showing rapid glucose consumption. **c** Fed-batch reaction with additions of 45 mM glucose, 1 mM NAD, and 2 mM ATP at 1.5 h intervals.

c. Extending the integrated cellular/cell-free metabolic engineering approach to alternative metabolites

After demonstrating the impact of using genetically rewired strains to increase flux for cell-free BDO production, we assessed whether the integrated *in vivo* metabolic

rewiring and *in vitro* biochemical transformation approach was generalizable for alternate products. Specifically, we sought to increase biological production of itaconic acid, a versatile biochemical precursor for plastics and resins[66, 67], and glycerol, a ubiquitous additive in commodity chemicals, food products, and industrial sectors[68, 69]. The selection of itaconic acid and glycerol can demonstrate yeast CFME for a low-flux, heterologous pathway and a high-flux, native pathway, respectively (**Fig. 4a**). The base strain for itaconic acid production expressed the *cis*-aconitate decarboxylase gene, *CAD1*, from *Aspergillus terreus*, and the enhanced strain expressed this gene in a background with genomic knockouts of *ade3* and *bna2*[67]. The base strain for glycerol production contained an empty plasmid backbone, while the enhanced strain contained a plasmid to overexpress *GPD1* and *GPP1*[69]. Extracts from base strains and metabolically rewired strains were established for both products using methods developed above. An initial pair of glycerol strains was generated through CRISPR-mediated metabolic rewiring with guide RNAs targeting positive effectors to upregulate *GPD1* and *GPP1*[69], and the resulting extract from the rewired strain produced 18.7% more glycerol than extract from the control strain. This demonstrates generalizability of *in vivo* CRISPR-dCas9 rewiring to increase *in vivo* biosynthesis, but these extracts were unable to consume high concentrations of glucose. For this reason, we carried out further glycerol biosynthesis reactions with extracts from the plasmid-rewired strains to maximize cell-free glycerol titers.

Initial reaction conditions reinforce the observations from the BDO extract optimizations that metabolic rewiring *in vivo* transfers to cell extracts with similarly altered flux. Cell-free reactions containing extract from the enhanced strains produced 94% more itaconic acid and 39% more glycerol than the corresponding reactions with extract from base strains (**Fig. 4b-c**). Optimization of reaction conditions further boosted these titers by more than 3-fold with tuned reaction conditions producing up to 0.85 ± 0.17 mM itaconic acid in 7 h from 240 mM glucose (**Fig. 4d**) and up to 472.4 ± 19.3 mM glycerol in 20 h from 500 mM glucose (**Fig. 4e**). Similar to BDO biosynthesis, the *in vitro* platform enables relatively high volumetric productivities of 0.12 ± 0.02 mM/h (15.9 ± 3.0 mg/L-h) itaconic acid and 54 ± 0.28 mM/h (4.98 ± 0.03 g/L-h) glycerol in the first 8 h when considering only the time of *in vitro* bioconversion, since glucose is also converted to product during cell growth.

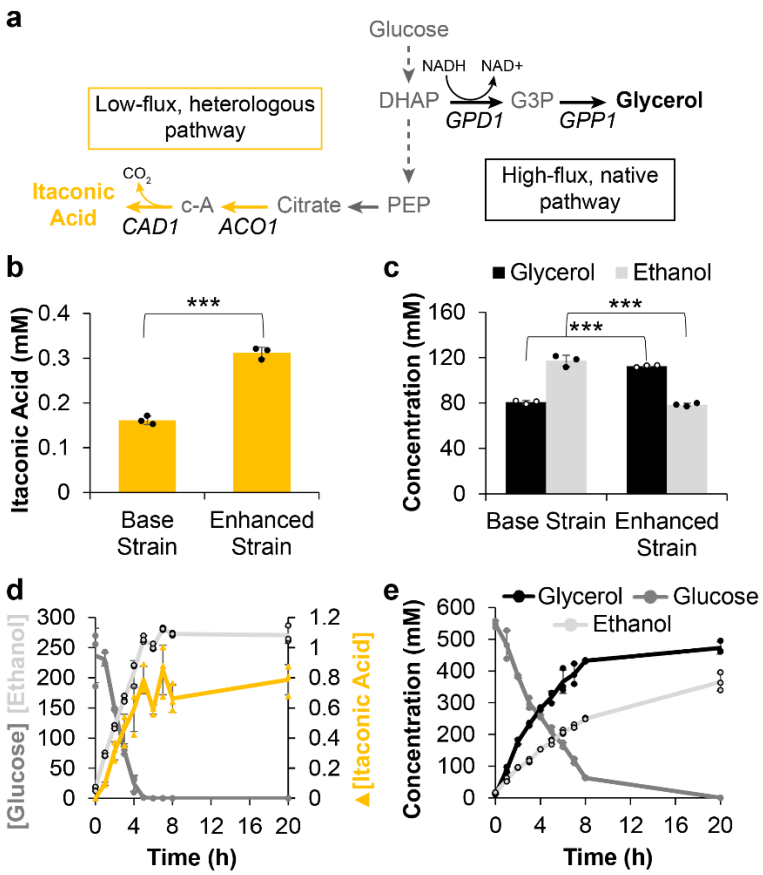

Figure 4. Cell-free reactions with extract from engineered yeast strains enable production of diverse metabolites. **a** Metabolic map showing the conversion of glucose to glycerol via dihydroxyacetone phosphate (DHAP) and glycerol-3-phosphate (G3P) or itaconic acid via citrate and cis-aconitate (c-A). **b-c** Initial cell-free reaction conditions confirm that strains engineered for increased product titers result in cell extracts with greater flux toward products of interest (***p ≤ 0.001 compared to base strain). **d-e** Yeast cell-free reactions with optimized conditions rapidly convert glucose into products, resulting in comparable concentrations to *in vivo* efforts in significantly less time.

This phase of the project demonstrated the biosynthesis of diverse metabolites using extracts enriched with heterologous enzymes during growth, highlighting the fact that cell-free metabolism reflects cellular engineering.

4. Establishing a versatile toolkit for pathway prototyping

Next, we sought to expand the product space and throughput of this cell-free biosynthesis approach using extracts derived from both *E. coli* and *S. cerevisiae* strains. The goal was to generate platform strains with enhanced carbon flux toward common nodes of metabolism and then use cell extracts derived from these platform strains to rapidly screen biosynthetic pathways consisting of purified enzymes for high-throughput prototyping and optimization (**Fig. 5**). Genes for several biosynthetic pathways were synthesized through the JGI Community Science Program, including 5-10 variants of each enzyme identified through BLAST (**Table. 1**), to make molecules prioritized by the Department of Energy. We sought to establish a toolkit for biochemical production, enzyme screening, and functional genomics across diverse branches of metabolism

through a suite of flux-enhanced strains of bacteria and yeast along with corresponding cell extracts that are metabolically active, shelf-stable, and capable of predicting the metabolic performance of cellular production strains.

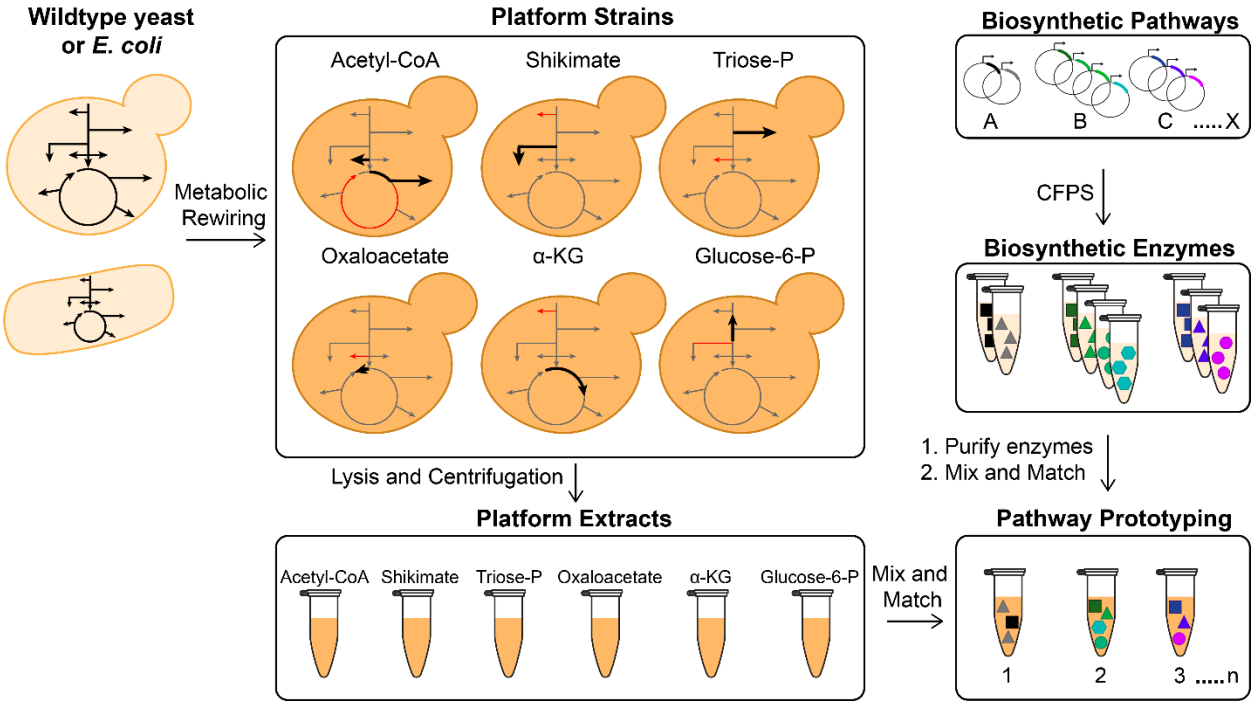

Fig. 5. Workflow for pathway prototyping toolkit generation and implementation. Cells are engineered for increased flux toward desired metabolic nodes, then these platform strains are converted into cell extracts to perform biosynthesis in the absence of growth or viability constraints. Panels of enzyme variants were synthesized by cell-free protein synthesis (CFPS), purified, and then mixed with the platform extracts for metabolite synthesis from glucose.

Table 1. Selected biosynthetic pathways and synthesized gene variants

Biosynthetic Pathways	Number of Enzymatic Steps	Number of Enzyme Variants Synthesized	Mix-and-Match Capability	Expected Optimal Lysate
Triacetic Acid Lactone	1	10	10	Acetyl-CoA
5-Aminolevulinic Acid	2	19	270	α-ketoglutarate
Myo-inositol	2	20	100	Glucose-6-P
Glucaric Acid	2 plus Myo-inositol pathway	40	10,000	Glucose-6-P
3-Hydroxypropinic Acid	2	20	100	Triose-P
Muconic Acid	3	62	8,250	Shikimate
Threonine	5	50	100,000	Oxaloacetate
n-Butanol	5	70	375,000	Acetyl-CoA
Isobutanol	5	44	40,000	Wild Type
Bisabolene	1 plus MEV pathway	28	15,309	Acetyl-CoA

a. Generation of platform strains and extracts

To increase the capacity of cell-free pathway prototyping, we diversified the cell-free metabolism with two approaches: 1) generating cell-free lysates from two organisms, and 2) pre-optimizing the cell-free lysates to have enhanced carbon flux towards key metabolic nodes (**Table 2**). To do so, we created different platform strains in both *S. cerevisiae* BY4741 and *E. coli* MG1655. These platform strains have been rewired at transcriptional level to activate (or overexpress) target genes while simultaneously repressing other target genes (**Table 2**), which resulted in a quantitative change of corresponding active enzymes. The change of enzymes can be preserved through the process of cell-free lysate preparation and enhance the cell-free carbon flux towards key metabolites. In order to expand the platforms flexibly if needed, the cellular rewiring applied here is portable and achieved with modular cloning.

Table 2. Selected gene targets for platform strain building

Platform Strains		Acetyl-CoA	Shikimate	Triose-P	Oxaloacetate	α-ketoglutarate	Glucose-6-P
Target Genes in *S. cerevisiae*	Upregulation	PDC1, ALD6, ACS1	TKL1, ARO4	GPD1/2	PYC1/2	PYC1/2, IDH2, IDP1, GDH2	PGI1, FBP1
	Downregulation	CIT2	ZWF1	TDH3, ADH1/3/5	MDH2, PDC1/5/6	GDH1	ZWF1
Target Genes in *E. coli*	Overexpression	aceE, aceF, acs	aroG (fbr)	tpiA	ppc	ppc, icd	pgi, fbp yggF, glpX
	Downregulation	gltA, glcB	pykA, pykF	gapA, adhE	mdh, aceE	gdhA	zwf

i. *S. cerevisiae* Strain Building and Validation

To build *S. cerevisiae* platform strains, a CRISPR-based multiplex regulation system was used with a single effector dCas9-VPR[52]. With specifically designed single-guide RNA (sgRNA), the dCas9-VPR can be directed to either the promoter regions of target genes to activate the transcription, or to the ORFs of target genes to block the transcription. Simultaneous multiplex regulation was achieved by co-expressing dCas9-VPR with a sgRNA cassette, which was assembled from several sgRNAs using a one-pot Golden Gate cloning method.

Although the ultimate goal is to rewire the carbon flux in the cell-free lysates, it was important to validate the effectiveness of the cellular rewiring. Different strategies were applied to validate *S. cerevisiae* platform strains (**Fig. 6**).

1. For acetyl-CoA platform strains, the enhanced carbon flux was validated by the increased TAL production. TAL is synthesized from acetyl-CoA by 2-pyrone synthase (g2ps1), which was introduced to both platform strains and control strains (containing control plasmid with only dCas9-VPR). The platform strains supported a 1.7-fold increase in TAL yield compared with control strains.
2. For shikimate platform strains, the enhanced carbon flux was validated by the increased 3-DHS production. 3-DHS is a key intermediate metabolite in the shikimate pathway and can be directly measured. The platform strains increased 3-DHS yield by 91% compared with control strains. Further

engineering (deleting ARO3 and replacing ARO4 with ARO4^{K229L}) of the platform strain led to a 6.8-fold increase in 3-DHS yield.

3. For triose-P platform strains, the enhanced carbon flux was validated by the increased glycerol production. The glycerol production is closely related to the availability of Triose-P and can be directly measured. The platform strains showed a 2.9-fold increase in glycerol yield compared with control strains.
4. For α-ketoglutarate platform strains, the enhanced carbon flux was validated by increased 5-ALA production. α-ketoglutarate is converted into succinyl-CoA in the TCA cycle, while 5-ALA is synthesized from glycine and succinyl-CoA by 5-ALA synthase (HemA), which was introduced to both platform strains and control strains. The platform strains supported a 7.5-fold increase in 5-ALA yield compared with control strains.
5. For glucose-6P platform strains, the enhanced carbon flux was validated by increased glucaric acid production. Glucaric acid is produced from Glucose-6P through a 4-enzyme pathway, which was introduced to both platform strains and control strains. The platform strains showed a 2-fold increase in glucaric acid yield compared with control strains.
6. However, for oxaloacetate platform strains, the cellular rewiring was not validated yet due to the lack of applicable methods or strategies.

ii. *E. coli* Strain Building and Validation

To build *E. coli* platform strains, we initially explored a similar CRISPR-based multiplex regulation system. However, while the multiplex repression was effective, the activation was not achievable. The single effector we used (dCas9ω)[70] needed to be precisely directed to a very specific location upstream the target genes for activation, which is practically infeasible due to the limited availability of native PAM sequences. We then applied a two-plasmid system to the platform strains, in which one plasmid was for the repression of target genes via CRISPR interference and another plasmid was for the IPTG-inducible overexpression of other target genes.

Similar strategies were applied to validate *E. coli* platform strains (**Figure. 6**). In the following experiment, strains containing two control plasmids were used as controls. In addition, only titer was compared across strains as the glucose consumption remained constant.

1. For acetyl-CoA platform strains, the enhanced carbon flux was validated by the increased TAL production. The platform strains showed a 1.7-fold increase in TAL titer compared with control strains.
2. For shikimate platform strains, the enhanced carbon flux was validated by the increased 3-DHS production. The platform strains showed a 2.2-fold increased 3-DHS titer compared with control strains.
3. For triose-P platform strains, the enhanced carbon flux was validated by the decrease of the acetate production. Initially, we tried to use the glycerol production to validate the platform strains by introducing the glycerol pathway into them. However, we found the glycerol production was very high and there was no difference between platform strains and control strains. We then measured the acetate titer as its carbon flux should be redirected to the triose-

P. The platform strains showed a 23% decrease in acetate titer compared with control strains.

4. For α-ketoglutarate platform strains, the enhanced carbon flux was validated by increased 5-ALA production. The platform strains supported a 1.7-fold increase in 5-ALA titer compared with control strains.
5. For glucose-6P platform strains, the enhanced carbon flux was validated by increased glucaric acid production. The platform strains showed a 2.4-fold increase in glucaric acid titer compared with control strains.
6. For oxaloacetate platform strains, the enhanced carbon flux was validated by increased aspartate production. This was only achievable in *E. coli* as it can grow in the minimal M9 media. Aspartate is converted from oxaloacetate by aspartate aminotransferase (AspC), which was introduced to both platform strains and control strains. The platform strains showed a 5.7-fold increase in aspartate titer compared with control strains.

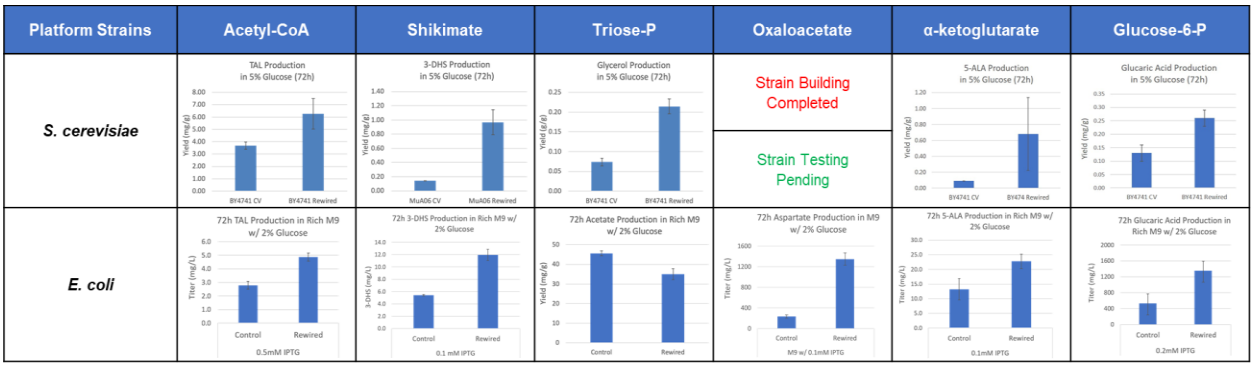

Fig. 6. Validation of metabolic rewiring in platform strains. All platform strains, except for the *S. cerevisiae* oxaloacetate strain, were validated with different strategies. Increased flux toward desired nodes of metabolism highlighted versatile platform strains for biosynthesis.

b. Preparation of platform extracts and cell-free biosynthesis

After validating the results of metabolic rewiring, all 12 platform strains and wildtype controls were converted into cell extracts by lysis and centrifugation to isolate the soluble biological machinery. *E. coli* cells were grown in 2xYTPG media to OD_{600} 3, pelleted and resuspended in an acetate-based buffer, lysed by homogenization, and centrifuged for 10 minutes at 12,000xg according to established protocols[71]. *S. cerevisiae* cells were grown in synthetic complete media with the appropriate amino acid dropouts for auxotrophic selection to OD_{600} 6-8, pelleted and resuspended in a glutamate-based buffer, lysed by homogenization, centrifuged for 5 minutes at 20,000xg, and dialyzed for 3 hours according to established protocols[55, 72]. This protocol was modified to omit the dialysis step for preparing the yeast platform extracts to better preserve cofactors and metabolic precursors synthesized during growth.

Cell-free metabolism was investigated in depth for two biochemical products using many enzyme variants. First, cell-free protein synthesis was performed by combining cell extract from *E. coli* BL21(DE3)-Star was with linear expression templates and the PanOx-SP reaction formulation according to established protocols[71, 73]. Enzymes were purified from cell-free reactions using Strep-tactin beads or resin and subsequently dialyzed into

a buffer more similar to cell-free reaction compositions (100 mM BisTris, 100 mM potassium glutamate, 10 mM sodium phosphate). Then cell-free metabolite synthesis was performed by combining extracts with glucose, cofactors (NAD, ATP, coenzyme A), buffer, and purified enzymes in 10-20 µL reactions mixed in 1.5-mL microcentrifuge tubes. Extracts enriched with enzymes during cell growth (used for initial validation of our approach and to assess extract stability) were not supplemented with exogenous enzymes. The reaction composition was tuned for each pathway investigated, including the concentrations of extract, cofactors, and purified enzymes. All reactions were incubated at 30 °C for up to 20 hours prior to precipitation with trichloroacetic acid and centrifugation for metabolite analysis.

c. Triacetic acid lactone pathway screening

Triacetic acid lactone (TAL) is a versatile biochemical that served as our test case for the combination of purified enzymes with metabolically tuned platform extracts. This pathway requires only 1 heterologous enzyme to convert central metabolites into TAL, enabling a simple comparison of the 6 engineered platform extracts and strains from both species for their metabolic potential. Cell-free reactions with a 2-pyrone synthase from literature[74] produced ~100-400 µM TAL depending on the platform extract employed for the conversion of glucose to acetyl- and malonyl-coA (**Fig. 7**). Although not statistically significant, the acetyl-coA platform extract resulted in higher TAL titers than wildtype extracts. But multiple platform extracts produced more TAL than the wildtype despite rewiring for different nodes of metabolism. We hypothesize that this is due to the centrality of acetyl-coA in metabolism, meaning that all extracts will maintain relatively high flux through this node. Although the yeast 3-DHS platform extract outperformed the acetyl-coA extract by ~25%, our approach still demonstrates increased flux through the desired node of metabolism. Further characterization of the 3-DHS platform is described below. We used the *E. coli* acetyl-coA platform extract to test the activity of a 2-pyrone synthase mutant from literature in addition to 8 uncharacterized enzymes identified through BLAST. The known mutant produced 35% as much TAL as the original enzyme, and the remaining enzymes showed 1-6% activity in all platform extracts.

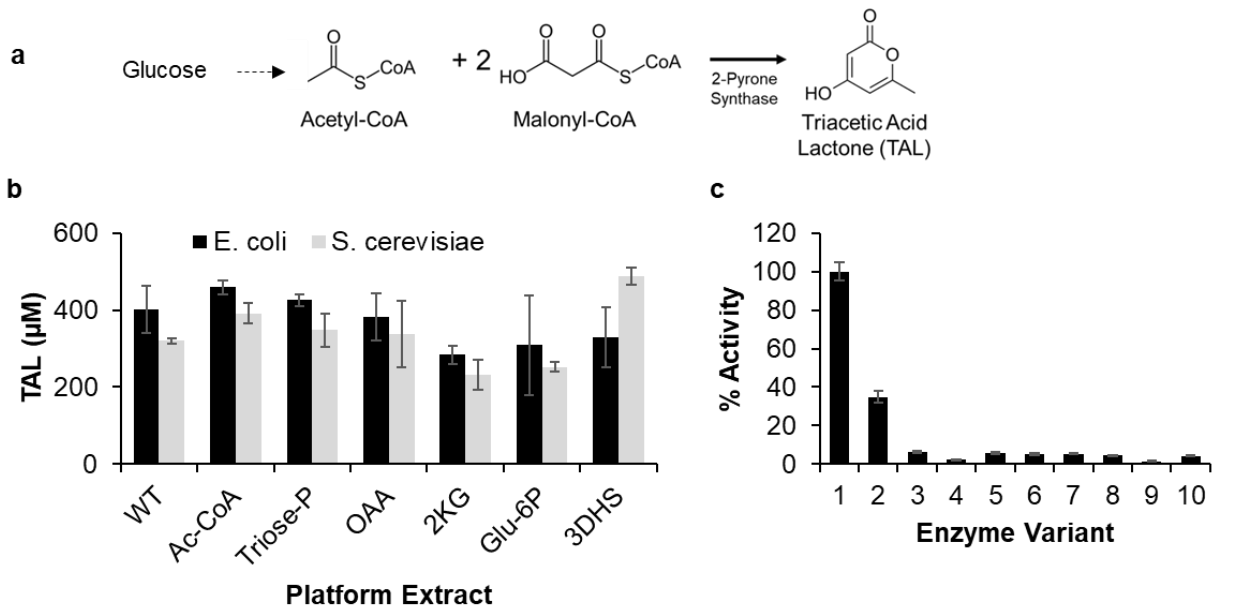

Fig. 7. Cell-free TAL synthesis. a The biosynthetic pathway for TAL requires endogenous glycolysis and 1 heterologous enzyme. **b** The acetyl-coA platform extract results in higher TAL titers than wildtype extracts for both *E. coli* and *S. cerevisiae*, and several other platform extracts also increase TAL synthesis. **c** Enzymes identified from literature or BLAST result in lower TAL titers than the canonical 2-pyrone synthase.

It was surprising that multiple platform extracts resulted in greater TAL titers despite rewiring toward different nodes of metabolism. To validate this result in the cellular environment, we introduced the TAL pathway into all *E. coli* platform strains and measured the TAL production (**Fig. 8**). Compared with control strains, acetyl-CoA platform strains showed significantly ($p<0.05$) higher TAL titer. While not statistically significant, shikimate platform strains supported TAL production, and α-ketoglutarate platform strains inhibited TAL production, which was predicted by the cell-free results. Further research is required to understand how the lack of regulation in cell-free reactions impacts metabolic flux, but we can still use platform extracts to rapidly explore enzymes prior to implementation in platform strains.

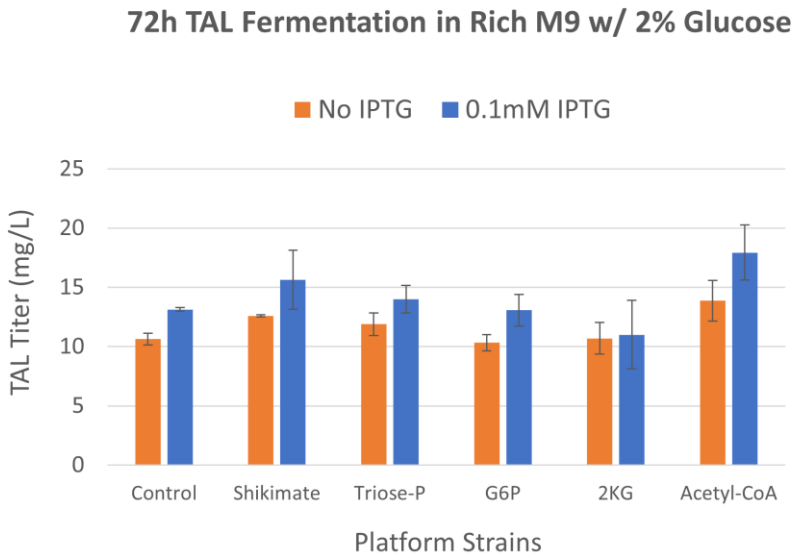

Figure 8. TAL Production across different platform strains. Metabolic rewiring for acetyl-coA provides a significant increase in cellular TAL synthesis, while apparent increases in other platform strains are not statistically significant.

d. Muconic acid pathway screening

To highlight the versatility of our cell-free biosynthesis workflow, we applied our toolkit of engineered strains and extracts for the synthesis of muconic acid from the energy-intensive shikimate pathway. This product requires 3 heterologous enzymes to convert the native 3-dehydroshikimate intermediate into muconate. We began with a set of enzymes from literature in extract from wildtype (WT) *E. coli*, which produced no detectable muconic acid despite the addition of cofactors. Only once we implemented the 3-DHS platform extract (generated from cells with increased flux through the shikimate pathway) was cell-free muconic acid biosynthesis observed. Furthermore, no platform extracts aside from 3-DHS produced significant concentrations of muconic acid, validating our approach of using specifically tailored cells and extracts for different branches of metabolism (**Fig. 9**). We used this platform extract to combinatorially screen 10 enzyme classes across the 3-step heterologous pathway and observed activity from a small subset (Pa4 or AroZ converting 3-DHS to protocatechuic acid (PCA), AroY converting PCA to catechol, and HQD2 or CatA converting catechol to muconic acid). This matrix of 32 unique enzyme combinations was built and tested in 3 days, which is significantly faster than screening the same test space *in vivo*.

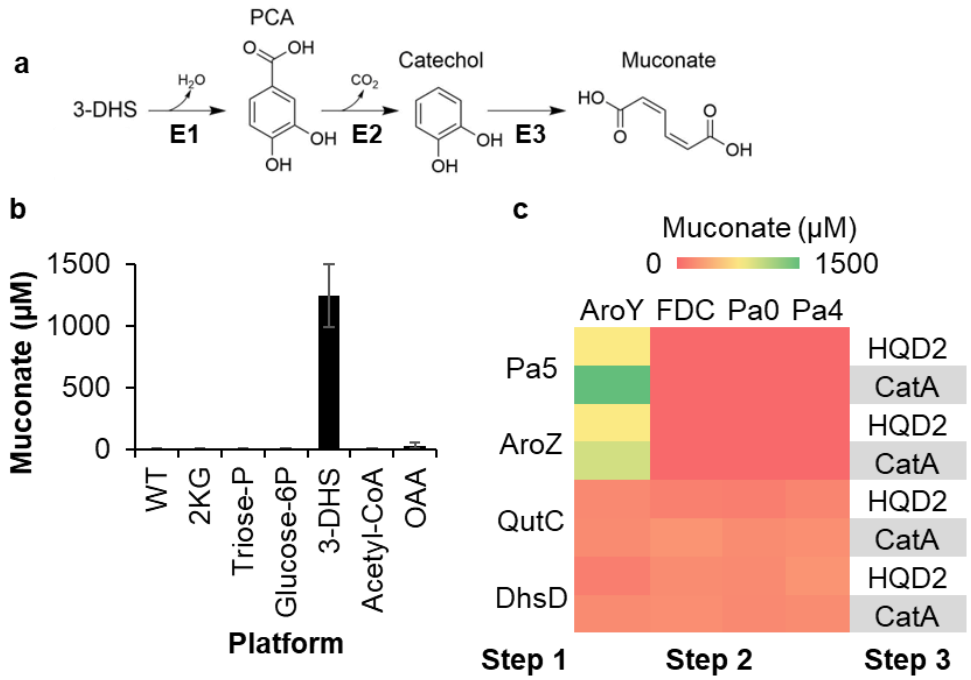

Fig. 9. Platform extracts enable cell-free muconate synthesis and enzyme screening. a Muconic acid biosynthesis requires three purified heterologous enzymes from central metabolism. **b** Muconic acid biosynthesis was not observed in extract from wildtype *E. coli*, and significant concentrations of the product were only observed in the platform extract with increased flux through the 3-dehydroshikimate intermediate. **c** Several classes of enzymes for each step of the pathway were assessed using the 3-DHS platform extract, revealing two functional classes for steps 1 and 3 but only one functional class for step 2.

The enzyme classes with demonstrated activity were investigated further by screening enzyme variants *in vitro* using both *E. coli* and *S. cerevisiae* platform extracts with enhanced 3-DHS flux. This is the first known example of cell-free metabolic prototyping using extracts from 2 distinct organisms. We explored 5 variants of AroZ and CatA and 10 variants of Pa5, AroY, and HQD2. Rather than assessing the full combinatorial space (2,250 unique combinations), we sequentially changed 1 enzyme from the best-performing combination in our initial comparison of enzyme classes (variant 1 of Pa5, AroY, and CatA). This allowed us to screen all enzyme variants through 45 enzyme combinations built and tested in 2 types of extract in only 5 days (**Fig. 10**). Reactions with *E. coli* extract made ~2000 μM muconic acid while those with yeast extract made ~150 μM, so the results are plotted as relative titer for ease of comparison on the same plot. Although the trends are qualitatively similar across the 3 steps in both organisms, some enzymes perform much better in one organism's extract. For example, yeast extract appears much more sensitive to the identity of the second enzyme, which could be a result of differences in cofactor regulation or other background metabolism in the extracts. This cell-free enzyme screening method can identify both more active enzyme variants and highlight metabolic nuances in different strains, providing deeper insights into strain design.

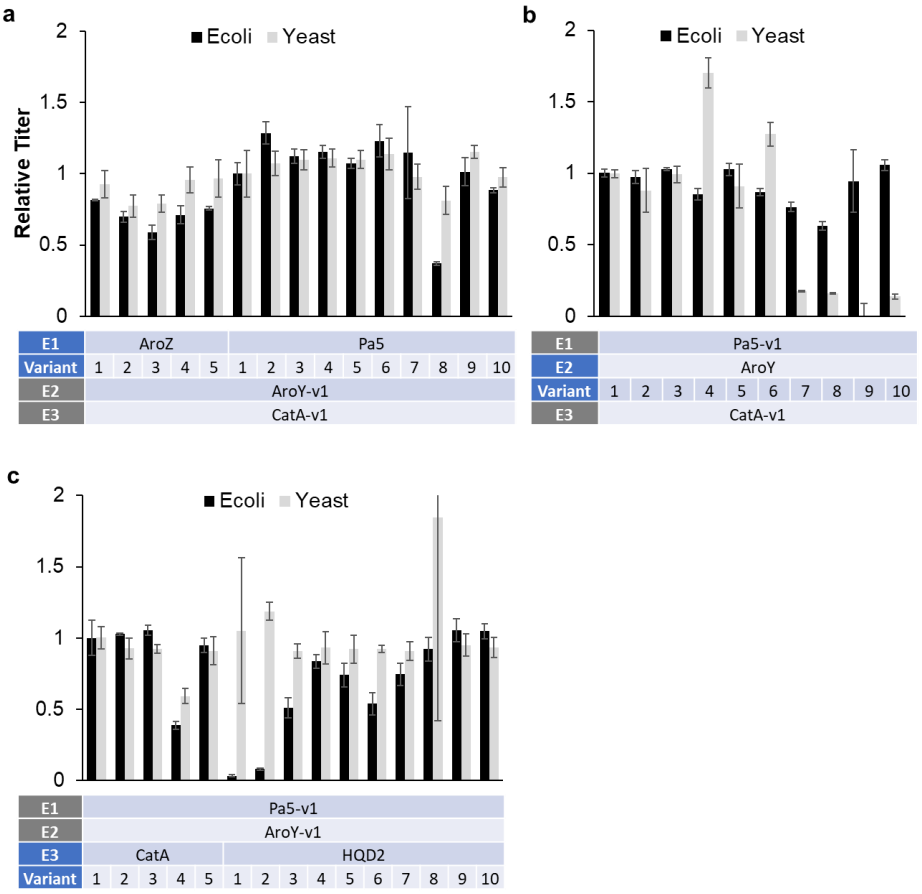

Fig. 10. Enzyme variants identified by BLAST and synthesized by JGI were sequentially screened in cell-free reactions containing platform extracts from *E. coli* or *S. cerevisiae*. Data are presented as relative titers compared to the baseline condition of ~2000 µM muconate with *E. coli* extract and ~150 µM muconate with yeast extract using Pa5-v1, AroY-v1, and CatA-v1 as the standard set of enzymes. Better variants were identified at each step in the pathway, including different optima for the different extracts.

Cell-free prototyping provided abundant information about the synthesized gene variants. To evaluate how much we can apply the information we learned from cell-free systems to inform cellular engineering, we selected 6 muconic acid pathway configurations and reconstituted them into shikimate *E. coli* platform strains and control strains. Notably, the most active pathway *in vitro* translated into the best-performing strain *in vivo* (**Fig. 11**). While this is a relatively small dataset, it is evident that cell-free prototyping can accelerate strain design by decreasing the test space required to find improved enzyme combinations. We will further compare cellular and cell-free performance by building more *E. coli* and yeast strains to correlate muconic acid titers between all 4 cellular and cell-free environments to better understand the predictive power of cell-free pathway prototyping.

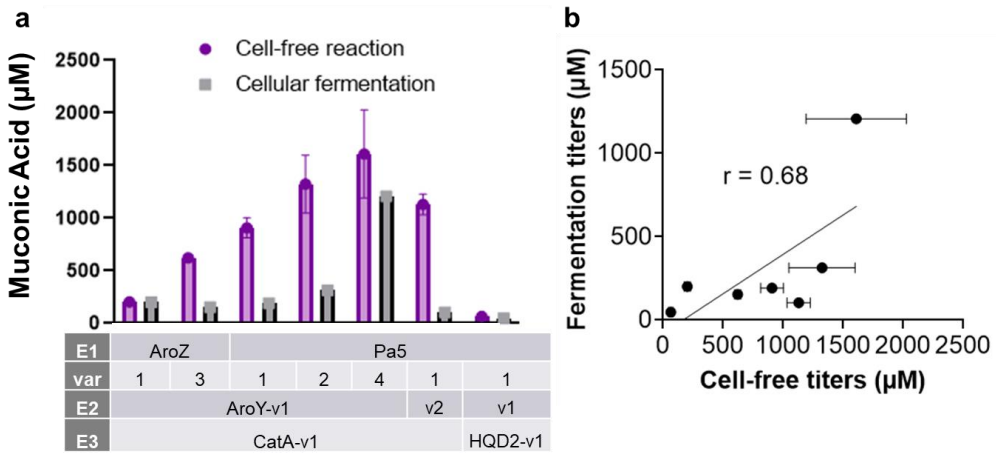

Fig. 11. Cell-free reaction performance informs cellular design. Several enzyme combinations validated in cell-free reactions were constructed in a platform *E. coli* strain. The best set of enzymes was the same *in vivo* and *in vitro*, highlighting the predictive power of cell-free biosynthesis.

e. Metabolomic analysis of cellular and cell-free systems

To further understand the differences between *in vivo* and *in vitro* systems, we compared the metabolomic information from cellular and cell-free systems samples. The cellular samples were taken from cellular fermentation using either shikimate platform strains or control strains, with or without muconic acid pathway. The cell-free samples were taken from cell-free reactions using either shikimate platform lysates or control lysates, with or without muconic acid pathway.

From principal component analysis (**Fig. 12**), it was evident that metabolomic information was different between cellular and cell-free systems. While the differences was larger in cellular samples between *S. cerevisiae* and *E. coli*, the metabolism of cell-free samples was more consistent. A more focused analysis on *E. coli* lysates with muconic acid pathway revealed differences between shikimate platform lysates or control lysates, indicating a metabolism change due to the pre-optimization. In further attempt to decipher the metabolomic differences between two systems, identify key metabolites, and explain the differences in pathway production in the previous experiments, we filtered through the thousands of features. However, the features are small unknown metabolites (m/z < 300) which cannot be identified from the database. A more targeted metabolomic analysis may be required to obtain the key information in these samples.

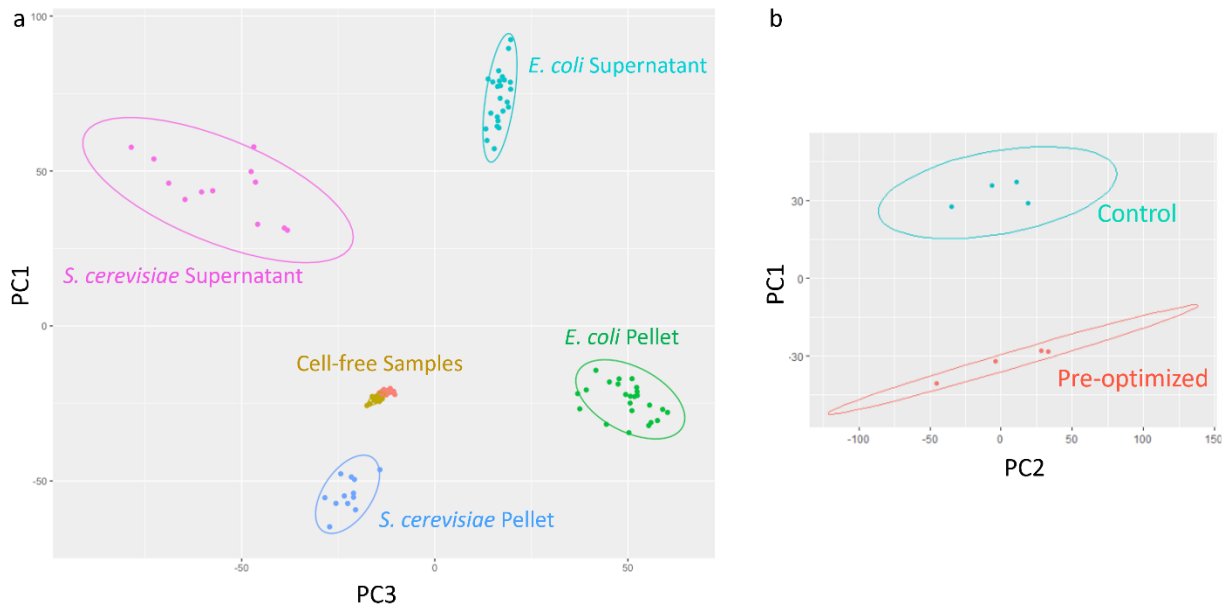

Fig. 12. Metabolomic analysis on cellular and cell-free samples. a. Principal component analysis on all samples. The principal components indicated distinct metabolomic information from cellular and cell-free systems. Within cellular samples, the metabolomic difference between *E. coli* and *S. cerevisiae* was also significant. **b.** Principal component analysis on *E. coli* cell-free samples with muconic acid pathway. The principal components revealed metabolomic change after the lysates were pre-optimized.

5. Long-term stability of cell extracts

For this toolkit to be broadly applicable as part of a user facility, batches of extracts must remain robust throughout long-term storage periods. We assessed metabolic stability using extracts from *E. coli* and *S. cerevisiae* enriched with enzymes for 2,3-BDO synthesis that had previously been validated in cell-free biosynthesis studies[36, 72]. The strains used for extract preparation expressed the same variants of *AlsD* and *AlsS*, but the final enzyme differed and produced unique stereoisomers of 2,3-BDO (*BudC* in E. coli and BDH1 in yeast producing meso- and R,R-BDO, respectively). Fresh batches of extract were prepared and utilized for BDO synthesis immediately. Then cell-free metabolism was assessed after 5 freeze-thaw cycles and at regular intervals over the course of 12 months in storage at -80 °C. The rates of catabolism and anabolism in these extracts remained comparable after repeating thawing and after extended storage, with the rate of BDO synthesis decreasing 25% in *E. coli* extract and 15% in yeast extract after the full year (**Fig. 13**).

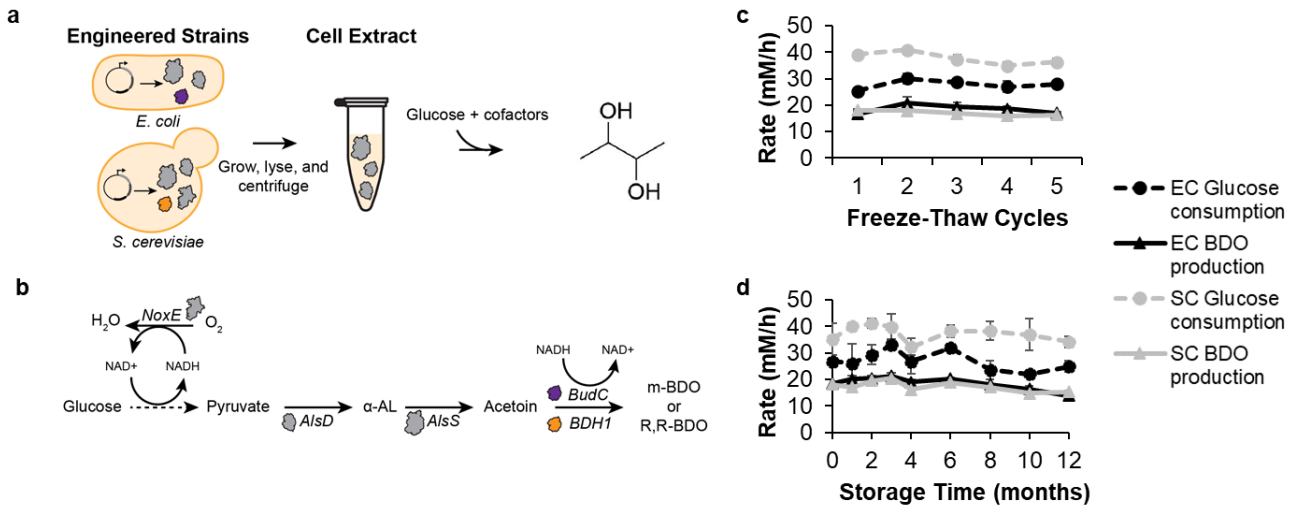

Fig. 13. Extracts retain metabolic activity after extended storage. **a** Strains of *E. coli* (EC) and *S. cerevisiae* (SC) were engineered to synthesize 2,3-BDO and converted into metabolically active cell extracts. **b** The extracts each produce a different isomer of BDO, and the yeast extract contains NoxE for cofactor recycling. **c** The rates of catabolism and anabolism remain consistent for both extracts after 5 freeze-thaw cycles. **d** Catabolism and anabolism slow by less than 25% after 12 months of storage, indicating that cell extract can provide a stable prototyping platform for several months after preparation.

In addition to the key parameters of glucose consumption and BDO synthesis, we observed the overall kinetics and titers of central metabolites. The primary byproducts of acetate and glycerol (from *E. coli* and yeast extract, respectively) are plotted alongside glucose, BDO, and the immediate precursor acetoin before and after 12 months of storage (**Fig. 14**). All reactions consumed 150 mM glucose in less than 9 hours and synthesized 80-114 mM BDO. An increase in acetoin and glycerol biosynthesis suggests altered cofactor balance or reduced regeneration after storage. However, the overall consistency of cell-free metabolism indicates that a single lot of extract can provide useful information for pathway prototyping, enzyme characterization, and biochemical conversions over many months before a new lot is required.

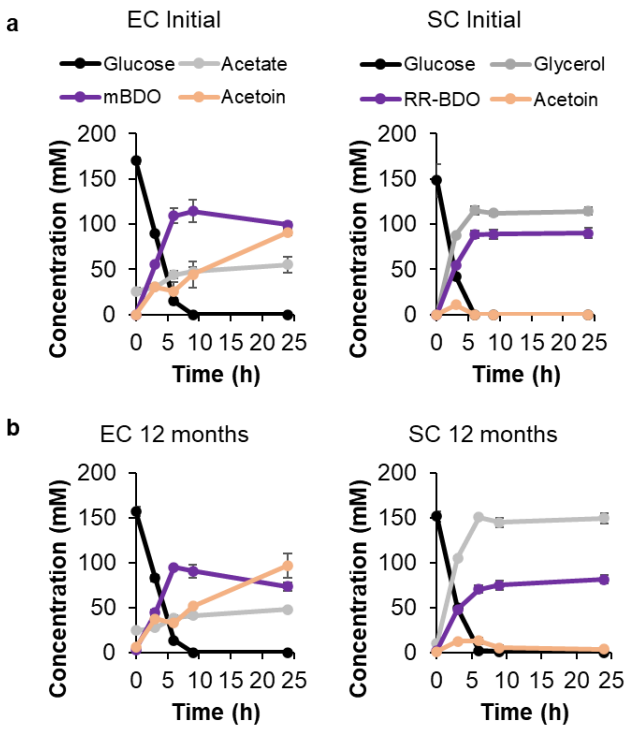

Fig. 14. Kinetics of cell-free metabolism remain qualitatively similar after storage for 1 year. a *E. coli* extract produced 114 mM BDO immediately after preparation, while the *S. cerevisiae* extract produced 90 mM BDO. **b** After 12 months of storage at -80 C, *E. coli*-based reactions produced ~20% less BDO (90 mM). Similarly, yeast-based reactions produced ~10% less BDO (81 mM).

Furthermore, cell-free reactions can be lyophilized in tubes or on paper, providing an additional layer of stability without the need for ultra-low temperature storage[75, 76]. Lyophilized reactions exhibit similar BDO synthesis capacity after rehydration in either format, again with increased acetoin concentrations suggesting differences in cofactor regeneration (**Fig. 15**). This method can be investigated further for the deployment of cell-free biosynthesis or larger-scale preparation of cell extracts without requiring extensive freezer space.

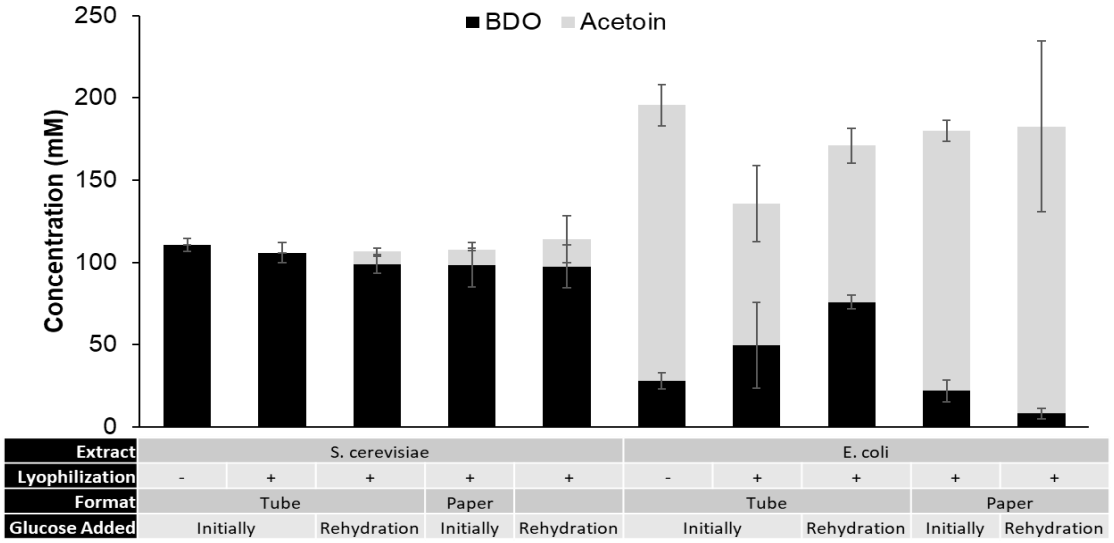

Fig. 15. Lyophilized cell extracts exhibit robust metabolism. Endpoint measurements of cell-free reactions without lyophilization (-) or after lyophilization (+) either in microcentrifuge tubes or on filter paper show continued BDO synthesis capacity.

6. Conclusions and outlook

In this study, we developed an integrated *in vivo/in vitro* metabolic engineering approach to activate cell-free metabolism by genetically modulating metabolic pathways in yeast source strains used for extract generation, either using heterologous enzymes produced during cell growth or purified enzymes subsequently added to extracts. We found that metabolic rewiring in these strains improves desired productivities *in vitro*. This reflects previous efforts to engineer strains for CFE to increase protein yields[27, 56, 57] and enable specialized applications[59-61] through gene knockouts and/or complementation, but the focus here was on reshaping metabolic flux for small molecule synthesis rather than translation. We demonstrated increased biosynthesis of TAL and muconic acid both *in vivo* and *in vitro* while highlighting the ability of cell-free reactions to reduce the time and effort required to screen many enzyme combinations for implementation in cells. The cell-free prototyping for muconic acid pathway was only achievable with pre-optimized lysates and resulted in improved muconic acid production from new pathway configurations. The cell-free production of TAL indicated the differences between *E .coli* and *S .cerevisiae* lysates and more information can be obtained using lysates from distinct organisms. As this project concludes, the techniques and workflows can easily be transferred to JGI to implement this biosynthetic screening toolkit as part of a user facility. We are providing guidance for JGI personnel to optimize cell extract preparation and cell-free protein synthesis on-site. Expanding these findings to different metabolic pathways enables rapid exploration of biochemical conversions *in vitro* to inform strain design in bacterial and fungal chassis strains, which will have broad impacts in the metabolic engineering community through accelerated design-build-test cycles for pathway optimization and metabolic exploration.

7. Outputs

a. Publications

i. Levine MZ, Gregorio NE, Jewett MC, Watts KR, Oza JP (2019) “Escherichia coli-based cell-free protein synthesis: protocols for a robust, flexible, and accessible platform technology” J. Vis. Exp. (144): e58882. https://dx.doi.org/10.3791/58882.
ii. Danielson N, McKay S, Bloom P, Dunn J, Jakel N, Bauer T, Hannon J, Jewett MC, Shanks B “Industrial Biotechnology—An Industry at an Inflection Point” (2020) Industrial Biotechnology, 16(6). https://doi.org/10.1089/ind.2020.29230.nda.
iii. Rasor BJ, Vögeli B, Landwehr GM, Bogart JW, Karim AS, Jewett MC (2021) “Toward sustainable, cell-free biomanufacturing” Current Opinion in Biotechnology, 69. https://doi.org/10.1016/j.copbio.2020.12.012.
iv. Heijstra BD, Fackler N, Rasor BJ, Brown S, Martin J, Ni Z, Shebek KM, Rosin RR, Simpson SD, Tyo KE, Giannone RJ, Hettich RL, Tschaplinski T, Leang C, Brown SD, Jewett MC, Köpke M (2021) “Stepping on the Gas to a Circular Economy: Accelerating Development of Carbon-Negative Chemical Production from Gas Fermentation” Annual Review of Chemical and Biomolecular Engineering, 12. https://doi.org/10.1146/annurev-chembioeng-120120-021122.
v. Rasor BJ, Yi X, Brown S, Alper HS, Jewett MC (2021) “An integrated cell/cell-free metabolic engineering approach to produce value-added compounds” Nature Communications 12, 5139. https://doi.org/10.1038/s41467-021-25233-y.
vi. Bomble YJ, Jewett MC (2021) “Editorial: Cell Free Biocatalysis for the Production of Bioproducts” Frontiers in Energy Research, 9:781552. https://doi.org/10.3389/fenrg.2021.781552.
vii. Rybnicky GA, Dixon RA, Kuhn M, Karim AS, Jewett MC (2022) “Development of a Freeze-Dried CRISP-Cas12 Sensor for Detecting Wolbachia in the Secondary Science Classroom” ACS Synthetic Biology, 11(2). https://doi.org/10.1021/acssynbio.1c00503.
viii. Yi X, Alper HS “Considering Strain Variation and Non-Type Strains for Yeast Metabolic Engineering Applications” (2022) Life, 12(4). https://doi.org/10.3390/life12040510.
ix. Manuscript in preparation: “Cell extracts from bacteria and yeast retain metabolic activity after repeated thawing and extended storage”
x. Manuscript in preparation: “Developing a toolkit of engineered cells and cell extracts for pathway prototyping”

b. Presentations

i. Northwestern Chemical and Biological Engineering Department Retreat (Talk) September 2022: “Cell-free biosynthesis for pathway prototyping and biomanufacturing”
ii. SIMB Annual Meeting (Invited Talk) August 2022: “Establishing cell-free systems for carbon negative biomanufacturing”

iii. SIMB Annual Meeting (Poster) August 2022: “Combining cellular and cell-free metabolic engineering for enhanced pathway prototyping”
iv. DOE Genomic Sciences Annual Meeting (Poster) March 2022: “Mechanistic insights into cell-free expression from an -omics analysis of extract preparation”
v. Central US Synthetic Biology Workshop (Poster) September 2021: “Integrated cellular / cell-free metabolic engineering for biosynthesis”
vi. SIMB Annual Meeting (Invited Talk) August 2021: “Cell-free synthetic biology platforms to accelerate biomanufacturing”
vii. NDSEG Fellows Conference (Poster) July 2021: “Integrated cellular / cell-free metabolic engineering for biosynthesis”
viii. Metabolic Engineering 14 (Poster) July 2021: “Integrated cellular / cell-free metabolic engineering for biosynthesis”

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