Kinetic-model-guided engineering of multiple *S. cerevisiae* strains improves *p*-coumaric acid production

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Abstract

The use of kinetic models of metabolism in design-build-learn-test cycles is limited despite their potential to guide and accelerate the optimization of cell factories. This is primarily due to difficulties in constructing kinetic models capable of capturing the complexities of the fermentation conditions. Building on recent advances in kinetic-model-based strain design, we present the rational metabolic engineering of an *S. cerevisiae* strain designed to overproduce *p*-coumaric acid (*p*-CA), an aromatic amino acid with valuable nutritional and therapeutic applications. To this end, we built nine kinetic models of an already engineered *p*-CA-producing strain by integrating different types of omics data and imposing physiological constraints pertinent to the strain. These nine models contained 297 mass balances involved in 303 reactions across four compartments and could reproduce the dynamic characteristics of the strain in batch fermentation simulations. We used constraint-based metabolic control analysis to generate combinatorial designs of 3 enzyme manipulations that could increase p-CA yield on glucose while ensuring that the resulting engineering strains did not deviate far from the reference phenotype. Among 39 unique designs, 10 proved robust across the phenotypic uncertainty of the models and could reliably increase *p*-CA yield in nonlinear simulations. We implemented these top 10 designs in a batch fermentation setting using a promoter-swapping strategy for down-regulations and plasmids for up-regulations. Eight out of the ten designs produced higher *p*-CA titers than the reference strain, with 19 – 32% increases at the end of fermentation. Importantly, these eight designs also maintained at least 90% of the growth of the reference strain; this indicates the importance of the phenotypic constraint. The high success rate of our in-silico designs in an experimental setting demonstrates the potential and utility of kinetic-model-based strain design. This work sets the foundation for accelerated design-build-test-learn cycles using large-scale kinetic models as a scaffold.

1 Introduction

Systems metabolic engineering enables us to rewire microorganisms to produce valuable chemicals, such as biofuels, aromatic amino acids, and other plant derivatives, in an environmentally friendly manner[1–3]. A common challenge in establishing microbial cell factories is optimizing the strain to ensure cost-effective production. There are two broad approaches to strain optimization: evolutionary and rational. Evolutionary approaches such as adaptive laboratory evolution[4] (ALE) and random mutagenesis[5] use the power of natural selection to choose overproducing strains. Conversely, rational strain design targets enzymes

deliberately chosen using existing knowledge of metabolic and regulatory networks, as well as computational tools[6]. Common rational strategies include the removal of inhibitory feedback loops, downregulating genes involved in reactions that divert resources away from the desired production pathway, or upregulating genes associated with the production pathway. Empirical designs based on detailed knowledge of the production and precursor pathways might not consider system-wide interactions and can, therefore, miss out on promising candidates in other parts of primary or secondary metabolism. Moreover, the nonlinear nature of metabolism makes it difficult to predict the systems-wide effects of simultaneous changes to multiple enzymes; this makes combinatorial design based on empirical knowledge challenging.

Computational models provide a systematic network-wide strategy for rational engineering by providing an efficient way to search the design space for suitable targets. Previous studies have relied on methods such as OptKnock[7], optGene[8], and optForce[9] to successfully steer organisms like *S. cerevisiae* toward the overproduction of different compounds[10–14]. These methods use constraint-based models to predict what an overproducing phenotype should look like; however, they have limited capacity to predict actual cellular behavior because they are just a snapshot of the cellular state and lack kinetic information[15]. As a result, most of the targets suggested by these algorithms went through a vetting process to ensure they were not detrimental to the transient behavior of the cell. Moreover, these constraint-based approaches are ill-suited for developing combinatorial designs as they cannot capture the nonlinear epistatic interactions of simultaneous interventions[16]. Kinetic models overcome these shortcomings by capturing the dynamic response of the entire metabolic network to single or combinatorial genetic perturbations[17–21]. In this manner, they can tell us how to steer an organism from its reference phenotype towards overproduction. Despite these advantages, kinetic models have not been widely used for strain design. Indeed, constructing large-scale kinetic models is challenging due to their parametric and structural uncertainty. Moreover, evaluating all possible combinations of genetic interventions that improve production is not trivial. To overcome these two hurdles, we recently developed NOMAD, a framework that employs nonlinear large-scale kinetic models for rational strain design[23].

In this study, we used NOMAD to suggest strategies that drive *S. cerevisiae* to overproduce *p*-coumaric acid (*p*-CA), an essential ingredient in the food, pharmaceutical, and cosmetic industries[24–26]. *S. cerevisiae* is widely used as a host organism due to its ability to grow at large scale, robustness across various conditions, and availability of advanced genetic engineering tools[27,28]. We began with a *S. cerevisiae* strain (ST10284), already engineered for *p*-CA production through multiple gene knockouts and heterologous pathway additions[29]. Based on

experimental physiological data, we built nine large-scale, multi-compartment kinetic models of ST10284 and used these models to identify combinatorial designs of three simultaneous enzyme targets that increase *p*-CA yield from glucose. We selected ten robust designs across all nine models and verified their performance in nonlinear batch fermentation simulations. We then utilized promoter swapping and plasmid-based overexpression strategies to implement these designs experimentally. Eight out of the ten recombinant strains exhibited increased *p*-CA titers in batch fermentations, demonstrating the potential of kinetic models in guiding microbial engineering toward desired phenotypes.

2 Methods and Materials

2.1 Strains, media, and chemicals.

We used a previously developed *S. cerevisiae* strain ST10284 as the reference strain for building kinetic models and further engineering. This strain is a derivative of the CEN.PK113-7D strain[30] that was engineered by incorporating heterologous pathways to produce *p*-CA from both L-tyrosine and L-phenylalanine. It includes numerous modifications designed to enhance flux within the pentose phosphate, shikimate, and aromatic amino acid biosynthetic pathways, as previously described[29] (Supplementary Table S4 and Supplementary Figure S2).

We used three kinds of medium: YPD (yeast extract peptone dextrose), SC (synthetic complete dextrose), and MM (minimal medium). YPD medium is composed of 10 g/L yeast extract, 20 g/L peptone, and 20 g/L glucose. When required, we added 200 mg/L of G418 (G8168, Merck Life Science) and 100 mg/L of nourseothricin (Jena Bioscience, AB-101) as antibiotics for selection. SC medium is composed of 6.7 g/L yeast nitrogen base without amino acids, 1.4 g/L yeast synthetic drop-out medium supplement without histidine, leucine, tryptophan, and uracil, and 20 g/L glucose. The nucleotide uracil (76 mg/L, U1128, Sigma) was added when *URA3* was the selection maker for yeast transformation. MM is composed of per liter 7.5 g $(NH_4)_2SO_4$, 14.4 g KH_2PO4, 0.5 g $MgSO_4$ $7H_2O$, 22 g dextrose, 2 mL trace metals solution (3.0 g/L $FeSO_4 \cdot 7H_2O$, 4.5 g/L $ZnSO_4 \cdot 7H_2O$, 4.5 g/L $CaCl_2 \cdot 2H_2O$, 0.84 g/L $MnCl_2 \cdot 2H_2O$, 0.3 g/L $CoCl_2 \cdot 6H_2O$, 0.3 g/L $CuSO_4 \cdot 5H_2O$, 0.4 g/L $Na_2MoO_4 \cdot 2H_2O$, 1.0 g/L H_3BO_3, 0.1 g/L KI, and 19.0 g/L $Na_2EDTA \cdot 2H_2O$), and 1 mL vitamins (0.05 g/L D-biotin, 0.2 g/L p-aminobenzoic acid, 1.0 g/L calcium D-pantothenate, 1.0 g/L thiamin–HCl, 1.0 g/L pyridoxine–HCl, 1.0 g/L nicotinic acid, and 25.0 g/L myo-inositol) with pH at 6. We also added 2% bacteriological agar (A5306, Sigma) to the above media when preparing agar plates. All the standards used for analysis were purchased from Sigma.

We used MM for the seed train and fed-batch cultivation of ST10284, as well as for the batch production cultivation of the engineered strains. For the fed-batch cultivation, we used a 10-fold higher concentration of the components and adjusted the pH to 5.6 using KOH to avoid the precipitation of salts. We used SC to detect the strength of the different promoters we evaluated and YPD to select strains after yeast transformations. Glucose was the carbon source in all the cultivations.

2.2 Fed-batch cultivation conditions

We used an established fed-batch cultivation setup to determine the parameters that characterized the phenotype of ST10284. For the seed strain, we streaked out ST10284 from -80 °C cryostocks onto YPD plates and cultivated it for 48 hours at 30 °C. We inoculated a single colony into 1 mL MM containing 6 g/L of glucose and grew it for 10 hours at 250 rpm. We transferred cells to an initial OD_{600} of 0.1 into 8 wells in the fed-batch fermentation plate.
We carried out the fed-batch fermentation in the microfluidic bioreactor Biolector Pro (m2p labs) using a 32 wells microfluidic flower plate (MTP-MF32C-BOH2) in the cultivation, with 800 µL of batch MM and 1.29 g/L of glucose in each well. We then filled two reservoir wells with 1800 µL of feed medium, with each reservoir feeding 4 cultivation wells, and triggered feeding after 18 hours using an exponential feeding profile, $F(t) = 1.23\, e^{0.1t}$ensuring no overflow metabolism or oxygen limitation. We fixed the temperature at 30 °C, stirring at 1,300 rpm, oxygen at 35% (enriched air), humidity at 85%, and left pH uncontrolled during the whole cultivation. We measured the glucose, biomass, *p*-CA, and ethanol concentrations over approximately 7 hours, covering about one doubling period, with data collected from four samples at regular intervals.

2.3 Plasmid construction and strain construction

We used *E. coli* competent cell *DH5α* for constructing and propagating all the plasmids corresponding to the different engineering strains (Supplementary Table S5). The *E. coli* strains grew at 37 °C on yeast extract tryptone (YT) medium with kanamycin (50 µg/mL), ampicillin (100 µg/mL), or chloramphenicol (25 ug/mL).
We compared the strength of the promoters of the target genes by amplifying the promoter region that was 1kb relative to the start codon location of the target genes using Phusion Hot Start II High-Fidelity PCR Master Mix (Thermo Scientific™, F565S) from *S. cerevisiae* genome. We assembled these amplified promoters, along with other selected promoters and the GFP coding sequence, into the pYTK096 vector[31] using the Golden Gate assembly method

as described in Shaw et al.'s work[32]. For gene knock-in, we amplified DNA fragments that were 500 bp upstream and downstream of the target genes from the *S. cerevisiae* genome and fused them with the corresponding weaker promoters via overlapping PCR to create repair templates. We constructed the corresponding guide RNA (gRNA) vectors for the target genes using Gibson Assembly Master Mix (New England BioLabs).

The designs proposed by the kinetic models included both native targets from *S. cerevisiae* and heterologous targets introduced during the construction of ST10284. We used Phusion U Hot Start PCR Master Mix (Thermo Scientific™, F533S) to amplify the native genes from *S. cerevisiae*. For the heterologous targets, we amplified *EcAroB* and *EcAroD* from the plasmid pCFB1955[33] and obtained *FjTAL* from Twist Bioscience (USA). We integrated these amplified target genes into ST10284 by cloning them into a set of vectors from the EasyClone 2.0 Yeast Toolkit using the USER cloning method[34]. We then verified all the constructed plasmids using Sanger sequencing (Eurofins Scientific SE).

We constructed the recombinant strains (Table S6) using a Cas9-assisted approach[34], conducted yeast transformations using the standard lithium acetate method3 and identified correct transformants using the Phire Plant Direct PCR Master Mix (Thermo Fisher Scientific, F160S).

2.4 Fluorescence measurement

We conducted single-cell fluorescence analysis using the NovoCyte Quanteon flow cytometry (Agilent). We diluted yeast cultures (200 µL) by tenfold in a 96-well microtiter plate before analysis, with GFP fluorescence excited by a 488 nm laser and detected through the 525/50 nm channel. For each sample, we collected data from 10,000 cells and processed them using the FlowJo software.

2.5 Growth detection using Growth Profiler

We monitored the growth of the engineered yeast strains using the Growth Profiler 960 (EnzyScreen BV) at 30 °C with shaking at 250 rpm, according to the manufacturer's recommended settings. We performed each experiment in triplicate, capturing images over a 72-hour cultivation period at 30-minute intervals with a 5ms shutter time. We then extracted the G-values from the central pixel of each well using the GP960Viewer software and converted them into OD_{600} based on established calibration curves.

2.6 Extraction and quantification of *p*-CA

We cultivated the strains for 5, 24, 30, 46, and 72 hours using the Growth Profiler 960. 1 mL of yeast culture was mixed with 1mL of absolute ethanol. We vortexed the mixture thoroughly for 10 seconds, centrifuged it at 17,000xg for 10 minutes, and then analyzed an aliquot of the resulting supernatant by high-performance liquid chromatography (HPLC) to determine the extracellular *p*-CA concentration. Additionally, we centrifuged 1 mL of yeast culture for 10 minutes and analyzed the ethanol and glucose concentrations in the supernatant by HPLC.

For *p*-CA analysis, the HPLC system was equipped with a Discovery HS F5 column (150 mm × 2.1 mm, 3 µm particle size, Supelco, 567503-U). We set the column oven to 30 °C with a flow rate of 0.7 mL/min and injected a 5 µL sample for quantification. The mobile phase consisted of Solvent A (10 mM ammonium formate, pH 3.0, adjusted with formic acid) and Solvent B (acetonitrile). The initial solvent composition was 95% A and 5% B, maintained for 0.5 minutes. We then applied a linear gradient, transitioning to 40% A and 60% B at 7.0 minutes, and held this composition constant for 2.5 minutes (7.0–9.5 min). We then returned the composition to the initial conditions (95% A, 5% B) at 9.6 minutes and held it there till the end of the run (9.6–12 min). We detected *p*-CA with a retention time of 4.7 minutes, using absorbance at 277 nm for quantification. We quantified glucose and ethanol using an Aminex HPX-87H column (Bio-Rad Laboratories, USA) under the conditions described by[36], but with a column temperature of 60 °C.

2.7 Kinetic model construction

We followed the NOMAD protocol to generate kinetic models representative of the *S.cerevisiae* strain ST10284[23]. In brief, we first defined the model's stoichiometry and generated steady states using thermodynamic flux balance analysis after integrating the available omics data. Next, we assigned kinetic mechanisms for the model's reactions and sampled sets of kinetic parameters consistent with the chosen steady state. Finally, we pruned these parameter sets for those that best represented the strain's physiology.

2.7.1 Model stoichiometry

We used redGEM[37] to reduce the Yeast8 genome-scale model (GEM)[38] around the central carbon metabolism subsystems: glycolysis, pentose phosphate pathway (PPP), cytosolic tricarboxylic acid cycle (TCA), mitochondrial TCA, shikimate pathway, and the anaplerotic reactions. We then lumped the biosynthetic reactions into a single reaction depicting growth using lumpGEM[39].

Next, we tailored the model to the strain ST10284 by incorporating the corresponding genetic modifications. We added reactions corresponding to the heterologously expressed tyrosine ammonia lyase (*FjTAL*), phenylalanine ammonia lyase (*PAL2*), cinnamate-4-hydroxylase (*C4H*), fructose 6-phosphate kinase (*FPK*), and cytochrome P450 reductase 2 (*CYP5*). We removed the reaction PPYRDC corresponding to the knockout of *PDC5*. The experimental strain ST10284 included the deletion of *Aro10*; however, we did not remove the two associated reactions in the model (INDPYRD and PYRDC) as other isoenzymes could catalyze them. We then included transport reactions for acetyl phosphate and *p*-CA and an extra reaction, FMN reductase (FMNRx), to ensure the resulting system was mass-balanced. The resulting model had 368 reactions involving 295 metabolites distributed across nine compartments.

2.7.2 Steady-state profiles consistent with multi-omics data

We integrated metabolomic, exofluxomic, and thermodynamic data using thermodynamics-based flux balance analysis (TFA) implemented in pyTFA[40]. We constrained the concentrations of all intracellular metabolites in each compartment to be within 5-fold of the reported values from Park et al.[41]. We then tailored the model to ST10284 by incorporating exometabolomic and exofluxomic data from the fermentation experiments. We also constrained the extracellular oxygen, glucose, and other mineral media components to within 20% of their experimental values and extracellular pH to 6, the starting pH value in fermentation experiments. We enforced an upper bound of 1 μmol on the concentrations of extracellular metabolites that were neither part of the medium nor reported to have been secreted. We required growth to be at least the reported lower bound, the secretion rates of *p*-CA and ethanol to be within the experimentally observed values, and the glucose uptake rate to be within 20% and 10% of the upper and lower bounds reported experimentally (Supplementary Table S6).

We then incorporated the glycolysis-PPP splitting ratio[42,43] in *S. cerevisiae* by constraining the ratio of the fluxes through glucose-6-phosphate isomerase (*PGI*) and glucose 6-phosphate dehydrogenase (G6pDH2r). We also ensured sufficient adenylate energy charge[44] by constraining the concentrations of the components of the cytosolic adenylate pool. We used equilibrator[45] to define bounds on the standard Gibbs free energy ($\Delta G'^0$) of the different reactions in the model. We assigned a $\Delta G'^0 = 0 \pm 2\ kcal/mol$ for reactions without any thermodynamic data. We also assumed that the intercompartmental transport reactions operate close to thermodynamic equilibrium. TFA uses these data to ensure that the sampled fluxes and concentrations are consistent with the second law of thermodynamics. Some reactions could

carry fluxes in both directions even after adding the different omics data. We manually curated the directions for these reactions by forcing the cyclic TCA, and the pentose phosphate pathway to have a catabolic non-oxidative branch[46].

We then used pyTFA to sample 5,000 steady-state profiles that produced at least 95% of the maximal growth under these constraints. We used the steady-state profile closest to the mean of all 5,000 profiles as the scaffold to build kinetic models.

2.7.3 Kinetic models consistent with integrated data

We defined kinetic mechanisms for each reaction, excluding the boundary reactions, and developed mass balance equations for all the metabolites in the model, except small molecules such as protons and inorganics. We then assigned volume fractions for the four (out of nine) compartments that contained at least one mass balance - the cytosol (70%), mitochondria (3%), endoplasmic reticulum (2%), and the endoplasmic reticulum membrane (0.5%). We constrained the Michaelis constants (K_Ms) to be within the minimum and maximum values across all reactions reported in BRENDA[47] for *S. cerevisiae*. We then sampled 15,000 sets of kinetic parameters that were consistent with the steady-state profile and yielded locally stable models around it.

We screened these locally stable models for those with dominant time constants at least three times smaller than the doubling time of the strain (7.3 hours) using the screening protocol defined in our previous works[18,20,23]. We further pruned these models for those that exhibited robust growth in batch fermentation simulations.

In the above protocol, we used steady-state data from fed-batch fermentation experiments to build the models. However, the final design strains would be implemented in a batch setting, where yeast exhibits a 3-fold increase in growth rate compared to the fed-batch conditions. We accounted for this altered phenotype by applying a 3-fold increase to the maximal velocities of all the reactions in the chosen models while keeping the parameters such as the Michaelis constants, thermodynamic constants, and the initial conditions fixed. We validated this approach by conducting a sensitivity analysis on kinetic models built around steady states constrained to have a 3-fold increase in exofluxomics and a 20% variability in the internal concentrations. This analysis showed that the distribution of maximal velocities in the resulting kinetic models had a median 3-fold increase compared to the models calibrated on fed-batch data, suggesting that this approach accurately captures the altered metabolic phenotype (Supplementary Note I).

2.8 Rational design generation for overproducing *p*-CA

Kinetic models enable the calculation of control coefficients, which quantify how a change in an enzyme activity or metabolite concentration impacts fluxes and concentrations throughout the network[48,49]. Using Network Response Analysis (NRA)[50], which relies on control coefficients, we identified engineering strategies to maximize the yield of *p*-CA from glucose while considering network-wide effects. Strategies adhered to the following constraints: (i) maximum 2-fold changes in enzyme activity, (ii) no more than 5-fold changes in concentrations, (iii) no more than 3 simultaneous target enzymes, and (iv) maximum 20% reduction in growth rate. We generated combinations of 3 enzymes for each kinetic model that were within 5% of the maximal NRA-predicted yield for that model.

2.8.1 Robustness evaluation

Designs generated with one model need not perform well across other models. Additionally, we do not know which of the kinetic models best represents the strain's true physiology. We accounted for these uncertainties by identifying designs that produce robust performance across the population of models. For each combination of design and model, we used NRA to maximize the yield of *p*-CA from glucose, permitting changes to only the 3 enzymes in the design. This procedure resulted in a population of predicted yield increases for each design across all the models. We then ranked the designs based on the mean of these predicted yield increases and chose the top 10 for further analysis and experimental implementation.

2.8.2 Verification through nonlinear simulations

We verified the top 10 NRA-predicted designs by implementing each design in full-scale nonlinear simulations. During the mid-exponential phase, we calculated the relative increase in *p*-CA yield from glucose when compared to the reference strain, ST10284.

For each combination of model and design from the Top 10, we adjusted the maximal velocities of the associated enzymes according to the NRA-suggested values. We then simulated batch fermentation for 48 hours, starting with an inoculum of 0.024 g/L and using the same medium as in the kinetic model development. After 5 hours, we measured the *p*-CA yield from glucose and calculated its increase relative to ST10284. We also recorded the relative increase in *p*-CA titer after 24 hours compared to ST10284.

3 Results

3.1 Kinetic models of a *p*-CA producing *S. cerevisiae* strain

We followed the protocol established by Narayanan et al.[23] to build a population of kinetic models representing *S. cerevisiae* ST10284 physiology (Supplementary Table S6) and can effectively simulate the strain's behavior in batch reactor conditions. The NOMAD framework identified nine models that could produce adequate growth in a batch fermentation setting. The resulting models captured the behavior of 297 metabolites connected by 303 reactions across the central carbon pathways such as glycolysis, the pentose phosphate pathway, the Krebs cycle, the Shikimate pathway, and the electron transport chain. The model parameters contained 1098 Michaelis constants (K_Ms) and 303 maximal velocities ($V_{max}s$). All nine models were locally stable and had dominant time constants that were at least three times faster than the doubling time of the strain; this ensured that they quickly returned to their homeostatic state when perturbed [18,20,23]. They also proved to be robust in a nonlinear setting by demonstrating growth and *p*-CA production in batch simulations.

3.2 Model-based designs for overproducing *p*-CA in *S. cerevisiae*

We applied NRA to the nine kinetic models to devise designs for improved yield of *p*-CA from glucose in the *p*-CA producing strain, ST10284. The design specifications limited each strategy to a maximum of three target enzymes, allowing up to a 2-fold increase in target enzymes' activities while permitting unlimited downregulation. To ensure that the introduced changes improve the desired trait without excessively disrupting other cellular functions, we restricted metabolite concentration changes to no more than a 5-fold difference. We then selected designs for each model based on their ability to achieve a *p*-CA yield increase within 5% of the model's maximal yield.

Across the nine kinetic models, we identified 39 unique designs, each defined by a distinct set of three target enzymes. Clustering analysis grouped these designs into four broad clusters based on the similarity of their enzyme targets (Figure 1). Group I contains two subgroups of two designs each; one subgroup targets glutamate dehydrogenase (GLUDy) and isocitrate dehydrogenase (ICDHyr) together, while the other targets sedoheptulose 1,7-bisphosphate D-glyceraldehyde-3-phosphate-lyase (FBA3). The 17 designs in Group II all increase the availability of phosphoenolpyruvate (pep), a precursor to the aromatic amino acid pathways, by decreasing its consumption by pyruvate kinase (*PYK*). Substitution of the PYK promoter has previously been shown to increase *p*-CA production in *S. cerevisiae*.[51] Ten out of the 17

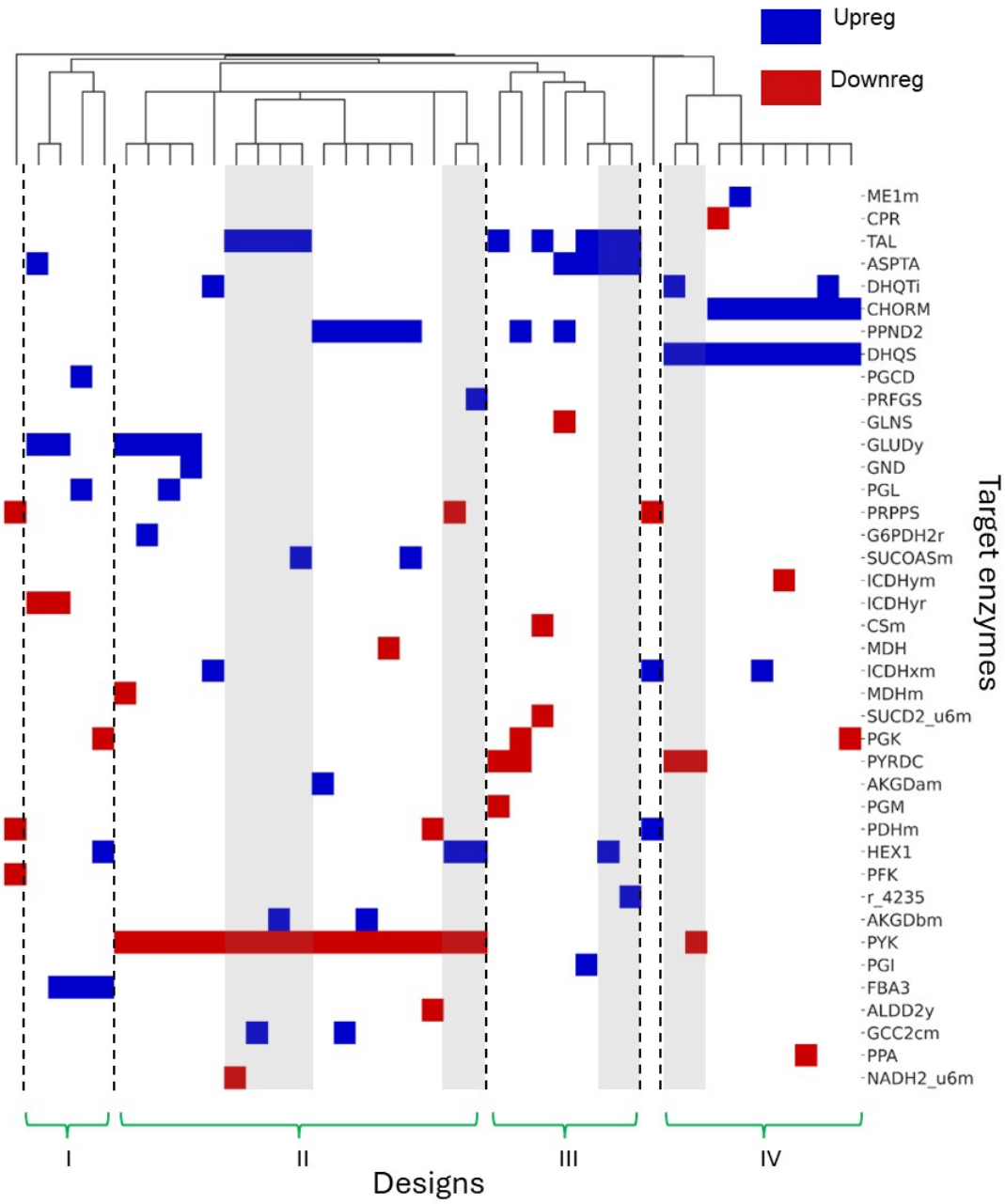

Figure 1: *Clustering analysis of the 39 unique designs (columns) generated using NRA and their associated enzymes (rows). Each column represents the three upregulated (blue) or downregulated (red) enzymes targeted by a single design. The target enzymes are contained mainly in the pentose phosphate pathway (PPP), the Krebs cycle, glycolysis, and the p-CA production pathway. Six of the 10 experimentally implemented designs (gray-shaded columns) lie within cluster II. The remaining 4 designs belong to clusters III and IV.* ***AKGDam:*** *Oxoglutarate dehydrogenase lipoamide,* ***AKGDbm:*** *Oxoglutarate dehydrogenase dihydrolipoamide S succinyltransferase,* ***ALDD2y:*** *Aldehyde dehydrogenase,* ***ASPTA:*** *Aspartate transaminase,* ***CHORM:*** *Chorismate mutase,* ***CPR:*** *Cytochrome P450 reductase 2,* ***CSm****: Citrate synthase,* ***DHQS:*** *3-dehydroquinate synthase,* ***DHQTi:*** *3-dehydroquinate dehydratase irreversible,* ***FBA3:*** *Sedoheptulose 1,7-bisphosphate D-glyceraldehyde-3-phosphate-lyase,* ***GCC2cm:*** *Glycine cleavage complex lipoamide mitochondrial,* ***GLNS:*** *Glutamine synthetase,* ***GLUDy:*** *Glutamate dehydrogenase,* ***GND:*** *Phosphogluconate dehydrogenase,* ***G6PDH2r:*** *Glucose 6-phosphate dehydrogenase,* ***HEX1****: Hexokinase,* ***ICDHxm:*** *Isocitrate dehydrogenase,* ***ICDHym:*** *Isocitrate dehydrogenase mitochondrial,* ***ICDHyr:*** *Isocitrate dehydrogenase,* ***MDH:*** *Malate dehydrogenase,* ***MDHm:*** *Malate dehydrogenase mitochondrial,* ***ME1m:*** *Malic enzyme mitochondrial,* ***NADH2_u6m:*** *NADH dehydrogenase mitochondrial,* ***PDHm:*** *Pyruvate dehydrogenase,* ***PFK:*** *Phosphofructokinase,* ***PGCD:*** *Phosphoglycerate dehydrogenase,* ***PGI:*** *Glucose-6-phosphate isomerase,* ***PGK:*** *Phosphoglycerate kinase,* ***PGL:*** *6-phosphogluconolactonase,* ***PGM:*** *Phosphoglycerate mutase,* ***PPA:*** *Inorganic diphosphatase,* ***PPND2:*** *Prephenate dehydrogenase,* ***PRFGS:*** *Phosphoribosylformylglycinamidine synthase,* ***PRPPS****: Phosphoribosylpyrophosphate synthetase,* ***PYK:*** *Pyruvate kinase,* ***PYRDC:*** *Pyruvate decarboxylase,* ***r_4235:*** *Glucokinase paralog EMI2,* ***SUCD2_u6m:*** *Succinate dehydrogenase ubiquinone 6 mitochondrial,* ***SUCOASm:*** *Succinate CoA ligase,* ***TAL:*** *Tyrosine Ammonia Lyase (heterologous).*

designs also increase activity through the production pathway by upregulating either the heterologous tyrosine ammonia lyase (TAL in our model), prephenate dehydrogenase (PPND2), or 3-dehydroquinate dehydratase (DHQTi). Groups III and IV focus on the *p*-CA production and shikimate pathways, respectively. All seven designs in group III suggest upregulating TAL, PPND2, or aspartate transaminase (ASPTA), with four designs containing an additional target among the three. In group IV, all nine designs target 3-dehydroquinate synthase (DHQS), with seven also upregulating chorismate mutase (CHORM).

We ranked these 39 designs by their mean NRA predicted performance across the models (Methods). The top 10 designs (Figure 1, gray shaded columns; Table 1) all predicted a mean increase of 12-14% in the *p*-CA yield from glucose relative to ST10284. Six of these designs

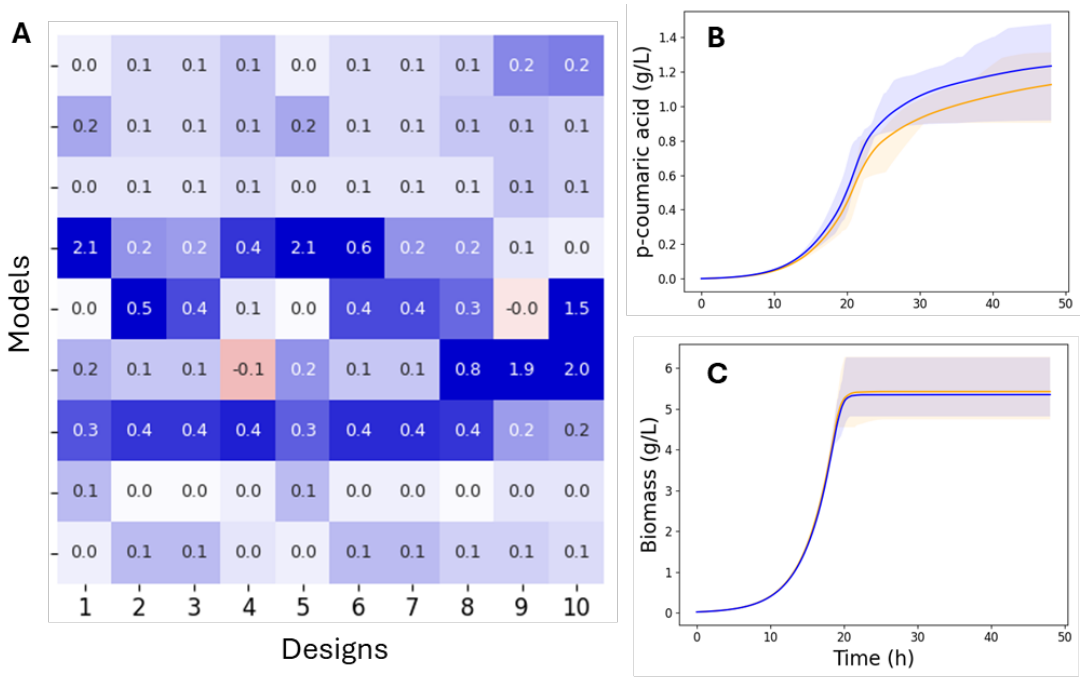

Figure 2: *Performance of the top 10 designs evaluated in nonlinear batch simulations. (A) Relative increase (blue) and decrease (red) in the p-CA yield from glucose for each combination of design and model, compared to ST10284. The yield was calculated in the mid-exponential phase, at t=5 hours. All 10 designs produced a higher yield for 8 out of 9 models, while 8 designs produced a higher yield for all 9 models. (B, C) Simulated p-CA and growth titers for the reference strain (orange) and the top 10 designs (blue) across the 39 combinations of models and designs that provided an increase in p-CA titer after 24 hours. The solid line is the mean across the models, and the shaded region represents the interquartile range.*

belonged to cluster II; four out of the six targeted TAL in addition to PYK, while two had hexokinase (HEX1) in common along with PYK. The remaining four top designs were equally split between clusters III and IV. The designs in cluster III targeted ASPTA, TAL, and HEX1 or glucokinase encoded by the gene *EMI2* (r_4235 in our model), while the designs from cluster IV targeted DHQS, pyruvate decarboxylase (PYRDC), and either PYK or DHQTi.

We simulated the top 10 designs in a batch fermentation setting to evaluate their performance in a nonlinear context. Most design-model combinations increased the *p*-CA yield, with a maximum increase of 210%. Eight out of the top 10 designs demonstrated improvement in all nine models, while the remaining two designs did so for eight out of the nine models (Figure 2A). This yield improvement aligns with the NRA predictions, strengthening confidence in the experimental viability of these designs. Moreover, 39 out of the possible 90 combinations of design and model provided an increase in overall *p*-CA titer after 24 hours in batch simulations (Figure 2B) despite this not being an explicit design objective.

Table 1: Top 10 NOMAD designs implemented experimentally. *Sets of 3 enzymes targeted by each design, along with their reaction names in the model, the genes targeted experimentally, and the name of the corresponding recombinant strain. We implemented design 7 without SUCOASm as implementing it experimentally caused yeast death.*

Design	Reaction	Enzyme	Genes	Strain
1	HEX1↑ PRPPS↓ PYK↓	Hexokinase Phosphoribosylpyrophosphate synthetase Pyruvate kinase	*HXK2* *PRS1* *PYK1*	ST14204
2	HEX1↑ PYK↓ PRFGS ↑	Hexokinase Pyruvate kinase Phosphoribosylformylglycinamidine synthase	*HXK2* *PYK1* *ADE6*	ST14210
3	PYK↓ AKGDbm↑ TAL↑	Pyruvate kinase Mitochondrial 2-oxoglutarate dehydrogenase complex Tyrosine ammonia-lyase	*PYK1* *KGD1, KGD2, LPD1, YMR31* *FjTAL*	ST14206
4	PYK↓ TAL↑ GCC2cm↑	Pyruvate kinase Tyrosine ammonia-lyase Glycine decarboxylase multienzyme complex	*PYK1* *FjTAL* *GCV1, GCV2, GCV3, LPD1*	ST14207
5	HEX1↑ TAL↑ ASPTA↑	Hexokinase Tyrosine ammonia-lyase Aspartate transaminase	*HXK2* *FjTAL* *AAT2*	ST14186
6	PYK ↓ DHQS↑ PYRDC↓	Pyruvate kinase 3-dehydroquinate synthase Pyruvate decarboxylase	*PYK1* *EcAroB* *PDC1*	ST14211
7	PYK↓ TAL↑ SUCOASm↑	Pyruvate kinase Tyrosine ammonia-lyase Mitochondrial succinyl-CoA synthetase complex	*PYK1* *FjTAL* ~~*LSC1*~~, ~~*LSC2*~~	ST14201

8	DHQTi↑ DHQS↑ PYRDC↓	3-dehydroquinate dehydratase 3-dehydroquinate synthase Pyruvate decarboxylase	*EcAroB, EcAroD* *EcAroB* *PDC1*	ST14212
9	PYK↓ TAL↑ NADH2_u6m↓	Pyruvate kinase Tyrosine ammonia-lyase NADH dehydrogenase mitochondrial	*PYK1* *FjTAL* *NDE1*	ST14195
10	TAL↑ ASPTA↑ r_4235↑	Tyrosine ammonia-lyase Aspartate transaminase Glucokinase	*FjTAL* *AAT2* *EMI2*	ST14205

3.3 Reconstruction of rationally engineered *S. cerevisiae* strains

The 10 designs (Table 1) suggested by the kinetic models required several simultaneous up and down regulations of enzyme activities. We first implemented strategies to downregulate the activities of pyruvate kinase (PYK, designs 1-4, 6, 7, and 9), phosphoribosylpyrophosphate synthetase complex (PRPPS, design 1), NADH dehydrogenase (NADH2_u6m, design 9), and pyruvate decarboxylase (PYRDC, designs 6 and 8). These enzymes all play pivotal roles in the homeostatic function of the strain. Pyruvate kinase has paralogs *PYK1* and *PYK2 and* has been reported as the primary enzyme in the glycolytic pathway[52,53]. NADH dehydrogenases *NDE1* and *NDE2* play roles in transferring cytosolic NADH to the mitochondrial respiratory chain[54,55]. PRPPS is crucial for the synthesis of nucleotides, with the *PRS1* subunit being key in enzyme activity encoding in *S. cerevisiae*[56]. PYRDC is encoded by three isozymes - *PDC1*, *PDC5*, and *PDC6*, with PDC1 being the major isozyme[57]. We used a promoter-swapping strategy to implement these four downregulations using the key genes for each enzyme - PYK1, NDE1, PRS1, and PDC1. We first evaluated the strength of various promoters compared to the native promoters of these target genes, considering the -1kb region upstream of the start codon as the native promoter region. We linked all the promoters with GFP using Golden Gate Assembly and measured their expression levels by flow cytometry. The native promoters *PrNDE1* and *PrPRS1* were already relatively weak, with only *PrRAD27* and *PrREV1* providing a lower fluorescence intensity (Figure 3). The weaker of the two, *PrREV1,* was chosen as their replacement. Conversely, the native promoters *PrPYK* and *PrPDC1* were stronger than all the candidates (Figure 3c). Therefore, they were replaced with their weakest compatible counterparts - *PrPRL18b* and *PrTEF*, respectively.

Next, we implemented the strategy for upregulating the design targets suggested by the kinetic

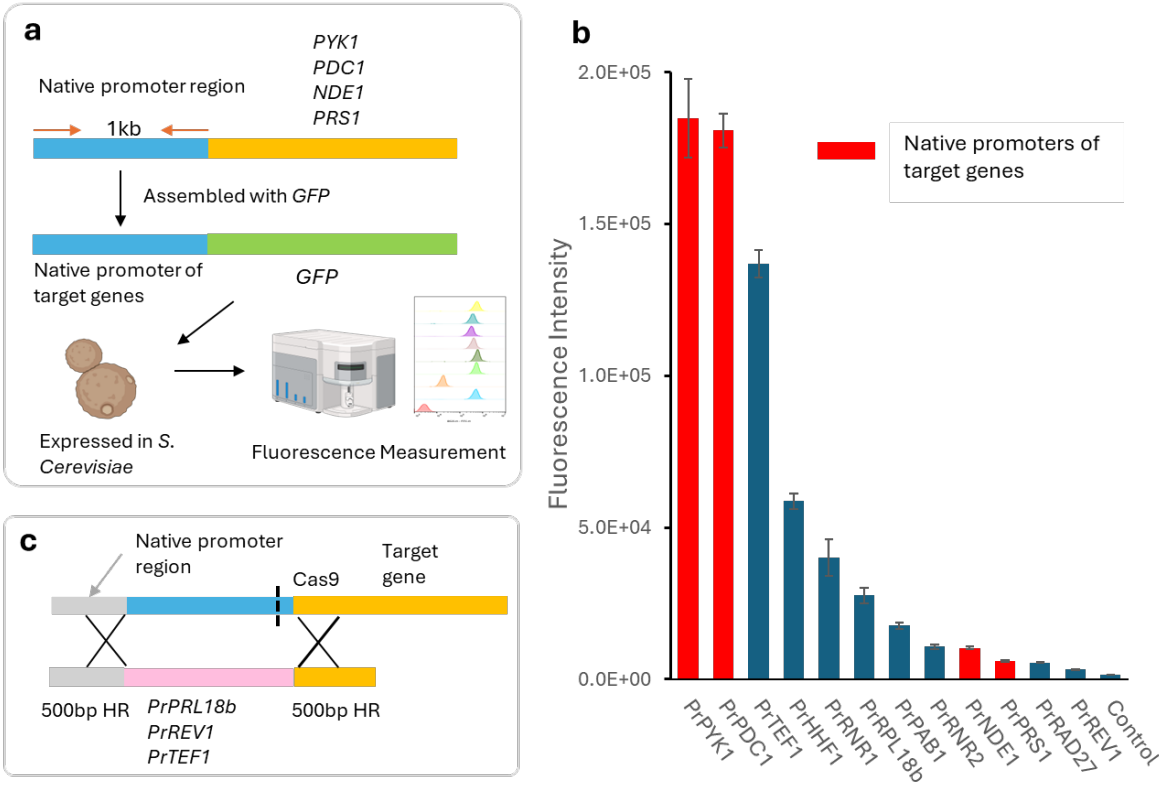

Figure 3: *Gene downregulation through a promoter swapping strategy.* ***(a)*** *Design for measuring the strength of the promoter of four target genes PYK1, PDC1, NED1 and PRS1.* ***(b)*** *Promoter strength measured by flow cytometry.* ***(c)*** *Downregulation of the four target genes through swapping of the native promoter with a weaker promoter using CRISPR-aid.*

models. Designs 3, 5, 7, 9, and 10 involved the upregulation of tyrosine ammonia-lyase, corresponding to TAL from *Flavobacterium johnsoniae* (FjTAL). Three of the targets were enzyme complexes formed by multiple subunits –the glycine decarboxylase multienzyme complex (GCC2cm, design 4), the mitochondrial succinyl-CoA synthetase complex (SUCOASm, design 7), and the mitochondrial 2-oxoglutarate dehydrogenase complex (AKGDbm, design 3). We chose to upregulate all the subunits involved in these complexes as upregulating only some of the subunits might not produce a significant effect. We upregulated 3-dehydroquinate synthase (DHQS, designs 6 and 8) and 3-dehydroquinate dehydratase (DHQTi, design 8) using the *E. coli* genes *EcAroB* and *EcAroD* instead of the native pentafunctional gene *ARO1* as this enabled us to target them specifically. For hexokinase (*HEX1*, designs 1, 2, and 5) and aspartate transaminase (ASPTA, designs 5 and 10), we used their dominant paralogs, *HEX2* and *AAT2*, as targets for upregulation. *HEX2* is the predominant isoenzyme for hexokinase during the growth on glucose, playing the central role in glucose phosphorylation[58]. Similarly, *AAT2* encodes the cytosolic form of aspartate transaminase, the

major contributor to total aspartate aminotransferase activity[59]. Finally, we used *S. cerevisiae*'s native gene *ADE6* for upregulating the activity of phosphoribosylformylglycinamidine synthase (PRFGS, design 2) and the gene *EMI2* for glucokinase (r_4235, design 10). We cloned all the target genes specific to each design into EasyClone vectors using USER cloning and expressed these targets in ST10284 using the CRISPR editing method. All designs could be implemented exactly except for design 7 (ST14201); we had to remove the overexpression of mitochondrial succinyl-CoA synthetase (SUCOASm) as it caused yeast death.

3.4 *p*-CA production in reconstructed strains

We tested the production capabilities of the recombinant strains (Table 1) in microplate batch fermentations using the Growth profiler shaker incubator. During the first 24 hours, all 11 strains – the reference strain and the 10 design strains – grew on glucose and produced ethanol (Supplementary Figures S3 and S4); these are the conditions under which we built the kinetic models and generated the *in-silico* designs. The strains then consumed ethanol for further growth and *p*-CA production. All the engineering strains, except ST14207 and ST14204, increased *p*-CA titers by 19-32% compared to the reference strain at the end of fermentation (Figure 4). ST14206 demonstrated the highest increase in titer (32%), while ST14210 provided the smallest increase (19%). These eight strains also produced more *p*-CA than the reference strain while growing on glucose alone, with increases ranging from 0.4% to 20% at 24 hours. Notably, all ten strains maintained a growth profile comparable to that of ST10284 during this period of growth on glucose; they attained biomass titers that were at least 85% of the reference strain at 24 hours. This adherence to the phenotypic behavior of the reference strain is pertinent as we explicitly constrained the generated designs to have minimal perturbation from the reference strain while satisfying the design objective. The eight strains that increased *p*-CA production maintained biomass production and reached final OD_{600} values of at least 90% of the reference OD_{600} at 72 hours. The two strains that did not increase *p*-CA titers,1) and ST14207 (design 4), reached substantially lower OD_{600} values. ST14207 maintained its growth and *p*-CA production during growth on glucose; however, it could not consume ethanol to fuel further growth and production (Supplementary Figure S4). ST14204, on the other hand, demonstrated impaired performance throughout the fermentation process. This is possibly due

to the downregulation of PRPPS, which disrupts the synthesis of nucleotides, which are the fundamental building blocks of the cell.

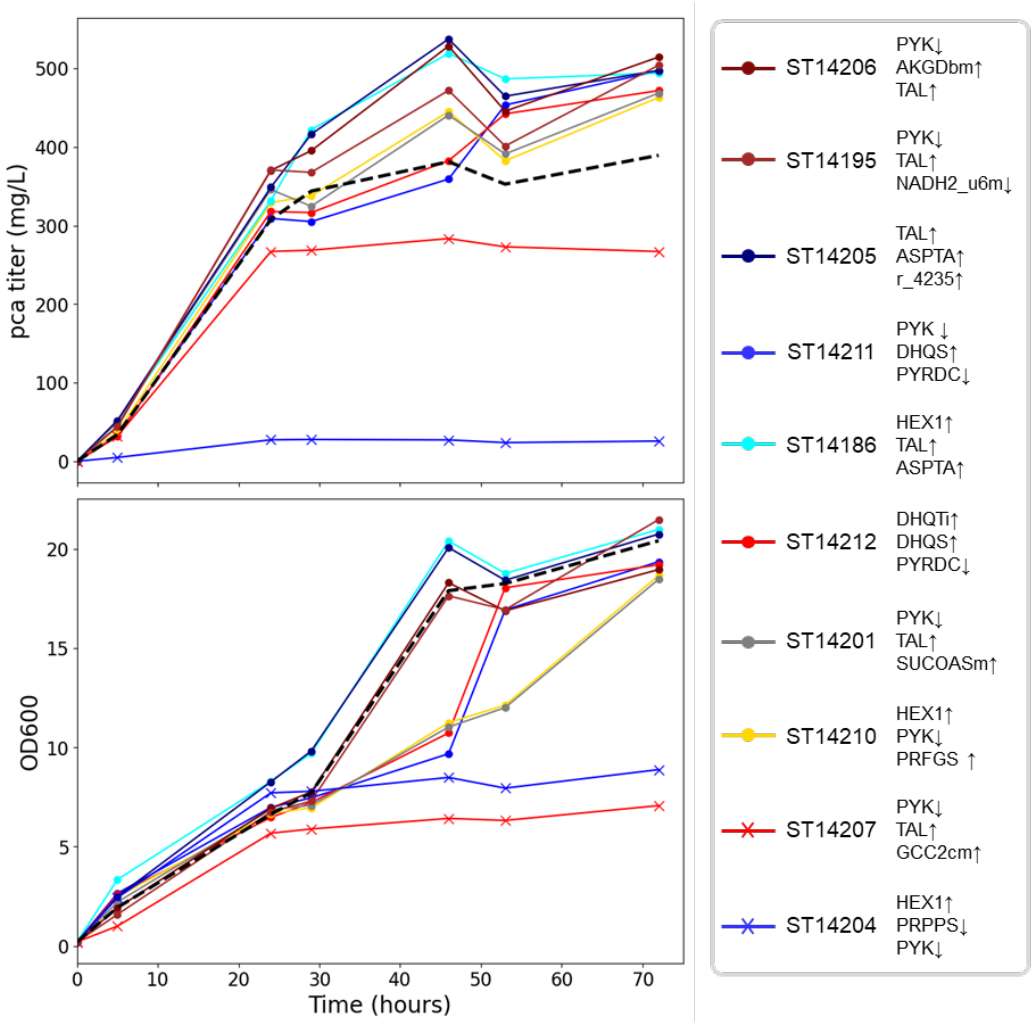

Figure 4: *Average p-CA titers (top) and OD600 (bottom) of all ten engineered strains and the reference strain (dashed black) in triplicate batch fermentation experiments. The strains in the legend are in descending order of the recorded p-CA titer at 72 hours. All ten strains adhered to the growth dynamics when growing on glucose; they produced at least 85% of the biomass concentration of ST10284 at 24 hours. Eight out of the ten strains (solid dots) produced higher p-CA titers than ST10284 during both growth phases. They displayed p-CA titer increases of 0.4-20% and 19-32% when compared to the reference strain at 24 hours and 72 hours respectively. The two strains that did not improve p-CA titers were ST14207 (red cross) and ST14204 (blue cross).*

4 Discussion

Advances in genetic engineering techniques have enabled multiple enzymatic interventions towards improved cellular performance. This work showed how model-guided strain design can successfully be integrated into a design-build-test-learn cycle. We used the NOMAD framework to devise strategies for improved *p*-CA production in *S. cerevisiae*. Our kinetic-model-based combinatorial designs

achieved a high success rate, with eight out of ten designs (80%) producing at least an 18% increase in *p*-CA titers at the end of fermentation.

This successful translation of in-silico designs into viable and high-performing strains was due to the use of (i) large-scale kinetic models that accurately capture the dynamic behavior of the reference strain, (ii) a constraint-based design that maintains the engineered strain's phenotype close to that of the reference strain, and (iii) experimental strategies capable of fine-tuning gene expression levels.

Large-scale kinetic models capture the network-wide effects of genetic interventions, including their impact on key cellular health indicators, such as the energy charge and redox potential. We selected the models most likely representing the reference strain by using nonlinear simulations and concepts from control theory[23]. With these models, we used NOMAD to identify the top 10 designs among 4.5 million combinations of three simultaneous enzyme modifications that could reliably increase *p*-CA production, allowing us to save time and resources. These models also enabled us to identify non-obvious, off-pathway targets, such as NADH dehydrogenase and 2-oxoglutarate dehydrogenase in the mitochondrion, out of 303 possible genetic interventions and their combinations.

NOMAD further ensured the viability of proposed designs by limiting how much the designed strains could deviate from the reference strain. This phenotypic constraint proved essential; we observed that the successful designs also maintained at least 90% of the growth of the reference strain at the end of fermentation. Moreover, we achieved an 80% success rate by choosing only those designs that remained robust across the phenotypic variation covered by the population of nine kinetic models.

Finally, the success of our top 10 designs relied on their precise experimental implementation as they included targets such as PYK and NADH2_u6m that are critical to the homeostatic functioning of the strain. We tackled this challenge by using a promoter-swapping strategy for downregulations and overexpressions for upregulation.

Our work demonstrates the potential of using kinetic models to explore the design space systematically prior to experimental implementation. These models can be used for targeted low-throughput screening when high-throughput screening (HTS) is impossible – for example, when biosensors do not exist. They can also be combined with high throughput screening first to identify combinatorial targets[29] and then optimize the promoter strengths of the chosen targets. Despite their promise, the complexities and challenges of building kinetic models using traditional methods[15,60–63] have meant that only a handful of large-scale models exist[64–67]. Recent advances in machine learning methods, such as RENAISSANCE[18], ReKindle[20], and iSCHRUNK[68–70], as well as other approaches like KETCHUP[21], have made it possible to parameterize and refine large-scale models rapidly. Coupled with the increasing availability of high-quality omics data, these developments will hasten the construction of large-scale models for a host of species. These models can then be used to develop designs and test

their viability in industrial settings by simulating their behavior in conditions that resemble real-world environments.

Overall, our work demonstrates that kinetic models offer a robust way to develop multi-target designs with a high success rate when implemented experimentally. It paves the way toward a future where libraries of large-scale kinetic models provide off-the-shelf solutions for various metabolic engineering applications, ultimately accelerating the development of microbial strains by reducing the number of design-build-test-learn cycles.

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Author contributions

Bharath Narayanan: Writing – original draft, Methodology, Conceptualization, Software, Formal analysis, Investigation, Visualization. **Wei Jiang**: Writing – original draft, Methodology, Conceptualization, Investigation, Formal Analysis, Visualization. **Shengbao Wang**: Writing – original draft, Methodology, Conceptualization, Investigation, Formal Analysis, Visualization. **Maria-Masid Barcon**: Methodology, Software. **Javier Sáez-Sáez**: Investigation, Analysis. **Viktor Hesselberg-Thomsen**: Investigation, Analysis. **Daniel Weilandt:** Methodology, Software. **Irina Borodina**: Resources, Conceptualization, Writing-Review and Editing, Supervision, Project administration, Funding acquisition. **Vassily Hatzimanikatis:** Resources, Formal analysis, Supervision, Funding acquisition. **Ljubisa Miskovic**: Conceptualization, Formal analysis, Writing-Review and Editing, Supervision, Project administration, Resources, Funding acquisition.

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