

1 **Genome-scale metabolic rewiring to achieve predictable titers rates and yield of a non-**
2 **native product at scale**

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32

33 **Abstract**

34 Achieving high titer rates and yields (TRY) remains a bottleneck in the production of
35 heterologous products through microbial systems, requiring elaborate engineering and many
36 iterations. Reliable scaling of engineered strains is also rarely addressed in the first designs of
37 the engineered strains. Both high TRY and scale are challenging metrics to achieve due to the
38 inherent trade-off between cellular use of carbon towards growth vs. target metabolite
39 production. We hypothesized that being able to strongly couple product formation with growth
40 may lead to improvements across both metrics. In this study, we use elementary mode analysis
41 to predict metabolic reactions that could be targeted to couple the production of indigoidine, a
42 sustainable pigment, with the growth of the chosen host, *Pseudomonas putida* KT2440. We
43 then filtered the set of 16 predicted reactions using -omics data. We implemented a total of 14
44 gene knockdowns using a CRISPRi method optimized for *P. putida* and show that the resulting
45 engineered *P. putida* strain could achieve high TRY. The engineered pairing of product
46 formation with carbon use also shifted production from stationary to exponential phase and the
47 high TRY phenotype was maintained across scale. In one design cycle, we constructed an
48 engineered *P. putida* strain that demonstrates close to 50% maximum theoretical yield (0.33 g
49 indigoidine/g glucose consumed), reaching 25.6 g/L indigoidine and a rate of 0.22g/l/h in
50 exponential phase. These desirable phenotypes were maintained from batch to fed-batch
51 cultivation mode, and from 100ml shake flasks to 250 mL ambr® and 2 L bioreactors.

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57 **Introduction**

58 Heterologous production of bioproducts has been demonstrated for a very large number of
59 compounds and in a wide variety of microbial hosts^{1,2}. Yet, even the most well-designed
60 heterologous pathway requires considerable additional work to reach titers, rates and yields
61 (TRY) necessary for the adoption of these systems by industry^{3,4}. In addition, the production
62 parameters of a strain at lab-scale is often not predictive of its performance and robustness
63 when cultivated in different modes or at larger scales. As a result, only a small fraction of
64 bioproduction strains have been successfully scaled and deployed².

65
66 Here we explore if it is possible to rewire the metabolism of the host strain such that production
67 of a final product or a key intermediate is coupled with the carbon source, and used to maximize
68 and maintain productivity at scale. Native microbial processes that take such growth coupled
69 routes include the generation of ethanol and organic acids during fermentation. Production of
70 these metabolites are required for carbon utilization during fermentative growth, and
71 correspondingly these compounds represent the most prominent examples of successful high-
72 volume bioproduction^{5,6}. We hypothesized that coupling production to growth is implementable
73 for a heterologous product, and that such a dependence could provide high TRY and the ability
74 to maintain production parameters across different growth modes and scales.

75
76 The availability of comprehensive metabolic models and genome editing tools in a wide variety
77 of microbes suitable for industrial use provides the foundation for our approach. We use the
78 production of indigoidine, a bipyridyl compound derived from glutamine, as the target
79 heterologous product. Both as a sustainable replacement for blue pigments⁷ in a wide array of
80 applications as well as a model non-ribosomal peptide⁸, this compound provides a valuable
81 target to explore. We used *Pseudomonas putida* KT2440 as our production host, leveraging the
82 availability of the iJN1462 genome scale model for *P. putida* KT2440⁹. We adapted elementary

83 mode analysis (EMA)¹⁰ to determine the constrained minimal cut set (cMCS) required to
84 minimize metabolic flux towards undesired products and link indigoidine formation to cell
85 viability¹¹. These analyses, combined with publicly available omics data^{12,13}, provided the set of
86 gene loci that represented the reactions necessary for removal. The corresponding set of gene
87 loci were repressed using multiplex CRISPR interference (CRISPRi) that we optimized for use
88 in *P. putida* KT2440. Our implementation resulted in a highly edited strain that, in a single
89 iteration of strain engineering, achieved close to 50% max theoretical yield of indigoidine in *P.*
90 *putida* KT2440 and TRY characteristics that maintain fidelity from laboratory to industrially
91 relevant scales.

92

93 **Results**

94

95 *Genome scale evaluation of P. putida for strong coupling*

96

97 To develop the product coupling approach (**Figure 1a**), we first identified the number of
98 metabolites represented in *P. putida* iJN1462⁹ model that can be made essential for growth.
99 For this we used the cMCS algorithm¹¹ that can identify minimal sets of reactions, the
100 elimination of which would cause production of a given metabolite to become essential for
101 growth. Aerobic conditions with glucose as the sole carbon source were used to model growth
102 parameters. We searched for gene knockdown sets to satisfy three potential constraints in
103 which the theoretical product yield was at a minimum of 10%, 50%, or 80% of the maximum
104 theoretical yield (MTY) for all producible metabolites in the model coupled to a minimum 10%
105 biomass yield. This analysis, completed for all 2145 metabolites in the genome scale model,
106 indicated that 979 organic metabolites could potentially be made essential for growth. In the first
107 pass, 98.6% of these 979 metabolites had the potential to satisfy this biomass-formation
108 constraint, with a minimum production threshold of 10% MTY. When the threshold for minimum

109 production was set to 50% MTY, 903 metabolites could be essential for growth; for an 80%
110 threshold MTY, only 444 metabolites could be made essential for growth, representing only 45%
111 of the total producible metabolites. This potential growth coupling for all metabolites is
112 consistent for similar calculation for other hosts¹¹ (see **Supplementary Table 1**). Setting a
113 higher demand for minimum product yield results in fewer metabolites that can be used to
114 implement a production obligatory regime.

115
116 As the framework proposed by von Kamp and Klamt required an extensive rewiring of microbial
117 physiology, *a priori* it was not obvious how to account for a heterologous end product, a
118 challenge to implement in practice. We began by adding an *in silico* reaction for the
119 heterologous product, indigoidine, to the genome scale metabolic model iJN1462⁹. This reaction
120 represents the biosynthesis of indigoidine from glutamine and accounts for all necessary
121 cofactors. The MTY for glutamine and indigoidine was calculated to be 1.141 mol/mol and 0.537
122 mol/mol respectively from glucose as the carbon source (**Table 1**). The MTY for glutamine in *P.*
123 *putida* was high relative to other hosts screened by us (**Supplementary Table 2**). As this
124 method accounts for the other physiological processes competing for resources, a MTY derived
125 from a genome scale model provided a more accurate assessment compared to simpler
126 methods, as is commonly done in the field^{14,15}.

127
128 In order to predict reactions that would be required to improve indigoidine production, we used
129 glutamine, its precursor, to conduct the analysis. Our process for determining the list of required
130 gene targets is diagrammed in **Figure 1**. The minimum theoretical product yield of glutamine
131 was set at 10%, 50% and 80% MTY to derive the reactions that would require knockout or
132 knockdown for product substrate paired growth. We eliminated potential target sets that needed
133 the removal of genes coding for multi-functional proteins, as we sought to limit additional
134 metabolic perturbations that could confound our analysis. Of the 1956 reactions in iJN1462 that

135 are associated with genes, only 60% have a single gene associated with them. If a metabolic
136 reaction was catalyzed by more than one gene product (genes coding for isozymes or multi-
137 subunit enzymes), we included both genes for inactivation. After implementing these filters, we
138 found that a threshold of 80% MTY could be achieved using the elimination of 14 cellular
139 reactions. These 14 metabolic reactions when mapped to their corresponding genes and gene
140 products represented 16 gene loci (**Figure 1b and Supplementary Dataset 1**).

141
142 Next, we used Flux Balance Analysis (FBA) and Flux Variability Analysis (FVA), to confirm the
143 16-gene cMCS strategy to be obligatory for glutamine production. Using our constructed cMCS
144 platform, we set the parameters to explore potential product-obligatory strategies to enhance
145 the production of indigoidine in *P. putida* when glucose is fed as the sole carbon source. This 16
146 gene set provided for glutamine was then extended to assess production paired growth for
147 indigoidine. FBA analyses confirmed that growth using glucose could be paired with indigoidine
148 production at 90% theoretical yield (0.48 mol/mol or 0.66 g/g of glucose). This analysis also
149 confirmed that we can adapt the work from von Kamp and Klamt¹¹ for non-native final products
150 and target specific genes rather than enzymatic reactions for intervention.

151
152 Since EMA requires the delineation of specific growth conditions, such as starting carbon
153 source, we examined if the gene cut set with glucose as a substrate, could maintain product
154 pairing with other known native carbon substrates for *P. putida*, such as *para*-coumarate and
155 lysine^{12,16}. FBA with these alternate carbon sources (i.e. lysine, *para*-coumarate) indicated that a
156 strain engineered using the 16-gene cMCS strategy for the glucose would fail to produce
157 glutamine (**Supplementary Table 3**). In contrast, this gene targeting set (**Supplementary**
158 **Dataset 1**) results in the desired production obligatory growth using galactose as a carbon
159 source because it shares the same downstream catabolism as glucose (**Figure 1b**).

160

161 *Building the multi-edit engineered strain:*

162

163 To test the predictions from the metabolic modeling described above, we built the control
164 engineered *P. putida* production systems. First we genomically integrated the heterologous
165 production pathway comprised of *sfp* and *bpsA*. BpsA is a non-ribosomal peptide synthetase
166 (NRPS) from *Streptomyces lavendulae* that catalyzes indigoidine formation from two molecules
167 of glutamine in an ATP-dependent manner¹⁷. Activation of BpsA requires a post-translational
168 pantetheinylation conferred by a promiscuous Sfp from *Bacillus subtilis*¹⁸. The genomically
169 integrated production strain harboring a plasmid-borne *dCpf1* and non-targeting gRNA serves
170 as the control production strain. The basal production of indigoidine in *P. putida* is 2.3 g/L
171 indigoidine from 10 g/L glucose after 24 hours (**Supplementary Figure 1a**). The bulk of
172 production occurred in stationary phase, approximately 12 hours after carbon depletion, as is
173 typically observed for *P. putida*^{19,20}. To test the use of galactose, we also engineered a
174 galactose utilization strain via genomic integration of a *galETKM* operon^{21,22} and here production
175 of indigoidine was negligible (**Supplementary Figure 1b**). Optimizing carbon/nitrogen ratio
176 yielded only modest improvements to indigoidine production for both glucose and ammonium
177 sulfate (**Supplementary Figure 1c-e**).

178

179 Prior to construction of the multi-gene edited production strain, we assessed if our gene set
180 contained essential genes. The iJN1462 model has an incomplete list of essential genes; in
181 addition we manually annotated genes as essential or dispensable using gene essentiality data
182 generated from a barcoded fitness library (RB-TnSeq)¹³ (**Supplementary Dataset 2**). Out of the
183 16 genes identified for knockdown, two genes were excluded because they are essential for
184 viability. By eliminating essential genes from the targeted gene set, we hypothesized that the
185 predicted metabolic rewiring is more consistent with product substrate pairing rather than growth
186 coupling.

187

188 To efficiently overcome technical limitations required to make 14 gene edits, we implemented a
189 multiplex CRISPRi/dCpf1 targeting strategy. We drew on our understanding of repetitive
190 element instability^{23,24} to minimize use of repeated DNA sequences to limit gRNA array loss.
191 While other reports have indicated technical challenges constructing multiplex gRNA arrays²⁵,
192 both native and synthetic repetitive arrays exist (including those of native CRISPR arrays)^{26,27}.
193 An endonuclease deficient class II CRISPR-Cas enzyme, FnCpf1²⁸, was chosen over Cas9 as
194 the Cpf1 crRNA is only 19 bp in size, compared to the corresponding crRNA (gRNA scaffold
195 sequence) from Cas9, which is 76 bp²⁸. Each gRNA was driven by a different *P. putida* tRNA
196 ligase promoter/terminator pair, and dCpf1 was placed under the control of the *lacUV5*
197 promoter. Minimal 100-bp promoter sequences from native tRNA ligases were sufficient to
198 express *mCherry* fluorescent protein, confirming that heterologous mRNA transcripts for gRNAs
199 would be generated (**Supplementary Figure 1f**).

200

201 In a successful deployment of the multiplex CRISPRi/dCpf1 we expect to see a decrease in
202 mRNA expression levels (and protein abundance) of the genes targeted with CRISPR
203 interference. We used RNAseq analysis to examine the engineered strain, and compared
204 normalized RNA expression levels between the control strain (**Figure 2a-c**). RNA expression
205 levels were sampled over the duration of a 72-hour time course. Expression of all 14 gRNAs
206 were detected by this analysis (**Figure 2a**). The multiplexed Cpf1 gRNAs in this array did not
207 efficiently terminate with endogenous terminator sequences and generated chimeric mRNAs.
208 Nonetheless, nine of the fourteen targeted gene loci exhibited decreased mRNA expression
209 levels, and at best showed a 50% decrease (**Figure 2b, Supplementary Figure 2**). Global
210 indirect changes in gene expression were also detected (**Figure 2c**). Partial reduction of protein
211 abundance was also observed for ten of the fourteen genes (**Figure 2b, Supplementary**
212 **Figure 2**).

213

214 A consequence of pairing production to the catabolism of specific carbon sources, results in
215 predictions that other carbon sources can no longer be metabolized (**Supplementary Table 3**).
216 We tested this prediction experimentally and observed that engineered strains for product
217 substrate pairing showed reduced growth when using either lysine or *para*-coumarate as the
218 sole carbon source, in agreement with the modeling (**Figure 2d**).

219

220 *Characterizing the multi-gene engineered production strain*

221

222 Redirecting metabolic flux to improve glucose paired glutamine/indigoidine formation is
223 quantifiable across several other metrics. We should observe high TRY for our desired product
224 since higher glutamine yields, to support growth, should result in more indigoidine yields. The
225 production of indigoidine would shift from stationary phase to exponential phase, as the
226 metabolism of glucose catabolism and glutamine production are paired. Finally, these
227 phenotypes should maintain fidelity across a range of growth modes and scales.

228

229 We tested to ensure that indigoidine production was improved in the engineered strain relative
230 to the controls in several laboratory cultivation formats. We tested production using both the
231 native glucose and engineered galactose pathways as carbon sources. Both strains were
232 cultivated with either 10 g/L glucose or galactose, as the same targeted reaction set would
233 function on either carbon source. In a deep well plate format, we observed that the engineered
234 strain produced nearly three-fold more indigoidine than the control strain when fed glucose
235 (**Figure 3a**). In a shake flask format, the engineered strain produced 30% more than the control
236 strain. Finally, when cells were cultivated with galactose in the deep well format, the same
237 engineered strain was able to produce indigoidine in contrast to the galactose utilization control
238 strain which only formed biomass (**Figure 3b**).

239

240 In a 2L bioreactor, cultivated in a batch-mode with glucose as the carbon source, we observed
241 improved titers at 12.5 g/L indigoidine from 60 g/L glucose. The control production strain, in
242 contrast, produced 5 g/L, and production of the final molecule was realized after glucose was
243 exhausted from the media. When galactose was fed, the engineered strain also exhibited
244 improved titers of 25.6 g/L of indigoidine from 60 g/L galactose as opposed to the control strain
245 that generated only around 900 mg/L of indigoidine; a 28-fold improvement in production was
246 observed in the engineered strain. Moving to an industrially relevant cultivation format did not
247 impact the final product titer, allowing us to further develop cultivation methods by switching to
248 fed-batch mode.

249

250 We realized greater improvements in final product titer as well as improvements in production
251 kinetics in the fed-batch mode using the ambr® 250 system. After administering an initial high
252 nutrient feed to increase biomass in the reactor, we reduced the feed rate to study indigoidine
253 product formation during exponential phase growth (**Figure 3a**, right hand panel, and
254 **Supplementary Figure 3**). During this phase, the engineered strain produced at a rate of 0.22
255 g/l/h, while the control strain accumulated no additional product. This observation is consistent
256 with our hypothesis that indigoidine formation would occur during exponential phase due to
257 pairing with glucose. In terms of yield, the engineered strain generated consistently higher
258 production than the control strain when cultivated with glucose (0.2 g/g compared to 0.1 g/g),
259 but was not as consistent when cultivated on galactose (**Figure 3c**). Together all aspects of the
260 phenotypes that were desirable for the engineered strain were found to be true.

261

262

263 **Discussion**

264 This study is the first implementation of cMCS predictions enabled with CRISPR interference
265 and resulted in a strain where production was paired with growth. Pairing the final desired
266 product with the carbon source used for growth, mimics native obligatory product formations
267 such as ethanol production and results in high productivity at scale. Further, to our knowledge,
268 there are no other reports where the production of a non-native molecule was shifted from
269 stationary phase to exponential phase as a result of strain engineering.

270

271 The competition between biomass accumulation and production of the target compound is a
272 well-recognized challenge in biomanufacturing. This trade-off impacts both TRY and scalability.
273 Approaches to address this tradeoff range from growth coupling²⁹ to growth decoupling.³⁰
274 Canonical approaches to growth coupling are FBA-based methods such as OptKnock³¹ that
275 identifies secondary pathways that reduce the pool of a key intermediate as means to increase
276 flux to the target of interest. This strategy has been termed as “weak” growth coupling³² where
277 growth still occurs even if the desired product is not formed. Yim *et al.* used a tailored solution
278 involving such computational methods to improve 1,3-BDO production to 18 g/L³³, but their
279 method cannot be generalized for other molecules. Others have described growth coupling as
280 the creation of a “driving force” such as ATP production or cofactor imbalance, and link the
281 driving force to the desired production pathway^{29,34–36}. Driving force coupling is also pathway
282 specific and requires additional strain engineering. Examples include 1-butanol production in *E.*
283 *coli* using NADH as the driving force³⁴ or media supplementation for butanone production in *E.*
284 *coli* linked to acetate assimilation²⁹.

285

286 In contrast to the examples described above, the multi-gene engineered product substrate
287 pairing we report here is an implementation of “strong” growth coupling. It relies on EMA based
288 methods that provide targets at the genome-scale level^{11,37,38} but predicts a large number of
289 enzymatic reactions for elimination. We used FBA to corroborate our optimal cMCS and

290 removed essential genes from targeted gene sets using -omics data to determine the genes that
291 should be targeted for CRISPRi. This genome scale approach also represents a valuable
292 paradigm for the evaluation of microbial hosts for their production capacity and could
293 significantly reduce the time taken to optimize carbon source conversion to the final product.
294 The appeal of this strategy is that the gene knockdown solution is scale-agnostic; the predicted
295 metabolic rewiring should apply even in the largest bioreactor formats.

296

297 In the context of TRY improvement alone, indigoidine itself is an example of a heterologous
298 product that has been demonstrated at high titers³⁹⁻⁴¹. The production of indigoidine was high in
299 the oleaginous yeast *Rhodospiridium toruloides* but remained low in the model yeast *S.*
300 *cerevisiae*, despite similar optimization of cultivation parameters. This comparison represents an
301 empirical example of the innate metabolic potential of a given host, and is consistent with our
302 calculated max theoretical yields for indigoidine (**Supplementary Table 2**). Genome scale
303 metabolic models can accurately predict how microbial hosts could be advantageous for the
304 production of a given metabolite. For indigoidine, the MTY from glucose in *P. putida* is 0.54
305 mol/mol and is comparable to that for *R. toruloides* (0.5 mol/mol), while *E. coli* (0.4 mol/mol) and
306 *S. cerevisiae* (0.079 mol/mol) are much lower. It is likely that every molecule will be different.
307 Thus, selecting the best host/ final product pair is a crucial aspect of developing the ideal
308 production platform.

309

310 While our engineered strains showed many desirable phenotypes, several aspects merit
311 additional discussion. The predicted EMA based cut set (cMCS) demands zero flux through
312 these reactions for strong growth coupling. We excluded two genes from the predicted gene set
313 due to their essentiality. Of the remaining gene targets, our RNAseq and proteomic data
314 indicates a partial gene knockdown, implying that a non-zero flux could occur through the
315 predicted reactions. The resulting yield space for indigoidine production is now different from

316 what was predicted by EMA (**Figure 3d**). This suggests that partial implementation of the EMA
317 predictions, still resulted in a shift of production from stationary to exponential phase while
318 maintaining desirable indigoidine TRY.

319

320 Our approach allowed us to achieve, in one cycle of strain engineering, a high and consistent
321 TRY for indigoidine across cultivation scales. With improvements in genetic tools and metabolic
322 models it may be possible to obtain the 90% MTY predicted by the EMA based cMCS. A better
323 understanding of the terminator sequence efficiency (as observed in this study and elsewhere in
324 *E. coli*²⁵) would enable more efficient CRISPR mediated gene knockdown. Similar fold
325 repression of targeted proteins by CRISPRi/dCas9 were recently reported⁴², suggestive of a
326 limitation for existing CRISPR systems in *P. putida*. Additionally, delineation of gene targets for
327 this approach relies on the availability of high-quality genome scale metabolic models, and also
328 calculated using a single carbon source. Future mechanistic studies of these strains will lead to
329 more refined genome scale models, enabling more accurate metabolic flux modeling when the
330 engineered strains are grown with these carbon sources. This approach cannot be used for
331 certain mixed carbon streams, such as glucose and xylose, as our calculations for glucose
332 pairing inactivates the pentose phosphate pathway. Similarly, there are metabolites that cannot
333 be made obligatory for growth¹¹. For final products derived from this class of metabolites,
334 alternative strategies or hosts would need to be explored. We also do not take into
335 consideration products or intermediates that may be toxic. As industrial processes also use
336 renewable carbon sources that may contain inhibitory byproducts, microbial hosts will require
337 some degree of tolerance engineering⁴³ to unlock its potential. Addressing these aspects will
338 further boost the usefulness of this product substrate pairing approach.

339

340 **Materials and Methods**

341 *Computation of constrained minimal cut sets (cMCS)*

342 *Pseudomonas putida* KT2440 genome scale metabolic model (GSM) iJN1462⁹ was used. The
343 ATP maintenance demand and glucose uptake were 0.97 mmol ATP/gDW/h and 6.3 mmol
344 glucose/gDW/h, respectively. Constrained minimal cut sets (cMCS) were calculated according
345 to the algorithm as previously described¹¹. Excretion of byproducts was initially set to zero,
346 except for the reported overflow metabolites for secreted products specific to *P. putida*
347 (gluconate, 2-ketogluconate, 3-oxoadipate, catechol, lactate, methanol, CO₂, and acetate).
348 Additional inputs including minimum demanded product yield (% of MTY) and minimum
349 demanded biomass yield at 10% of maximum biomass yield were also specified in order to
350 constrain the desired design space. Knockouts of export reactions and spontaneous reactions
351 were not allowed. The algorithm computed for all minimal combinations of reaction knockouts
352 blocking all undesired flux distributions and maintaining at least one of the desired metabolic
353 flux distributions. With the specifications used herein each calculated knockout strategy (cMCS)
354 will ensure that growth is not feasible without biosynthesis of glutamine. All cMCS calculations
355 were done using API functions of CellNetAnalyzer⁴⁴ on MATLAB 2017b platform using CPLEX
356 12.8 as the MILP solver. A summary of common potential growth coupled reactions and the
357 number of targeted reactions to satisfy coupling restraints is included (**Supplementary Figure**
358 **4**). Once all the cMCS were enumerated, we used the decision workflow (**Figure 1a**) to identify
359 an optimal engineering strategy for experimental validation.

360 *Constraint Based methods to confirm the cMCS*

361 iJN1462 was extended to account for indigoidine biosynthesis pathway and checked for strong
362 growth coupling to confirm the chosen engineering strategy for experimental implementation.
363 Flux Balance Analysis (FBA) was used to calculate the maximum theoretical yield (MTY) from
364 reaction stoichiometry and redox balance and also for single gene deletion analysis. Flux
365 variability analysis (FVA) was used along with FBA to check for minimum and maximum
366 glutamine or indigoidine flux under the identified cMCS strategy to confirm product obligatory

367 growth. COBRA Toolbox v.3.0⁴⁵ in MATLAB R2017b was used for FBA and FVA simulations
368 with the GLPK (<https://gnu.org/software/glpk>), an open-source linear optimization solver.
369 Production envelope was obtained using the internal COBRA Toolbox function,
370 *productionEnvelope()* and plotted for *P. putida* (**Figure 3d**) as a fraction of maximum theoretical
371 product yield on y-axis and maximum theoretical biomass yield on x-axis.

372

373 *Chemicals, media and culture conditions*

374 All chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless
375 mentioned otherwise. When cells were cultivated in a microtiter dish format, plates were sealed
376 with a gas-permeable film (Sigma-Aldrich, St. Louis, MO). Tryptone and yeast extract were
377 purchased from BD Biosciences (Franklin Lakes, NJ). Engineered strains were grown on M9
378 Minimal Media as described previously⁴⁶ with slight modifications. Carbon sources (glucose or
379 galactose) were used at 56mM and (NH₄)₂SO₄ was used at 2 g/L, unless indicated otherwise.

380

381 *Strains and strain construction*

382 *Pseudomonas putida* KT2440 was used as the host for strain engineering. All strains used in
383 this study are described in **Supplementary Table 4** and are available upon request from public-
384 registry.jbei.org. Upon publication, plasmid sequences are available at public-registry.jbei.org.
385 Specific DNA sequences used to design the gRNA array are described in **Supplementary**
386 **Dataset 1**. Electroporation with the respective plasmid was performed using a Bio-Rad (Bio-
387 Rad Laboratories, Hercules, CA) MicroPulser preprogrammed EC2 setting (0.2 cm cuvettes with
388 100 μ L cells, ~5msec pulse and 2.5kV) with slight modifications⁴⁷. Cells transformed with
389 replicative plasmid DNA were allowed to recover at 25 °C for 2.5 hours before plating on
390 selective agar media at 23 °C for overnight incubation. Cells transformed with non-replicative
391 (integrating) plasmids were allowed to recover for 4-8 hours in LB media before plating on
392 selective agar media at 23 °C for an additional 24 hours. Kanamycin sulfate or gentamicin

393 sulfate (Sigma-Aldrich, St. Louis, MO) was used at a concentration of 50 µg/mL or 30 µg/mL,
394 respectively. Integration of the *Ec.galETKM* operon or heterologous indigoidine gene pathway
395 was implemented using a sucrose counterselectable plasmid for allelic exchange⁴⁸. Positive
396 clones were confirmed for the genotype by colony PCR using Q5 Polymerase enzyme (NEB,
397 Ipswich, MA). The dCpf1/CRISPRi system was adapted for use in *P. putida* by subcloning an
398 endonuclease dead *Francisella tularensis* subsp. *Novicida* *Cpf1*⁴⁹ into a pBBR1 backbone and
399 placed under the LacUV5 promoter. The synthetic gRNA array was constructed using gene
400 synthesis techniques (Genscript, Piscataway, NJ) and cloned into the dCpf1/CRISPRi backbone
401 using isothermal DNA assembly. All plasmid constructs were verified with Sanger sequencing
402 before transformation into *Pseudomonas putida*.

403

404 *Analytics/ Sugar Quantification - HPLC*

405 Glucose and organic acids from cell cultures were measured by an 1100 Series HPLC system
406 equipped with a 1200 Series refractive index detector (RID) (Agilent Technologies, Santa Clara,
407 CA) and Aminex HPX-87H ion-exclusion column (300 mm length, 7.8 mm internal diameter).
408 300 µL aliquots of cell cultures were removed at various time points during production and
409 filtered through a spin-cartridge with a 0.45-µm nylon membrane, and 10 µL of the filtrate was
410 eluted through the column at 50°C with 4 mM sulfuric acid at a flow rate of 600 µL/min for 30
411 min. Metabolites were quantified with an external standard calibration with authentic standards.

412 *Indigoidine Extraction and Quantification*

413 Indigoidine was purified from *P. putida* with slight modifications as previously described⁵⁰. Cells
414 were lysed in 1% SDS and 100 mM NaCl and then centrifuged at 14,000 xg for 3 minutes. The
415 supernatant was discarded and the pellet was washed with three rounds of methanol,
416 isopropanol, water, ethanol, and hexane to remove contaminating proteins and metabolites. The
417 pellet was allowed to dry overnight and then resuspended in DMSO at a final concentration of

418 1mg/mL. Indigoidine purity was characterized by NMR. A standard curve correlating indigoidine
419 concentration to OD₆₁₂ was generated using this reagent (**Supplementary Figure 5**). The purity
420 of extracted indigoidine (**Supplementary Figure 6**) from both *E. coli* and *P. putida* were cross-
421 validated by NMR as previously described⁴¹.

422

423 To rapidly quantify indigoidine production in a high throughput manner, the colorimetric assay
424 was used to determine indigoidine production. Briefly, pelleted 100uL of cells by centrifugation
425 at 15000 rpm for 2 min. The supernatant was discarded and 500uL DMSO was added to the
426 pellet. The solution was vortexed vigorously for 10 minutes to dissolve indigoidine. After
427 centrifugation at 15000 rpm for 2 min, 100μL of DMSO extracted indigoidine was added to 96-
428 well flat- bottomed microplates (BD Biosciences, San Jose CA). Indigoidine was quantified by
429 measuring the optical density at using a microplate reader (Molecular Devices, San Jose, CA)
430 preheated to 25°C and applying standard curve generated from indigoidine. The equation used
431 was Y (g/L of Indigoidine) = $0.212 * A_{612} - 0.0035$. DMSO-solubilized cell lysate from wild-type *P.*
432 *putida* does not contribute any signal when measured at OD₆₁₂.

433

434 To correlate indigoidine yields with biomass yields, the dry cell weight was determined using
435 OD₆₁₂ to biomass conversion estimates as previously described⁵¹.

436

437 *RNAseq and data analyses*

438 Total RNA was prepared following the manufacturer's protocol⁵² for Trizol-based RNA extraction
439 with several modifications. RNA from trizol treated lysates were bound to a silica column (Direct-
440 zol RNA MiniPrep Plus Kit, Zymo Research, Irvine CA) and its integrity confirmed using a
441 Bioanalyzer RNA 6000 Nano assay (Agilent Technologies, Santa Clara, CA). rRNA was
442 removed from 100 ng of total RNA using Ribo-Zero(TM) rRNA Removal Kit (Illumina
443 Biotechnology, San Diego, CA). Stranded cDNA libraries were generated using the Illumina

444 Truseq Stranded mRNA Library Prep kit. The rRNA depleted RNA was fragmented and
445 reversed transcribed using random hexamers and SSII (Invitrogen-ThermoFisher, Carlsbad,
446 CA) followed by second strand synthesis. The fragmented cDNA was treated with end-pair, A-
447 tailing, adapter ligation, and 10 cycles of PCR amplification. Prepared libraries were quantified
448 using KAPA Biosystem's next-generation sequencing library qPCR kit (Kapa Biosystems /
449 Roche AG, Basel, Switzerland) and run on a Roche LightCycler 480 real-time PCR instrument.
450 Sequencing of the flowcell was performed on the Illumina NovaSeq sequencer using NovaSeq
451 XP V1 reagent kits, following a 2x150nt indexed run protocol. Reported gene expression values
452 are the total normalized transcripts per million (TPM). All raw data is available through NCBI-
453 SRA associated with NCBI-Bioproject (Accession IDs: PRJNA580539 - PRNJA580574) and the
454 DOE-JGI IMG database (Project ID: 505977).

455 *Targeted proteomics by LC-MS/MS*

456 A targeted SRM (selected reaction monitoring) method was developed to quantify relative levels
457 of pathway proteins in samples under the various tested conditions in a 60 mL cultivation
458 format. At the time points indicated, 1 mL of each sample was pelleted by centrifugation at
459 14,000g and flash frozen with liquid nitrogen at -80 °C until ready for processing. Cells were
460 lysed in 100 mM NaHCO₃ using 0.1 mm glass beads using a Biospec Beadbeater (Biospec
461 Products, Bartlesville, OK) with 60 s bursts at maximum power and repeated three times. Cell
462 lysates were cooled on ice between each round. The clarified supernatant was harvested by
463 centrifugation at 14,000g. The lysate protein concentration was estimated following the
464 manufacturer's directions for the BCA method (ThermoFisher Scientific/Pierce Biotechnology,
465 Waltham, MA). The SRM-targeted proteomic assays and analyses were performed as
466 described previously^{53,54}. The SRM methods and data are available at Panoramaweb
467 (shorturl.at/rsAK3).

468 *Cultivation at different scales*

469 Cultures from glycerol stocks were struck to single colonies on LB agar media with the
470 appropriate antibiotic as necessary. Single colonies were used to inoculate overnight cultures in
471 LB with the appropriate antibiotic. Saturated overnight LB cultures were then back-diluted
472 1/100x into M9 minimal media with the appropriate carbon source as indicated. Cultures were
473 back-diluted and adapted twice to ensure robust cell growth before heterologous pathway
474 induction. Adaptation of *P. putida Ec.galETKM* strains for growth in M9 minimal salt media with
475 galactose had a long initial adaptation phase of around 96-120 hours before cultures showed
476 turbidity. All cultures were incubated with shaking at 200 rpm and 30°C. To prepare cells for
477 pathway induction, M9 adapted cultures were back-diluted to a starting OD600 of 0.1, at which
478 point IPTG and arabinose were added as appropriate. Production cultures grown in 24 well
479 deep well plates (Axygen Scientific, Union City, CA) inoculated into a 200 µL culture volume and
480 incubated InFors Multitron HT Double Stack Incubator Shaker (Infors HT, Bottmingen-Basel,
481 Switzerland) set to 999 rpm linear shaker, and 70% humidity. For shake flask experiments, 60
482 mL cultures were grown in 250mL unbaffled Erlenmeyer shake flask and incubated at 200 rpm
483 with orbital shaking. For all experiments, the indigoidine pathway was induced with 0.3% w/v L-
484 arabinose, and dCpf1 mediated gene repression was induced with 500 µM IPTG. Production
485 assays were performed in independent biological triplicate and repeated at least twice, except
486 for the scale up experiments (described below), which were performed in biological duplicate.

487 *Batch experiments at 2 L bioreactor scale*

488 Batch experiments were performed using a 2 L bioreactor equipped with a Sartorius BIOSTAT
489 B® fermentation controller (Sartorius Stedim Biotech GmbH, Goettingen, Germany) , fitted with
490 two Rushton impellers fixed at an agitation speed of 800 rpm. Initial reactor volume was 1 L M9
491 Minimal Media (10g/L Glucose, 0.3% w/v L-arabinose, 30mM NH₄⁺), and 50 mL overnight pre-
492 culture in the same media. Feeding solution contained 100 g/L glucose, 300mM NH₄⁺ along with

493 L-arabinose and kanamycin. The temperature was held constant at 30°C. The bioreactor pH
494 was monitored using the Hamilton EasyFerm Plus PHI VP 225 Pt100 (Hamilton Company,
495 Reno, NV) and was maintained at a pH of 7 using 10 M sodium hydroxide. Dissolved oxygen
496 concentration was monitored using Hamilton VisiFerm DO ECS 225 H0.

497

498 *Advanced micro bioreactor method: 250 mL ambr® 250 bioreactor cultivations*

499 Fed-batch bioreactor experiments were carried out in a 12-way ambr® 250 bioreactor system
500 equipped with 250 mL single-use, disposable bioreactors (microbial vessel type). The vessels
501 were filled with 150 mL M9 minimal salt media containing 10 g/L glucose as carbon source.
502 Temperature was maintained at 30 °C throughout the fermentation process and agitation was
503 set constant to 1300 RPM. Airflow was set constant to 0.5 VVM based on the initial working
504 volume and pH was maintained at 7.0 using 4 N NaOH. Reactors were inoculated manually with
505 5 mL of pre-culture cell suspension. After an initial batch phase of 12 hours, cultures were fed
506 with a concentrated glucose feed solution (600 g/L glucose, 120 g/L ammonium sulfate, 50
507 µg/mL kanamycin, 3 g/L arabinose and 500 µM IPTG) by administering feed boluses every two
508 hours restoring glucose concentrations to 10 g/L (feed parameters: 3.1 min @ 50 mL/h). After
509 observing glucose accumulation, feed addition was paused and resumed at reduced feed rates
510 when glucose levels dropped below 10 g/L (1 min @ 50 mL/h). Experiments with a continuous
511 feeding regime were initially fed at 1.3 mL/h (0.3 mL/h after seeing glucose accumulation).
512 Samples were taken 1-2 times every day (2 mL) and stored at -20 °C. The ambr® 250 runtime
513 software and integrated liquid handler was used to execute all process steps unless stated
514 otherwise.

515

516

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528 that the United States Government retains a non-exclusive, paid-up, irrevocable, world-wide
529 license to publish or reproduce the published form of this manuscript, or allow others to do so,
530 for United States Government purposes. In accordance with the journal's conflict of interest
531 policy, AM, TE, and DB have submitted a patent related to some of the work in this study.

532

533 **Contributions**

534 Conceptualization of the project: AM, TE, DB. Development and implementation of
535 Computational Methods: DB. Strain construction, molecular biology, analytical chemistry: TE,
536 AL, RH, BW. Contributed critical reagents: TE, YS, RH, CP. Proteomic analysis: YC, CJP.
537 RNAseq pipeline: VRS. NMR analysis: AL, YL. Interpreted results: TE, AL, DB, YC, JDK, AM.
538 Bioreactors and Scaleup: AL, JPP, TE, AL. Drafted the manuscript: DB, TE, CJP, JDK, AM.
539 Raised funds: AM and JDK. All authors contributed to and provided feedback on the manuscript.
540 All authors read and approved the final manuscript.

541

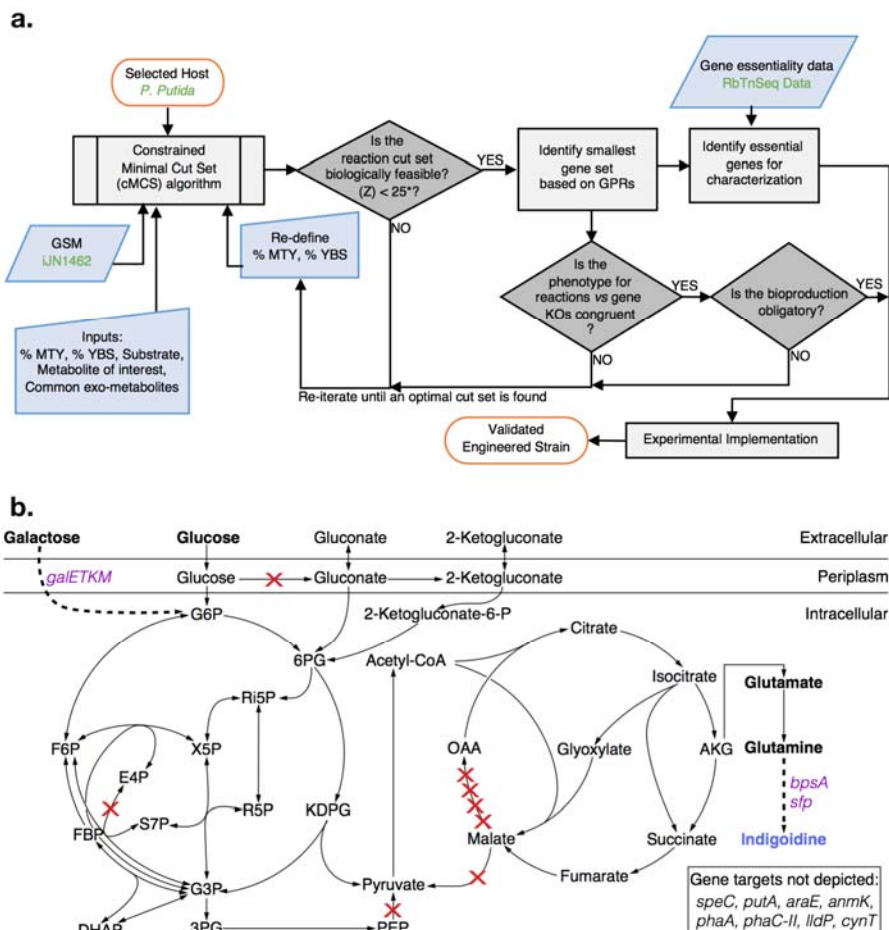
542 **Competing Financial Interest**

543 J.D.K. has a financial interest in Amyris, Lygos, Demetrix, Napigen, Maple Bio, Berkeley
544 Brewing Sciences, Ansa Biotech and Apertor Labs.

545

546

547 **Figure 1.**



548

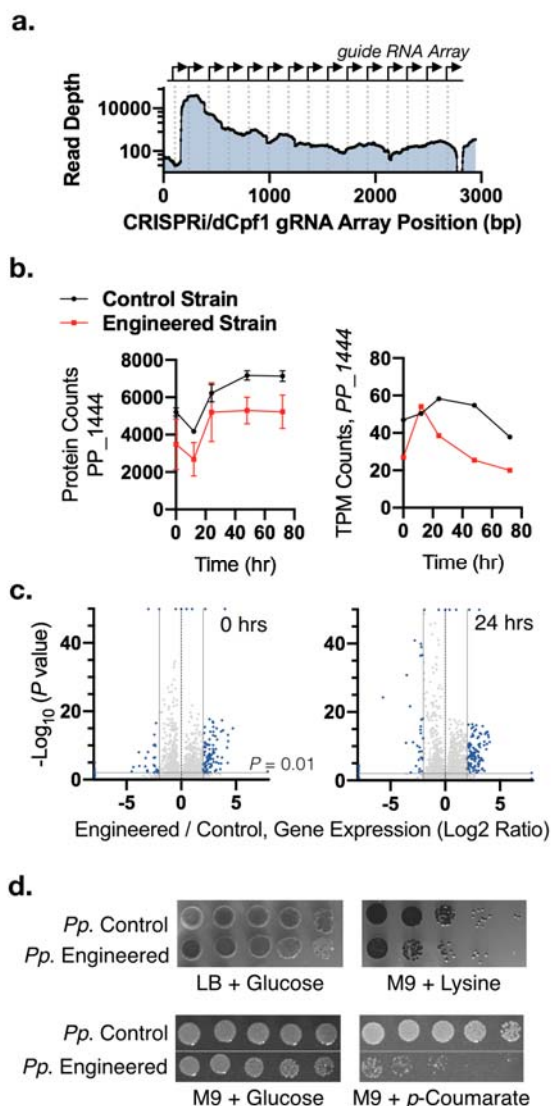
549

550 **Figure 1: Computationally Guided Predictions for Metabolic Rewiring in *P. putida*.** **a,**
 551 Modeling and engineering workflow diagram. This approach can potentially be extended to any
 552 carbon source, host and/or metabolite. Input specific to this specific host/final product work is
 553 marked in green font. **b,** The central metabolism of *Pseudomonas putida* engineered to produce
 554 indigoidine from either glucose or galactose. Heterologous genes are indicated in purple text.
 555 Indigoidine is derived from the TCA intermediate alpha-ketoglutarate (AKG) via two molecules
 556 of glutamine. The genes targeted in *P. putida* central metabolism for knockdown by
 557 dCpf1/CRISPRi are indicated with red X marks. Additional gene targets outside of *P. putida*
 558 central metabolism are indicated in the box on the bottom right. A total of 14 genes were
 559 targeted for CRISPR interference excluding *mgo-I* and *cynT*, as the latter are essential by
 560 genome-wide transposon mutagenesis (RB-TnSeq). Refer to **Supplementary Table 4** for more
 561 information.
 562

563

564

565 **Figure 2.**

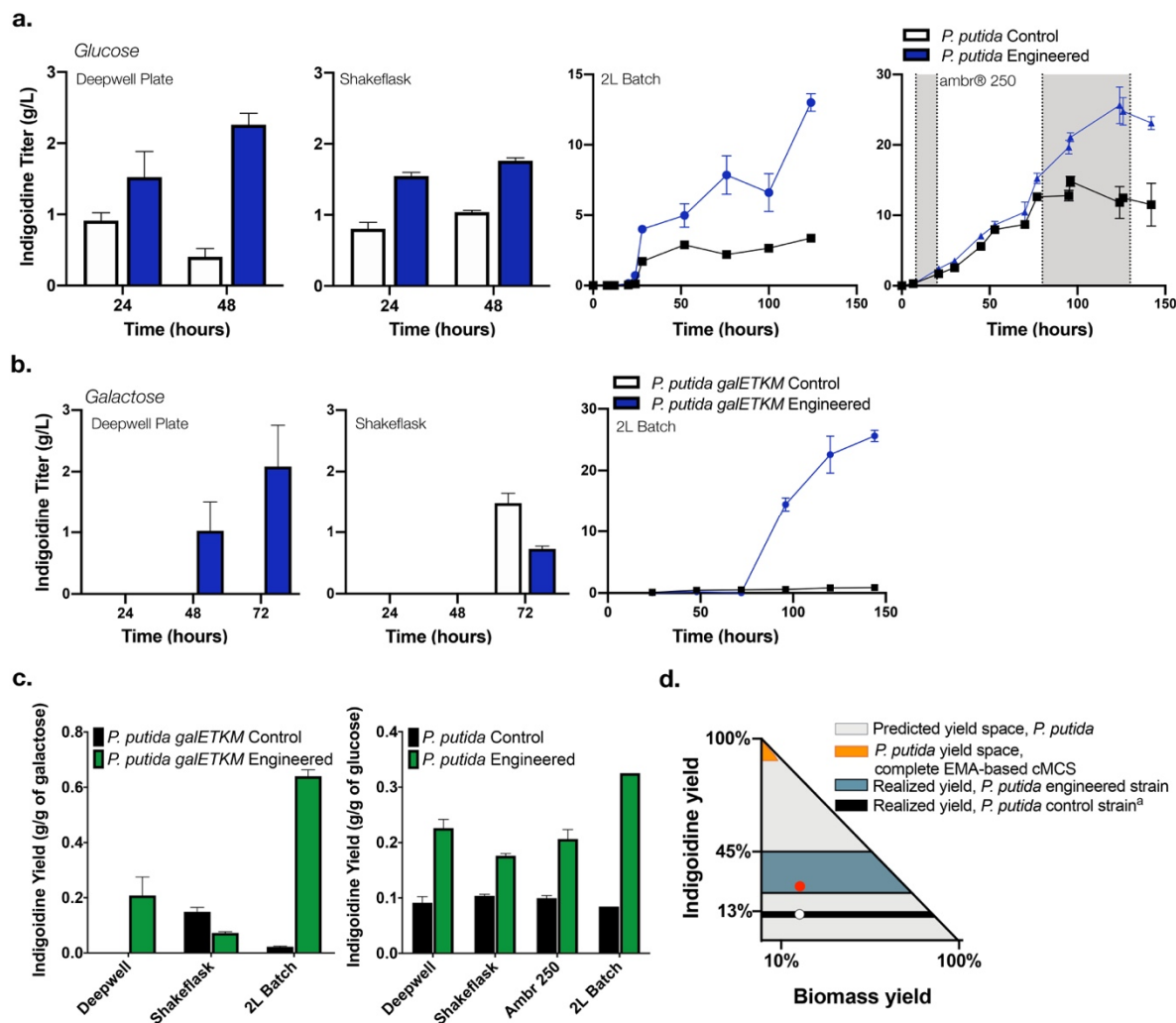


566

567 **Figure 2: Characterization of the multi-gene engineered strain via RNAseq and**
568 **Proteomics. a-c,** *P. putida* harboring a genomically integrated indigoidine expression cassette
569 and either an empty vector (control strain) or a dCpf1/CRISPRi targeting array examined for
570 gene knockdown efficiency. **a,** RNAseq analysis of plasmid-borne gRNA array in *P. putida*. **b,**
571 Knockdown efficiency of a representative gene locus targeted for inhibition over a 72-hour time
572 course. RNA expression levels (right hand panel) were validated with targeted proteomic
573 analysis (left hand panel). Proteomic samples were analyzed in at least biological triplicate.
574 RNAseq analysis for the control sample was completed in biological duplicate for the control
575 and biological quadruplicate for the engineered strain. **c,** dCpf1/CRISPR interference causes
576 global RNA expression level changes. Volcano plot of mRNA expression levels compared at t =
577 0 h and t = 24 h between multi-gene engineered and control strains. 184 datapoints (0 hr) and
578 391 datapoints (24 hrs) out of 5369 datapoints are outliers and are some are displayed on the
579 edge of the axes. **d,** Validation of carbon source rewiring. Genome-scale modeling predicts that
580 glucose/indigoidine rewiring blocks growth of engineered strains on lysine as a carbon source.

581

582 **Figure 3.**



583

584 **Figure 3: The product substrate pairing approach can improve Titer, Rate and Yield (TRY)**

585 **across two carbon sources. A.** Analysis of *P. putida* galETKM multi-gene engineered strains

586 and a control strain (*P. putida* galETKM, empty vector plasmid) for production of indigoidine

587 using glucose (a) or galactose (b) as the sole carbon source in M9 minimal medium. The culture

588 format assessed is indicated above each panel. A fed-batch mode of cultivation was

589 implemented in the ambr® 250 cultivation format. Glucose feeding is indicated by the gray

590 shaded area. Control samples indicated with black outlined bars or black dots. The multi-gene

591 engineered strains are indicated with blue bars or blue dots. c, Analysis of indigoidine yield

592 across cultivation formats for both glucose-fed and galactose-fed strains. Yield from the control

593 strain is indicated with black bars, and the multi-gene engineered strain is indicated with green

594 bars. d, Predicted production envelope using genome scale model and constraint-based

595 methods represented as theoretical yields of indigoidine as a function of biomass yields.

596 Possible yield space for control strain is represented in grey. The possible yield space for 16

597 gene cMCS predicted by EMA is represented in orange. The range of observed experimental

598 yield space for either the control or engineered strain across different production formats is

599 represented with black and teal fill. A red dot indicates the realized production yield vs biomass
600 yield in exponential phase under optimized conditions. The phase shift in production from
601 stationary phase to exponential is not depicted.
602

603 **Table 1:** Maximum theoretical yield (MTY) of glutamine and indigoidine from two different
604 substrates glucose and galactose with respect to stoichiometry and redox balance in *P. putida*
605

| Metabolite | Maximum theoretical Yield (MTY) | | | |
|---------------------|---------------------------------|---------------------------|-------------------------------|---------------------------|
| | Glucose | | Galactose | |
| | mol product/ mol substrate | g product/ g substrate | mol product/ mol substrate | g product/ g substrate |
| Alpha-ketoglutarate | 1.320 | 1.07 | 1.366 | 1.11 |
| Glutamine | 1.141 | 0.93 | 1.181 | 0.96 |
| Indigoidine | 0.537 | 0.74 | 0.556 | 0.77 |

606

607

608 **Description of Supplemental Figures, Tables, and Datasets.**

609

610 **Supplementary Figure 1:** Characterization of Indigoidine Production Kinetics in *Pseudomonas*
611 *putida*.

612

613 **Supplementary Figure 2:** Quantification of CRISPRi efficacy in *Pseudomonas putida*.

614

615 **Supplementary Figure 3.** Replicate fed-batch ambr250 cultivation (continuous feeding regime)
616 of CRISPRi engineered product substrate paired indigoidine production strategy.

617

618 **Supplementary Figure 4.** Output from Computational Growth Coupling Metabolic Modeling.

619

620 **Supplementary Figure 5:** Characterization of Indigoidine.

621

622 **Supplementary Figure 6:** Analysis of indigoidine purity by H-NMR.

623

624 **Supplementary Table 1:** Potential for product substrate pairing for all metabolites in
625 *Pseudomonas putida* KT2440 and *E. coli* MG1655 using glucose as the sole carbon source.

626

627 **Supplementary Table 2:** Comparison of industrially relevant hosts for glutamine and
628 indigoidine production.

629

630 **Supplementary Table 3:** Analysis of suitable starting carbon sources to determine compatible
631 carbon sources for cultivation for substrate-product pairing with indigoidine.

632

633 **Supplementary Table 4:** Strains Used in this Study.

634

635 **Supplementary Data Set 1:** Gene Sequences Used Design of Synthetic CRISPR Interference
636 gRNA Array.

637

638 **Supplementary Data Set 2:** Identification of essential genes in *P. putida* using barcoded
639 transposon mutagenesis (RB-TnSeq).

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