

1 Heterologous caffeic acid biosynthesis in *Escherichia coli* is
2 affected by choice of tyrosine ammonia lyase and redox partners
3 for bacterial Cytochrome P450

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5 Kristina Haslinger¹ and Kristala L.J. Prather¹

6

7 ¹Department of Chemical Engineering, Massachusetts Institute of Technology,
8 Cambridge, USA

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10 Correspondence should be directed to kljp@mit.edu

11 Abstract

12 **Background:** Caffeic acid is industrially recognized for its antioxidant activity and
13 therefore its potential to be used as an anti-inflammatory, anticancer, antiviral,
14 antidiabetic and antidepressive agent. It is traditionally isolated from lignified plant
15 material under energy-intensive and harsh chemical extraction conditions. However,
16 over the last decade bottom-up biosynthesis approaches in microbial cell factories have
17 been established, that have the potential to allow for a more tailored and sustainable
18 production. One of these approaches has been implemented in *Escherichia coli* and
19 only requires a two-step conversion of supplemented L-tyrosine by the actions of a
20 tyrosine ammonia lyase and a bacterial Cytochrome P450 monooxygenase. Although
21 the feeding of intermediates demonstrated the great potential of this combination of
22 heterologous enzymes compared to others, no *de novo* synthesis of caffeic acid from
23 glucose has been achieved utilizing the bacterial Cytochrome P450 thus far.

24 **Results:** The herein described work aimed at improving the efficiency of this two-step
25 conversion in order to establish *de novo* caffeic acid formation from glucose. We
26 implemented alternative tyrosine ammonia lyases that were reported to display superior
27 substrate binding affinity and selectivity, and increased the efficiency of the Cytochrome
28 P450 by altering the electron-donating redox system. With this strategy we were able to
29 achieve final titers of more than 300 μ M or 47 mg/L caffeic acid over 96 h in an
30 otherwise wild type *E. coli* MG1655(DE3) strain with glucose as the only carbon source.
31 We observed that the choice and gene dose of the redox system strongly influenced the
32 Cytochrome P450 catalysis. In addition, we were successful in applying a tethering

33 strategy that rendered even an initially unproductive Cytochrome P450/ redox system
34 combination productive.

35 **Conclusions:** The caffeic acid titer achieved in this study is about 25% higher than
36 titers reported for other heterologous caffeic acid pathways in wildtype *E. coli* without L-
37 tyrosine supplementation. The tethering strategy applied to the Cytochrome P450
38 appears to be particularly useful for non-natural Cytochrome P450/redox partner
39 combinations and could be useful for other recombinant pathways utilizing bacterial
40 Cytochromes P450.

41 [Keywords](#)

42 Caffeic acid, Cytochrome P450, tethering, PUPPET, recombinant pathway

43 [Background](#)

44 Caffeic acid is widely recognized for its medicinal potential due to its antidepressive [1],
45 antihyperglycemic [2], anti-inflammatory [3], antioxidant [2, 4], anti-coagulatory [3],
46 anticancer [5] and antiviral [6] properties. It is readily produced in plants as a key
47 intermediate in phenylpropanoid biosynthesis. In this pathway, phenylalanine is diverted
48 from primary metabolism by a phenylalanine ammonia lyase associated with the
49 endoplasmic reticulum and transformed into *trans*-cinnamic acid. Cinnamic acid is
50 then hydroxylated by the membrane-anchored Cytochrome P450 enzymes cinnamate
51 4-hydroxylase (C4H) and *p*-coumarate 3-hydroxylase to *p*-coumarate and caffeic acid,
52 respectively [7, 8]. From there a range of molecules can be produced that serve as
53 lignin building blocks or precursors for secondary metabolites such as tannins,
54 (iso)flavonoids, anthocyanins, stilbenes and coumarins [9]. All of these compounds
55 have high market value but are difficult to isolate because they are of low natural

56 abundance (e.g. stilbenes and coumarins), or challenging to extract (e.g. lignin-derived
57 aromatics) [10]. Therefore, over the last decade various strategies have been
58 developed to implement biosynthetic pathways in microbial cell factories that promise
59 their tailored biosynthesis in a sustainable manner. Recent examples are the production
60 of stilbenoids and flavonoids in *Corynebacterium glutamicum* [11, 12], and curcumin
61 [13, 14] and caffeic acid [14–24] in *Escherichia coli*. For the biosynthesis of *p*-coumaric
62 acid in *E. coli*, it was found that using L-tyrosine as a pathway precursor was superior
63 over phenylalanine [25], since activity of the plant Cytochrome P450 enzyme C4H could
64 not be reconstituted as of recently [26]. Based on this finding, two major strategies have
65 been devised to produce caffeic acid that employ microbial tyrosine ammonia lyases
66 (TAL) to generate *p*-coumaric acid followed by either (1) a flavin-dependent HpaBC-type
67 oxidoreductase complex (4-hydroxyphenylacetate 3-hydroxylase, PFAM PF03241) from
68 *Saccharothrix espanaensis* [14–18], *E. coli* [19–21], *Thermus thermophilus* HB8 [20] or
69 *Pseudomonas aeruginosa* [22, 23], or (2) a bacterial cytochrome P450 enzyme
70 CYP199A2 F185L from *Rhodopseudomonas palustris* [14, 18, 24]. In all of these
71 studies it became evident that the caffeic titers are rather low unless L-tyrosine or *p*-
72 coumaric acid are added to the growth media, or the aromatic amino acid pathway is
73 engineered to increase intracellular L-tyrosine levels. For the pathways utilizing HpaBC-
74 type oxidoreductases, the highest titer reported for *de novo* synthesis in wild-type *E. coli*
75 to date is 42 mg/L (*S. espanaensis* TAL and HpaBC) [17]. However, to our knowledge
76 no *de novo* synthesis has been reported for pathways utilizing CYP199A2 F185L.
77 In this study, we established *de novo* biosynthesis of caffeic acid from glucose through
78 the actions of TAL and CYP199A2 F185L $\Delta 7$. In order to achieve this goal, we tested

79 TALs from three different organisms and explored strategies to enhance the activity of
80 CYP199A2 F185L NΔ7. We found that driving the binding equilibrium of the electron-
81 donating redox partners to CYP199A2 F185L NΔ7 towards the bound state improves
82 pathway titers and enabled us to produce ~47 mg/L caffeic acid from glucose in wildtype
83 *E. coli* MG1655(DE3). This titer is slightly higher than the titers reported for the HpaBC-
84 based pathways in wildtype *E. coli* with glucose as the only carbon source [17, 19].

85 Results

86 In an earlier study Rodrigues et al. demonstrated the two-step conversion of 3 mM L-
87 tyrosine to caffeic acid in *E. coli* MG1655(DE3) expressing the enzymes RgTAL and
88 CYP199A2 F185L NΔ7 with redox partners, without reporting *de novo* production of
89 caffeic acid from glucose (Figure 1) [18]. In this study we set out to improve these
90 enzymatic steps to establish caffeic acid production from glucose without supplementing
91 L-tyrosine. When examining the two-step conversion more closely, we determined that
92 both pathway steps needed improvement. First, the efficiency of the committed step, the
93 conversion of L-tyrosine to *p*-coumaric acid, determines how much L-tyrosine is
94 withdrawn from primary metabolism and fed into the pathway. Therefore, we
95 hypothesized that TAL variants with higher selectivity and affinity for L-tyrosine would
96 improve pathway flux. Second, the hydroxylation of *p*-coumaric acid to caffeic acid
97 catalyzed by CYP199A2 F185L NΔ7 appears to be a bottleneck in the pathway, since *p*-
98 coumaric acid accumulates in the fermentation [18]. This accumulation is thought to be
99 detrimental because *p*-coumaric acid has been shown to inhibit TAL activity and to be
100 cytotoxic [27, 28]. Since a common problem with Cytochrome P450 catalyzed reactions
101 is the protein-protein interaction with redox partners, which is strictly required for

102 electron transfer and substrate turnover [29], we hypothesized that driving the assembly
103 of the redox complex would lead to higher product titers.

104 To improve the first pathway step, we selected two homologous tyrosine ammonia
105 lyases with supposedly superior characteristics compared to RgTAL, namely a stronger
106 selectivity for L-tyrosine over L-phenylalanine, higher substrate affinity (K_m) and superior
107 catalytic efficiency (k_{cat}/K_m) (Supporting Information Table S1) [30]. We chose FjTAL
108 from *Flavobacterium johnsoniae* and SeSam8 from *Saccharothrix espanaensis* and
109 obtained the synthetic genes codon-optimized for expression in *E. coli*. In a first pass,
110 utilizing these two TALs in the same three plasmid expression system as used by
111 Rodrigues et al., and providing glucose as the only carbon source, we observed
112 accumulation of caffeic acid 72 h post induction (p. i.). The highest titers of caffeic acid
113 and *p*-coumaric acid are seen with the FjTAL enzyme (Figure 2A, strain s02). In a
114 parallel experiment, where 3 mM L-tyrosine was fed in addition to glucose, the final
115 caffeic acid titers were comparable among the three strains (Figure 2B). This indicates
116 that all enzymes are able to efficiently route L-tyrosine into the caffeic acid pathway at
117 high L-tyrosine concentrations, whereas FjTAL outperforms the other enzymes under
118 low L-tyrosine conditions and is therefore a strong candidate for this pathway.

119 Next, we sought to improve the efficiency of the second pathway step, the hydroxylation
120 of *p*-coumaric acid to caffeic acid catalyzed by CYP199A2 F185L NΔ7, by enhancing
121 the efficiency of the electron transfer step from the two redox partner proteins to
122 CYP199A2 F185L NΔ7. To achieve this goal, we tested three strategies: 1. the use of
123 alternative redox partners, 2. the tethering of the redox complex by creating genetic
124 fusions with high-affinity tethering domains, and 3. the supply of extra gene copies

125 coding for one of the redox partners. To facilitate cloning from here on in the study, we
126 use both multiple cloning sites of the pETDuet vector for the genes encoding redox
127 enzymes rather than the bicistronic pKVS45 vector (see Tables 1 and 2).

128 For class I Cytochromes P450, two redox partners are required to provide two electrons
129 from NAD(P)H: an iron-sulfur cluster containing ferredoxin (Fdx) and a flavin-dependent
130 ferredoxin reductase (FdR) [31]. Rodrigues et al. utilized a redox system composed of
131 palustrisferredoxin (Pux) and putidaredoxin reductase (PdR), which had been used in the
132 original characterization of CYP199A2 [32]. This is, however, not the natural redox
133 system for CYP199A2, since the palustrisferredoxin reductase PuR was only identified and
134 characterized a few years later [33]. Although the Pux/PdR redox system has been
135 proven to support substrate turnover, it remained unclear whether the assembly of the
136 trimeric complex and the respective redox potentials of the proteins supported optimal
137 electron transfer. Therefore, we decided to test the natural redox system (Pux/PuR)
138 alongside a well-characterized surrogate redox system (Pdx/PdR). We determined the
139 caffeic acid titers 72 h p. i. with supplementation of *p*-coumaric acid for three strains
140 expressing CYP199A2 F185L N Δ 7 and one of the three respective redox systems
141 Pux/PdR (hybrid, s04), Pux/PuR (natural, s05), Pdx/PdR (surrogate, s06). We observed
142 the highest titers for the natural redox system (s05) and no turnover with the full
143 surrogate system composed of Pdx/PdR (Figure 3A). This suggests that the electron
144 transfer from ferredoxin to CYP199A2 F185L N Δ 7 is severely impaired with the
145 surrogate ferredoxin Pdx, whereas the electron transfer from PdR to Pux in the hybrid
146 system appears to sufficiently support substrate turnover. The native redox complex
147 Pux/PuR, however, displays the highest catalytic power and a titer of 1.6 +/- 0.32 mM

148 caffeic acid was observed which corresponds to 53% conversion of the fed *p*-coumaric
149 acid. These results indicate that the careful choice of redox system is crucial for this
150 pathway step.

151 With our second strategy, we sought to further improve these redox systems by
152 generating genetic fusions of the enzymes with the subunits of the heterotrimeric DNA
153 sliding clamp PCNA (Proliferating Cell Nuclear Antigen) of *Sulfolobus solfataricus* P2
154 [34]. This PCNA complex has been shown to tolerate the fusion of other genes to the '3
155 ends (C-termini) [35] of its three subunits, while maintaining their high binding affinity
156 towards each other: the PCNA1/PCNA2 dimer has a dissociation constant in the low
157 picomolar range and the PCNA1/PCNA2/PCNA3 trimer in the high nanomolar range
158 [34]. This fusion strategy has been shown to be highly efficient for the *in vitro*
159 reconstitution of Cytochrome P450 activity and was termed PUPPET by the inventors
160 (PCNA-utilized protein complex of P450 and its two electron transfer-related proteins)
161 [35–40]. To our knowledge, this strategy hasn't been used in whole-cell catalysis to
162 date. Initially, we tested fusion proteins analogous to the previously described PUPPET
163 fusions with FdR fused to the C-terminus of PCNA domain 1, Fdx to PCNA2 and the
164 Cytochrome P450 to PCNA3 (tether design I, strains s07-s09; Figure 3B). When feeding
165 3 mM *p*-coumaric acid, we observed higher titers of caffeic acid for all tethered redox
166 systems than compared to the respective free enzymes. The effect was more
167 pronounced with the hybrid and surrogate systems, where a 6-fold increase in titer was
168 observed for Pux/PdR (s07) and an 8-fold increase for Pdx/PdR (s09). Overall, the
169 highest titer was observed with the tethered version of Pux/PdR (s07, titer: 2.3 +/- 0.07
170 mM). Next, we investigated whether these titers could be further improved by

171 generating a new arrangement of the fusion partners. Based on the published
172 dissociation constants for the well-studied Cytochrome P450 CYP101A1 and its redox
173 partners [41, 42], we assumed that the affinity of Fdx to FdR is about 100-fold higher
174 than the affinity of Fdx to the Cytochrome P450. We hypothesized that the high affinity
175 interaction between PCNA1 and PCNA2 might be even more beneficial to the low
176 affinity interaction between the Cytochrome P450 and Fdx than between Fdx and FdR.
177 Therefore, we generated a second set of fusion genes (tether design II), where
178 CYP199A2 F185L NΔ7 is fused to PCNA1, Fdx to PCNA2 and FdR to PCNA3, while
179 maintaining the linker arrangements that had previously been optimized for the
180 respective elements of the redox complex [39] (s 10-s12, Fig 3C). With these alternative
181 tethering constructs, the highest final caffeic acid titers were obtained with the surrogate
182 Pdx/PdR redox system (s10, titer: 2.1 +/- 0.35 mM), while the titers obtained with the
183 other redox systems were lower than in the previous experiments. This indicates that
184 the domain arrangements in the second tether design supports the weaker protein-
185 protein interactions in the surrogate redox complex better than the other tether design,
186 whereas it disturbs catalysis with the two redox systems that already led to high titers
187 with free redox partners and tether design I.

188 Next, we tested the best redox partner constructs in the context of the full pathway with
189 FjTAL as the first pathway enzyme (Figure 3D). We observed the highest caffeic acid
190 titers with the untethered, natural redox partners (Pux/PuR, s15, titer: 0.14 +/-
191 0.028 mM). Although strains s07, s08 and s12 had slightly outperformed s05 in the one-
192 step conversion, the corresponding strains expressing FjTAL (s14, s16, s17,
193 respectively) yielded lower caffeic acid titers in the two-step recombinant pathway. The

194 cost for expressing the additional tethering domains may offset the positive effects of
195 the enhanced enzymatic activity. In all of the fermentations, lower final titers of *p*-
196 coumaric acid are measured than in the initial test of FjTAL (s02), which indicates that
197 the changes made to the second pathway step allow for an almost complete conversion
198 to the final product.

199 Lastly, we tested whether additional copies of the palustrisredoxin encoding gene, *pux*,
200 would further improve the performance of the so far best pathway configuration with
201 FjTAL and the natural redox partners of CYP199A2 F185L NΔ7 (Pux/PuR redox
202 system). Therefore, we inserted *pux* into MCS1 of plasmid IR64 pCDFDuet::₆His-
203 CYP199A2 F185L NΔ7, yielding plasmid c84 pCDFDuet::₆His-Pux_6His-
204 CYP199A2F185L NΔ7. Based on the supplier's reports (Novagen), the copy numbers of
205 pETDuet and pCDFDuet are in a similar range so that the incorporation of an additional
206 gene copy into pCDFDuet should lead to an estimated doubling of the gene dose and
207 potentially the level of protein expressed. When comparing the strain harboring this set
208 of plasmids (s18) to the RgTAL control strain (s13) and the strain expressing FjTAL and
209 Pux/PuR (s15), we observed an increase in caffeic acid titer with full consumption of the
210 intermediate *p*-coumaric acid (Figure 4A). This indicates that the availability of Pux was
211 previously insufficient and that a higher expression level of this protein supports better
212 Cytochrome P450 performance. Despite the improvements in final caffeic acid titer, we
213 observed an accumulation of *p*-coumaric acid in early fermentation until 48 h p. i. and
214 then a sharp drop in titer until it is fully converted to caffeic acid at 96 h p. i. (Figure 4B).
215 This indicates that in early fermentation the first pathway step is still faster than the
216 second pathway step. In late fermentation, the conversion of *p*-coumaric acid to caffeic

217 acid is faster than the formation of the intermediate, or no additional *p*-coumaric acid is
218 formed. This could be caused by the lack of available L-tyrosine once the cultures reach
219 stationary phase, although we did not observe increased titers when spiking the cultures
220 with 3 mM L-tyrosine at 48 h p. i. (I Figure S1). Therefore, we are inclined to suggest
221 that the tyrosine ammonia lyase has lost its activity by that time. Potential causes could
222 be structural instability of the TAL enzyme or its inhibition by the intermediate as
223 described previously [27]. Overall, with the exchange of RgTAL for FjTAL and the
224 change of the redox system from Pux/PdR to Pux/PuR with an additional gene copy of
225 *pux*, we improved this recombinant pathway and were able to produce caffeic acid from
226 glucose without feeding L-tyrosine. The highest final titer after 96 h of fermentation was
227 47 mg/L, which is slightly higher than caffeic acid titers achieved with other recombinant
228 pathways without L-tyrosine supplementation [17, 19]. Furthermore, the improved
229 pathway is able to convert >50% of fed L-tyrosine to caffeic acid (SI Figure S1), which
230 indicates that it should be able to produce high amounts of caffeic acid in a tyrosine-
231 producer strain.

232 Discussion

233 Building microbial cell factories for the production of plant polyphenols has been a major
234 goal for metabolic engineers over the last decade [43, 44]. The low abundance of these
235 compounds and their occurrence in complex mixtures of variable composition in plants,
236 makes recombinant microbial cell factories an attractive source for industrial
237 applications. However, the strict regulation of the aromatic amino acid metabolism,
238 which provides precursors to most recombinant polyphenol-producing pathways, limits
239 the overall pathway efficiency. For recombinant polyphenol-producing pathways in

240 *E.coli*, it has been observed that overcoming the precursor bottleneck by metabolic
241 engineering of the aromatic amino acid pathway, often reveals bottlenecks further down
242 the recombinant pathway [45–47]. Therefore, it is crucial to optimize the recombinant
243 pathway itself before moving into a microbial chassis with deregulated aromatic amino
244 acid production. In this study, we optimized the two-step conversion of L-tyrosine to
245 caffeic acid. Here it is important to ensure high efficiency of the second pathway step to
246 avoid accumulation of *p*-coumaric acid, which has been shown to severely inhibit the
247 activity of the first pathway enzyme, TAL [27]. The three strategies we tested focused
248 on the electron-donating redox partners rather than the Cytochrome P450 enzyme itself.
249 Previous *in vitro* studies of this particular Cytochrome P450 and others have shown that
250 the right choice of redox system, in particular the ferredoxin, is crucial for efficient
251 electron transfer and enzyme catalysis [29, 33]. As expected, we observed the highest
252 caffeic acid titers with the natural redox system composed of Pux and PuR in the one-
253 step conversion with untethered redox partners. However, when we applied tethering
254 strategies to increase the affinity of the Cytochrome P450 and the redox partners
255 towards each other, we observed higher titers with the non-natural redox partners.
256 Tethering strategies have previously been applied to several Cytochrome P450
257 enzymes, both *in vitro* [35, 42, 48–51] and *in vivo* [42, 48]. The *in vitro* studies showed
258 that tethered redox complexes are able to overcome the need to use an excess of redox
259 partners over the Cytochrome P450 enzyme, to compensate for low protein-protein
260 affinities (typically a five- to twenty-fold molar excess of ferredoxin is used *in vitro*).
261 Furthermore, kinetic studies showed that at low enzyme concentrations, the tethered
262 complexes outperform the 1:1:1 mixtures of free enzymes. These reports and our

263 findings for our versions of the PUPPET tether indicate that tethering strategies in
264 whole-cell catalysis may be particularly useful in two scenarios: (A) if the expression
265 levels of the Cytochrome P450 and redox partners are low (poor protein expression,
266 expression from genomic gene copies or as part of a multi-enzyme recombinant
267 pathway), or (B) if the natural redox partners are unknown and surrogate systems are
268 used to reconstitute the Cytochrome P450 activity.

269 To our knowledge, this study is the first one to use the PUPPET tether in whole-cell
270 catalysis and also the first one to use any of the known Cytochrome P450 tethers in the
271 context of a recombinant pathway. In the caffeic acid pathway, the tethered Cytochrome
272 P450 complexes were slightly outperformed by the free, natural redox complex, in
273 particular in the presence of extra copies of the *pux* gene (s18). This may indicate that
274 the metabolic burden of expressing the PCNA subunits in addition to the pathway
275 enzymes and the three resistance genes required for plasmid maintenance represents a
276 disadvantage of the strains expressing the tethered Cytochrome P450 complexes
277 compared to the ones expressing the free, natural redox complex (s15 and s18). The
278 fact that s18 outperforms s15 indicates that the availability of Pux is limiting in s15, and
279 is in good agreement with observations made in other whole-cell conversions [52, 53].
280 Since our strategy only doubled the gene dose of *pux*, it is very well possible that
281 rearranging the genes in the vector system to achieve higher protein levels of Pux
282 relative to the other enzymes could lead to even better results than described in this
283 study. Our optimization efforts of the second pathway step in combination with the use
284 of FjTAL for the first pathway step, enabled us to demonstrate the *de novo* production of
285 caffeic acid in an otherwise wild type *E. coli* background. FjTAL had previously been

286 seen to be beneficial for the production of *p*-coumaric acid and its derivatives in other
287 microbes [11, 54, 55], however, to our knowledge it has not been used in *E. coli*. It
288 appears that this enzyme allows for a more efficient routing of L-tyrosine into the caffeic
289 acid pathway than RgTAL at low L-tyrosine concentrations. Under high L-tyrosine
290 conditions, at levels that we would expect in tyrosine producer strains [56], our
291 fermentation strains expressing FjTAL achieve slightly higher caffeic acid titers than the
292 strains expressing RgTAL and lower titers of *p*-coumaric acid. This indicates that the
293 optimized pathway is more balanced so that less *p*-coumaric acid accumulates but
294 overall less L-tyrosine is converted into *p*-coumaric acid. To further improve these
295 results, it is necessary to investigate the stability and activity of the FjTAL enzyme over
296 time, since it appears to be inactive after 48h of fermentation.

297 Conclusions

298 In this study we established *de novo* synthesis of caffeic acid by expressing tyrosine
299 ammonia lyase from *Flavobacterium johnsoniae* and CYP199A2 F185L N⁷ from
300 *Rhodopseudomonas palustris* with its redox partners palustrisredoxin and
301 palustrisredoxin reductase. We found that compared to earlier versions of this pathway,
302 changes made to the redox partners, namely the use of palustrisredoxin reductase
303 instead of putidaredoxin reductase and the duplication of the palustrisredoxin gene
304 dose, as well as the use of FjTAL instead of RgTAL, enhanced the pathway
305 performance under low L-tyrosine conditions as encountered in otherwise wild type *E.*
306 *coli*. Furthermore, we observed that applying a tethering strategy to the Cytochrome
307 P450-catalyzed pathway step based on the PUPPET system [35] increases caffeic acid
308 titers in strains expressing non-natural redox systems. This indicates that this strategy

309 can be useful for pathways containing orphan bacterial Cytochromes P450. The
310 optimized caffeic acid pathway could now be transferred into a tyrosine-producer *E. coli*
311 strain for more in-depth characterization or process engineering.

312 **Materials and Methods**

313 **Bacterial strains and plasmids**

314 All molecular cloning and plasmid propagation steps were performed in chemically
315 competent *Escherichia coli* *E. coli*® 10G (F- *mcrA* Δ (*mrr-hsdRMS-*
316 *mcrBC)* *endA1 recA1* Φ 80*dlacZ* Δ M15 Δ *lacX74 araD139*
317 Δ (*ara,leu*)7697*galU galK rpsL nupG* λ -*tonA*) produced by Lucigen (Middleton, WI,
318 USA). Gene expression under the control of T7 promoters was performed in *E. coli* K-12
319 MG1655(DE3) [57]. Plasmids were constructed with a range of strategies summarized
320 in the Supplementary Information (SI) Table S2. All genes in the final constructs were
321 fully sequenced (Eton Bioscience, Charlestown, MA). The FjTAL, SeSam8 and PCNA1-
322 PdR genes were codon optimized for *E. coli* and synthesized as gblocks® gene
323 fragments by Integrated DNA Technologies (Coralville, IA, USA) (sequence provided in
324 SI). Plasmids pHSG-PCNA2 and pHSG-PCNA3 were a gift from Teruyuki Nagamune
325 obtained through Addgene (Cambridge, MA, USA) (Addgene plasmid # 66126;
326 <http://n2t.net/addgene:66126>; RRID:Addgene_66126) and (Addgene plasmid # 66127;
327 <http://n2t.net/addgene:66127>; RRID:Addgene_66127) [35]. Plasmid pACYCDuet-
328 PuR/Pux was a gift from Dr. Stephen G. Bell (University of Adelaide, Australia). The
329 construction of plasmids IR54 and IR64 is described in Rodrigues et al 2015 [18].
330 The peptide linkers connecting the PCNA subunits with the respective enzymes were
331 designed based on the optimized linkers described in Haga et al. 2013 [39] (tether

332 design I: PCNA1-(GGGS)₂-FdR, PCNA2-GGGSP₂₀G-Fdx, PCNA3-GGS-Cytochrome
333 P450; tether design II: PCNA1-GGS-Cytochrome P450, PCNA2-GGGSP₂₀G-Fdx,
334 PCNA3-(GGGS)₂-FdR).

335 Fermentation

336 Plasmids and strains used in fermentations are described in Table 1 and Table 2,
337 respectively. *E. coli* K-12 MG1655(DE3) made chemically competent according to the
338 protocol by Inoue et al [58] was sequentially transformed with appropriate plasmids. The
339 correct identity of strains was confirmed by colony PCR. Starter cultures were prepared
340 from three individual colonies of the final strains in 5 mL Lysogeny broth (LB)
341 supplemented with carbenicillin (100 µg/mL), spectinomycin (50 µg/mL) and kanamycin
342 (50 µg/mL, only s01-s03 and s13-s18) in round-bottom polystyrene tubes, incubated
343 over night at 37°C with agitation and used to inoculate the main cultures (7 mL LB with
344 antibiotics; round-bottom polystyrene tubes). After 4 h of growth at 37°C, 250 rpm,
345 OD₆₀₀ was measured and the appropriate volume of each culture pelleted and
346 resuspended in modified, selective M9 including substrates and 4% glucose to obtain
347 15 mL cultures at OD₆₀₀ of 0.7 or 20 mL cultures at OD₆₀₀ of 0.5 to 0.7 (time course
348 experiment) in sterile glass tubes. These cultures were incubated at 26°C, 160 rpm for
349 72 h or 96 h (time course experiment). For the time course experiment samples of 1000
350 µL were taken every 24 h, for all other experiments samples of 2000 µL were taken
351 after 72 h and either stored at -20°C until further processing or extracted with ethyl
352 acetate immediately.

353 M9 medium composition (1x) prepared from sterile stocks: M9 salts (Millipore-Sigma,
354 used as 5x stock), Trace Mineral Supplement (ATCC® MD-TMS™, used as 200x
355 stock), vitamin mix (from 100x stock; final: riboflavin 0.84 mg/L, folic acid 0.084 mg/L,

356 nicotinic acid 12.2 mg/L, pyridoxine 2.8 mg/L, and pantothenic acid 10.8 mg/L), biotin
357 (from 1000x stock; final: 0.24 mg/L), thiamine (from 1470x stock; final: 340 mg/L), δ -
358 Aminolevulinic acid (from 1000x stock in MeOH, final: 7.5 μ g/mL), IPTG (from 1000x
359 stock, final: 1 mM), aTc (from 1000x stock, final: 100 ng/mL; only included in
360 fermentations of s01-s03), carbenicillin (from 1000x stock, final: 100 μ g/mL),
361 spectinomycin (from 1000x stock, final: 50 μ g/mL), kanamycin (from 1000x stock, final:
362 50 μ g/mL, only strains s01-s03 and s13-s18), 4% (w/v) glucose (from 50% w/v stock).
363 Optional: *p*-coumaric acid (from fresh 100x stock in MeOH, final 3 mM) or L-tyrosine
364 (from fresh 100x stock in 1M HCl).

365 [Product extraction](#)

366 The samples were acidified with 6N HCl (pH<3) and split into two tubes as technical
367 duplicates. Samples were extracted twice with equal volumes of ethylacetate. The
368 organic phases of both extraction steps were combined and evaporated under a stream
369 of air or nitrogen. The dried material was resuspended in 100 μ L Acetonitrile with 0.1%
370 Trifluoroacetic acid (10x concentrated compared to culture) or 80 μ L Acetonitrile with
371 0.1% Trifluoroacetic acid (5x concentrated compared to culture) for the time course
372 experiment. Samples were transferred into HPLC vials with conical glass inserts and
373 analyzed by HPLC.

374 [HPLC analysis](#)

375 10 μ L of the samples were analyzed by reversed-phase HPLC (instrument: Agilent
376 1100, column: Agilent Zorbax Eclipse XDB-C18 80Å, 4.6 x 150 mm, 5 μ m; detector:
377 Agilent diode array detector G1315B, λ =310nm, gradient: 10% to 20% Acetonitrile with
378 0.1% Trifluoroacetic acid over 17 min. The *p*-coumaric acid and caffeic acid peaks were
379 identified by comparing the retention times to authentic standards and by mass

380 spectrometry (Agilent G6120, quadrupole MS). The integrated peak areas were
381 converted to concentrations in mM based on calibration curves generated with authentic
382 standards.

383 [List of abbreviations](#)

384 FdR – ferredoxin reductase

385 Fdx – ferredoxin

386 FjTAL – *F. johnsoniae* tyrosine ammonia lyase

387 PdR – *P. putida* putidaredoxin reductase

388 Pdx – *P. putida* putidaredoxin

389 PCNA - Proliferating Cell Nuclear Antigen = heterotrimeric DNA sliding clamp; used as
390 tether

391 p. i. – post induction

392 PuR – *R. palustris* palustrisredoxin reductase

393 Pux – *R. palustris* palustrisredoxin

394 RgTAL – *R. glutinis* tyrosine ammonia lyase

395 TAL – tyrosine ammonia lyase

396 [Declarations](#)

397 [Ethics approval and consent to participate](#)

398 Not applicable.

399 [Consent for publication](#)

400 Not applicable.

401 [Availability of data and materials](#)

402 The datasets used and/or analyzed during the current study are available from the
403 corresponding author on reasonable request.

404 [Competing interests](#)

405 The authors declare that they have no competing interests.

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410 [Authors' contributions](#)

411 K.H. conceived the study and wrote the manuscript with support and guidance by
412 K.L.J.P. K.H. performed and analyzed the experiments.

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608 **Tables**

609 **Table 1: List of plasmids used in caffeic acid production strains.**

Plasmid name	source
IR54 pKVS45::PdR-Pux operon	[18]
IR64 pCDFduet::_6His-CYP199A2 F185L NΔ7	[18]
c22 pRSFduet::6His-RgTAL	This study
c25 pCDFduet::_PCNA3-CYP199A2 F185L NΔ7	This study
c28 pETduet::6His-PCNA2-Pux_PCNA1-PdR (opt)	This study
c50 pETduet::6His-Pux_PdR (opt)	This study
c62 pETduet::6His-Pux_PuR	This study
c63 pETduet::6His-PCNA2-Pux_PCNA1-PuR	This study
c71 pRSFduet::6His-FJTAL	This study
c72 pRSFduet::6His-SeSam8	This study
c84 pCDFduet::6His-Pux_6His-CYP199A2F185L NΔ7	This study
c86 pETduet::6His-Pdx_PdR (opt)	This study
c88 pETduet::6His-PCNA2-Pdx_PCNA1-PdR(opt)	This study
c96 pCDFduet::_PCNA1-GGS-CYP199A2 F185L NΔ7	This study
c97 pETduet::6His-PCNA2-Pux_PCNA3-(GGGS)2-PdR (opt)	This study
c98 pETduet::6His-PCNA2-Pux_PCNA3-(GGGS)2-PuR	This study
c106 pETduet::6His-PCNA2-Pdx_PCNA3-GGS-PdR (opt)	This study

610 Table 2: List of *E. coli* MG1655(DE3) strains used in fermentation experiments.

identifier	Plasmid name	source
s01	IR64 pCDFduet:: ₆ His-CYP199A2 F185L NΔ7 IR54 pKVS45::PdR-Pux operon c22 pRSFduet:: ₆ His-RgTAL	This study
s02	IR64 pCDFduet:: ₆ His-CYP199A2 F185L NΔ7 IR54 pKVS45::PdR-Pux operon c71 pRSFduet:: ₆ His-FjTAL	This study
s03	IR64 pCDFduet:: ₆ His-CYP199A2 F185L NΔ7 IR54 pKVS45::PdR-Pux operon c72 pRSFduet:: ₆ His-SeSam8	This study
s04	IR64 pCDFduet:: ₆ His-CYP199A2 F185L NΔ7 c50 pETduet:: ₆ His-Pux_PdR (opt)	This study
s05	IR64 pCDFduet:: ₆ His-CYP199A2 F185L NΔ7 c62 pETduet:: ₆ His-Pux_PuR	This study
s06	IR64 pCDFduet:: ₆ His-CYP199A2 F185L NΔ7 c86 pETduet:: ₆ His-Pdx_PdR (opt)	This study
s07	c25 pCDFduet:: ₆ PCNA3-CYP199A2 F185L NΔ7 c28 pETduet:: ₆ His-PCNA2-Pux_PCNA1-PdR (opt)	This study
s08	c25 pCDFduet:: ₆ PCNA3-CYP199A2 F185L NΔ7 c63 pETduet:: ₆ His-PCNA2-Pux_PCNA1-PuR	This study
s09	c25 pCDFduet:: ₆ PCNA3-CYP199A2 F185L NΔ7 c88 pETduet:: ₆ His-PCNA2-Pdx_PCNA1-PdR (opt)	This study
s10	c96 pCDFduet:: ₆ PCNA1-GGS-CYP199A2 F185L NΔ7 c97 pETduet:: ₆ His-PCNA2-Pux_PCNA3-(GGGS) ₂ -PdR (opt)	This study
s11	c96 pCDFduet:: ₆ PCNA1-GGS-CYP199A2 F185L NΔ7 c98 pETduet:: ₆ His-PCNA2-Pux_PCNA3-(GGGS) ₂ -PuR	This study
s12	c96 pCDFduet:: ₆ PCNA1-GGS-CYP199A2 F185L NΔ7 c106 pETduet:: ₆ His-PCNA2-Pdx_PCNA3-GGS-PdR (opt)	This study
s13	IR64 pCDFduet:: ₆ CYP199A2 F185L NΔ7 c50 pETduet:: ₆ His-Pux_PdR (opt) c22 pRSFduet:: ₆ His-RgTAL	This study
s14	c25 pCDFduet:: ₆ PCNA3-CYP199A2 F185L NΔ7 c28 pETduet:: ₆ His-PCNA2-Pux_PCNA1-PdR (opt) c71 pRSFduet:: ₆ His-FjTAL	This study
s15	IR64 pCDFduet:: ₆ CYP199A2 F185L NΔ7 c62 pETduet:: ₆ His-Pux_PuR c71 pRSFduet:: ₆ His-FjTAL	This study
s16	c25 pCDFduet:: ₆ PCNA3-CYP199A2 F185L NΔ7 c63 pETduet:: ₆ His-PCNA2-Pux_PCNA1-PuR c71 pRSFduet:: ₆ His-FjTAL	This study

s17	c96 pCDFduet:: _{PCNA1} -GGs-CYP199A2 F185L NΔ7 c106 pETduet:: _{6His-PCNA2-Pdx_PCNA3-GGS-PdR} (opt) c71 pRSFduet:: _{6His-FjTAL}	This study
s18	c84 pCDFduet:: _{6His-Pux_6His-CYP199A2F185L NΔ7} c62 pETduet:: _{6His-Pux_PuR} c71 pRSFduet:: _{6His-FjTAL}	This study

611 **Figure captions**

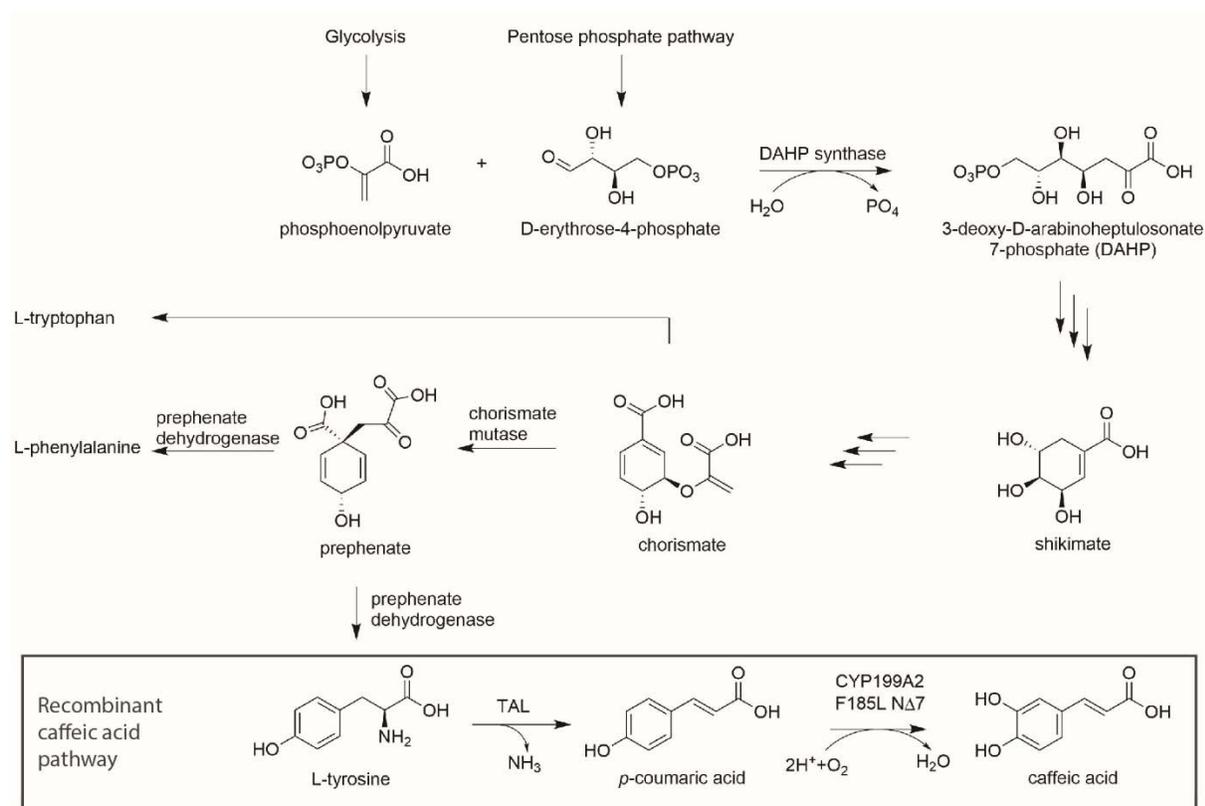
612 Figure 1: Aromatic amino acid anabolism and recombinant caffeic acid pathway with L-
613 tyrosine as a branchpoint, and TAL and CYP199A2 F185L N⁷ catalyzing the two
614 pathway steps.

615
616 Figure 2: Titters of *p*-coumaric acid and caffeic acid from glucose without (A) and with
617 (B) L-Tyr supplementation (stacked histograms, error bars= standard deviation of
618 biological replicates, n≥3).

619
620 Figure 3: The choice of redox partners and tethering strategies for redox partners leads
621 to higher caffeic acid titters from *p*-coumaric acid (panels A-C) and from glucose (panel
622 D). A-C, caffeic acid titters from 3 mM *p*-coumaric acid 72 h p. i.: untethered/free redox
623 partners (A), tether design I analogous to PUPPET [35] (B), tether design II (C).
624 Stacked histograms of *p*-coumaric and caffeic acid titters after 72 h of fermentation for
625 select strains expressing the two-step pathway (D). (Error bars= standard deviation of
626 biological replicates, n≥3; Pictograms of tether designs: P450=Cytochrome P450
627 enzyme, Fdx=ferredoxin (Pux or Pdx), FdR=ferredoxin reductase (PuR or PdR)).

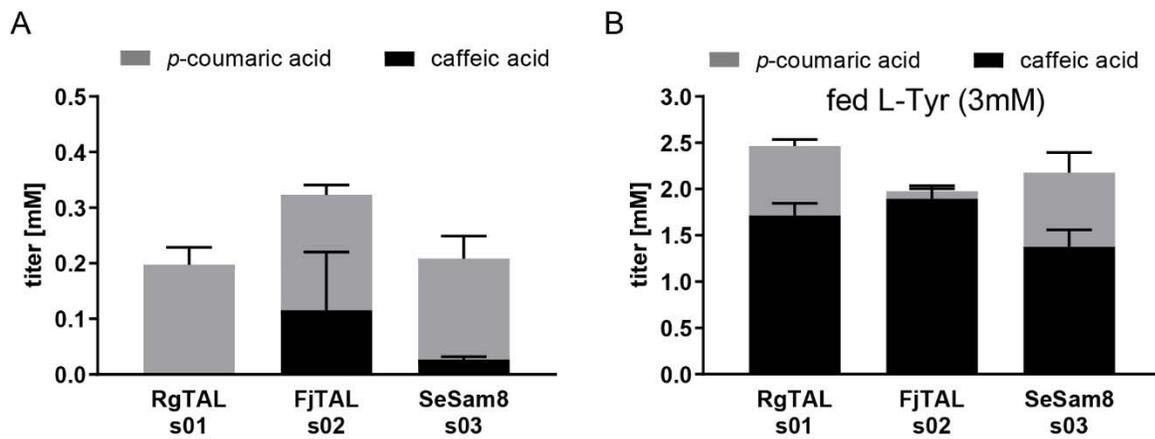
628
629 Figure 4: Duplication of the *pux* gene copy number further increases caffeic acid titters.
630 Stacked histograms of *p*-coumaric and caffeic acid titters after 72 h of fermentation with
631 glucose as the only carbon source for select strains expressing the two-step pathway
632 (A). Titters plotted over time of a 96 h fermentation of s18 (B). (Error bars= standard
633 deviation of biological replicates, n≥3.)

634 Figures



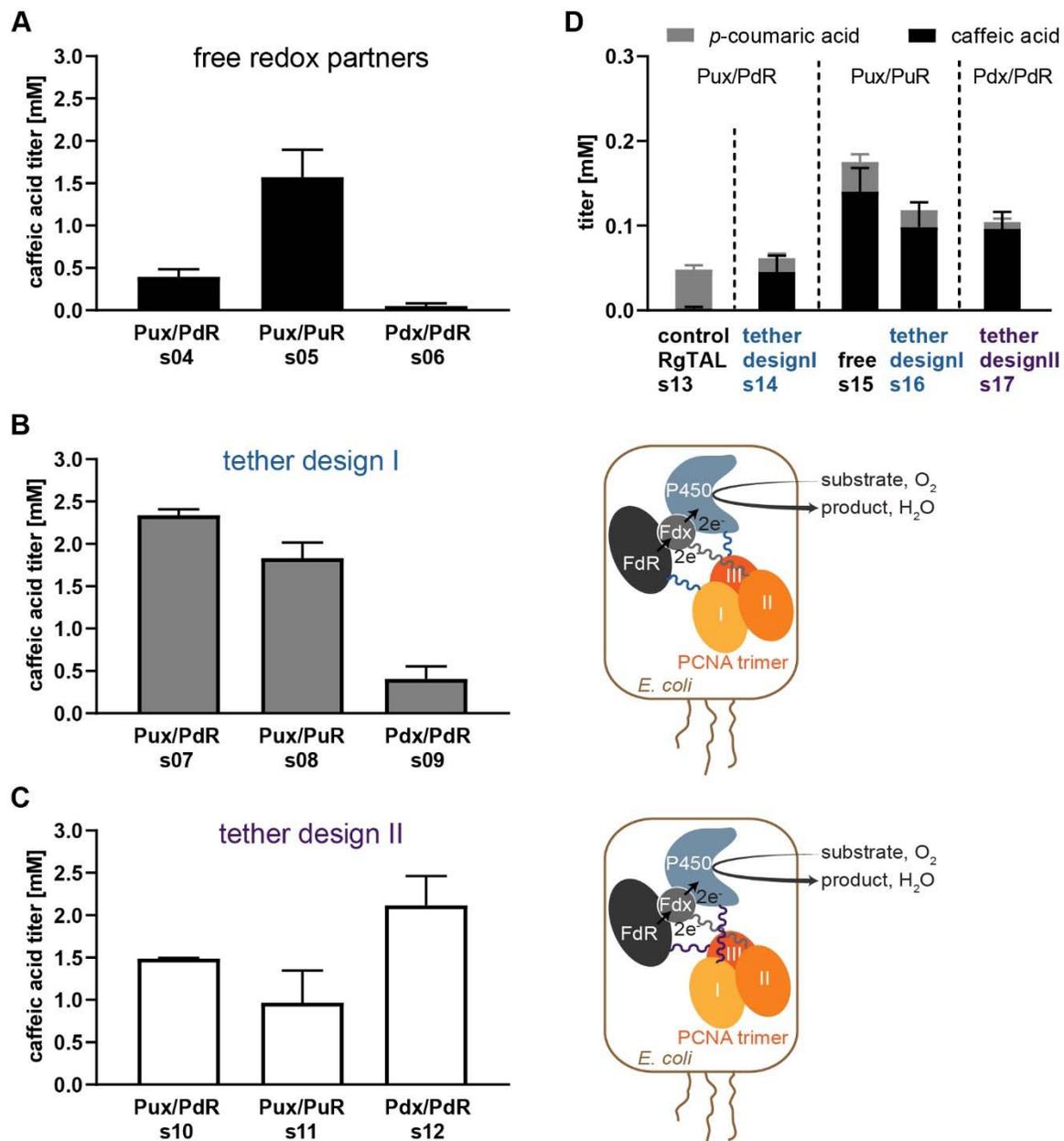
635

636 Figure 1



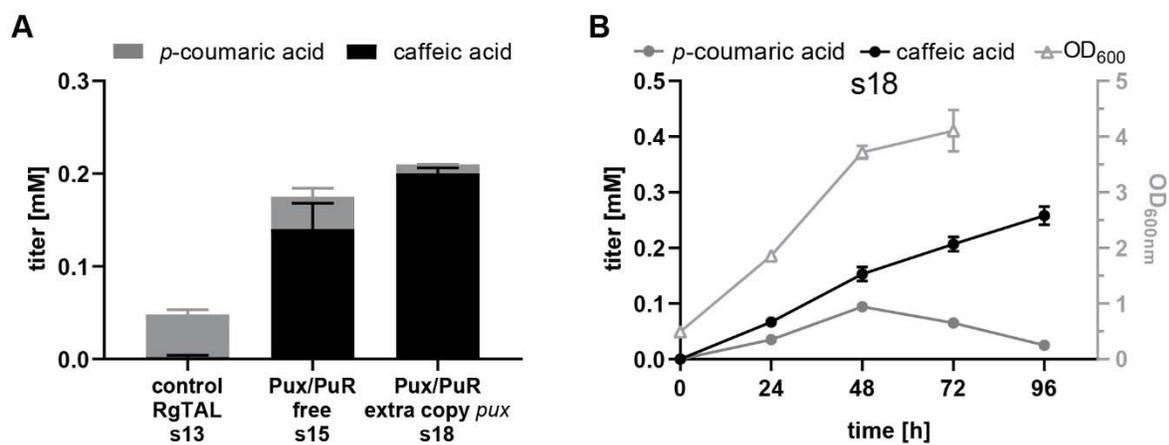
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638 Figure 2



639

640 Figure 3



641

642 Figure 4