Caffeic acid production from glucose using metabolically engineered Escherichia coli

Sakae, Kosuke ; Nonaka, Daisuke ; Kishida, Mayumi ; Hirata, Yuuki ; Fujiwara, Ryosuke ; Kondo, Akihiko ; Noda, Shuhei ; Tanaka, Tsutomu

(Citation)
Enzyme and Microbial Technology,164:110193

(Issue Date)
2023-03

(Resource Type)
journal article

(Version)
Accepted Manuscript

Caffeic acid production from glucose using metabolically engineered *Escherichia coli*

Kosuke Sakae[a], Daisuke Nonaka[a], Mayumi Kishida[a], Yuuki Hirata[a], Ryosuke Fujiwara[a], Akihiko Kondo[b,c], Shuhei Noda[b], and Tsutomu Tanaka[a]*

[a]Department of Chemical Science and Engineering, Graduate School of Engineering, Kobe University, 1-1 Rokkodai, Nada, Kobe 657-8501, Japan

[b]Center for Sustainable Resource Science, RIKEN, 1-7-22 Suehiro-cho, Tsurumi-ku, Yokohama, Kanagawa 230-0045, Japan

[c]Graduate School of Science, Technology and Innovation, Kobe University, 1-1 Rokkodai, Nada, Kobe 657-8501, Japan

*Corresponding author: Tsutomu Tanaka,

E-mail: tanaka@kitty.kobe-u.ac.jp

Tel/fax: +81-78-803-6202

Abstract

Caffeic acid (3,4-dihydroxycinnamic acid) is a precursor for high-valued compounds with anticancer, antiviral activities, and anti-inflammatory making it an important substance in the food additive, cosmetics, and pharmaceutical industries. Here, we developed an engineered *Escherichia coli* strain capable of directly producing high levels of caffeic acid from glucose. Tyrosine ammonia-lyase from *Rhodotorula glutinis* (RgTAL) and *p*-coumaric acid 3-hydroxylase from *Saccharothrix espanaensis* (SeC3H) were expressed. Next, feedback-resistant chorismate mutase/prephenate dehydrogenase, was introduced to promote L-tyrosine synthesis. This engineered strain CA3 produced 1.58 g/L of caffeic acid from glucose without tyrosine supplemented to the medium. Furthermore, to reduce *p*-coumaric acid accumulation, 4-hydroxyphenylacetate 3-hydroxylase from *Pseudomonas aeruginosa* (PaHpaBC) was introduced. Finally, an engineered strain CA8 directly produced 6.17 g/L of caffeic acid from glucose using a jar fermenter. The *E. coli* developed in this study would be helpful as a chassis strain to produce value-added caffeic acid-derivatives.

Keywords: caffeic acid, *E. coli*, metabolic engineering, shikimate pathway, *p*-coumaric acid

1. Introduction

Caffeic acid (3,4-dihydroxycinnamic acid) is a type of phenylpropanoid, a naturally occurring organic compound of plant origin. Caffeic acid has pharmacological activities, such as antioxidant [1], antiviral [2], anticancer [3], and antidepressant [4] effects, making it an important substance in the food additive, cosmetics, and pharmaceutical industries. The most common method of its production is extraction, mainly from plants, but the yield is low due to low accumulation in plant cells [5-7], also separation and purification are complicated and inefficient [8]. Therefore, as demand increases, the establishment of more efficient methods is desired, and biosynthesis via metabolically engineered microorganisms is attracting interest as a promising alternative [9].

Escherichia coli is widely used to produce many kinds of chemical compounds. Figure 1 shows the engineered metabolic pathway of caffeic acid biosynthesis in *E. coli*. Caffeic acid can be synthesized from tyrosine via a two-step reaction by tyrosine ammonia-lyase and *p*-coumaric acid 3-hydroxylase (C3H). In plants, cinnamic acid is formed from L-phenylalanine by phenylalanine ammonia-lyase, followed by two cytochrome P450 monooxygenases, cinnamic acid 4-hydroxylase (C4H) and *p*-coumaric acid 3-hydroxylase (C3H), which in turn produce *p*-coumaric acid, before it is converted to caffeic acid [10]. However, cytochrome P450 monooxygenases are difficult to express in several microbial systems [11-14]. In microbial systems, sam5-encoded SeC3H (from *Saccharothrix espanaensis*) can be used, allowing caffeic acid production in *Escherichia coli* [15,16]. Other effective hydroxylase complexes 4HPA3H for caffeic acid synthesis are EcHpaBC (from *Escherichia coli*) [11, 17], TtHpaBC (from *Thermus thermophilus HB8*) [18], RpHpaBC (from *Rhodopseudomonas palustris*) and PaHpaBC (from *Pseudomonas aeruginosa*) [19,20]. They have been reported, to show a high affinity for *p*-coumaric acid.

Previously, studies have been conducted to synthesize caffeic acid using the precursors *p*-coumaric acid and L-tyrosine as substrates [16,21]. However, caffeic acid synthesis relying on direct precursor

57 supply is undesirable due to high production costs. Therefore, a production process that allows
58 utilization of a simple carbon source such as glucose has been developed [12, 16]. The reported caffeic
59 acid titer using glucose as a carbon source is 1.03 g/L in *Escherichia coli* [22], 7.92 g/L in a 5-L
60 fermenter using *Escherichia coli* [23] and 5.5 g/L in *Saccharomyces cerevisiae* [24, 25].
61
62 This study constructed caffeic acid-producing *E. coli* strain using metabolically engineering
63 techniques. We previously developed a CFT1 strain [26,28], useful for aromatic compound
64 biosynthesis via the shikimate (SHK) pathway. Therefore, it was selected as the parent strain. To
65 construct the caffeic acid synthesis pathway, RgTAL (from *Rhodotorula glutinis*) and SeC3H (from
66 *Saccharothrix espanaensis*) were expressed using plasmids. Next, to construct a series of processes to
67 produce caffeic acid from glucose, $tyrA^{fbr}$, encoding a feedback-resistant chorismate
68 mutase/prephenate dehydrogenase, was introduced. Constructed tyrosine overproducing strain CA3
69 produced 1.58 ± 0.09 g/L of caffeic acid at a yield of 0.08 ± 0.00 g/g-glucose. Strain CA8, in which
70 PaHpaC (from *Pseudomonas aeruginosa*) was additionally introduced for pathway enhancement,
71 produced 3.61 ± 0.10 g/L of caffeic acid at a yield of 0.19 ± 0.01 g/g-glucose, which significantly
72 increased productivity. Furthermore, strain CA8 was cultured in a bioreactor and produced 6.17 ± 0.19
73 g/L of caffeic acid in 90 hours of culture. The production volume and yield obtained in this study are
74 the highest values reported in previous caffeic acid biosynthesis studies. They may even be applicable
75 in studies involving ferulic acid and L-dopa, which are caffeic acid derivatives.
76
77 **2. Materials and Methods**
78 2.1 Strains and plasmids construction
79 The strains and plasmids used in this study are listed in Table 1. ATCC31882 and its derivative
80 strains were used for caffeic acid production. NovaBlue competent cells (Novagen, Cambridge, MA,
81 USA) were used for gene cloning. Polymerase chain reaction was performed using KOD FX Neo
82 (TOYOBO, Osaka, Japan). Custom DNA oligonucleotide primers were synthesized by Invitrogen
83 Custom DNA Oligos (Thermo Fisher Scientific, Tokyo, Japan) and are listed in Table S1. Codon-
84 optimized foreign gene fragments RgTAL, SeC3H, PaHpaB, and PaHpaC were synthesized by

85 Invitrogen GeneArt Gene Synthesis service (Thermo Fisher Scientific).

86 Plasmids named pZE12-RS, pTrcHisB-RS, pHLA-RS, and pSAK-RS were prepared. Briefly, RgTAL
87 gene fragment was amplified by PCR using the primer pair pZS-RgTAL_Fw and pZS-RgTAL_Rv
88 with the RgTAL synthetic gene as a template. SeC3H gene fragment was amplified by PCR using the
89 primer pair pZS-SeC3H_Fw and pZS-SeC3H_Rv with SeC3H synthetic gene as a template. The
90 amplified fragments were cloned between the KpnI/HindIII site of pZE12-MCS or pSAK [27] and the
91 resulting plasmids were designated pZE12-RS or pSAK-RS. RgTAL–SeC3H gene fragment was
92 amplified by PCR using the primer pairs pTrc-RgTAL_Fw and pTrc-SeC3H_Rv, pHLA-RgTAL_Fw
93 and pHLA-SeC3H_Rv with pZE12-RS as a template. The amplified fragment was cloned between the
94 *Bgl*II/*Eco*RI site of pTrcHisB or between the *Bgl*II/*Xho*I site of pHLA [28] and the resulting plasmids
95 were designated pTrcHisB-RS or pHLA-RS.

96 Plasmids named pSAK-BC, pSAK-B, and pSAK-C were prepared as follows. Briefly, PaHpaB gene
97 fragment was amplified by PCR using the primer pair pS-PaHpaB_Fw and pS-PaHpaB_Rv with
98 PaHpaB synthetic gene as a template. PaHpaC gene fragment was amplified by PCR using the primer
99 pair pS-PaHpaC_Fw and pS-PaHpaC_Rv with PaHpaC synthetic gene as a template. The plasmids
100 obtained by cloning the PaHpaBC, PaHpaB, and PaHpaC fragments between the KpnI/HindIII sites of
101 pSAK, respectively, were designated pSAK-BC, pSAK-B, and pSAK-C, respectively.

102 pSAK-aroG^{fbr} and pSAK-tktA were prepared as follows. Briefly, *aroG^{fbr}* was amplified by PCR using
103 the primer pair pS-aroG^{fbr}_Fw and pS-aroG^{fbr}_Rv with *aroG^{fbr}* synthetic gene as a template. *tktA* was
104 amplified by PCR using the primer pair pS-tktA_Fw and pS-tktA_Rv with ATCC31882 genomic DNA
105 as a template. The plasmids obtained by cloning the *aroG^{fbr}* or *tktA* fragments between the
106 KpnI/HindIII sites of pSAK were designated pSAK-aroG^{fbr} or pSAK-tktA.

107 A plasmid for *pheA* gene disruption named pTΔpheA was constructed as follows. Briefly, pTargetF
108 [25] served as a template and was amplified by PCR of linearized fragments using the primer pairs
109 del_pheA.N20_Fw and del_pheA.N20_Rv. The amplified fragments were self-ligated, and the
110 resulting plasmid was designated pTFΔpheA. The upstream and downstream DNA sequences of *pheA*
111 were amplified using the primer pairs del_pheA.UP_Fw and del_pheA.UP_Rv, del_pheA.DOWN_Fw
112 and del_pheA.DOWN_Rv with ATCC31882 genome DNA as template. The amplified fragments were

113 cloned into the EcoRI/HindIII site of pTFΔpheA, and the resulting plasmid was designated pTΔpheA.
114 pTΔpheA::$P_{A1lacO1}$-PaHpaB was constructed as follows. Briefly, pSAK-B served as a template and
115 was amplified by PCR using the primer pairs del_pheA.pSAKinsert_Fw and
116 del_pheA.pSAKinsert_Rv. The resulting fragment was cloned between the Spe I sites of pTΔpheA and
117 the resulting plasmid was designated pTΔpheA::$P_{A1lacO1}$-PaHpaB. pTΔpheA::$P_{A1lacO1}$-PaHpaC,
118 pTΔpheA::$P_{A1lacO1}$-aroG^{fbr}, and pTΔ pheA::$P_{A1lacO1}$-tktA were constructed by the same procedure.
119 Disruption of *trpE*, *pheA*, *pykA* and *pykF* and transduction of *tyrA^{fbr}*, PaHpaB, PaHpaC, *aroG^{fbr}* and
120 *tktA* were performed using the CRISPR-CAS9-plasmid system [29] with the following pTΔtrpE,
121 pTΔpheA, pTΔpykA, pTΔpykF, pTΔtrpE::P_{trc}-tyrA^{fbr}, pTΔtrpE::$P_{A1lacO1}$-tyrA^{fbr} [26, 30],
122 pTΔpheA::$P_{A1lacO1}$-PaHpaB, pTΔpheA::$P_{A1lacO1}$-PaHpaC, pTΔpheA::$P_{A1lacO1}$-aroG^{fbr}, and pTΔ
123 pheA::$P_{A1lacO1}$-tktA. The deletion strains were constructed according to previous report [31].
124
125 2.2 Medium
126 LB medium (10 g/L tryptone, 5 g/L yeast extract, and 5 g/L NaCl) was used for pre-culture and
127 culture during strain genetic manipulation. M9Y medium (20 g/L glucose, 5 g/L yeast extract, 40 mg/L
128 L-tryptophan, 40 mg/L L-phenylalanine, 0.5 g/L NaCl, 1 g/L NH_4Cl, 3 g/L of KH_2PO_4, 6.7 g/L of
129 Na_2HPO_4, 2.78 mg/L $FeSO_4 \cdot 7H_2O$, 14.7 mg/L $CaCl_2 \cdot 2H_2O$, 246 mg/L $MgSO_4 \cdot 7H_2O$, 10 mg/L of
130 thiamine hydrochloride, 0.1 mM IPTG, 100 mg/L of ampicillin (and 30 mg/L of chloramphenicol))
131 was used for the caffeic acid production. The medium for bioreactor culture contained 50 g/L glucose.
132 If needed, 0.5 g/L of L-tyrosine was added in the culture medium.
133
134 2.3 Culture conditions
135 *E. coli* colonies from LB plates were inoculated into test tubes containing 5 mL of LB medium and
136 preincubated at 37°C for 24 h with shaking at 220 rpm. For test tube scale incubation, test tubes
137 containing 5 mL of M9Y medium were inoculated with the pre-culture solution to an OD_{600} of 0.10,
138 incubated at 37°C at 220 rpm, and 300 μL of the solution was collected at 24 and 48 h, which was then
139 centrifuged at 10000 rpm for 20 min, and the supernatant was analyzed. For bioreactor cultures, 5 mL

140 of the pre-cultures in LB medium were transferred into flasks with 20mL of fresh medium and
141 incubated at 37°C for an additional 8 h with shaking at 220 rpm. The pre-culture solution was then
142 inoculated into a bioreactor containing 500 mL of M9Y medium to an OD_{600} of 0.10, incubated at
143 37°C. The pH was maintained at 7.00 by the automated addition of 28% (v/v) NH_3. Foam formation
144 was suppressed using KM-70 (Shin-Etsu Chemical, Co., Ltd., Tokyo, Japan). Dissolved oxygen was
145 maintained at >20% by controlling agitation. The solution (300 μL) was collected every predetermined
146 time up to 96 h.
147
148 2.4 Analytical methods
149 Cell proliferation was evaluated using a UVmini-1240 spectrophotometer (Shimadzu Corporation,
150 Kyoto, Japan) at an optical density of OD_{600}. For glucose analysis, a Prominence HPLC System
151 (Shimadzu Corporation) equipped with a Shodex SUGAR KS-801 column (particle size 6 μm, L ×
152 I.D. 300 × 8.0 mm, Shodex) was used. The HPLC profile was monitored using a refractive index
153 detector. Caffeic acid and *p*-coumaric acid were analyzed using HPLC equipped with a MSII column
154 (particle size 5 μm, L × I.D. 250 × 4.6 mm, Nacalai Tesque). A two-component solvent system was
155 used. Solvent A was 0.2% phosphate buffer, and solvent B was methanol. The flow rate of the mobile
156 phase was 1.0 mL min^{-1} and the column was maintained at 40°C. The gradient was started with an
157 80:20 mixture of A and B (0–15 min), shifted to a 50:50 mixture of A and B (15–20 min), and back to
158 an 80:20 mixture of A and B (20–25 min). HPLC profiles were obtained using a 240 nm UV–VIS
159 detector. L-Tyrosine was analyzed using HPLC equipped with a PBr column (particle size 5 μm, L ×
160 I.D. 250 × 4.6 mm, Nacalai Tesque). A two-component solvent system was used. Solvent A was 0.2%
161 phosphate buffer, and solvent B was methanol. The flow rate of the mobile phase was 1.0 mL min^{-1}
162 and the column was maintained at 40°C. The gradient was started with 90:10 mixture of A and B (0–6
163 min), shifted to a 50:50 mixture of A and B (6–8 min), and back to 90:10 mixture of A and B (11–13
164 min). HPLC profiles were obtained using a 240 nm (caffeic acid and *p*-coumaric acid) or 220 nm (L-
165 tyrosine) UV–VIS detector. The retention time of caffeic acid, *p*-coumaric acid and L-tyrosine were
166 4.0 min, 5.0 min and 10.0 min, respectively.

167

168 **3. Results and Discussion**

169 3.1 Construction of a caffeic acid production pathway in *E coli*

170 The previously developed *E. coli* CFT1 strain derived from ATCC 31882 was selected as the parent
171 strain for this study. The endogenous phosphotransferase system (PTS) of CFT1 was replaced with a
172 galactose permease/glucokinase system (GalP/Glk system). This modification increased intracellular
173 phosphoenolpyruvate (PEP) utilization, an important factor in aromatic compound biosynthesis. First,
174 we generated the CA0 strain based on CFT1 by disrupting *pheA*, which encodes prephenate
175 dehydratase. PheA catalyzes competitive reactions in caffeic acid synthesis such as phenylalanine
176 production. Subsequently, RgTAL and SeC3H were expressed in plasmids to construct a caffeic acid
177 production pathway. Next, RgTAL and SeC3H were cloned into pZE12, pTrcHisB, pHLA, and pSAK
178 vectors to create plasmids pZE12-RS, pTrcHisB-RS, pHLA-RS, and pSAK-RS. pZE12, pTrcHisB and
179 pHLA are high-copy plasmids carrying LlacO1 promoter, Trc promoter and HCE promoter. pSAK is a
180 low-copy plasmid carrying A1lacO1 promoter. These plasmids were respectively transformed into
181 CA0. The caffeic acid conversion capacity of each strain was evaluated by adding 0.5 g/L of L-
182 tyrosine to the medium. The results of the cultivation are shown in Figure 2. A strain CA0 harboring
183 pZE12-RS showed the highest caffeic acid conversion capacity (0.47 ± 0.02 g/L) from L-tyrosine. The
184 amount of p-coumaric acid was less than 0.1 g/L in all strains (data not shown). It indicates LlacO1
185 promoter was suitable for expression of RgTAL and SeC3H. Thus, pZE12-RS was selected as the
186 plasmid for RgTAL and SeC3H expression. All of the following experiments were performed without
187 the addition of tyrosine.

188

189 3.2 Production from glucose by $tyrA^{fbr}$ overexpression

190 Caffeic acid production using the strain CA0 harboring pZE12-RS was only observed when L-tyrosine
191 was added. This strain did not produce caffeic acid from glucose (data not shown). This suggests that
192 the amount of intracellular L-tyrosine must be increased to produce caffeic acid from glucose.
193 Therefore, to promote L-tyrosine synthesis, $tyrA^{fbr}$, encoding feedback-resistant chorismate
194 mutase/prephenate dehydrogenase, was introduced into the CA0 strain in three ways. A low-copy

195 plasmid for *tyrA*^{fbr} expression under the Trc promoter (pSAK-Ptrc-tyrA^{fbr}) control, was constructed. A
196 CA0 strain harboring pZE12-RS and pSAK-Ptrc-tyrA^{fbr} was named CA1. A strain in which *tyrA*^{fbr} was
197 integrated into the *trpE* locus of the genome under the Trc promoter named CA2 and a strain in which
198 *tyrA*^{fbr} was introduced into the same locus under the A1lacO1 promoter was named CA3. Caffeic acid
199 production after 48 h using glucose as a sole carbon source is shown in Figure 3. All strains introduced
200 with *tyrA*^{fbr} successfully produced caffeic acid from glucose. Particularly, CA3 produced 1.58 ± 0.09
201 g/L, approximately 1.3-times more than strains CA1 and CA2. Therefore, CA3 was selected as the
202 caffeic acid producing strain.
203
204 3.3 Enhancement of the caffeic acid production pathway by PaHpaB and PaHpaC overexpression
205 After CA3 cultivation, the accumulation of *p*-coumaric acid (1.20 ± 0.10 g/L), caffeic acid precursor,
206 was observed (Figure 3). This suggests that the reaction to convert *p*-coumaric acid to caffeic acid is
207 the bottleneck of caffeic acid production. We attempted to enhance the reaction that converts *p*-
208 coumaric acid to caffeic acid, however, additional expression of SeC3H could not improve caffeic acid
209 production (data not shown). Other than SeC3H (from *Saccharothrix espanaensis*), EcHpaBC (from
210 *Escherichia coli*) and TtHpaBC (from *Thermus thermophilus HB8*) have been reported to catalyze this
211 reaction. EcHpaBC has high activity than TtHpaBC [18], and EcHpaBC-mediated caffeic acid
212 producing pathway produced 12.1 mg/L of caffeic acid from glucose [11]. When yeast was used as a
213 host, 569.0 mg/mL of caffeic acid was produced [24]. We focused on PaHpaBC (from *Pseudomonas*
214 *aeruginosa*), which showed high oxidation activity [19], but they have not previously been used for
215 the production of caffeic acid from glucose. Therefore, we introduced them into CA3. A CA3 strain
216 expressing PaHpaBC and using plasmid pSAK-PaBC was named CA4. A strain CA5, with PaHpaB
217 introduced into the genome of CA3 under the control of the A1lacO1 promoter, was also constructed.
218 A strain CA6 is a CA3 strain carrying plasmid pSAK-PaB for PaHpaB expression only. Similarly, a
219 CA7 strain was constructed by introducing PaHpaC in its genome under the control of the A1lacO1
220 promoter, and a CA8 strain carries a plasmid pSAK-PaC for HpaC expression. The results of the
221 culture are shown in Figure 4. CA4, which has PaHpaBC introduced as a complex, showed a slight
222 production decrease, while CA5-CA8, which had PaHpaB/PaHpaC independently introduced, showed

223 decreased *p*-coumaric acid accumulation and increased caffeic acid production. Particularly, CA8
224 produced 3.61 ± 0.09 g/L of caffeic acid, approximately 2.3-times more than CA3. CA7 produced 3.11
225 g/L of caffeic acid, which is higher than CA6 (1.88 g/L) and CA5 (1.91 g/L). The accumulation of *p*-
226 coumaric acid were less than 0.6 g/L among all strains (Figure 4) and no L-tyrosine was detected in
227 the medium (data not shown). Strains CA7 and CA8 overexpressed PaHpaC and CA5 and CA6
228 overexpressed PaHpaB. SeC3H is a single-component $FADH_2$-utilizing hydroxylases and FAD is
229 regenerated such as a flavin reductase in *E. coli* [32]. PaHpaBC are a two-component flavin-dependent
230 monooxygenase consists of reductase (PaHpaC) and oxygenase (PaHpaB) [33]. Generally,
231 optimization of $FADH_2$ transfer within or between flavin-dependent enzymes is important to decrease
232 the extravagant consumption of reducing agents NADH. From the Figure 4, the enhancement of
233 reduced flavin supply (i.e. PaHpaC overexpression) improved caffeic acid production.
234
235 3.4 Enhancement of upstream pathways related to E4P/PEP
236 After caffeic acid biosynthesis pathway was established in *E. coli* through metabolic engineering of
237 the downstream pathway after chorismic acid, we focused on enhancing the upstream pathway to
238 achieve even higher caffeic acid production. The methods of shikimate pathway enhancement have
239 been reported previously [27]. The first step of the SHK pathway is the condensation of erythrose 4-
240 phosphate (E4P) and PEP to produce 3-deoxy-d-arabinoheptulosonic acid 7-phosphate (DAHP) by the
241 action of DAHP synthase. DAHP synthase of *E. coli* is composed of three isozymes, AroG, AroF, and
242 AroH, whose activity is strictly regulated by allosteric regulation and transcriptional repression [35].
243 Therefore, to enhance DAHP synthesis response, $aroG^{fbr}$, encoding feedback-resistant AroG, was
244 introduced into CA8 to create a CA9 strain. Furthermore, the CA8 strain shortened the intermediate
245 substrates, E4P and/or PEP. Therefore, to promote E4P supply, the CA10 strain was generated by
246 introducing *tktA*, which encodes a transketolase that catalyzes the E4P synthesis reaction, into CA8. In
247 a report, PEP accumulation can be effectively controlled by the inactivation of pyruvate kinase I
248 (*pykF*)/pyruvate kinase II (*pykA*), which catalyzes the reaction that converts PEP to pyruvate [35, 36].
249 Therefore, we generated a CA11 strain, which had *pykF*/*pykA* disrupted. Since the inactivation of
250 pyruvate kinase I/pyruvate kinase II weakens the TCA cycle and affects bacterial growth, 2 g/L of

251 sodium pyruvate was added to the culture medium. The results of the cultivation are shown in Figure
252 5. Caffeic acid production was almost at similar levels among these strains, suggesting that E4P and
253 PEP supply is not a limiting factor in caffeic acid production from glucose.
254
255 3.5 Caffeic acid production using a jar fermenter
256 The CA8 strain was cultured in a jar fermenter to demonstrate the potential for scaled-up production
257 for industrialization. Modified M9Y medium with 50 g/L glucose was used. Figure 6 shows the culture
258 profiles in the jar fermenter. Caffeic acid production using jar fermenter was 6.17 ± 0.19 g/L after 90 h
259 cultivation, which is the highest titer from glucose reported to date. Almost all glucose was consumed
260 during caffeic acid production. Less than 0.6 g/L of p-coumaric acid accumulated, but it was
261 consumed during the cultivation. These results indicates that the caffeic acid-producing strains
262 developed in this study are effective and have the potential to be scaled-up for industrialization.
263
264 **4. Conclusion**
265 We succeeded in constructing a caffeic acid-producing pathway from glucose in *E. coli* by metabolic
266 engineering. We demonstrated that enhancement of PaHpaC expression effectively increased caffeic
267 acid production. Using a jar fermenter, we succeeded in producing 6.17 g/L caffeic acid, the highest
268 value obtained from glucose as a carbon source using *E. coli*. We believe that establishing the caffeic
269 acid production process can serve as an effective platform technology for the synthesis of derivatives
270 of other shikimate pathway intermediates.
271
272 **Funding**
273 This work was supported by the JST-Mirai Program (Grant Number JPMJMI17EI), Japan (to S.N. and
274 T.T.), the Japan Society for the Promotion of Science (JSPS) Grant-in-Aid for Scientific Research (A)
275 (Grant Number 20H00321), Japan (to T.T.).
276
277 **Conflicts of interest**
278 The authors declare no commercial or financial conflict of interest.

Acknowledgments

The authors would like to thank Enago (www.enago.jp) for the English language review.

CRediT authorship contribution statement

Kosuke Sakae: Conceptualization, Methodology, Investigation, Writing–Original Draft. Daisuke Nonaka: Investigation. Mayumi Kishida: Investigation. Yuuki Hirata: Investigation. Ryosuke Fujiwara: Methodology, Investigation, Writing–Review & Editing. Akihiko Kondo: Methodology. Shuhei Noda: Conceptualization, Methodology, Writing–Review & Editing. Tsutomu Tanaka: Conceptualization, Methodology, Investigation, Writing–Review & Editing.

References

[1] D.K. Maurya, T.P. Devasagayam, Antioxidant and prooxidant nature of hydroxycinnamic acid derivatives ferulic and caffeic acids, Food Chem Toxicol. ;48 (2010) 3369-3373. doi:10.1016/j.fct.2010.09.006

[2] N. Rajendra Prasad, A. Karthikeyan, S. Karthikeyan, B.V. Reddy, Inhibitory effect of caffeic acid on cancer cell proliferation by oxidative mechanism in human HT-1080 fibrosarcoma cell line. Mol Cell Biochem. 349 (2011) 11-19. doi:10.1007/s11010-010-0655-7

[3] K. Ikeda, K. Tsujimoto, M. Uozaki, M. Nishide, Y. Suzuki, A.H. Koyama, H. Yamasaki, Inhibition of multiplication of herpes simplex virus by caffeic acid. Int J Mol Med. 28 (2011) 595-598. doi:10.3892/ijmm.2011.739

[4] H. Takeda, M. Tsuji, M. Inazu, T. Egashira, T. Matsumiya, Rosmarinic acid and caffeic acid produce antidepressive-like effect in the forced swimming test in mice. Eur J Pharmacol. 449 (2002) 261-267. doi: 10.1016/s0014-2999(02)02037-x.

[5] Z.L. Fowler, M.A. Koffas, Biosynthesis and biotechnological production of flavanones: current state and perspectives. Appl Microbiol Biotechnol. 83 (2009) 799-808. doi:10.1007/s00253-009-2039-z

[6] E. Leonard, Y. Yan, Z.L. Fowler, Z. Li, C.G. Lim, K.H. Lim, M.A. Koffas, Strain improvement of

307 recombinant *Escherichia coli* for efficient production of plant flavonoids. Mol Pharm. 5 (2008)
308 257-265. doi: 10.1021/mp7001472.
309 [7] S. Horinouchi, Combinatorial biosynthesis of plant medicinal polyketides by microorganisms. Curr
310 Opin Chem Biol. 13 (2009) 197-204. doi:10.1016/j.cbpa.2009.02.004
311 [8] J.C. Ye, M.W. Hsiao, C.H. Hsieh, W.C. Wu, Y.C. Hung, W.C. Chang. Analysis of caffeic acid
312 extraction from *Ocimum gratissimum* Linn. by high performance liquid chromatography and its
313 effects on a cervical cancer cell line. Taiwan J Obstet Gynecol. 49 (2010) 266-271.
314 doi:10.1016/S1028-4559(10)60059-9
315 [9] M. Cao, M. Gao, M. Suástegui, Y. Mei, Z. Shao, Building microbial factories for the production of
316 aromatic amino acid pathway derivatives: From commodity chemicals to plant-sourced natural
317 products. Metab Eng. 58 (2020) 94-132. doi:10.1016/j.ymben.2019.08.008
318 [10] Y.H. Kim, T. Kwon, H.J. Yang, W. Kim, H. Youn, J.Y. Lee, B. Youn, Gene engineering,
319 purification, crystallization and preliminary X-ray diffraction of cytochrome P450 p-coumarate-3-
320 hydroxylase (C3H), the Arabidopsis membrane protein. Protein Expr Purif. 79 (2011) 149-155.
321 doi: 10.1016/j.pep.2011.04.013.
322 [11] Y. Lin, Y. Yan, Biosynthesis of caffeic acid in *Escherichia coli* using its endogenous hydroxylase
323 complex. Microb Cell Fact. 11 (2012) 42. doi: 10.1186/1475-2859-11-42.
324 [12]T. Furuya, Y. Arai, K. Kino, Biotechnological production of caffeic acid by bacterial cytochrome
325 P450 CYP199A2. Appl Environ Microbiol. 78 (2012), 6087–6094. doi.org/10.1128/AEM.01103-
326 12
327 [13] K. Haslinger, K.L.J. Prather, Heterologous caffeic acid biosynthesis in *Escherichia coli* is affected
328 by choice of tyrosine ammonia lyase and redox partners for bacterial Cytochrome P450. Microb
329 Cell Fact 19, (2020) 26. doi.org/10.1186/s12934-020-01300-9
330 [14] J. L. Rodrigues, R.G. Araújo, K.L. Prather, L.D. Kluskens, L.R. Rodrigues, Heterologous
331 production of caffeic acid from tyrosine in *Escherichia coli*. Enzyme Microb Technol. 71 (2015),
332 36–44. doi.org/10.1016/j.enzmictec.2015.01.001
333 [15] O. Choi, C.Z. Wu, S. Y. Kang, J.S. Ahn, T.B. Uhm, Y.S. Hong, Biosynthesis of plant-specific
334 phenylpropanoids by construction of an artificial biosynthetic pathway in *Escherichia coli*. J Ind

335 Microbiol Biotechnol. 38 (2011) 1657-1665. doi:10.1007/s10295-011-0954-3
336 [16] H. Zhang, G. Stephanopoulos. Engineering *E. coli* for caffeic acid biosynthesis from renewable
337 sugars. Applied microbiology and biotechnology, 97, (2013), 3333–3341.
338 doi.org/10.1007/s00253-012-4544-8
339 [17] J. Wang, M. Mahajani, S.L. Jackson, Y. Yang, M. Chen, E.M. Ferreira, Y. Lin, Y. Yan,
340 Engineering a bacterial platform for total biosynthesis of caffeic acid derived phenethyl esters and
341 amides. Metab Eng. 44 (2017) 89-99. doi:10.1016/j.ymben.2017.09.011
342 [18] Q. Huang, Y. Lin, Y. Yan, Caffeic acid production enhancement by engineering a phenylalanine
343 over-producing *Escherichia coli* strain. Biotechnol Bioeng. 110 (2013) 3188-3196.
344 doi:10.1002/bit.24988
345 [19] T. Furuya, K. Kino K, Catalytic activity of the two-component flavin-dependent monooxygenase
346 from *Pseudomonas aeruginosa* toward cinnamic acid derivatives. Appl Microbiol Biotechnol. 98
347 (2014) 1145-1154. doi:10.1007/s00253-013-4958-y
348 [20] H. Kawaguchi, Y. Katsuyama, D. Danyao, P. Kahar, S. Nakamura-Tsuruta, H. Teramura, K.
349 Wakai, K. Yoshihara, H. Minami, C. Ogino, Y. Ohnishi, A. Kondo, Caffeic acid production by
350 simultaneous saccharification and fermentation of kraft pulp using recombinant *Escherichia coli*.
351 Appl Microbiol Biotechnol. 101 (2017) 5279-5290. doi: 10.1007/s00253-017-8270-0.
352 [21] A. Sachan, S. Ghosh, S. K. Sen, A. Mitra, Co-production of caffeic acid and p-hydroxybenzoic
353 acid from p-coumaric acid by *Streptomyces caeruleus* MTCC 6638. Appl Microbiol Biotechnol.
354 71 (2006) 720-727. doi:10.1007/s00253-005-0197-1
355 [22] J.A. Jones, V.R. Vernacchio, S.M. Collins, A.N. Shirke, Y. Xiu, J.A. Englaender, B.F. Cress, C.C.
356 McCutcheon, R.J. Linhardt, R.A. Gross, M.A.G. Koffas, Complete biosynthesis of anthocyanins
357 using *E. coli* polycultures. mBio. 8 (2017) e00621-17. doi: 10.1128/mBio.00621-17.
358 [23] L. Wang, N. Li, S. Yu, J. Zhou, Enhancing caffeic acid production in Escherichia coli by
359 engineering the biosynthesis pathway and transporter. Bioresour Technol. 368(2023) 128320. doi:
360 10.1016/j.biortech.2022.128320.
361 [24] P. Zhou, C. Yue, B. Shen, Y. Du, N. Xu, L. Ye, Metabolic engineering of *Saccharomyces*
362 *cerevisiae* for enhanced production of caffeic acid. Appl Microbiol Biotechnol. 105 (2021) 5809-

363 5819. doi:10.1007/s00253-021-11445-1
364 [25] R. Chen, J. Gao, W. Yu, X. Chen, X. Zhai, Y. Chen, L. Zhang, Y.J. Zhou, Engineering cofactor
365 supply and recycling to drive phenolic acid biosynthesis in yeast. Nat Chem Biol. 18 (2022) 520-
366 529. doi: 10.1038/s41589-022-01014-6.
367 [26] S. Noda, T. Shirai, S. Oyama, A. Kondo, Metabolic design of a platform *Escherichia coli* strain
368 producing various chorismate derivatives. Metab Eng. 33 (2016) 119-129.
369 doi:10.1016/j.ymben.2015.11.007
370 [27] S. Noda, A. Kondo, Recent advances in microbial production of aromatic chemicals and
371 derivatives. Trends Biotechnol. 35 (2017) 785-796. doi:10.1016/j.tibtech.2017.05.006
372 [28] T. Tanaka, H. Kawabata, C. Ogino, A. Kondo, Creation of a cellooligosaccharide-assimilating
373 *Escherichia coli* strain by displaying active beta-glucosidase on the cell surface via a novel
374 anchor protein. Appl Environ Microbiol. 77 (2011) 6265-6270. doi:10.1128/AEM.00459-11
375 [29] Y. Jiang, B. Chen, C. Duan, B. Sun, J. Yang, S. Yang, Multigene editing in the *Escherichia coli*
376 genome via the CRISPR-Cas9 system Appl Environ Microbiol. 81 (2015) 2506-2514.
377 doi:10.1128/AEM.04023-14
378 [30] R. Fujiwara, S. Noda, T. Tanaka, A. Kondo, Metabolic engineering of *Escherichia coli* for
379 shikimate pathway derivative production from glucose-xylose co-substrate, Nat Commun. 11
380 (2020) 279. doi:10.1038/s41467-019-14024-1
381 [31] R., Fujiwara, M. Nakano, Y. Hirata, C. Otomo, D. Nonaka, S. Kawada, H. Nakazawa, M. Umetsu,
382 T. Shirai, S. Noda, T. Tanaka, A. Kondo. G6P-capturing molecules in the periplasm of
383 *Escherichia coli* accelerate the shikimate pathway. Metab Eng. 72 (2022) 68-81. doi:
384 10.1016/j.ymben.2022.03.002.
385 [32] K.T. Heo, B. Lee, S. Son, J.S. Ahn, J.H. Jang, Y.S. Hong, Production of bioactive 3'-
386 hydroxystilbene compounds using the flavin-dependent monooxygenase Sam5. J Microbiol
387 Biotechnol. 28 (2018) 1105-1111. doi:10.4014/jmb.1804.04007
388 [33] S. Chakraborty, M. Ortiz-Maldonado, B. Entsch, D.P. Ballou, Studies on the mechanism of p-
389 hydroxyphenylacetate 3-hydroxylase from *Pseudomonas aeruginosa*: a system composed of a
390 small flavin reductase and a large flavin-dependent oxygenase. Biochemistry. 49 (2010) 372-385.

391 doi:10.1021/bi901454u

392 [34] G.A. Sprenger, From scratch to value: engineering *Escherichia coli* wild type cells to the
393 production of L-phenylalanine and other fine chemicals derived from chorismate. Appl Microbiol
394 Biotechnol. 75(2007) 739-749. doi:10.1007/s00253-007-0931-y

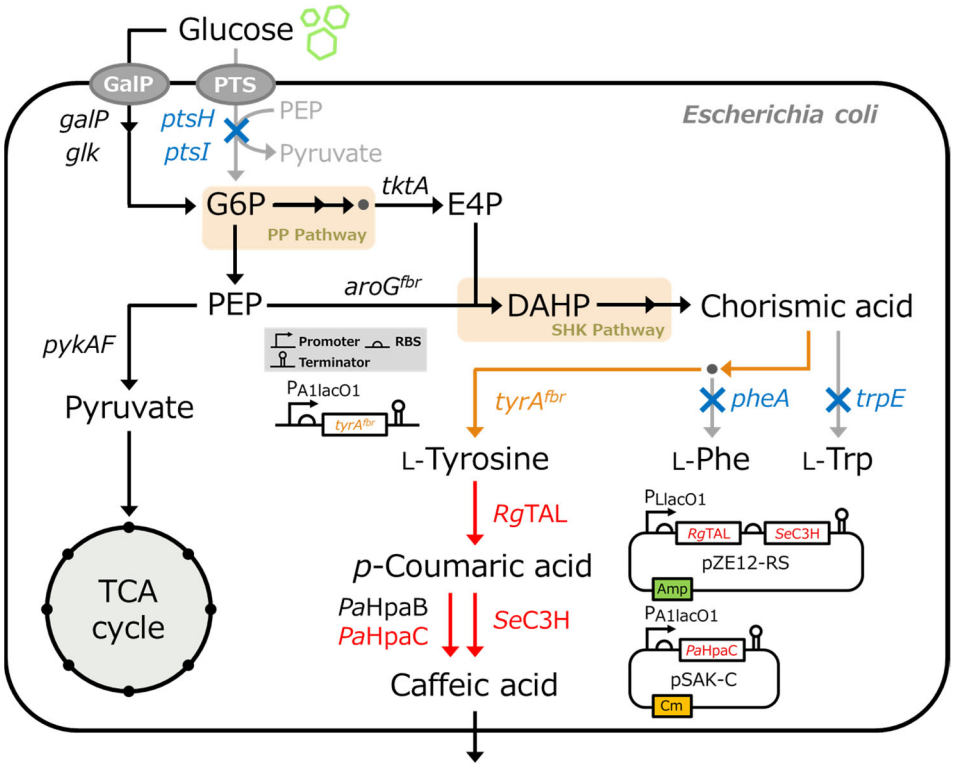
395 [35] Y.F. Yao, C.S. Wang, J. Qiao, G.R. Zhao, Metabolic engineering of *Escherichia coli* for
396 production of salvianic acid A via an artificial biosynthetic pathway. Metab Eng. 19 (2013) 79-87.
397 doi:10.1016/j.ymben.2013.06.001

398 [36] M. Weiner, C. Albermann, K. Gottlieb, G.A. Sprenger, D. Weuster-Botz, Fed-batch production of
399 L-phenylalanine from glycerol and ammonia with recombinant *Escherichia coli*. Biochem Eng J.
400 83 (2014) 62–69.doi 10.1016/j.bej.2013.12.001.

401

402

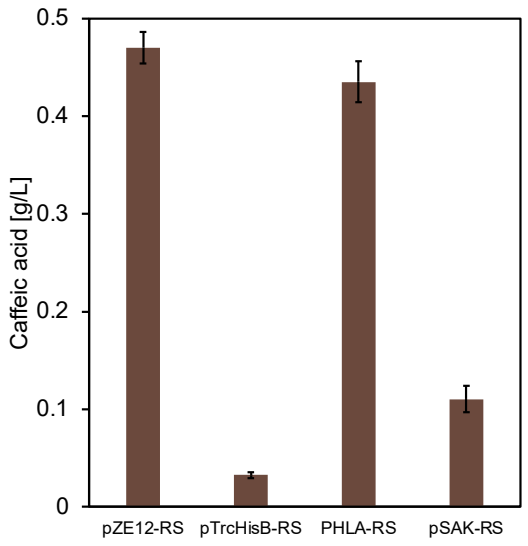
403 **Figures**



404

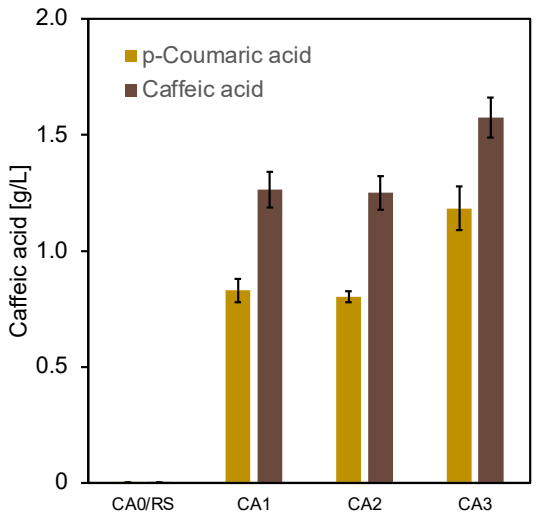
405 **Figure 1.** Metabolic engineering of caffeic acid producing *E. coli*. The blue X indicates disruption of
406 the *ptsH*, *ptsI*, *trpE* and *pheA* genes. Red or orange shows genes involved in caffeic acid synthesis
407 overexpressed by plasmid or genomic integration. G6P, glucose 6-phosphate; PEP,
408 phosphoenolpyruvate; E4P, erythrose 4-phosphate; DAHP, 3-deoxy-d-arabinoheptulosonic acid 7-
409 phosphate; PP pathway, pentose–phosphate pathway; SHK pathway, shikimate pathway; RgTAL,
410 tyrosine ammonia-lyase from *Rhodotorula glutinis*; SeC3H, *p*-coumaric acid 3-hydroxylase from
411 *Saccharothrix espanaensis*; PaHpaBC, hydroxylase complexes 4HPA3H from *Pseudomonas*
412 *aeruginosa*; *tyrA*^{fbr}, feedback-resistant chorismate mutase/prephenate dehydrogenase; *galP*, d-
413 galactose transporter; *glk*, glucokinase; *ptsH*, phosphocarrier protein HPr; *ptsI*, phosphoenolpyruvate-
414 protein phosphotransferase; *pykF*, pyruvate kinaseI; *pykA*, pyruvate kinaseII; *tktA*, transketolase;
415 *aroG*^{fbr}, feedback-resistant 3-deoxy-7-phosphoheptulonate synthase; *trpE*, anthranilate synthase
416 component I; *pheA*, chorismate mutase/prephenate dehydratase.

417



418

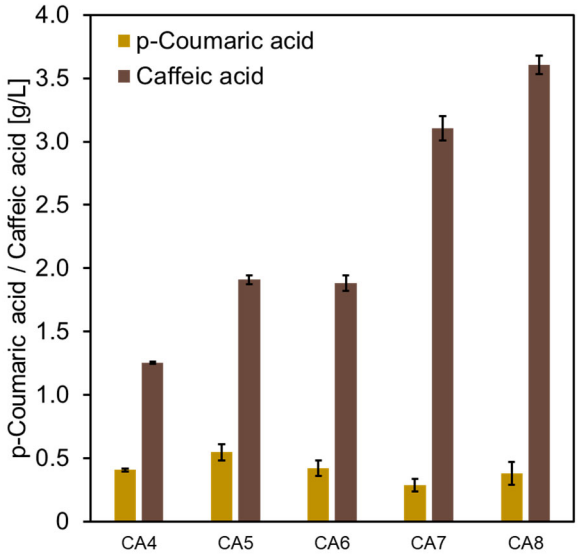
419 **Figure 2.** Caffeic acid production after 48 h of cultivation in M9Y medium containing 0.5 g/L L-
420 tyrosine and 20 g/L glucose using the strains harboring plasmid pZE12-RS, pTrcHisB-RS, pHLA-RS,
421 or pSAK-RS. The data shown are as the means and standard deviations of three independent
422 experiments.



423

424 **Figure 3.** Caffeic acid production after 48 h of cultivation in M9Y medium containing 20 g/L glucose
425 using strains CA1, CA2, and CA3. The data shown are as the means and standard deviations of three
426 independent experiments

427

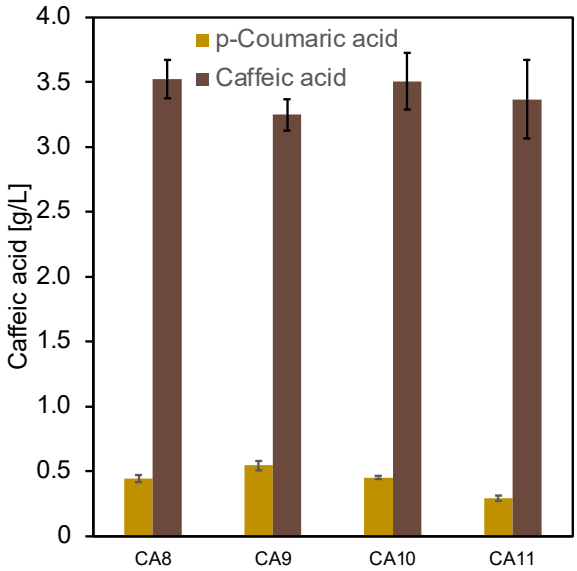


428

429 **Figure 4.** Caffeic acid production after 48 h of cultivation in M9Y medium containing 20 g/L glucose

430 using the strains CA4-CA8. The data shown are as the means and standard deviations of three

431 independent experiments.



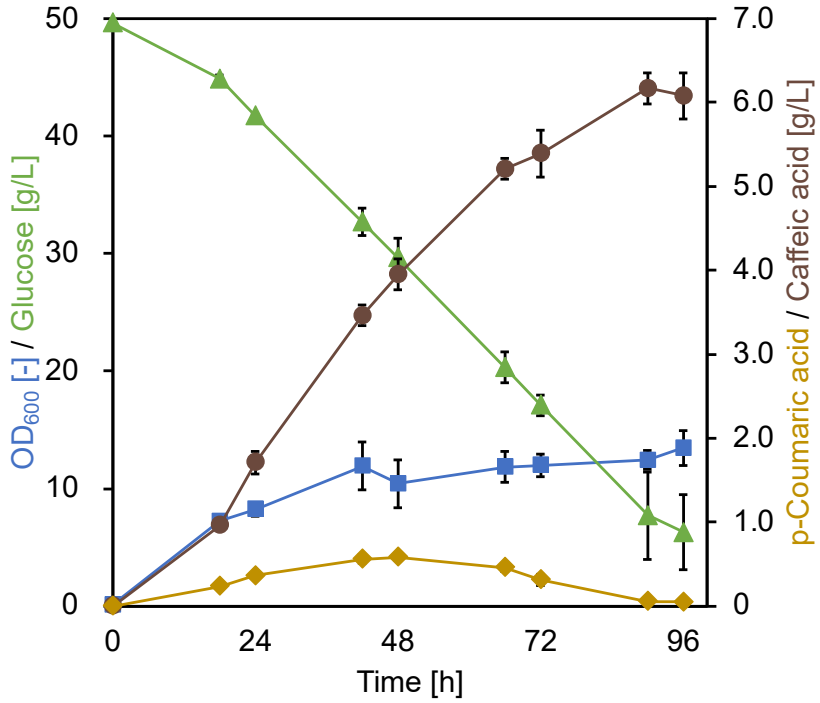
432

433 **Figure 5.** Caffeic acid production after 48 h of cultivation in M9Y medium containing 20 g/L glucose

434 using the strains CA8-CA11. The data shown are as the means and standard deviations of three

435 independent experiments

436



437

438 **Figure 6.** CA8 culture profiles in a jar fermenter. Blue squares, green triangles, light-brown and dark-
439 brown symbols indicate cell growth, glucose concentration, *p*-coumaric acid concentration and caffeic
440 acid concentration, respectively. The data shown are as the means and standard deviations of three
441 independent experiments.

442

443 **Table 1.** Strains and plasmids used in this study.

Strains and Plasmids		
Strains	**Genotype**	**Reference**
NovaBlue	*endA1 hsdR17*(*rK12-mK12*$^{+}$) *supE44 thi-1 gyrA96 relA1 lac recA1*/F [*proAB*$^{+}$ *laclqZ*Δ*M15 Tn10*(*TetR*)]	Novagen
ATCC31882	L-Phenylalanine-overproducing strain (aroF aroG tyrR pheA tyrA trpE)	ATCC
CFT1	ATCC31882Δ*ptsHI*::$P_{A1lacO1}$-*glk-galP*	Noda et al
CA0	CFT1Δ*pheA*	This study
CA0/RS	CFT1Δ*trpE*Δ*pheA*/pZE12-RS	This study
CA1	CFT1Δ*trpE*Δ*pheA*/pZE12-RS+pSAK-Ptrc-tyrA^{fbr}	This study
CA2	CFT1Δ*trpE*::P_{trc}-*tyrA*^{fbr}Δ*pheA*/pZE12-RS	This study
CA3	CFT1Δ*trpE*::$P_{A1lacO1}$-*tyrA*^{fbr}Δ*pheA*/pZE12-RS	This study
CA4	CFT1Δ*trpE*::$P_{A1lacO1}$-*tyrA*^{fbr}Δ*pheA*/pZE12-RS+pSAK-BC	This study
CA5	CFT1Δ*trpE*::$P_{A1lacO1}$-*tyrA*^{fbr}Δ*pheA*::$P_{A1lacO1}$-PaHpaB/pZE12-RS	This study
CA6	CFT1Δ*trpE*::$P_{A1lacO1}$-*tyrA*^{fbr}Δ*pheA*/pZE12-RS+pSAK-B	This study
CA7	CFT1Δ*trpE*::$P_{A1lacO1}$-*tyrA*^{fbr}Δ*pheA*::$P_{A1lacO1}$-PaHpaC/pZE12-RS	This study
CA8	CFT1Δ*trpE*::$P_{A1lacO1}$-*tyrA*^{fbr}Δ*pheA*/pZE12-RS+pSAK-C	This study
CA9	CFT1Δ*trpE*::$P_{A1lacO1}$-*tyrA*^{fbr}Δ*pheA*::$P_{A1lacO1}$-*aroG*^{fbr}/pZE12-RS+pSAK-C	This study
CA10	CFT1Δ*trpE*::$P_{A1lacO1}$-*tyrA*^{fbr}Δ*pheA*::$P_{A1lacO1}$-*tktA*/pZE12-RS+pSAK-C	This study
CA11	CFT1Δ*trpE*::$P_{A1lacO1}$-*tyrA*^{fbr}Δ*pheA*Δ*pykAF*/pZE12-RS+pSAK-C	This study
Plasmids	**Characteristic**	**Reference**

pZE12-MCS	P_{LlacO1}, *colE ori*, and Amp^r	Expressys
pTrcHisB	P_{trc}, *pBR322 ori*, and Amp^r	Life Technologies
pHLA	P_{HCE}, *colE1 ori*, and Amp^r	Tanaka et al., 2011
pSAK	$P_{A1lacO1}$, *SC101 ori*, and Cm^r	Noda et al., 2017
pZE12-RS	pZE12 containing RgTAL and SeC3H	This study
pTrcHisB-RS	pTrcHisB containing RgTAL and SeC3H	This study
pHLA-RS	pHLA containing RgTAL and SeC3H	This study
pSAK-RS	pSAK containing RgTAL and SeC3H	This study
pSAK-Ptrc-tyrA^{fbr}	pSAK-P_{trc} containing $tyrA^{fbr}$	Fujiwara et al., 2020
pSAK-BC	pSAK containing PaHpaBC	This study
pSAK-B	pSAK containing PaHpaB	This study
pSAK-C	pSAK containing PaHpaC	This study
pSAK-aroG^{fbr}	pSAK containing $aroG^{fbr}$	This study
pSAK-tktA	pSAK containing *tktA*	This study
pTargetF	Constitutive expression of sgRNA	Addgene
pCas	Constitutive expression of cas9 and inducible expression of λ RED and sgRNA	Addgene
pTΔtrpE	Constitutive expression of sgRNA with donor editing template DNA for *trpE* disruption	Fujiwara et al., 2020
pTΔtrpE::P_{trc}-tyrA^{fbr}	Constitutive expression of sgRNA with donor editing template DNA for P_{trc}-$tyrA^{fbr}$ into *trpE* gene loci	Fujiwara et al., 2020
pTΔtrpE::$P_{A1lacO1}$-tyrA^{fbr}	Constitutive expression of sgRNA with donor editing template DNA for $P_{A1lacO1}$-$tyrA^{fbr}$ into *trpE* gene loci	Fujiwara et al., 2020

pTΔpykA	Constitutive expression of sgRNA with donor editing template DNA for *pykA* disruption	Noda et al., 2016
pTΔpykF	Constitutive expression of sgRNA with donor editing template DNA for *pykF* disruption	Noda et al., 2016
pTΔpheA	Constitutive expression of sgRNA with donor editing template DNA for *pheA* disruption	This study
pTΔpheA::$P_{A1lacO1}$-PaHpaB	Constitutive expression of sgRNA with donor editing template DNA for $P_{A1lacO1}$-PaHpaB into *pheA* gene loci	This study
pTΔpheA::$P_{A1lacO1}$-PaHpaC	Constitutive expression of sgRNA with donor editing template DNA for $P_{A1lacO1}$-PaHpaC into *pheA* gene loci	This study
pTΔpheA::$P_{A1lacO1}$-aroG^{fbr}	Constitutive expression of sgRNA with donor editing template DNA for $P_{A1lacO1}$-*aroG*^{fbr} into *pheA* gene loci	This study
pTΔpheA::$P_{A1lacO1}$-tktA	Constitutive expression of sgRNA with donor editing template DNA for $P_{A1lacO1}$-*tktA* into *pheA* gene loci	This study

Graphical abstract