SCIEPublish

Article

Hydroxybenzoic Acid Production Using Metabolically Engineered *Corynebacterium glutamicum*

Misa Doke [1], Mayumi Kishida [1], Yuuki Hirata [1], Mariko Nakano [1], Mayo Horita [1], Daisuke Nonaka [1], Yutaro Mori [1], Ryosuke Fujiwara [2], Akihiko Kondo [2,3], Shuhei Noda [3,4] and Tsutomu Tanaka [1,*]

[1] Department of Chemical Science and Engineering, Graduate School of Engineering, Kobe University, 11 Rokkodai, Nada, Kobe 657-8501, Japan; 219t441t@stu.kobe-u.ac.jp (M.D.); caomaruco@people.kobe-u.ac.jp (M.K.); yhirata@puppy.kobe-u.ac.jp (Y.H.); nakanonakan@phoenix.kobe-u.ac.jp (M.N.); 229t449t@stu.kobe-u.ac.jp (M.H.); 204t441t@stu.kobe-u.ac.jp (D.N.); yutaro.mori@hawk.kobe-u.ac.jp (Y.M.)

[2] RIKEN Center for Sustainable Resource Science, 1722 Suehirocho, Tsurumiku, Yokohama, Kanagawa 230-0045, Japan; ryosuke.fujiwara@riken.jp (R.F.); akondo@kobe-u.ac.jp (A.K.)

[3] Graduate School of Science, Technology, and Innovation, Kobe University, 1-1, Rokkodai, Nada, Kobe 657-8501, Japan; shuhei.noda@opal.kobe-u.ac.jp (S.N.)

[4] Japan Science and Technology Agency (JST), PRESTO 4-1-8, Honcho, Kawaguchi-shi, Saitama 332-0012 Japan

* Corresponding author. E-mail: tanaka@kitty.kobe-u.ac.jp (T.T.)

Received: 30 May 2023; Accepted: 14 July 2023; Available online: 19 July 2023

ABSTRACT: Hydroxybenzoic acids (HBAs), including 4-HBA, 3-HBA, and 2-HBA, are valuable platform chemicals for production of commodity materials and fine chemicals. Herein, we employed metabolic engineering techniques to enhance the production of these HBAs in *Corynebacterium glutamicum* ATCC 13032. Our approach augmented the shikimate pathway and eliminated genes associated with HBA degradation, particularly phenol 2-monooxygenase encoded by *cg2966*. Increased titers of 3-HBA and 4-HBA were achieved via selection of suitable promoters for 3-hydroxybenzoate synthase and chorismate pyruvate lyase. A tac-M1 promoter was suitable for chorismate pyruvate lyase expression and 8.3 g/L of 4-HBA production was achieved. Efficient production of 2-HBA was enabled by maintaining a balanced expression of isochorismate synthase and isochorismate pyruvate lyase. Consequently, strains KSD5-tacM1-H and KSD5-J2-PE exhibited production levels of 19.2 g/L of 3-HBA and 12.9 g/L of 2-HBA, respectively, using 1 L jar fermenter containing 80 g/L of glucose. Therefore, this engineered strain platform holds significant potential for production of other valuable products derived from chorismate.

Keywords: *Corynebacterium glutamicum*; Hydroxybenzoic acid; 2-hydroxybenzoic acid; 3-hydroxybenzoic acid; Metabolic engineering; Chorismate derivatives

1. Introduction

Recent decades have seen a substantial expansion in using microorganisms to produce fuels and chemicals derived from renewable resources [1–3]. This trend reflects mounting concerns regarding global warming and the limited availability of fossil fuels [4,5]. Multiple studies have shown that microorganisms can synthesize diverse classes of compounds [6–8], and metabolic engineering has helped develop microbial strains that can produce useful compounds, including pigments [9], terpenoids [10], and amino acids [11], with exceptional efficiency.

Hydroxybenzoic acids (HBAs), comprising 4-HBA, 3-HBA, and 2-HBA, are widely used to produce polymer materials, food additives, and pharmaceuticals [12–14]. Furthermore, these compounds exhibit a range of functional biological properties, such as anticancer, antiaging, antiviral, and anti-inflammatory activities [15–18]. Given their widespread application in toiletries, food, and pharmaceutical industries, their production from renewable biomass is an area of growing interest. HBAs can be biosynthesized from chorismite, the end product of the shikimate pathway [19] and starting point in the aromatic amino acids biosynthesis (tyrosine, phenylalanine, and tryptophan) in microorganisms [20].

Microbial synthesis of HBAs from glucose has been investigated using metabolically engineered microorganisms [21–26]. Enzymatic production of 4-HBA, 3-HBA, and 2-HBA is catalyzed by chorismate pyruvate lyase (EC 4.1.3.4), 3-hydroxybenzoate synthase (EC 4.1.3.4), isochorismate pyruvate lyase (EC 4.2.99.21), respectively, which all release pyruvate as a byproduct. To

https://doi.org/10.35534/sbe.2023.10010

enhance the production of HBAs in *Escherichia coli*, a platform strain CFT5 was developed with replacement of the phosphotransferase system with the GalP transporter and glucokinase *glk* genes and deletion of the pyruvate kinase genes to improve phosphoenolpyruvate supply [21]; the phenylalanine and tyrosine synthesis pathways were also inactivated to eliminate competing pathways. Test tube cultivation of CFT5 produced 1.82, 2.18, and 1.48 g/L of 4-HBA, 3-HBA, and 2-HBA, respectively [21], and an improved strain produced 3.01 g/L of 2-HBA with a yield of 0.51 mol/mol. The same pathway has been used in *Pseudomonas putida* [27] and *Saccharomyces cerevisiae* [23] for 4-HBA production; however, the titer and yield were lower than those achieved in *E. coli*, indicating the need for further optimization in these organisms.

Corynebacterium glutamicum, a gram-positive nonpathogenic bacterium, is widely used for production of industrial amino acids, including L-glutamate, L-lysine, L-phenylalanine, and L-tyrosine [11]. This microbe represents a highly promising microbial platform to produce various fuels and chemicals [28], such as diols [29], lactams [30], and organic acids [31]. The optimization of the aromatic amino acid production in *C. glutamicum* requires the detailed study of individual reactions within the shikimate pathway and pathway regulation. More recently, *C. glutamicum* has been genetically engineered to produce shikimate pathway intermediates, including shikimate, and derivative aromatic compounds, including p-aminobenzoate [22]. *C. glutamicum* has a high tolerance to 4-HBA (to 300 mM), making this an attractive host for 4-HBA production [25].

Herein, we engineered enhanced HBA production in *C. glutamicum* using the SA-7 strain that had previously been engineered for shikimate overproduction [32]. Our strategy involved the inactivation of the HBA degradation gene clusters, and the removal of phenol 2-monooxygenase gene (*cg2966*) significantly improved HBA production. Additionally, we optimized promoter use to achieve elevated production of 3-HBA and of 4-HBA to 12 g/L, which represents the highest titer for this HBA reported from *C. glutamicum*.

2. Materials and Methods

2.1. Bacterial Strains and Media

Bacterial strains employed throughout this study are shown in Table 1. Recombinant *C. glutamicum* ATCC 13032 strains were cultivated aerobically at 30 °C in either Brain Heart Infusion (BHI) medium or a defined CGXIIY (CGXII medium containing 4 g/L of yeast extract) medium plus 50 g/L of glucose and 100 mg/L of aromatic amino acids [32]. *E. coli* NovaBlue was routinely cultured in Luria–Bertani medium (containing 10 g/L peptone, 5 g/L yeast extract, and 10 g/L NaCl) at 37 °C. Kanamycin (25 μg/mL for *C. glutamicum* strains and 50 μg/mL for *E. coli*) was added as required.

2.2. Plasmid and Strain Construction

Strains and plasmids used in this study are listed in Table 1. PCR was performed using KOD FX Neo (TOYOBO, Osaka, Japan). Custom DNA oligonucleotide primers were synthesized by Invitrogen (Thermo Fisher Scientific, Tokyo, Japan) and are listed in Table S1. The plasmid for the *pheA* gene deletion was constructed as follows. The upstream and downstream regions of the *C. glutamicum pheA* gene were PCR amplified from ATCC 13032 using the primer pairs EcoRI_pheA_Up_for/pheA_Up_re and pheA_down_for/pheA_UP_re, respectively. The amplified fragments were ligated into EcoRI/BamHI-digested pK18 mobsacB using the Infusion HD Cloning kit. Plasmids for other gene deletions were constructed similarly.

The *hyg5*-expression plasmid under the control of the dap-e10 promoter was constructed as follows. PCR was performed using pCC-H36-cgR0949-Tfu0937 [33] as a template with the primer pair dap-A16-1_Nhe_cgR0949_for/H36_dap-A16-1_re. The amplified fragment was used as a template in a second round of PCR using the primer pairs dap-e10_A16_for/dap-e10_re. The resulting fragment was self-ligated and then digested with NheI and XhoI. Simultaneously, a codon-optimized *hyg5* from *Streptomyces hygroscopicus* (purchased from Life Technologies) was PCR amplified with primer pair NheI_Hyg5_for/XhoI_Hyg5_re. These two fragments were ligated together to generate plasmid pCC-e10-hyg5 plasmid. Other plasmids were constructed similarly. The strains/plasmids during the current study are available from the corresponding author on reasonable request.

2.3. Culture Conditions

Precultures (5 mL BHI medium in test tubes) were inoculated with single colonies and grown overnight at 30 °C with shaking at 220 rpm. Cultures were centrifuged at 4000× *g* for 2 min, and cells were resuspended in 1 mL of CGXII medium supplemented with 100 mg/L each of L-phenylalanine, L-tyrosine, L-tryptophan, and p-aminobenzoate. The resulting suspension (400 μL) was inoculated into 5 mL of CGXII medium or CGXIIY medium containing 40 g/L of glucose and incubated at 30 °C at 220 rpm for 48 h. The performance of 3-HBA- or 2-HBA-producing strains was evaluated in a batch process conducted in 1 L bioreactors (ABLE Co. & Biott Co., Tokyo, Japan) with a 500 mL working volume. The batch medium contained 80 g/L of glucose. A 40 mL volume of preculture was used to inoculate 500 mL of culture medium in the jar fermenter. To maintain the pH at 7.0 during cultivation, NH_4OH (7% solution) was automatically added to the fermenter. The dissolved oxygen was maintained at >30% saturation by automatically regulating the agitation speed (300–800 rpm) and supplementing with air when necessary.

2.4. Analysis of Substrates and Products

Concentrations of 3-HBA, 4-HBA, and 2-HBA were determined using HPLC (Shimadzu, Kyoto, Japan) equipped with a COSMOSIL PBr column (5 μm, 4.6 × 250 mm, I.D. × L, Nacalai Tesque, Kyoto, Japan). For 3-HBA and 2-HBA, a mobile phase of 50:50 mixture of 0.2% phosphoric acid and methanol was employed at 1.0 mL/min, whereas for 4-HBA, a 60:40 mixture was used. The column temperature was maintained at 40 °C. The UV-VIS detector was set at 236 nm for 3-HBA and 2-HBA and at 254 nm for 4-HBA. Cell growth was determined by measuring the optical density at 600 nm on a Shimadzu UVmini-1240 spectrophotometer. The glucose concentration was analyzed using an HPLC system equipped with a Shodex SUGAR KS-801 column (6 μm, 300 × 8.0 mm I.D. × L, Shodex, Yokohama, Japan) with a column temperature of 50 °C and a mobile phase of water at a flow rate of 0.8 mL/min. The HPLC profile was monitored using refractive index detector.

Table 1. Strains used in this study.

Corynebacterium glutamicum **Strains**	**Genotype**	**Source or Reference**
ATCC 13032	Wild-type strain	ATCC
SA-7	ATCC13032, Δ*cgMRR*(*cg1966-cg1998*), Δ*qsuD*, Δ*qsuB*, Δ*nagD*, Δ*cg2392*, gnd^{S361F}, $aroG^{gtg\text{-}atg}$, *pta*::P_{H36}-*aroG*, *aroK*::P_{H36}-*aroB*	32
KSD1	ATCC13032, Δ*cgMRR*(cg*1966*-cg*1998*), Δ*qsuD*, Δ*qsuB*, Δ*nagD*, Δ*cg2392*, gnd^{S361F}, $aroG^{gtg\text{-}atg}$, *pta*::P_{H36}-*aroG*, Δ*pheA*, Δ*tyrA*, Δ*cg0975*	This study
KSD2	KSD1, Δ*pobA*, Δ*cg0344-cg0347*, Δ*cg3349-cg3354*, Δ*cg2624-cg2640*	This study
KSD3	KSD2, Δ*pqo*	This study
KSD4	KSD2, Δ*ldh*	This study
KSD5	KSD2, Δ*cg2966*	This study
KSD1-H36-H	KSD1 harboring pCC-H36-hyg5	This study
KSD2-H36-H	KSD2 harboring pCC-H36-hyg5	This study
KSD3-H36-H	KSD3 harboring pCC-H36-hyg5	This study
KSD4-H36-H	KSD4 harboring pCC-H36-hyg5	This study
KSD5-H36-H	KSD5 harboring pCC-H36-hyg5	This study
KSD1-H36-U	KSD1 harboring pCC-H36-ubiC	This study
KSD2-H36-U	KSD2 harboring pCC-H36-ubiC	This study
KSD3-H36-U	KSD3 harboring pCC-H36-ubiC	This study
KSD4-H36-U	KSD4 harboring pCC-H36-ubiC	This study
KSD5-H36-U	KSD5 harboring pCC-H36-ubiC	This study
KSD5-e10-H	KSD5 harboring pCC-e10-hyg5	This study
KSD5-e11-H	KSD5 harboring pCC-e11-hyg5	This study
KSD5-e12-H	KSD5 harboring pCC-e12-hyg5	This study
KSD5-tacM1-H	KSD5 harboring pCC-tacM1-hyg5	This study
KSD5-J2-H	KSD5 harboring pCC-J2-hyg5	This study
KSD5-J3-H	KSD5 harboring pCC-J3-hyg5	This study
KSD5-J4-H	KSD5 harboring pCC-J4-hyg5	This study
KSD5-e10-U	KSD5 harboring pCC-e10-ubiC	This study
KSD5-e11-U	KSD5 harboring pCC-e11-ubiC	This study
KSD5-e12-U	KSD5 harboring pCC-e12-ubiC	This study
KSD5-tacM1-U	KSD5 harboring pCC-tacM1-ubiC	This study
KSD5-J2-U	KSD5 harboring pCC-J2-ubiC	This study
KSD5-J3-U	KSD5 harboring pCC-J3-ubiC	This study
KSD5-J4-U	KSD5 harboring pCC-J4-ubiC	This study
KSD5-e10-P	KSD5 harboring pCC-e10-pchB	This study
KSD5-e11-P	KSD5 harboring pCC-e11-pchB	This study
KSD5-e12-P	KSD5 harboring pCC-e12-pchB	This study
KSD5-tacM1-P	KSD5 harboring pCC-tacM1-pchB	This study
KSD5-J2-P	KSD5 harboring pCC-J2-pchB	This study
KSD5-J3-P	KSD5 harboring pCC-J3-pchB	This study
KSD5-J4-P	KSD5 harboring pCC-J4-pchB	This study
KSD5-e10-eP	KSD5 harboring pCC-e10-entC-pchB	This study
KSD5-e11-eP	KSD5 harboring pCC-e11-entC-pchB	This study
KSD5-e12-eP	KSD5 harboring pCC-e12-entC-pchB	This study
KSD5-tacM1-eP	KSD5 harboring pCC-tacM1-entC-pchB	This study
KSD5-J2-eP	KSD5 harboring pCC-J2-entC-pchB	This study
KSD5-J3-eP	KSD5 harboring pCC-J3-entC-pchB	This study
KSD5-J4-eP	KSD5 harboring pCC-J4-entC-pchB	This study
KSD5-e10-PE	KSD5 harboring pCC-e10-pchB-entC	This study
KSD5-e11-PE	KSD5 harboring pCC-e11-pchB-entC	This study
KSD5-e12-PE	KSD5 harboring pCC-e12-pchB-entC	This study
KSD5-tacM1-PE	KSD5 harboring pCC-tacM1-pchB-entC	This study
KSD5-J2-PE	KSD5 harboring pCC-J2-pchB-entC	This study
KSD5-J3-PE	KSD5 harboring pCC-J3-pchB-entC	This study
KSD5-J4-PE	KSD5 harboring pCC-J4-pchB-entC	This study

3. Results and Discussion

3.1. Construction of a 3-HBA Biosynthetic Pathway Using a Shikimate-producing Strain of C. glutamicum

We selected the SA-7 strain given its prior use in shikimate biosynthesis and deleted three genes encoding DHS dehydratase *qsuB* (*cg0502*), QA/SA dehydratase *qsuD* (*cg0504*), and DHAP phosphatase *nagD* (*cg2474*). The point mutation Ser361Phe of the 6-phosphogluconate dehydrogenase gene (*gnd*; *cg1643*) increased L-lysine production due to enhancement of NADPH supply [34]. To improve the activity of the shikimate dehydrogenase AroE, which requires NADPH as a cofactor, we introduced this point mutation. To enhance the expression of AroG, we changed the start codon of *aroG* from GTG to ATG (*aroG*$^{gtg\rightarrow atg}$) and deleted *cg2392* while also introducing an extra copy of *aroG* chromosomally into the *pta* region. We reintroduced *aroK* gene to account for the disrupted expression of shikimate kinase AroK in the SA-7 strain. Additionally, we deleted the *pheA*, *tyrA*, and chorismite mutase genes (*cg0937*) to prevent phenylalanine and tyrosine biosynthesis to generate strain KSD1. Furthermore, we deleted genes involved in the 3-HBA degradation pathway [26]: *phdBCDE* (*cg0344-47*), *pobA* (*cg1226*), *pcaFDO–pcaCBGH–cg2633–catCBA–benABCD* (*cg2625-40*), and *nagIKL–nagR–nagT–genH* (*cg3349-54*). The resulting strain was named KSD2, and the engineered metabolic pathway is illustrated in Figure 1a.

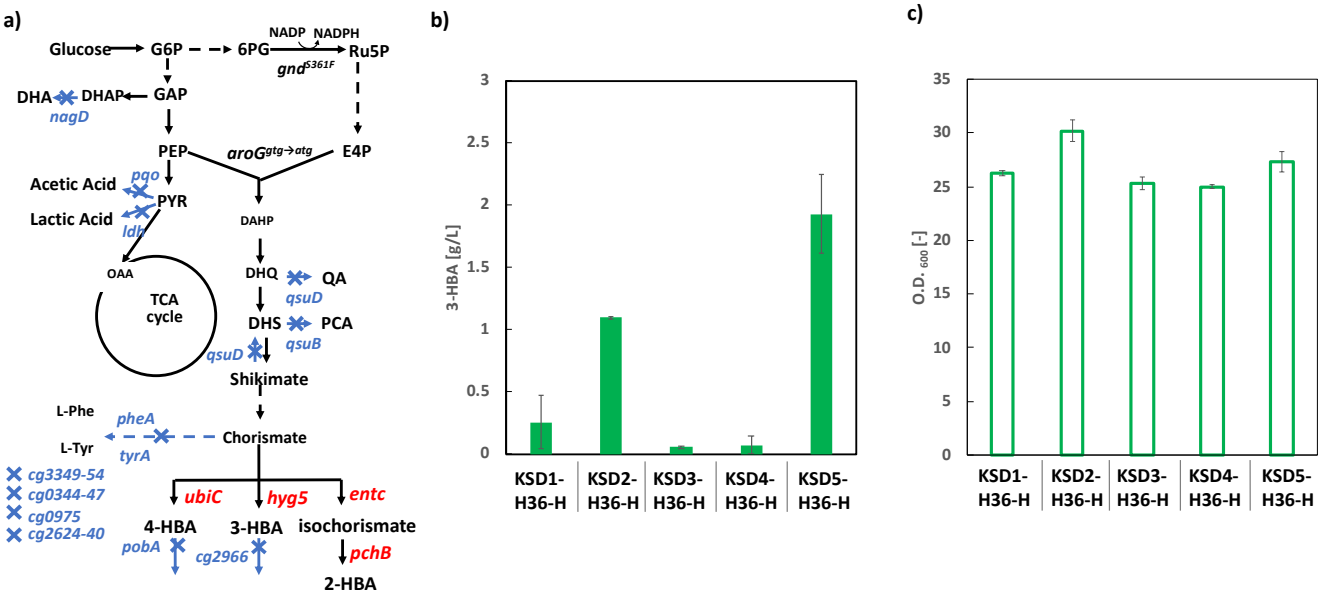

Figure 1. (**a**) Schematic illustration of the hydroxybenzoic acid (HBA) production pathway in *C. glutamicum*. Metabolic engineering of *C. glutamicum* for HBA production. The blue X indicates gene deletion. Genes involving hydroxybenzoic acids synthesis pathways are indicated in red. G6P, glucose-6-phosphate; GAP, glyceraldehyde-3-phosphate; DHAP, 1,3-dihydroxyacetone phosphate; DHA, 1,3-dihydroxyacetone; PEP, phosphoenolpyruvate; PYR, pyruvate; OAA, oxaloacetate; Ru5P, ribulose-5-phosphate; E4P, erythrose 4-phosphate; DAHP, 3-deoxy-D-arabinoheptulosonate-7-phosphate; DHQ, 3-dehydroquinate; DHS, 3-dehydroshikimate; PCA, protocatechuate; *aroG*, DAHP synthase; *qsuB*, DHS dehydratase; *qsuD*, QA/shikimate dehydrogenase; *gnd*, 6-phosphogluconate dehydrogenase; *pheA*, prephenate dehydratase; *tyrA*, prephenate dehydrogenase; *pobA*; p-hydroxybenzoate hydroxylase; *cg0975*, chorismate mutase; *cg2966*, phenol 2-monooxygenase. (**b**) 3-HBA production and (**c**) cell growth among KSD strains harboring the *hyg5* expression plasmid under the control of the H36 promoter after 48 h cultivation. Data are presented as the average of three independent experiments, and error bars indicate the standard deviation.

To produce 3-HBA, we selected the 3-hydroxybenzoate synthase gene *hyg5* from *S. hygroscopicus* and used KSD1 and KSD2 as host strains. To express *hyg5*, we used the synthetic constitutive promoter P_{H36} [35], which has been successfully used to produce recombinant single-chain variable fragments [36], gamma-aminobutyrate [37], 1,5-diaminopentane [33], and glutaric acid [38]. KSD2 carrying pCC-H36-hyg5 produced 1.1 g/L of 3-HBA, which was approximately 3-fold higher than the titer produced by KSD1 (Figure 1b). Throughout cultivation, we observed the formation of a small (nonsignificant) amount of lactic acid and acetic acid (data not shown). However, deletion of the *pqo* and *ldh* genes to reduce organic acid accumulation and enhance carbon flux into the shikimate pathway produced a significant decrease in 3-HBA production by the resultant strains KSD3 and KSD4 carrying pCC-H36-hyg5 (Figure 1b). Therefore, we focused on *cg2966*, the phenol 2-monooxygenase gene, as a possible candidate for hydroxybenzoate degradation and deleted this gene in the KSD2 strain. The resultant strain KSD5 carrying pCC-H36-hyg5 produced 1.93 g/L of 3-HBA after 48 h of cultivation (Figure 1b). The cell growth among KSD strains were almost same (Figure 1c), suggesting less effects of these gene deletion on cell growth.

3.2. Improvement of 3-HBA Production by Selection of an Appropriate Promoter for hyg5 Expression

To enhance the titer of 3-HBA, we identified suitable promoters for *hyg5* expression. Various promoter libraries consisting of native and/or fully synthetic promoters have been employed in *C. glutamicum*. For *hyg5* overexpression, we screened a set of potent promoters (dap-e10, dap-e11, dap-e12, tac-M1, J2, J3, and J4) that have been previously used for demonstrating arginine biosynthesis [39]. The dap-e12 promoter has the highest strength, followed by that of dap-e11, dap-10, and tac-M1 [39]. The J2 promoters, J3 and J4 have similar activity with the tac-M1 promoter [39]. Figure 2a shows 3-HBA production using different promoters. Strain KSD5-tacM1-H exhibited a significant increase in 3-HBA production up to 5.9 g/L after 48 h of cultivation, and use of the tac-M1 promoter produced approximately 4-fold higher titers compared with those of the H36 promoter (Figure 1b). Promoter J4 also increased 3-HBA production to 5.35 g/L. Thus, these promoters could be used for plasmid-based gene overexpression. Previously reported 3-HBA production titers were approximately 2.5 g/L from 20 g/L of glucose (0.125 g/g-glucose) using *E. coli* [21] and 2.0 g/L from 40 g/L of glucose (0.05 g/g-glucose) using *C. glutamicum* [27], whereas strain KSD5-tacM1-H produced 5.9 g/L of 3-HBA from 50 g/L of glucose (0.118 g/g-glucose) (Figure 2a), which is more than twice the production titer of those strains. The cell growth among these strains were almost same (Figure 2b), suggesting less effects of employed promoters on cell growth.

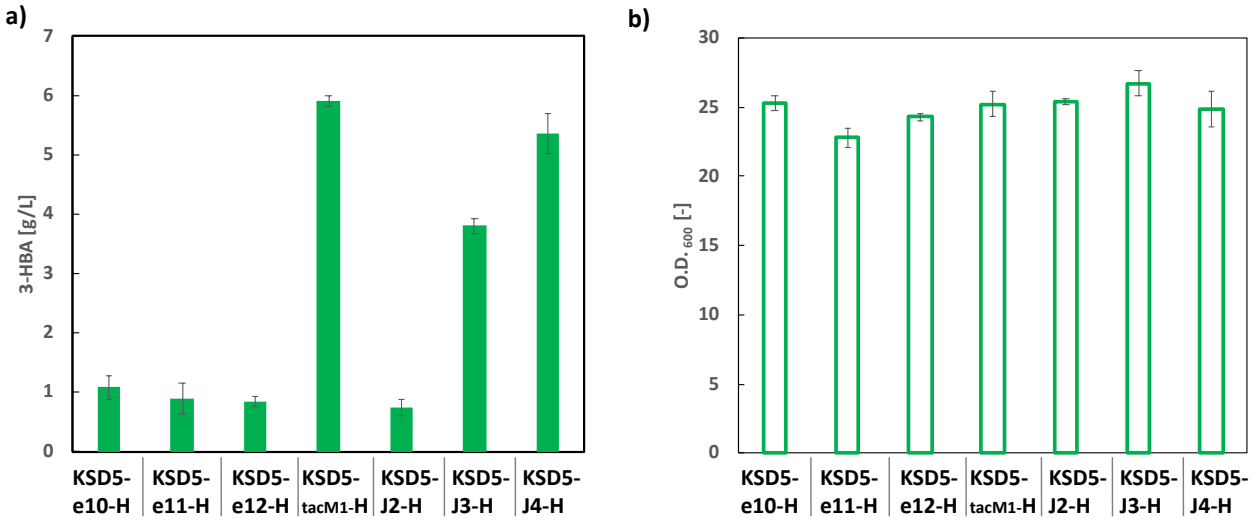

Figure 2. (**a**) 3-HBA production and (**b**) cell growth using the KSD-5 strains harboring hyg5-espressing plasmids with different promoters after 48 h of cultivation in CGXIIY medium containing 50 g/L glucose. Data shown are mean and standard deviations of three independent experiments.

3.3. 4-HBA Production Using KSD Strains and Promoter Variants

4-HBA is synthesized from chorismite by chorismate pyruvate lyase (EC 4.1.3.40) derived from *E. coli* (UbiC). To enhance 4-HBA production, we tested strains KSD1 to KDS5. KSD5 carrying pCC-H36-UbiC produced 6.37 g/L of 4-HBA (Figure 3a). Interestingly, strains KSD3 and KSD4 also exhibited increased 4-HBA production compared with that of KSD2. One possible reason is that both reactions producing 3-HBA and 4-HBA produce pyruvate simultaneously. Although the pathway to the precursor chorismate is the same, produced pyruvate might affect the carbon flux into PEP or TCA cycle. We evaluated seven different promoters for 4-HBA production using the HB-5 strain as a host (Figure 3b). Promoters e11, tac-M1, and J2 were suitable for UbiC expression and produced 8.3 g/L of 4-HBA after 48 h of cultivation. The cell growth among these strains were almost same (Figure 3c). In a previous report on 4-HBA production, UbiC was expressed using the gapA [25], IPTG-inducible T7 [26], or sodM promoters [40]. Direct comparison of the titers of these strains is difficult because of the differences in cultivation conditions (growth-arrested culture, baffled Erlenmeyer flask, or conventional fed-batch culture). Our results suggest that the appropriate promoter for overexpression is gene dependent as indicated by the comparison with Figures 1b and 2.

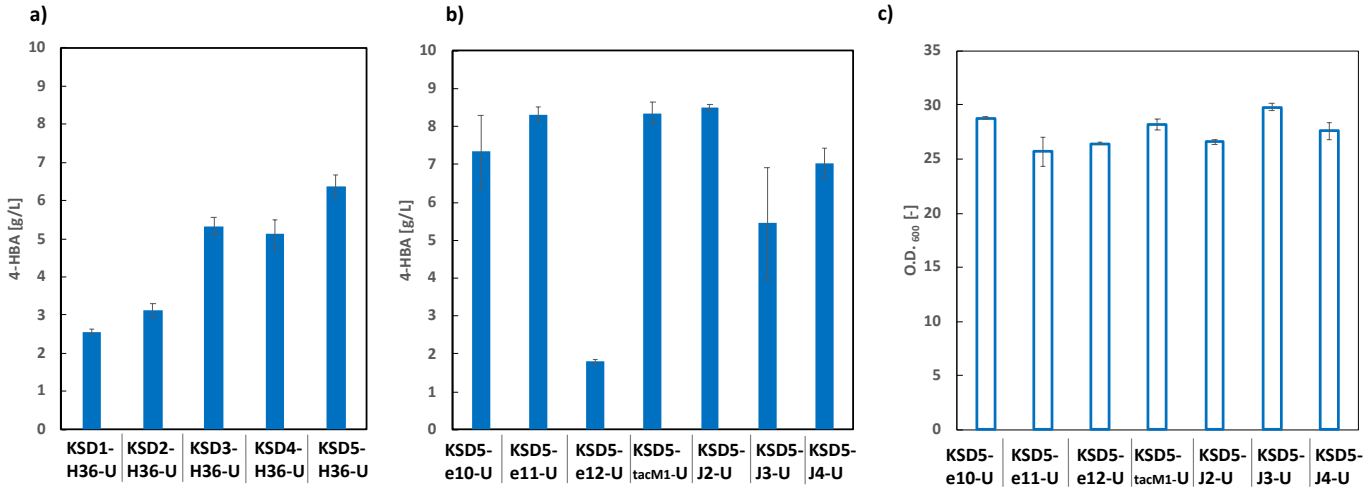

Figure 3. (**a**) 4-HBA production using the KSD1, KSD2, KSD3, KSD4, and KSD5 strains harboring plasmid pCC-H36-UbiC after 48 h of cultivation in CGXIIY medium containing 50 g/L glucose (**b**) 4-HBA production and (**c**) cell growth using the KSD5 strains harboring UbiC expression plasmids with different promoter after 48 h of cultivation in CGXIIY medium containing 50 g/L glucose. Data shown are mean and standard deviations of three independent experiments.

3.4. 2-HBA Production by entC and pchB Cooverexpression

Encouraged by the results for 3-HBA and 4-HBA production, we sought to produce 2-HBA (salicylic acid) using KSD5 strains and the above-mentioned promoters. Since *C. glutamicum* possesses an endogenous isochorismate synthase encoded by *entC*, we solely focused on expressing the isochorismate pyruvate lyase from *Pseudomonas aeruginosa* encoded by *pchB*. This specific enzyme has been employed for 2-HBA synthesis in *E. coli*, where co-expression of isochorismate synthase is necessary due to the absence of native isochorismate synthase in *E. coli* [21]. However, 2-HBA titers using the KSD5 strains were <0.15 g/L across all *pchB* expression plasmids (Figure 4a). We hypothesized that expression levels of native *entC* were insufficient for 2-HBA production. Therefore, we consequently constructed a co-expression vector for *entC* and *pchB*. Both genes (*entC* and *pchB*) were connected through a consensus ribosome binding site (RBS) sequence (GAAAGGAGGCCCTTCAG) and expressed under the control of the seven promoters and 2-HBA production were evaluated (Figure 4b). Use of plasmid pCC-tacM1-entC-pchB increased 2-HBA production to 0.8 g/L (Figure 4b). The dap-e12 promoter failed to produce any 2-HBA. Although the titer showed a slight improvement, it was also suggested that the expression level of *entC* was sufficient under these conditions, whereas the expression level of *pchB* was inadequate. Subsequently, when pCC-J2-pchB-entC was used, 2-HBA production significantly improved to 5.3 g/L (Figure 4c). Interestingly, the tac-M1 promoter, which is one of the suitable promoters for 3-HBA and 4-HBA production, could not produce 2-HBA at all (Figure 4c). These results suggest that a strong promoter is not necessarily suitable for all gene expressions, and the optimal promoter should be selected for each target gene. Furthermore, achieving a balance in the transcription levels of both *pchB* and *entC* genes proved to be crucial for 2-HBA production. Previously reported 2-HBA production titers were approximately 0.3 g/L [27] using only the bifunctional salicylate synthase. We attempted to express the bifunctional enzyme irp9 from *Yersinia enterocolitica*, but no 2-HBA was produced (data not shown). It is likely that the appropriate promoters for *irp9* expression were not among the seven promoters we tested.

2-HBA, 3-HBA, and 4-HBA are derived from a precursor, chorismate. The variation in production levels among these hydroxybenzoic acids may be attributed to the specific gene introduced (e.g., *hyg5*, *ubic*, or *pchB-entC*) that impacts the conversion of chorismate. In contrast, studies on the commonly utilized host *E. coli* have shown almost same production levels for 2-HBA, 3-HBA, and 4-HBA [21]. This suggests that chorismate production may be the limiting factor in *E. coli*, whereas the downstream reaction from chorismate could be the bottleneck in *C. glutamicum*. The investigation of promoters and expression systems conducted in this study may offer a promising approach to address this bottleneck effectively.

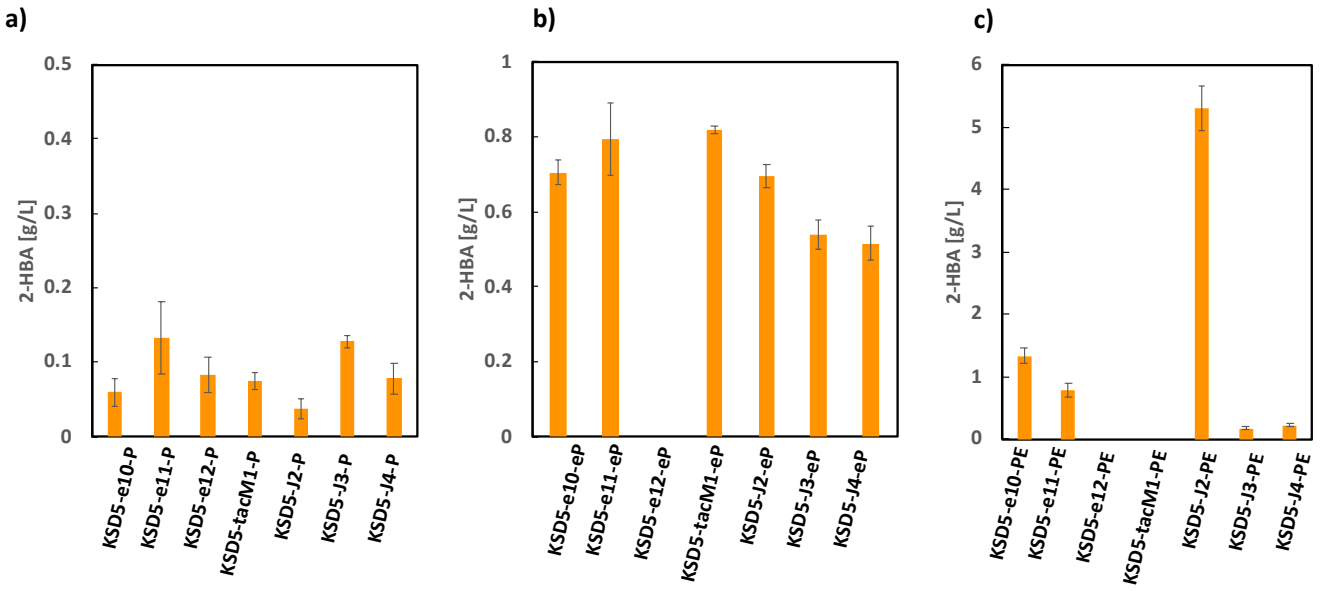

Figure 4. (**a**) 2-HBA production using the KSD-5 strains harboring plasmid pCC-(promoter)-pchB. (**b**) 2-HBA production using the KSD-5 strains harboring plasmid pCC-(promoter)-entC-RBS-pchB. (**c**) 4-HBA production using the KSD-5 strains harboring plasmid pCC-(promoter)-pchB-RBS-entC. 2-HBA and 4-HBA production was evaluated after 48 h of cultivation in CGXIIY medium containing 50 g/L glucose for all strains. Data shown are mean and standard deviations of three independent experiments.

3.5. Production of 3-HBA and 2-HBA Using a Jar Fermentor

Batch culture using a jar fermenter was conducted at an initial glucose concentration of 80 g/L. The culture profiles of KSD5 carry pCC-tacM1-hyg5 demonstrate that the production of 3-HBA reached 19.2 g/L after 41 h of cultivation (Figure 5a). The culture profiles of KSD5 harboring pCC-J2-PE show that 2-HBA production reached the highest yield of 12.9 g/L after 45 h of cultivation (Figure 5b). Glucose consumption was complete within 50 h for both 3-HBA and 2-HBA, and only trace amounts of organic acids (<1 g/L) were detected during cultivation in both cases. Although the *nagIKL–nagR–nagT–genH* gene cluster in the gentisate pathway, which is responsible for 3-HBA degradation, was disrupted in the KSD5 strain (Figure 1a), a decrease in 3-HBA production was observed in the later stages of cultivation (Figure 5a). One possibility is that there may be other genes involved in 3-HBA degradation, which might be caused by lower glucose concentrations or glucose depletion. A method of producing 3-HBA while supplying glucose in fed-batch culture may be one possible solution.

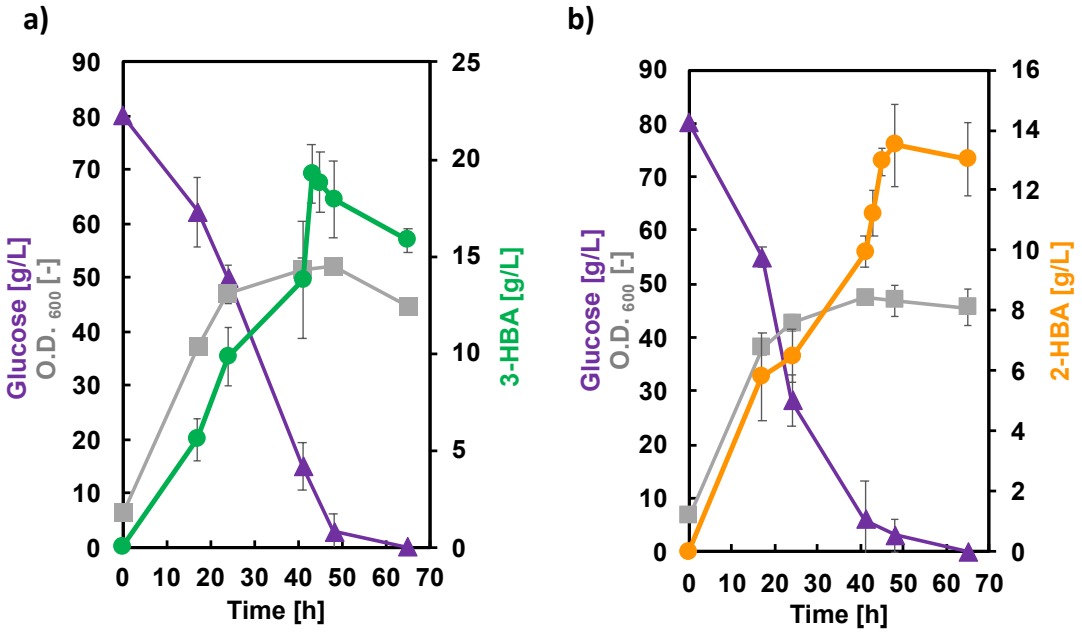

Figure 5. Culture profiles of (**a**) KSD5-tacM1-H and (**b**) KSD5-J2-PE grown using a jar fermenter. The concentrations of 3-HBA (green circles) and 2-HBA (orange circles), cell growth (gray squares), and glucose consumption (purple triangles) are shown. Data shown are mean and standard deviations of three independent experiments.

4. Conclusions

We demonstrated the versatility of the *C. glutamicum* platform for the efficient production of HBAs. Through deletion of genes associated with HBA degradation gene *cg2966* and careful selection of suitable promoters for *hyg5* and *UbiC* as well as balancing expression levels of *pchB* and *entC*, we engineered a *C. glutamicum* strain that produced 19.2 g/L of 3-HBA and 12.9 g/L of 2-HBA in jar fermenter, which surpasses the titers reported for previously studied *C. glutamicum* strains [26]. We also acheved 8.3 g/L of 4-HBA in a test tube culture. These significant advancements made in our study highlight the potential and progress of using *C. glutamicum* as a platform for HBAs production.

Supplementary Materials

The following supporting information can be found at: https://www.sciepublish.com/index/journals/article/sbe/25.html/id/52, Table S1: Plasmids used in this study; Table S2: Primers used in this study.

Acknowledgments

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

Author Contributions

Conceptualization, M.D. and T.T.; Investigation, M.D., M.K., Y.H., M.N., M.H., D.N., T.T.; Resources, A.K.; Writing–Original Draft Preparation, M.D. and T.T.; Writing–Review & Editing, M.D.; Y.M.; R.F.; S.N.; T.T.; Project Administration, T.T.; Funding Acquisition, T.T.

Ethics Statement

Not applicable.

Informed Consent Statement

Not applicable.

Funding

This research was supported by the JST-Mirai Program (grant number JPMJMI17EI), Japan (to T.T.), the Japan Society for the Promotion of Science (JSPS) Grant-in-Aid for Scientific Research (B) (grant number 19H02526), Japan (to T.T.), and the New Energy and Industrial Technology Development Organization (grant number JPNP22101006-0).

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

References

1. Kim JY, Ahn YJ, Lee JA, Lee SY. Recent advances in the production of platform chemicals using metabolically engineered microorganisms. *Curr. Opin. Green Sustain. Chem*. **2023**, *40*, 100777.
2. Madhavan A, Arun KB, Sindhu R, Nair BG, Pandey A, Awasthi MK, et al. Design and genome engineering of microbial cell factories for efficient conversion of lignocellulose to fuel. *Bioresour. Technol*. **2023**, *370*, 128555.
3. Geng B, Jia X, Peng X, Han Y. Biosynthesis of value-added bioproducts from hemicellulose of biomass through microbial metabolic engineering. *Metab. Eng. Commun*. **2022**, *15*, e00211.
4. Gómez-Sanabria A, Kiesewetter G, Klimont Z, Schoepp W, Haberl H. Potential for future reductions of global GHG and air pollutants from circular waste management systems. *Nat. Commun*. **2022**, *13*, 106.
5. Olabi A, Abdelkareem MA. Renewable energy and climate change. *Renew. Sustain. Energy Rev*. **2022**, *158*, 112111.
6. Ding Q, Ye C. Microbial cell factories based on filamentous bacteria, yeasts, and fungi. *Microb. Cell Fact*. **2023**, *22*, 20.
7. Liang P, Cao M, Li J, Wang Q, Dai Z. Expanding sugar alcohol industry: Microbial production of sugar alcohols and associated chemocatalytic derivatives. *Biotechnol. Adv*. **2023**, *64*, 108105.
8. Zhou S, Ding N, Han R, Deng Y. Metabolic engineering and fermentation optimization strategies for producing organic acids of the tricarboxylic acid cycle by microbial cell factories. *Bioresour. Technol*. **2023**, *379*, 128986.
9. Xu S, Gao S, An Y. Research progress of engineering microbial cell factories for pigment production. *Biotechnol. Adv*. **2023**, *65*, 108150.
10. Jiang H, Wang X. Biosynthesis of monoterpenoid and sesquiterpenoid as natural flavors and fragrances. *Biotechnol. Adv*. **2023**, *65*, 108151.
11. Zha J, Zhao Z, Xiao Z, Eng T, Mukhopadhyay A, Koffas MA, et al. Biosystem design of *Corynebacterium glutamicum* for bioproduction. *Curr. Opin. Biotechnol*. **2023**, *79*, 102870.

12. Wang S, Bilal M, Hu H, Wang W, Zhang X. 4-Hydroxybenzoic acid - a versatile platform intermediate for value-added compounds. *Appl. Microbiol. Biotechnol*. **2018**, *102*, 3561–3571.
13. Wang Y, Meng X, Tian Y, Kim KH, Jia L, Pu Y, et al. Engineered sorghum bagasse enables a sustainable biorefinery with *p*-hydroxybenzoic acid-based deep eutectic solvent. *ChemSusChem* **2021**, *14*, 5235–5244.
14. Kuatsjah E, Johnson CW, Salvachúa D, Werner AZ, Zahn M, Szostkiewicz CJ, et al. Debottlenecking 4-hydroxybenzoate hydroxylation in *Pseudomonas putida* KT2440 improves muconate productivity from p-coumarate. *Metab. Eng*. **2022**, *70*, 31–42.
15. Khadem S, Marles RJ. Monocyclic phenolic acids; hydroxy- and polyhydroxybenzoic acids: occurrence and recent bioactivity studies. *Molecules* **2010**, *15*, 7985–8005.
16. Sroka Z, Cisowski W. Hydrogen peroxide scavenging, antioxidant and anti-radical activity of some phenolic acids. *Food Chem. Toxicol*. **2003**, *41*, 753–758.
17. Khan SA, Shyam C, Vikas K. Potential anti-stress, anxiolytic and antidepressant like activities of mono-hydroxybenzoic acids and aspirin in rodents: a comparative study. *Austin J. Pharmacol. Ther*. **2015**, *3*, 1073.
18. Juurlink BH, Azouz HJ, Aldalati AM, AlTinawi BM, Ganguly P. Hydroxybenzoic acid isomers and the cardiovascular system. *Nutr. J*. **2014**, *13*, 63.
19. Chung H, Yang JE, Ha JY, Chae TU, Shin JH, Gustavsson M, et al. Bio-based production of monomers and polymers by metabolically engineered microorganisms. *Curr. Opin. Biotechnol*. **2015**, *36*, 73–84.
20. Noda S, Kondo A. Recent advances in microbial production of aromatic chemicals and derivatives. *Trends Biotechnol*. **2017**, *35*, 785–796.
21. Noda S, Shirai T, Oyama S, Kondo A. Metabolic design of a platform *Escherichia coli* strain producing various chorismate derivatives. *Metab Eng*. **2016**, *33*, 119–129.
22. Kubota T, Watanabe A, Suda M, Kogure T, Hiraga K, Inui M. Production of para-aminobenzoate by genetically engineered *Corynebacterium glutamicum* and non-biological formation of an N-glucosyl byproduct. *Metab. Eng*. **2016**, *38*, 322–330.
23. Averesch NJH, Prima A, Krömer JO. Enhanced production of para-hydroxybenzoic acid by genetically engineered *Saccharomyces cerevisiae*. *Bioprocess Biosyst. Eng*. **2017**, 40, 1283–1289.
24. Lee JH, Wendisch VF. Biotechnological production of aromatic compounds of the extended shikimate pathway from renewable biomass. *J. Biotechnol*. **2017**, *257*, 211–221.
25. Kitade Y, Hashimoto R, Suda M, Hiraga K, Inui M. Production of 4-hydroxybenzoic acid by an aerobic growth-arrested bioprocess using metabolically engineered *Corynebacterium glutamicum*. *Appl. Environ. Microbiol*. **2018**, *84*, e02587-17.
26. Kallscheuer N, Marienhagen J. *Corynebacterium glutamicum* as platform for the production of hydroxybenzoic acids. *Microb. Cell Fact*. **2018**, *17*, 70.
27. Meijnen JP, Verhoef S, Briedjlal AA, de Winde JH, Ruijssenaars HJ. Improved p-hydroxybenzoate production by engineered *Pseudomonas putida* S12 by using a mixed-substrate feeding strategy. *Appl. Microbiol. Biotechnol*. **2011**, *90*, 885–893.
28. Xiao S, Wang Z, Wang B, Hou B, Cheng J, Bai T, et al. Expanding the application of tryptophan: Industrial biomanufacturing of tryptophan derivatives. *Front. Microbiol.* **2023**, *14*, 1099098.
29. Kou M, Cui Z, Fu J, Dai W, Wang Z, Chen T. Metabolic engineering of *Corynebacterium glutamicum* for efficient production of optically pure (2R,3R)-2,3-butanediol. *Microb. Cell Fact*. **2022**, *21*, 150.
30. Zhao X, Wu Y, Feng T, Shen J, Lu H, Zhang Y, et al. Dynamic upregulation of the rate-limiting enzyme for valerolactam biosynthesis in *Corynebacterium glutamicum*. *Metab. Eng*. **2023**, *77*, 89–99.
31. Weiland F, Barton N, Kohlstedt M, Becker J, Wittmann C. Systems metabolic engineering upgrades *Corynebacterium glutamicum* to high-efficiency cis, cis-muconic acid production from lignin-based aromatics. *Metab. Eng*. **2023**, *75*, 153–169.
32. Sato N, Kishida M, Nakano M, Hirata Y, Tanaka T. Metabolic Engineering of Shikimic acid-producing *Corynebacterium glutamicum* from glucose and cellobiose retaining its phosphotransferase system function and pyruvate kinase activities. *Front. Bioeng. Biotechnol*. **2020**, *8*, 569406.
33. Matsuura R, Kishida M, Konishi R, Hirata Y, Adachi N, Segawa S, et al. Metabolic engineering to improve 1,5-diaminopentane production from cellobiose using β-glucosidase-secreting *Corynebacterium glutamicum*. *Biotechnol. Bioeng*. **2019**, *116*, 2640–2651.
34. Ohnishi J, Katahira R, Mitsuhashi S, Kakita S, Ikeda M. A novel *gnd* mutation leading to increased L-lysine production in *Corynebacterium glutamicum*. *FEMS Microbiol. Lett*. **2005**, *242*, 265–274.
35. Yim SS, An SJ, Kang M, Lee J, Jeong KJ. Isolation of fully synthetic promoters for high-level gene expression in *Corynebacterium glutamicum*. *Biotechnol. Bioeng*. **2013**, *110*, 2959–2969.
36. Yim SS, Choi JW, Lee SH, Jeong KJ. Modular optimization of a hemicellulose-utilizing pathway in *Corynebacterium glutamicum* for consolidated bioprocessing of hemicellulosic biomass. *ACS Synth. Biol*. **2016**, *5*, 334–343.
37. Choi JW, Yim SS, Lee SH, Kang TJ, Park SJ, Jeong KJ. Enhanced production of gamma-aminobutyrate (GABA) in recombinant *Corynebacterium glutamicum* by expressing glutamate decarboxylase active in expanded pH range. *Microb. Cell Fact.* **2015**, *14*, 21.
38. Kim HT, Khang TU, Baritugo KA, Hyun SM, Kang KH, Jung SH, et al. Metabolic engineering of *Corynebacterium glutamicum* for the production of glutaric acid, a C5 dicarboxylic acid platform chemical. *Metab. Eng*. **2019**, *51*, 99–109.
39. Duan Y, Zhai W, Liu W, Zhang X, Shi JS, Zhang X, et al. Fine-tuning multi-gene clusters via well-characterized gene expression regulatory elements: Case study of the arginine synthesis pathway in *C. glutamicum*. *ACS Synth. Biol*. **2021**, *10*, 38–48.
40. Syukur Purwanto H, Kang MS, Ferrer L, Han SS, Lee JY, Kim HS, et al. Rational engineering of the shikimate and related pathways in *Corynebacterium glutamicum* for 4-hydroxybenzoate production. *J. Biotechnol*. **2018**, *282*, 92–100.