1
2
3
4 **High-yield production of vanillin from ferulic acid by a coenzyme-independent**
5 **decarboxylase/oxygenase two-stage process**
6
7

8 Toshiki Furuya, Misa Miura, Mari Kuroiwa and Kuniki Kino
9

10
11 Department of Applied Chemistry, Faculty of Science and Engineering,
12 Waseda University, 3-4-1 Ohkubo, Shinjuku-ku, Tokyo 169-8555, Japan
13

14 Tel.: +81 3 5286 3211; fax: +81 3 3232 3889.
15
16
17
18
19

20 -----
21 Corresponding author: Furuya, T. (tfuruya@fuji.waseda.jp), Kino, K.
22 (kkino@waseda.jp)
23
24
25

Abstract

Vanillin is one of the world's most important flavor and fragrance compounds in foods and cosmetics. Recently, we demonstrated that vanillin could be produced from ferulic acid via 4-vinylguaiacol in a coenzyme-independent manner using the decarboxylase Fdc and the oxygenase Cso2. In this study, we investigated a new two-pot bioprocess for vanillin production using the whole-cell catalyst of *Escherichia coli* expressing Fdc in the first stage and that of *E. coli* expressing Cso2 in the second stage. We first optimized the second-step Cso2 reaction from 4-vinylguaiacol to vanillin, a rate-determining step for the production of vanillin. Addition of FeCl₂ to the cultivation medium enhanced the activity of the resulting *E. coli* cells expressing Cso2, an iron protein belonging to the carotenoid cleavage oxygenase family. Furthermore, a butyl acetate-water biphasic system was effective in improving the production of vanillin. Under the optimized conditions, we attempted to produce vanillin from ferulic acid by a two-pot bioprocess on a flask scale. In the first stage, *E. coli* cells expressing Fdc rapidly decarboxylated ferulic acid and completely converted 75 mM of this substrate to 4-vinylguaiacol within 2 h at pH 9.0. After the first-stage reaction, cells were removed from the reaction mixture by centrifugation, and the pH of the resulting supernatant was adjusted to 10.5, the optimal pH for Cso2. This solution was subjected to the second-stage reaction. In the second stage, *E. coli* cells expressing Cso2 efficiently oxidized 4-vinylguaiacol to vanillin. The concentration of vanillin reached 52 mM (7.8 g L⁻¹) in 24 h, which is the highest level attained to date for the biotechnological production of vanillin using recombinant cells.

Keywords

Coenzyme-independent, Decarboxylase, Ferulic acid, Oxygenase, Two-pot bioprocess, Vanillin

Introduction

Vanillin (4-hydroxy-3-methoxybenzaldehyde) is one of the most widely used flavor and fragrance compounds in the world and is a versatile building block in organic reactions [1,2]. The annual demand for vanillin exceeds 10,000 tons [2,3]. Currently, vanillin is mainly produced from guaiacol by chemical synthesis, and less than 1% of vanillin production is derived from natural sources, i.e., the beans of the vanilla orchid [1,2]. Biotechnological or biocatalytic production of vanillin using microorganisms and enzymes has attracted much attention as an alternative to conventional methods [4,5]. Biotechnological methods potentially provide environmentally benign and efficient processes for the production of vanillin. Furthermore, vanillin produced from renewable resources by biotechnological methods can be labeled as “natural flavor” under European and US legislation, thus representing a high value product compared with the synthetic flavor [3,6]. Biotechnological processes also offer vanillin as a “biobased building block” for the synthesis of various chemicals. Ferulic acid is one of the practical starting materials for the biotechnological production of vanillin, because a large amount of this compound can be recovered from renewable resources, e.g., agro-industrial wastes including wheat and rice bran [6,7]. Many microorganisms that generate vanillin from ferulic acid have been isolated [4,5]. Especially actinomycetous

1 strains such as *Amycolatopsis* sp. ATCC 39116 and *Streptomyces* sp. V-1 were able to
2 efficiently produce vanillin from ferulic acid [8-11]. Recombinant strains also have high
3 potential for vanillin production as a new alternative to wild-type strains such as
4 actinomycetous strains [12-14]. In particular, although wild-type strains generally
5 include vanillin degradation pathways, which lead to undesirable by-products (e.g.,
6 vanillic acid and guaiacol) and low production yield [9-10,15-17], host cells of
7 recombinant strains such as *Escherichia coli* have no vanillin degradation pathways and
8 can avoid the generation of by-products [12-14].

9 Recently, we developed a novel biocatalytic method to produce vanillin from ferulic
10 acid in a coenzyme-independent manner by using a recombinant *E. coli* strain [18]. A
11 well-known metabolic pathway consists of a feruloyl-CoA synthetase (Fcs), which
12 catalyzes the conversion of ferulic acid to feruloyl-CoA, and then enoyl-CoA
13 hydratase/aldolase (Ech), which catalyzes the conversion of feruloyl-CoA to vanillin
14 [12-17]. The former enzyme requires ATP and CoA as coenzymes. In contrast, the
15 developed artificial pathway does not require any coenzymes; the novel pathway
16 consists of a coenzyme-independent decarboxylase (Fdc) that converts ferulic acid to
17 4-vinylguaiacol (2-methoxy-4-vinylphenol), and a subsequent step with a
18 coenzyme-independent oxygenase (Cso2) that converts 4-vinylguaiacol to vanillin (Fig.
19 1). The ferulic acid decarboxylase Fdc from *Bacillus pumilus* catalyzes the nonoxidative
20 decarboxylation of aromatic carboxylic acids [19,20]. The 4-vinylguaiacol oxygenase
21 Cso2 from *Caulobacter segnis* belongs to the carotenoid cleavage oxygenase family and
22 catalyzes the oxidative cleavage of a conjugated C=C bond [18,21]. The genes encoding
23 Fdc and Cso2 were coexpressed in *E. coli* cells. We demonstrated that the recombinant
24 *E. coli* whole-cell catalyst was able to produce vanillin (8.0 mM, 1.2 g L⁻¹) from ferulic
25 acid via 4-vinylguaiacol in one pot [18]. We also found that in this two-step route, the

second-step Cso2 reaction limited the rate of vanillin production [18].

In this study, we investigated a new two-pot bioprocess using Fdc and Cso2 for high-yield vanillin production (Fig. 1). One-pot processes are generally easy-to-operate compared with two-pot processes, but in one-pot processes it is difficult to achieve high-yield production when the optimal conditions for the first-step reaction are largely different from those for the second-step reaction. In contrast, two-pot processes are often more practical from the viewpoint of high-yield production, because both the first- and second-step reactions can be performed under individually optimized conditions. Herein, we first optimized the second-step Cso2 reaction, a rate-determining step for the production of vanillin. Under the optimized conditions, we attempted to produce vanillin from ferulic acid by a two-pot bioprocess on a flask scale.

Materials and methods

Preparation of whole cells

Whole cells of the recombinant *E. coli* BL21 (DE3) (Novagen, San Diego, CA, USA) carrying pETDfdc were used for the first-step Fdc reaction, and those of the recombinant *E. coli* BL21 (DE3) carrying pETDcso2 and pGro7 were used for the second-step Cso2 reaction. The pETDfdc and pETDcso2 plasmids were previously constructed [18]. The pGro7 plasmid (Takara Bio, Tokyo, Japan) was used for the coexpression of the chaperonin GroEL and the cochaperonin GroES, as described previously [18]. The recombinant *E. coli* cells were cultivated at 37°C in LB medium (1% Bacto tryptone, 0.5% Bacto yeast extract, and 1% NaCl, pH 7.0) supplemented with ampicillin (50 µg mL⁻¹). For recombinant *E. coli* cells carrying pGro7, the LB

medium was supplemented with chloramphenicol ($30\ \mu\text{g mL}^{-1}$) and arabinose ($4\ \text{mg mL}^{-1}$) in addition to ampicillin ($50\ \mu\text{g mL}^{-1}$). After cultivation for 3 h ($\text{OD}_{600} = 0.8\text{-}1.0$), isopropyl- β -D-thiogalactopyranoside (IPTG; $0.1\ \text{mM}$) was added to the medium. When required, FeCl_2 ($1\ \text{mM}$) was also added together with IPTG. Cultivation was continued for an additional 16 h at 25°C . Cells were harvested by centrifugation ($5000\ \text{g}$, 10 min, 4°C) and washed with potassium phosphate buffer ($100\ \text{mM}$, pH 7.5) containing glycerol (10% v/v). These cells were used for whole-cell reactions.

Reactions using whole cells

For the optimization of the second-step Cso2 reaction, the reaction mixture (1 mL) contained *E. coli* cells expressing Cso2 (50 g wet cell weight per liter), the substrate 4-vinylguaiacol (25-100 mM; Sigma-Aldrich, St. Louis, MO, USA), dimethylsulfoxide (DMSO; 10% v/v), and glycine-NaOH buffer ($100\ \text{mM}$, pH 10.5) containing glycerol (10% v/v). When the effect of organic solvents on the reaction was examined, DMSO was replaced with ethyl acetate, butyl acetate, or hexyl acetate. The reactions were performed at 20°C with vigorous shaking for 24 h.

Vanillin production by a two-pot bioprocess

The two-pot production of vanillin was performed in a 500-mL flask. In the first-stage reaction, the reaction mixture (20 mL) contained *E. coli* cells expressing Fdc (50 g wet cell weight per liter), the substrate ferulic acid ($75\ \text{mM}$; Wako Pure Chemicals, Osaka, Japan), butyl acetate (10% v/v), and glycine-NaOH buffer ($100\ \text{mM}$, pH 9.0) containing glycerol (10% v/v). After the first-stage reaction, cells were removed from the reaction

mixture by centrifugation (5000 g, 10 min, 15°C), and the pH of the resulting supernatant was adjusted to 10.5. In the second-stage reaction, *E. coli* cells expressing Cso2 (100 g wet cell weight per liter) were added to the solution prepared by the first-stage reaction. The reactions were performed at 20°C with rotary shaking at 300 rpm.

Product analysis

HPLC analysis was performed using a 10AVP system (Shimadzu, Kyoto, Japan) with a Wakosil-II 5C18 HG column (4.6 mm × 150 mm; particle size, 4.2-4.7 µm; Wako Pure Chemicals), as described previously [18]. The reaction mixture (1 mL) was acidified by the addition of HCl (pH 2 to 3), and ethanol (1 mL) was added to the reaction mixture. In a biphasic system, 2-propanol (1 mL) was added to the reaction mixture instead of ethanol. After vigorous shaking and centrifugation, the resulting supernatant was appropriately diluted and injected into the HPLC system. Mobile phases A and B were 0.1% formic acid in water and in methanol, respectively. The samples were eluted at a flow rate of 0.5 mL min⁻¹. Mobile phase B was held at 40% for 8.5 min, increased to 85% for 1 min, and held at 85% for 15.5 min. Compounds were detected spectrophotometrically at a wavelength of 280 nm.

Results

Effect of substrate concentration on the second-step Cso2 reaction

To optimize the second-step Cso2 reaction, we first examined the effect of substrate

concentration on vanillin production by Cso2. In our previous study, we found that *E. coli* cells coexpressing the decarboxylase Fdc and the oxygenase Cso2 produced vanillin in one pot most efficiently at pH 9.5 [18]. This optimal pH was due to the fact that the first-step Fdc exhibited high activity in the pH range 6.0-9.0 and that the second-step Cso2 exhibited high activity under alkaline conditions, with the highest activity at pH 10.5 [18]. Here, we examined the effect of the concentration of the second-step substrate, 4-vinylguaiacol, at the optimal pH for Cso2, 10.5 (Fig. 2). Whole-cell reactions were performed using *E. coli* cells expressing Cso2 that were harvested and suspended in glycine-NaOH buffer (pH 10.5). The reaction temperature was maintained at 20°C, because Cso2 was stable at 20°C compared with 30°C, as reported previously [18]. As the initial concentrations of 4-vinylguaiacol increased, the amount of vanillin production increased. The recombinant *E. coli* cells produced 29 mM vanillin from 75 mM 4-vinylguaiacol in 24 h. In contrast, the production of vanillin suddenly decreased at the initial concentration of 100 mM 4-vinylguaiacol. These results suggested that a substrate concentration higher than 100 mM inhibited the Cso2 reaction.

Effect of Fe²⁺ addition on the second-step Cso2 reaction

We next examined the effect of Fe²⁺ addition on the second-step Cso2 oxygenase, because enzymes in the carotenoid cleavage oxygenase family contain iron as a prosthetic group [21]. The addition of FeCl₂ (1 mM or 10 mM) to the reaction mixture did not exhibit any positive effects on the activity of Cso2 (data not shown). In contrast, when FeCl₂ (1 mM) was added to the cultivation medium at the same time as IPTG addition, the resulting *E. coli* cells expressing Cso2 more efficiently converted

1 4-vinylguaiacol to vanillin compared with cells prepared in the absence of FeCl₂ (Fig.
2 2). The recombinant *E. coli* cells produced 34 mM vanillin from 75 mM 4-vinylguaiacol
3 in 24 h, representing 18% higher production than that by cells prepared in the absence
4 of FeCl₂. Thus, the recombinant *E. coli* cells prepared in the presence of FeCl₂ were
5 used hereafter.

6 7 Second-step Cso2 reaction in an organic solvent-water biphasic system

8
9 We also investigated an organic solvent-water biphasic system to further improve the
10 productivity of the second-step Cso2 reaction. An organic solvent that does not mix with
11 water could partly extract the hydrophobic substrate (i.e., 4-vinylguaiacol) and product
12 (i.e., vanillin) from the aqueous phase containing cells and enzymes during the reaction,
13 and alleviate the toxic and inhibitory effects of the substrate/product on cells and
14 enzymes. The substrate 4-vinylguaiacol was added with 10% v/v of DMSO, which is
15 miscible with water, in the experiments presented above. Here, esters immiscible with
16 water, including ethyl acetate, butyl acetate, and hexyl acetate, were tested (Fig. 3).
17 These esters have been used in several studies on an organic solvent-water biphasic
18 system [22-25]. When 10% v/v of butyl acetate was added to the reaction mixture
19 instead of DMSO, *E. coli* cells expressing Cso2 efficiently converted 4-vinylguaiacol to
20 vanillin. The recombinant *E. coli* cells produced 37 mM vanillin from 75 mM
21 4-vinylguaiacol in the presence of butyl acetate in 24 h, which was 7.4% higher than
22 that by the cells in the presence of DMSO. Interestingly, although 100 mM of the
23 substrate strongly inhibited the production of vanillin in the presence of DMSO, the
24 esters tested here lifted the inhibition. The recombinant *E. coli* cells produced only 4.9
25 mM vanillin from 100 mM 4-vinylguaiacol in the presence of DMSO, whereas the cells

produced 39 mM vanillin in the presence of butyl acetate. Butyl acetate concentrations higher than 10% did not further enhance the production of vanillin (data not shown).

Vanillin production by a two-pot bioprocess

Under the optimized conditions, we attempted to produce vanillin from ferulic acid by a coenzyme-independent decarboxylase/oxygenase two-pot process on a flask scale (Fig. 1). In the first stage, 75 mM ferulic acid was incubated with *E. coli* cells expressing Fdc at pH 9.0 at 20°C. Butyl acetate (10% v/v) was added to the reaction mixture from the first stage. The recombinant *E. coli* cells rapidly decarboxylated ferulic acid and completely converted this substrate to 4-vinylguaiacol within 2 h (Fig. 4). After the first-stage reaction, cells were removed from the reaction mixture by centrifugation, and the pH of the resulting supernatant was adjusted to 10.5. This solution containing the intermediate, 4-vinylguaiacol, was subjected to the second-stage incubation with *E. coli* cells expressing Cso2. In the second stage, the recombinant *E. coli* cells oxidized 4-vinylguaiacol to produce 36 mM of vanillin in 8 h (Fig. 4). The production of vanillin increased temporally and reached 52 mM (7.8 g L⁻¹) in 24 h.

Discussion

To date, several attempts have been made to biotechnologically produce vanillin from ferulic acid using recombinant strains expressing Fcs and Ech (Table 1). These recombinant strains have high potential for vanillin production. In particular, the recombinant strains are promising biocatalysts for use in the production of high-purity vanillin in high yield, as mentioned in the introduction section. Barghini et al. and Yoon

et al. reported that recombinant *E. coli* strains expressing Fcs and Ech produced 0.58-2.52 g L⁻¹ of vanillin [12-14] (Table 1). *Pseudomonas* strains are also practical candidates for host cells to produce vanillin. Di Gioia et al. engineered *Pseudomonas fluorescens* to efficiently produce vanillin from ferulic acid. The engineered *P. fluorescens* strain expressing Fcs and Ech produced 1.28 g L⁻¹ of vanillin [26] (Table 1). One limitation of the metabolic pathway consisting of Fcs and Ech is its requirement for the coenzymes, CoA and ATP. In biocatalytic processes using whole cells or resting cells, coenzymes are basically not regenerated after coenzymes in cells are completely consumed by coenzyme-dependent enzymes. Thus, whole cells of the recombinant strains require addition of coenzymes or energy sources (e.g., glucose) to regenerate coenzymes or construction of a coenzyme regeneration system for high-yield production of vanillin. Lee et al. attempted to improve the productivity by constructing a CoA regeneration system in *E. coli* cells. The constructed system in combination with the use of a vanillin adsorbent resin enhanced the production of vanillin up to 5.14 g L⁻¹ [27] (Table 1).

In this study, we investigated a new two-pot bioprocess for vanillin production using *E. coli* cells expressing Fdc in the first stage and cells expressing Cso2 in the second stage (Fig. 1). These two enzymes are both coenzyme-independent. We found that addition of FeCl₂ to the cultivation medium enhanced the activity of the resulting *E. coli* cells expressing Cso2 (Fig. 2). We reasoned that the FeCl₂ addition would increase the amount of active Cso2 containing iron as a prosthetic group in *E. coli* cells. This strategy may be applicable to the *E. coli* expression of other enzymes in the carotenoid cleavage oxygenase family. Furthermore, a butyl acetate-water biphasic system was effective in improving the production of vanillin (Fig. 3). Under the optimized conditions, we succeeded in producing 7.8 g L⁻¹ of vanillin in the two-pot bioprocess

(Fig. 4). This production is 6.5 times higher than that in the one-pot bioprocess that was reported previously [18], and is the highest level attained to date for the biotechnological production of vanillin using recombinant cells (Table 1). This bioprocess can achieve high-yield production of vanillin without addition of coenzymes or energy sources or construction of a coenzyme regeneration system. Furthermore, the two-pot bioprocess is simple and efficient, because the production of vanillin can be attained by pH adjustment alone after the first-stage reaction, without the need for costly intermediate purification steps. It would be difficult to employ the two-pot bioprocess for the consecutive reaction of Fcs and Ech, as the intermediate feruloyl-CoA is relatively unstable and is too large to permeate cell membranes. The easy applicability of the coenzyme-independent decarboxylase/oxygenase pair, Fdc and Cso2, to the two-pot bioprocess expands possibilities for the practical bioproduction of vanillin. In addition to the whole-cell process presented here, taking advantage of the coenzyme-independent feature, we are now investigating the two-stage cell-free process based on the immobilized enzymes.

Acknowledgments

This work was supported by the Japan Science and Technology Agency (JST), Adaptable & Seamless Technology Transfer Program through Target-driven R&D (A-STEP) AS251Z01150N to T.F.

References

[1] Rao SR, Ravishankar GA. Vanilla flavour: production by conventional and

- biotechnological routes. J Sci Food Agric 2000;80:289–304.
- [2] Walton NJ, Mayer MJ, Narbad A. Molecules of interest: vanillin. Phytochemistry 2003; 63:505-515.
- [3] Wenda S, Illner S, Mella A, Kragl U. Industrial biotechnology—the future of green chemistry? Green Chem 2011;13:3007-3047.
- [4] Kaur B, Chakraborty D. Biotechnological and molecular approaches for vanillin production: a review. Appl Biochem Biotechnol 2013;169:1353-1372.
- [5] Priefert H, Rabenhorst J, Steinbüchel A. Biotechnological production of vanillin. Appl Microbiol Biotechnol 2001;56:296-314.
- [6] Di Gioia D, Sciubba L, Setti L, Luziatelli F, Ruzzi M, Zanichelli D, Fava F. Production of biovanillin from wheat bran. Enzym Microb Technol 2007;41:498-505.
- [7] Rosazza JP, Huang Z, Dostal L, Volm T, Rousseau B. Review: biocatalytic transformations of ferulic acid: an abundant aromatic natural product. J Ind Microbiol 1995;15:457-471.
- [8] Fleige C, Hansen G, Kroll J, Steinbüchel A. Investigation of the *Amycolatopsis* sp. strain ATCC 39116 vanillin dehydrogenase and its impact on the biotechnical production of vanillin. Appl Environ Microbiol 2013;79:81-90.
- [9] Hua D, Ma C, Song L, Lin S, Zhang Z, Deng Z, Xu P. Enhanced vanillin production from ferulic acid using adsorbent resin. Appl Microbiol Biotechnol 2007;74:783-790.
- [10] Ma XK, Daugulis AJ. Transformation of ferulic acid to vanillin using a fed-batch solid-liquid two-phase partitioning bioreactor. Biotechnol Prog 2014;30:207-214.
- [11] Ma XK, Daugulis AJ. Effect of bioconversion conditions on vanillin production by *Amycolatopsis* sp. ATCC 39116 through an analysis of competing by-product formation. Bioprocess Biosyst Eng 2014;37:891-899.
- [12] Yoon SH, Li C, Lee YM, Lee SH, Kim SH, Choi MS, Seo WT, Yang JK, Kim JY,

- Kim SW. Production of vanillin from ferulic acid using recombinant strains of *Escherichia coli*. *Biotechnol Bioprocess Eng* 2005;10:378-384.
- [13] Yoon SH, Li C, Kim JE, Lee SH, Yoon JY, Choi MS, Seo WT, Yang JK, Kim JY, Kim SW. Production of vanillin by metabolically engineered *Escherichia coli*. *Biotechnol Lett* 2005;27:1829-1832.
- [14] Barghini P, Di Gioia D, Fava F, Ruzzi M. Vanillin production using metabolically engineered *Escherichia coli* under non-growing conditions. *Microb Cell Fact* 2007;6:13.
- [15] Achterholt S, Priefert H, Steinbüchel A. Identification of *Amycolatopsis* sp. strain HR167 genes, involved in the bioconversion of ferulic acid to vanillin. *Appl Microbiol Biotechnol* 2000;54:799-807.
- [16] Masai E, Harada K, Peng X, Kitayama H, Katayama Y, Fukuda M. Cloning and characterization of the ferulic acid catabolic genes of *Sphingomonas paucimobilis* SYK-6. *Appl Environ Microbiol* 2002;68:4416-4424.
- [17] Narbad A, Gasson MJ. Metabolism of ferulic acid via vanillin using a novel CoA-dependent pathway in a newly-isolated strain of *Pseudomonas fluorescens*. *Microbiology* 1998;144:1397-1405.
- [18] Furuya T, Miura M, Kino K. A coenzyme-independent decarboxylase/oxygenase cascade for the efficient synthesis of vanillin. *Chembiochem* 2014;15:2248-2254.
- [19] Barthelmebs L, Diviès C, Cavin JF. Expression in *Escherichia coli* of native and chimeric phenolic acid decarboxylases with modified enzymatic activities and method for screening recombinant *E. coli* strains expressing these enzymes. *Appl Environ Microbiol* 2001;67:1063-1069.
- [20] Yang J, Wang S, Lorrain MJ, Rho D, Abokitse K, Lau PC. Bioproduction of lauryl lactone and 4-vinyl guaiacol as value-added chemicals in two-phase

1 biotransformation systems. Appl Microbiol Biotechnol 2009;84:867-876.

2 [21] Kloer DP, Schulz GE. Structural and biological aspects of carotenoid cleavage. Cell
3 Mol Life Sci 2006;63:2291-2303.

4 [22] Shimizu S, Kataoka M, Katoh M, Morikawa T, Miyoshi T, Yamada H.
5 Stereoselective reduction of ethyl 4-chloro-3-oxobutanoate by a microbial aldehyde
6 reductase in an organic solvent-water diphasic system. Appl Environ Microbiol
7 1990;56:2374-2377.

8 [23] Toda H, Imae R, Itoh N. Efficient biocatalysis for the production of enantiopure
9 (S)-epoxides using a styrene monooxygenase (SMO) and Leifsonia alcohol
10 dehydrogenase (LSADH) system. Tetrahedron: Asymmetry 2012;23:1542-1549.

11 [24] Wangrangsimagul N, Klinsakul K, Vangnai AS, Wongkongkatep J, Inprakhon P,
12 Honda K, Ohtake H, Kato J, Pongtharangkul T. Bioproduction of vanillin using an
13 organic solvent-tolerant *Brevibacillus agri* 13. Appl Microbiol Biotechnol
14 2012;93:555-563.

15 [25] Yamada M, Okada Y, Yoshida T, Nagasawa T. Vanillin production using
16 *Escherichia coli* cells over-expressing isoeugenol monooxygenase of *Pseudomonas*
17 *putida*. Biotechnol Lett 2008;30:665-670.

18 [26] Di Gioia D, Luziatelli F, Negroni A, Ficca AG, Fava F, Ruzzi M. Metabolic
19 engineering of *Pseudomonas fluorescens* for the production of vanillin from ferulic
20 acid. J Biotechnol 2010;156:309-316.

21 [27] Lee EG, Yoon SH, Das A, Lee SH, Li C, Kim JY, Choi MS, Oh DK, Kim SW.
22 Directing vanillin production from ferulic acid by increased acetyl-CoA consumption
23 in recombinant *Escherichia coli*. Biotechnol Bioeng 2009;102:200-208.

TABLE 1 Biotechnological production of vanillin from ferulic acid using recombinant strains.

Host cell	Introduced gene	Vanillin production (g L ⁻¹)	Reference
<i>E. coli</i>	<i>fcs</i> and <i>ech</i> from <i>Amycolatopsis</i> sp.	0.58	12
<i>E. coli</i>	<i>fcs</i> and <i>ech</i> from <i>Amycolatopsis</i> sp.	1.1	13
<i>E. coli</i>	<i>fcs</i> and <i>ech</i> from <i>P. fluorescens</i>	2.52	14
<i>P. fluorescens</i>	<i>fcs</i> and <i>ech</i> from <i>P. fluorescens</i>	1.28	26
<i>E. coli</i>	<i>fcs</i> and <i>ech</i> from <i>Amycolatopsis</i> sp.	5.14	27
<i>E. coli</i>	<i>fdc</i> from <i>B. pumilus</i> and <i>cso2</i> from <i>C. segnis</i>	1.2	18
<i>E. coli</i>	<i>fdc</i> from <i>B. pumilus</i> and <i>cso2</i> from <i>C. segnis</i>	7.8	This study

Figure legends

FIGURE 1 Production of vanillin from ferulic acid by a coenzyme-independent decarboxylase/oxygenase two-stage process.

FIGURE 2 Effect of substrate concentration and Fe^{2+} addition on the second-step Cso2 reaction. The recombinant *E. coli* cells prepared in the absence (white bars) or presence (gray bars) of FeCl_2 were reacted with each initial concentration of the substrate, 4-vinylguaiacol, for 24 h, and the concentration of the product, vanillin, was determined. Data are the average of two independent experiments, and error bars indicate standard deviation of the mean.

FIGURE 3 Effect of the addition of organic solvents on the second-step Cso2 reaction. The recombinant *E. coli* cells were reacted with 75 mM (white bars) or 100 mM (gray bars) of the substrate, 4-vinylguaiacol, in the presence of each organic solvent (10% v/v) for 24 h, and the concentration of the product, vanillin, was determined. Data are the average of two independent experiments, and error bars indicate standard deviation of the mean.

FIGURE 4 Flask-scale production of vanillin from ferulic acid by a coenzyme-independent decarboxylase/oxygenase two-pot process. In the first stage, the substrate, ferulic acid, (white circles) was reacted with *E. coli* cells expressing Fdc, and converted to 4-vinylguaiacol (triangles). In the second stage, the resulting intermediate 4-vinylguaiacol was reacted with *E. coli* cells expressing Cso2, and converted to vanillin (black circles). Data are the average of determinations performed in duplicate,

1 and error bars indicate standard deviation of the mean.

2

3

4

Figure 1

Figure 2

Figure 3

Figure 4

Furuya et al., Figure 4