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High-yield production of vanillin from ferulic acid by a coenzyme-independent decarboxylase/oxygenase two-stage process

Toshiki Furuya, Misa Miura, Mari Kuroiwa and Kuniki Kino

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

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 $FeCl_2$ 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.

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, that is, 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, for example, 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 strains such as *Amycolatopsis* sp. ATCC 39116 and *Streptomyces* sp. V-1 were able to efficiently produce vanillin from ferulic acid [8–11]. Recombinant strains also have

Corresponding authors: Furuya, T. (tfuruya@fuji.waseda.jp), Kino, K. (kkino@waseda.jp)

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high potential for vanillin production as a new alternative to wild-type strains such as actinomycetous strains [12–14]. In particular, although wild-type strains generally include vanillin degradation pathways, which lead to undesirable by-products (e.g., vanillic acid and guaiacol) and low production yield [9,10,15–17], host cells of recombinant strains such as *Escherichia coli* have no vanillin degradation pathways and can avoid the generation of by-products [12–14].

Recently, we developed a novel biocatalytic method to produce vanillin from ferulic acid in a coenzyme-independent manner by using a recombinant *E. coli* strain [18]. A well-known metabolic pathway consists of a feruloyl-CoA synthetase (Fcs), which catalyzes the conversion of ferulic acid to feruloyl-CoA, and then enoyl-CoA hydratase/aldolase (Ech), which catalyzes the conversion of feruloyl-CoA to vanillin [12–17]. The former enzyme requires ATP and CoA as coenzymes. In contrast, the developed artificial pathway does not require any coenzymes; the novel pathway consists of a coenzyme-independent decarboxylase (Fdc) that converts ferulic acid to 4-vinylguaiacol (2-methoxy-4-vinylphenol), and a subsequent step with a coenzyme-independent oxygenase (Cso2) that converts 4-vinylguaiacol to vanillin (Fig. 1). The ferulic acid decarboxylase Fdc from *Bacillus pumilus* catalyzes the nonoxidative decarboxylation of aromatic carboxylic acids [19,20]. The 4-vinylguaiacol oxygenase Cso2 from *Caulobacter segnis* belongs to the carotenoid cleavage oxygenase family and catalyzes the oxidative cleavage of a conjugated C=C bond [18,21]. The genes encoding Fdc and Cso2 were coexpressed in *E. coli* cells. We demonstrated that the recombinant *E. coli* whole-cell catalyst was able to produce vanillin (8.0 mM, 1.2 g L^{-1}) from ferulic 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 (100 μg mL^{-1}). For recombinant *E. coli* cells carrying pGro7, the LB medium was supplemented with chloramphenicol (30 μg mL^{-1}) and arabinose (4 mg mL^{-1}) in addition to ampicillin (100 μg mL^{-1}). After cultivation for 3 h (OD_{600} = 0.8–1.0), isopropyl-β-D-thiogalactopyranoside (IPTG; 0.1 mM) was added to the medium. When required, $FeCl_2$ (1 mM) was also added together with IPTG. Cultivation was continued for an additional 16 h at 25°C. Cells were harvested by centrifugation (5000 *g*, 10 min, 4°C) and washed with potassium phosphate buffer (100 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 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

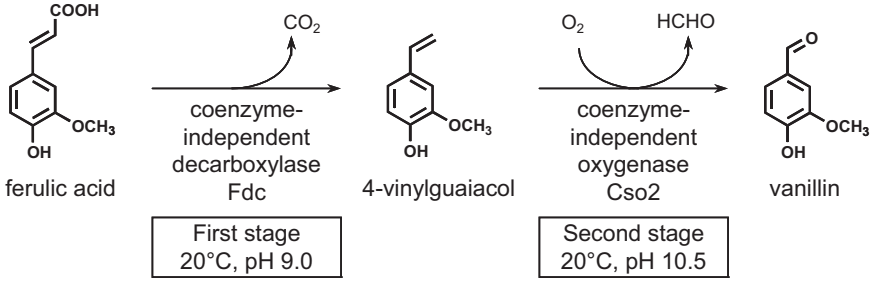

FIGURE 1

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