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Short communication

Biotechnological production of vanillin using immobilized enzymes

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ABSTRACT

Vanillin is an important and popular plant flavor, but the amount of this compound available from plant sources is very limited. Biotechnological methods have high potential for vanillin production as an alternative to extraction from plant sources. Here, we report a new approach using immobilized enzymes for the production of vanillin. The recently discovered oxygenase Cso2 has coenzyme-independent catalytic activity for the conversion of isoeugenol and 4-vinylguaiacol to vanillin. Immobilization of Cso2 on Sepabeads EC-EA anion-exchange carrier conferred enhanced operational stability enabling repetitive use. This immobilized Cso2 catalyst allowed 6.8 mg yield of vanillin from isoeugenol through ten reaction cycles at a 1 mL scale. The coenzyme-independent decarboxylase Fdc, which has catalytic activity for the conversion of ferulic acid to 4-vinylguaiacol, was also immobilized on Sepabeads EC-EA. We demonstrated that the immobilized Fdc and Cso2 enabled the cascade synthesis of vanillin from ferulic acid via 4-vinylguaiacol with repetitive use of the catalysts. This study is the first example of biotechnological production of vanillin using immobilized enzymes, a process that provides new possibilities for vanillin production.

Vanillin (4-hydroxy-3-methoxybenzaldehyde) is the major aroma component of vanilla, giving it a sweet and creamy odor. Its flavor and fragrance properties have made this compound important around the world, especially to the foods and cosmetics industries (Rao and Ravishankar, 2000; Walton et al., 2003). Vanillin is available from the beans of the vanilla orchid through extraction. However, the yield of vanillin is very low because it accumulates at low levels in the plant (Rao and Ravishankar, 2000; Walton et al., 2003). Biotechnological production of vanillin using microorganisms has attracted much attention as an alternative to extraction from vanilla beans (Gallage and Møller, 2015; Kaur and Chakraborty, 2013; Priefert et al., 2001). For example, vanillin can be produced from isoeugenol, which occurs in some essential oils, by microorganisms (Priefert et al., 2001; Wangrangsimagul et al., 2012; Yamada et al., 2007). Ferulic acid is a more practical starting material for the biotechnological production of vanillin, because a large amount of this compound can be recovered from agroindustrial wastes including wheat and rice bran (Di Gioia et al., 2007; Rosazza et al., 1995). Many microorganisms that convert ferulic acid to vanillin have been isolated and extensively studied for vanillin production (Priefert et al., 2001; Hua et al., 2007; Ma and Daugulis, 2014).

In addition to microorganisms, use of immobilized enzymes shows promise in methods used for vanillin production. Enzymes in microbial cells and cell-free enzymes are often unstable when used as biocatalysts. In contrast, immobilized enzymes may be more operationally stable due to rigidification of their structure via direct interaction with proper carriers. Furthermore, immobilized enzymes can be easily separated from reaction solutions, allowing repetitive, cost-effective use (Brady and Jordaan, 2009; Hilterhaus et al., 2008; Sheldon and van Pelt, 2013). Although vanillin production using microorganisms has been extensively studied, there have been no reports concerning production using immobilized enzymes. This is partly due to the complexity of the enzyme system in microorganisms that convert ferulic acid to vanillin. A well-known pathway in microorganisms consists of the conversion of ferulic acid to feruloyl-CoA, catalyzed by feruloyl-CoA synthetase, followed by the conversion of feruloyl-CoA to vanillin by enoyl-CoA hydratase/aldolase; the first step requires ATP and CoA as coenzymes (Achterholt et al., 2000; Barghini et al., 2007; Lee et al., 2009; Narbad and Gasson, 1998). These expensive coenzymes have to be supplied to continue the coupled enzymatic reactions when the synthetase and hydratase/aldolase are used as immobilized catalysts.

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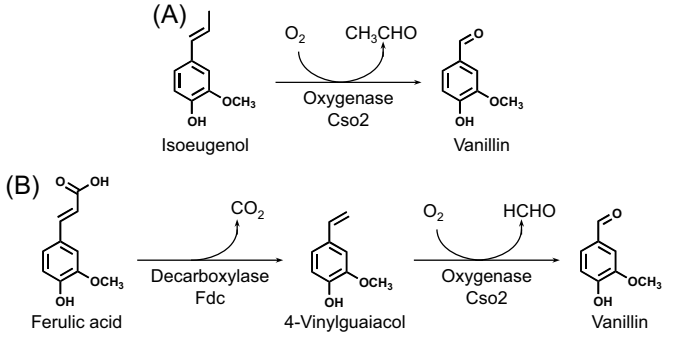

Fig. 1. Coenzyme-independent synthesis of vanillin. Vanillin is synthesized from isoeugenol by oxygenase Cso2 (A) and from ferulic acid via 4-vinylguaiacol by decarboxylase Fdc and oxygenase Cso2 (B).

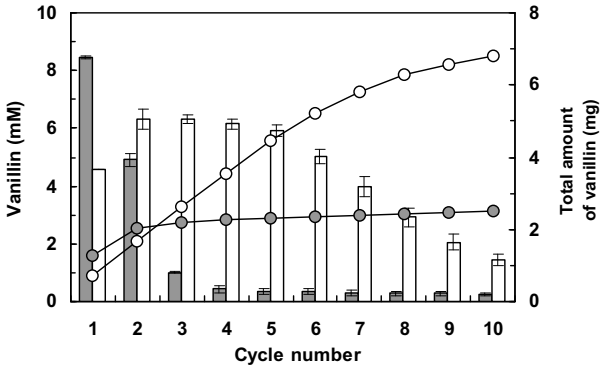

Fig. 2. The vanillin production from isoeugenol by repetitive use of immobilized Cso2. Isoeugenol (10 mM) was incubated with 50 mg whole cells or 7 mg protein immobilized on 100 mg of the carrier in the reaction mixture (1 mL) for 24 h in each cycle. The amount of protein (7 mg) was almost equal to that obtained through disruption of 50 mg whole cells. Vanillin produced in each cycle by the whole cells (gray bars) and the immobilized enzyme (white bars) is shown. Total amount of vanillin produced by the whole cells (gray circles) and the immobilized enzyme (white circles) is also shown. Data are the average of three independent experiments, and error bars indicate standard deviation of the mean.

Recently, we developed a route to vanillin from ferulic acid that does not require any coenzymes (Fig. 1). This artificial 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 (Furuya et al., 2014; Furuya et al., 2015; Muschiol et al., 2015). The ferulic acid decarboxylase Fdc from *Bacillus pumilus* catalyzes the nonoxidative decarboxylation of aromatic carboxylic acids (Barthelmebs et al., 2001; Yang et al., 2009). 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 (Furuya et al., 2014; Kloer and Schulz, 2006). We demonstrated that *Escherichia coli* cells expressing Fdc and Cso2 produced vanillin from ferulic acid via 4-vinylguaiacol (Furuya et al., 2014). Cso2 also converts isoeugenol to vanillin in a coenzyme-independent manner (Furuya et al., 2014) (Fig. 1). This coenzyme-independent feature would be advantageous to the production of vanillin using immobilized enzymes. In the present study, we investigated biotechnological production of vanillin using immobilized Fdc and Cso2.

A proper carrier for immobilization of Cso2, a key enzyme for vanillin production, which is relatively labile, was first examined. The oxygenase Cso2 exhibits high activity in the pH range 9.0–10.5, whereas the isoelectric point of Cso2 is pH 5.3 (Furuya et al., 2014). This property indicates that Cso2, with a negative charge at the optimal pH, would be immobilized on anion-exchange carriers. Four anion-exchange carriers were tested. The matrix, functional group and particle size of each carrier are shown in Table 1. When each carrier was incubated with Cso2 solution, all were able to adsorb protein (0.04–0.10 mg mg-carrier^{-1}) (Fig. S1A in Supplementary data). In addition, we examined vanillin-producing activity of these carriers after adsorbing Cso2. When each was incubated with isoeugenol, Cso2 immobilized on Sepabeads EC-EA efficiently converted this substrate to vanillin (Fig. S1B in Supplementary data). In contrast, Cso2 on the other carriers lost catalytic activity. These results suggested that Cso2 was compatible with the polymethacrylate matrix and the ethylamine group of Sepabeads EC-EA (Table 1).

We then attempted to produce vanillin from isoeugenol using Cso2 immobilized on Sepabeads EC-EA (Fig. 1A). The activity of the immobilized Cso2 catalyst was compared with that of a whole-cell catalyst, *E. coli* expressing Cso2. Each catalyst was incubated with a solution containing the substrate isoeugenol. The whole cells produced 8.4 mM vanillin from 10 mM isoeugenol during 24 h biotransformation (Fig. S2A in Supplementary data), whereas the immobilized enzyme produced only 4.6 mM vanillin (Fig. S2B in Supplementary data). We confirmed that a portion of the substrate isoeugenol and the product vanillin bound to the carrier, which led to lower yield of vanillin by the immobilized enzyme than the whole cells. After the first reaction cycle, the whole cells and the immobilized enzyme were collected from the reaction mixture and added to a fresh reaction mixture (Fig. 2). In the second reaction cycle, the whole cells produced only 4.9 mM vanillin. After the third reaction cycle, the activity of the whole cells was almost lost. Although the oxygenase Cso2 exhibits high activity in the reaction pH 9.0, the enzyme is relatively unstable under the alkaline condition (Furuya et al., 2014). However, the immobilized enzyme enabled to produce 6.3 mM vanillin during the second reaction cycle. Furthermore, more than 50% of the activity was maintained even in the seventh reaction cycle. These results indicated that the stability of Cso2 was significantly enhanced by immobilizing on Sepabeads EC-EA. The total amount of vanillin produced from isoeugenol by the immobilized Cso2 catalyst reached 6.8 mg at the 1 mL scale after ten reaction cycles, but the whole cells produced only 2.5 mg (Fig. 2).

Since we confirmed that Cso2 immobilized on Sepabeads EC-EA had superior operational stability, we used the immobilized Cso2 catalyst in the cascade synthesis of vanillin from ferulic acid via 4-vinylguaiacol (Fig. 1B). We found that the first-step Fdc was as easily immobilized on Sepabeads EC-EA as Cso2 was. We confirmed that 5 mg of protein immobilized on 50 mg of the carrier efficiently converted ferulic acid to 4-vinylguaiacol (Fig. S3 in Supplementary data). Thus, the immobilized Fdc catalyst and the immobilized Cso2 catalyst were added to a solution containing the substrate ferulic acid. Although a portion of the substrate and the product bound to the carrier as described above, the immobilized Fdc and Cso2 efficiently converted ferulic acid to vanillin via 4-vinylguaiacol. The immobilized enzymes produced 2.8 mM vanillin during 24 h biotransformation (Fig. S4 in Supplementary data). After the first reaction cycle, the immobilized enzymes were collected from the reaction mixture and added to a fresh reaction mixture (Fig. 3). In the second reaction cycle, the immobilized enzymes produced 3.5 mM vanillin. In the third and fourth reaction cycles, the immobilized enzymes maintained their activity, producing 3.1 mM and 2.4 mM vanillin, respectively. In subsequent reaction cycles, the production of vanillin gradually decreased. We confirmed that the intermediate 4-vinylguaiacol accumulated in the reaction mixture. The total amount of vanillin produced from ferulic acid by the immobilized Fdc and Cso2 catalysts reached 2.5 mg at the 1 mL scale after ten reaction cycles (Fig. 3). The amount of vanillin produced from ferulic acid was lower than that produced from isoeugenol.