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Enhancement of protocatechuate decarboxylase activity for the effective production of muconate from lignin-related aromatic compounds

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ABSTRACT

The decarboxylation reaction of protocatechuate has been described as a bottleneck and a rate-limiting step in *cis*,*cis*-muconate (ccMA) bioproduction from renewable feedstocks such as sugar. Because sugars are already in high demand in the development of many bio-based products, our work focuses on improving protocatechuate decarboxylase (Pdc) activity and ccMA production in particular, from lignin-related aromatic compounds. We previously had transformed an *Escherichia coli* strain using *aroY*, which had been used as a protocatechuate decarboxylase encoding gene from *Klebsiella pneumoniae* subsp. *pneumoniae* A170-40, and inserted other required genes from *Pseudomonas putida* KT2440, to allow the production of ccMA from vanillin. This recombinant strain produced ccMA from vanillin, however the Pdc reaction step remained a bottleneck during incubation. In the current study, we identify a way to increase protocatechuate decarboxylase activity in *E. coli* through enzyme production involving both *aroY* and *kpdB*; the latter which encodes for the B subunit of 4-hydroxybenzoate decarboxylase. This permits expression of Pdc activity at a level approximately 14-fold greater than the strain with *aroY* only. The expression level of AroY increased, apparently as a function of the co-expression of AroY and KpdB. Our results also imply that ccMA may inhibit vanillate demethylation, a reaction step that is rate limiting for efficient ccMA production from lignin-related aromatic compounds, so even though ccMA production may be enhanced, other challenges to overcome vanilate demethylation inhibition still remain.

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1. Introduction

Cis,*cis*-muconic acid (ccMA) has potential use as a platform chemical to synthesize a variety of plastics including polyamides (*e.g.* nylon-6,6), polyurethanes, and polyethylene terephthalate (PET) (Weber et al., 2012; Curran et al., 2013; Polen et al., 2013; Lin et al., 2014). Bio-based muconic acid production has been reported as a $22 billion market (Myriant Corporation, 2014). *Cis*,*cis*-muconic acid can be produced *via* the *ortho*-cleavage of catechol, which is one of the intermediates of aromatic compound metabolism. Some bacteria can convert benzoic acid to catechol *via* benzoate 1,2-dioxygenase and 1,2-dihydroxybenzoate dehydrogenase, and further metabolize catechol *via* ccMA by the beta-ketoadipate pathway (Harwood and Parales, 1996; Polen et al., 2013). The production of ccMA from benzoic acid by microbial action has also been reported (van Duuren et al., 2011, 2012; Polen et al., 2013). Although this represents a bioconversion process, the benzoic acid source has typically been petroleum-based and it is preferable to use a renewable feedstock. It has previously been reported that ccMA can be produced from sugar using an engineered *Escherichia coli* (Draths and Frost, 1994; Lin et al., 2014) and *Saccharomyces cerevisiae* (Weber et al., 2012; Curran et al., 2013). In these reports, the genes for 3-hydroxyshikimate dehydratase, protocatechuic acid (PA, 3,4-dihydroxybenzoate) decarboxylase, and catechol 1,2-dioxygenase were introduced into the microorganisms, and refinements were made to overproduce aromatic amino acids and related compounds in order to increase the supply of precursors for ccMA production. The resulting engineered strains were

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able to synthesize ccMA from 3-dehydroxyshikimate. Although advancements in the biological production of ccMA from sugars have occurred, two major issues remain. One of which is the activity of protocatechuate decarboxylase (Pdc, EC 4.1.1.63); and in previous studies, it has been demonstrated that low activity levels of Pdc remains a bottleneck for ccMA production from sugars (Draths and Frost, 1994; Weber et al., 2012; Curran et al., 2013). Only a few publications are listed in the comprehensive enzyme information system (BRENDA; http://www.brenda-enzymes.org/) relative to this Pdc activity. Among these reports, He and Wiegel (1996), noted that Pdc is a bidirectional enzyme, but that at equilibrium carboxylation is favored (He and Wiegel, 1996). Decarboxylation of PA is what is desired relative to the effective production of ccMA from renewable feedstocks so a mechanism or reaction conditions that shifts the equilibrium of the enzymatic action in this direction is desired. Although there are relatively few reports that discuss the isolation of Pdc from bacteria, Pdc derived from *Sedimentibacter* (*Clostridium*) *hydroxybenzoicus* has been isolated and reported as a homopentamer (He and Wiegel, 1996). In the previous studies, the *aroY* (accession no. AB479384) (Draths and Frost, 1994) or the gene encoding Pdc (AB364296) from *Enterobacter cloacae* P241 (Yoshida et al., 2010) has also been used to encode Pdc in ccMA production from glucose. Protocatechuate decarboxylase belongs to the family of hydroxyarylic acid decarboxylases and is composed of multiple subunits encoded by B, C and D genes as typified by 4-hydroxybenzoate decarboxylase which is encoded by *bsdB*, *bsdC* and *bsdD* (Lupa et al., 2005). It has been also reported that these genes form a gene cluster (Lupa et al., 2005).

Another issue is the availability of feedstock: With sugars already in high demand for food, and other bio-based products such as biofuels, it is desirable to use a feedstock for the production of ccMA that does not compete with the food chain, and that also is of lower cost than sugar. Protocatechuate, a precursor to ccMA, is also an intermediate in the metabolic pathway for degradation of lignin-related aromatic compounds observed in some species of *Pseudomonas* (Jimenez et al., 2002), *Acinetobacter* (Delneri et al., 1995), and *Sphingobium* (Kamimura et al., 2010). Although this suggests that ccMA could potentially be produced from purified lignin-related compounds such as ferulic acid and vanillin *via* PA, and catalytic pathways for mixed production of ccMA have been reported (Evtuguin et al., 2009; Ma, 2013), no reports of ccMA production from lignin-derived aromatics *via* biosynthetic pathways have been previously published. The most important aspect of future work for ccMA production from lignin-related compounds is therefore the development of a way to enhance Pdc production and/or activity. In the current work, we grapple with this issue, focusing on a method to enhance Pdc production, and then evaluating the effectiveness of this method on ccMA production from vanillin, which served as a proxy in this work for other lignin-derived aromatics.

2. Materials and methods

2.1. Bacterial strains and plasmids

E. coli strain XL-1 Blue, supplied by National Institute of Genetics in Mishima, Japan, was used as the recombinant host. The plasmids and primers used in this study are listed in Table 1. We used *aroY* from *Klebsiella pnuemoniae* subsp. *pneumoniae* A170-10 which encodes Pdc. *AroY* (accession no. AB479384) was amplified from pKD136 which was isolated from the genomic library of *K. pneumoniae* subsp. *pneumoniae* A170-10 (ATCC25597) (Draths and Frost, 1994), and the *kpdB, kpdC and kpdD* encoding subunits of the putative 4-hydroxybenzoate decarboxylase were amplified from the genome of *K. pneumoniae* NBRC14940 (AB920346). Nucleotide sequencing of the gene harboring plasmid pKD136 was performed to identify any previously unreported subunits and identify any unreported open reading frames (ORF) potentially associated with the *aroY* gene.

Vdh encoding vanillin dehydrogenase, *vanA* and *vanB* encoding alpha- and beta-subunits, respectively, of vanillate demethylase, and *catA* encoding catechol dioxygenase were amplified from the genome of *Pseudomonas putida* NBRC100650 (KT2440) (AE01541). Ampicillin (50 μg/ml) or chroramphenicol (10 μg/ml) (or both) were added to the media when these transformants were cultured.

2.2. In vivo assay of the Pdc reaction using whole cell biotransformation

E. coli XL-1blue strain was transformed using the plasmids listed in Table 1 to evaluate the decarboxylation abilities of PA by the whole cell reaction. Each resulting transformant, XL-1Blue/pTS032, XL-1Blue/pTS035, XL-1Blue/pTS039, XL-1Blue/pTS044, XL-1Blue/pTS045, XL-1Blue/pTS046, XL-1Blue/pTS047, XL-1Blue/pTS048, XL-1Blue/pTS051, XL-1Blue/pTS036 + pUC118, XL-1Blue/pTS036 + pTS039, XL-1Blue/pTS036 + pTS044, XL-1Blue/pTS036 + pTS045, XL-1Blue/pTS036 + pTS046, XL-1Blue/pTS036 + pTS047, XL-1Blue/pTS036 + pTS048, XL-1Blue/pTS036 + pTS051, XL-1Blue/pTS036, XL-1Blue/pTS052 and XL-1Blue/pTS053, was grown in 10 ml of LB medium containing appropriate antibiotics, and shake-incubated (Maniatis et al., 1982). Two hundred microliters of each culture was then used to inoculate to 10 ml of fresh LB medium containing appropriate antibiotics and 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) to induce the expression of the recombinant proteins, and these cultures were then shake-incubated at 30 °C until an OD_{600} of approximately 1.5 was achieved. Total cells from each culture were then collected by centrifugation (5 min, 6000 × *g*, 4 °C) and the pelleted cells were then washed twice using an ice-cold M9 liquid medium (Maniatis et al., 1982). The pellet was then re-suspended in 1 ml of M9 medium containing 1 mM protocatechuic acid (PA, 95.0%, Wako Pure Chemical Industries, Ltd., Osaka, Japan) and the suspension was shake-incubated at 30 °C for 3 h.

2.3. Analytical conditions of metabolites

A 0.12 ml aliquot of each cell culture was centrifuged for 5 min at 16,000 × *g*. Benzoic acid (0.1 ml, 10 mM, BA, 99.5%, Wako Pure Chemical Industries, Ltd.) was added to 0.1 ml of the supernatant as an internal standard. The mixture was acidified with HCl, and then extracted twice with ethyl acetate. The ethyl acetate fraction was collected, dried, and the extract dissolved in a solvent consisting of 10% (v/v) CH_3OH and 1% (v/v) CH_3COOH in H_2O. An 0.1 ml aliquot of the solution was analyzed using high performance liquid chromatography (HPLC, Agilent Technologies Inc., CA, USA) equipped with a ZORBAX Eclipse Plus C18 column (reverse phase, 4.6 mm in diameter, 150 mm in length, 0.5 μm particle size) run at 40 °C using a mobile phase gradient (Solvent A: 10% (v/v) CH_3OH and 1% (v/v) CH_3COOH in H_2O. Solvent B: 50% (v/v) CH_3OH and 1% (v/v) CH_3COOH in H_2O introduced 10 min after injection and ramped from 0 to 100% in 10 min). The flow rate of the mobile phase was 1.0 ml/min and the detection wavelength was 280 nm. Vanillin (Vn, 98.0%) was purchased from Nakalai Tesque, Inc. (Kyoto, Japan). Vanillic acid (VA, 95.0%) and catechol (CL, 99.0%) were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) and *cis*, *cis*-muconic acid (ccMA) was purchased from Sigma-Aldrich (St Louis, MO).

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