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High-yield Production of *cis*,*cis*-Muconic Acid from Catechol in Aqueous Solution by Biocatalyst

Aya Kaneko, Yoshitaka Ishii, and Kohtaro Kirimura*
Department of Applied Chemistry, Faculty of Science and Engineering, Waseda University, 3-4-1 Ohkubo, Shinjuku-ku, Tokyo 169-8555

(Received January 7, 2011; CL-110022; E-mail: kkohtaro@waseda.jp)

A fed-batch process was used to produce *cis*,*cis*-muconic acid from catechol by recombinant *Escherichia coli* cells expressing the *catA* gene, which encodes the *Pseudomonas putida* mt-2 catechol 1,2-dioxygenase responsible for catalyzing *ortho*-cleavage of catechol, as biocatalysts. We succeeded in producing 415 mM (59.0 g L^{-1}) *cis*,*cis*-muconic acid in aqueous solution without generation of by-products in 12 h under the optimal conditions with successive addition of 10 mM catechol. The molar conversion yield based on the amount of consumed catechol was the theoretical value of 100% (mol mol^{-1}).

cis,*cis*-Muconic acid (ccMA, 1,3-butadiene-1,4-dicarboxylic acid) has a dicarboxylic acid structure with conjugated double bonds (Figure 1). This compound is expected to gain widespread use as a raw material for new functional resins, pharmaceuticals, and agrochemicals.[1] For example, ccMA can be easily converted to adipic acid, which is used as a commodity chemical for production of nylon-6,6 by hydrogenation at 50 psi for 3 h at room temperature.[2] Furthermore, highly stereoregular polymers can be produced through topochemical polymerization of muconic acid esters.[3] These polymers are useful as functional resins. Verrucarin is an antibiotic that can be synthesized from ccMA by organic synthesis.[4]

ccMA had been reported to be synthesized from phenol by peracetic acid oxidation,[5] but the reaction also generates by-products. Therefore, biocatalytic methods have been recently developed based on site-specific oxidation of aromatic compounds such as benzoate and toluene using enzymes and microbial cells,[6–8] because they enable selective production of ccMA under environmentally benign conditions. For example, Mizuno et al. reported that a mutant strain of *Arthrobacter* sp., which lacks muconate-lactonizing enzyme, could produce 44.1 g L^{-1} in 48 h in a 30-1 jar fermenter by successive feeding of benzoate.[6] However, biocatalytic methods have not yet been applied in industrial production of ccMA, probably due to low yields, formation of by-products, and/or long reaction periods.

As shown in Figure 1, ccMA production by catechol 1,2-dioxygenase (CatA; EC 1.13.11.1), which catalyzes the *ortho*-cleavage of CA, is a direct 1-step conversion, and this enzymatic reaction is efficient and advantageous for recovery and purification of ccMA in practical processes because it does not generate any by-product. However, there has been no report concerning high-yield production of ccMA using CA as a starting substrate by enzymatic or whole-cell reactions using CatA.

In this report, we describe the production of ccMA without by-product through whole-cell reaction of recombinant *Escherichia coli* cells highly expressing the gene (*catA*) encoding CatA. To our knowledge, this is the first report of high-yield and rapid production of ccMA by a biocatalyst in aqueous solution under environmentally benign conditions.

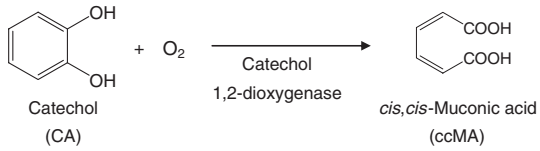

Figure 1. Enzymatic production of ccMA from CA by catechol 1,2-dioxygenase (CatA; EC1.13.11.1) from *P. putida* mt-2.

As prerequisites for ccMA production, we examined the enzymatic properties of the recombinant His-tagged CatA in comparison to those of native CatA reported previously.[9] The purified His-tagged CatA yields a single 34-kDa band in SDS–polyacrylamide gel electrophoresis (data not shown). The specific activity of the purified His-tagged CatA was 25.7 μmol min^{-1} mg^{-1}-protein. This specific activity is the same level as that (31.6 μmol min^{-1} mg^{-1}-protein) of a purified native CatA previously reported.[9] The optimal temperature of the purified His-tagged CatA was 35 °C although the optimal temperature of the purified native CatA has not yet been reported. The optimal pH of His-tagged CatA was 7.5 and is identical to that of the purified native CatA.[9]

We confirmed that whole cells of recombinant *E. coli* BL21(DE3) expressing *catA*, instead of the purified His-tagged CatA or a cell-free extract of the recombinant *E. coli* cells, can be directly used to produce ccMA from CA. This whole-cell reaction is advantageous for practical production of ccMA because no additional pretreatment, such as disruption of recombinant *E. coli* cells or purification of the recombinant His-tagged CatA, is necessary for the reaction. We confirmed that *E. coli* BL21(DE3) cells without pEcatA show no activity toward ccMA or CA, and that they did not consume ccMA or CA in whole-cell reactions.

To optimize conditions for ccMA production from CA in whole-cell reaction, we examined the effects of several parameters.[10] Because the optimum temperature of the purified His-tagged CatA is 35 °C, the whole-cell reaction was first performed at 35 °C. Effects of CA concentration on the whole-cell reaction by *E. coli* cells BL21 (DE3) with pEcatA were examined at 35 °C. As shown in Figure 2, below CA concentrations of 40 mM, CA was stoichiometrically converted to ccMA, and the pH was decreased from 7.5 to 6.2 with accompanying production of ccMA. At 50 mM, CA was not completely degraded to ccMA. Above CA concentration of 60 mM, very little CA was degraded, and ccMA production was essentially stopped. These effects may be due to the toxic effects of CA against the enzymes and microbial cells.[11] Thus, under

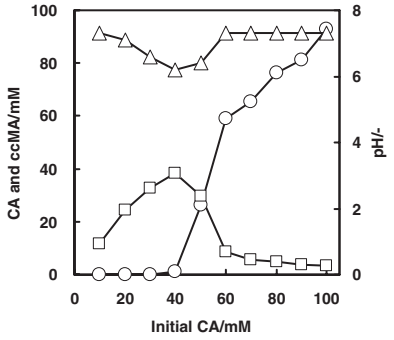

Figure 2. Effects of initial CA concentration on ccMA production by whole-cell reaction using *E. coli* BL21 (DE3)/pEcatA cells. ccMA production was performed under the following conditions. The reaction mixture contained CA at each concentration, 10–100 mM, and *E. coli* cells ($O.D._{600} = 10$) in 500 mM Tris-acetate buffer (pH 7.5) in 18 mmϕ test tube (total volume of 0.6 mL). Each reaction was performed at 35 °C with shaking at 160 rpm for 2 h. Symbols; square, ccMA; circle, CA; triangle, pH.

the conditions tested, feeding of CA at concentrations less than 40 mM was found to be effective for high-yield production of ccMA. Effects of shaking speed in relation to oxygen supply on the rate of ccMA production were also examined, and we confirmed that shaking speeds above 180 rpm were necessary for rates of ccMA production higher than 1.5 mM min^{-1} (Supporting Information; SI, Figure S1[10]). Effects of temperature on ccMA production were examined, and we confirmed that the ccMA production rates were almost constant within a temperature range of 10–30 °C (SI, Figure S2[10]).

Effects of temperature on continuous CA degradation through the fed-batch process with successive additions of CA were therefore examined in the range of 10–30 °C.[10] The terminal point of successive CA addition for the fed-batch process was determined as the point at which CA degradation is no longer observed and the concentration of CA exceeds 5 mM in the reaction mixture. The terminal points of the fed-batch process with successive additions of CA at 10, 20, and 30 °C were 31, 32, and 24 additions of CA (7.75, 8.00, and 6.00 h), respectively (SI, Table S1[10]). Although ccMA production rates were almost constant over the temperature range of 10–30 °C (Figure S2[10]), the maintenance of CA degradation in the fed-batch process was affected by temperatures in the range of 10–30 °C, and the optimum temperature was determined to be 20 °C. Based on these results, further ccMA production was performed at 20 °C with rotary shaking at 180 rpm and successive additions of 10 mM CA.

CatA contains nonheme iron(III) as a cofactor in its active center.[12] Wu et al. reported that addition of Fe(III)–EDTA complex was effective to increase ccMA production from benzoate by *Sphingobacterium* sp. strain GCG.[13] Effects of the addition of Fe(II) and Fe(III) on continuous CA degradation through the fed-batch process were examined with successive additions of CA. We used 1 mM $FeCl_3$, 1 mM $FeCl_3$–EDTA complex, 1 mM $FeSO_4$–10 mM ascorbic acid, and 10 mM ascorbic acid as additives. Among the tested compounds containing Fe(II) or Fe(III), addition of $FeCl_3$ showed a clear effect on prolongation of continuous CA degradation. The

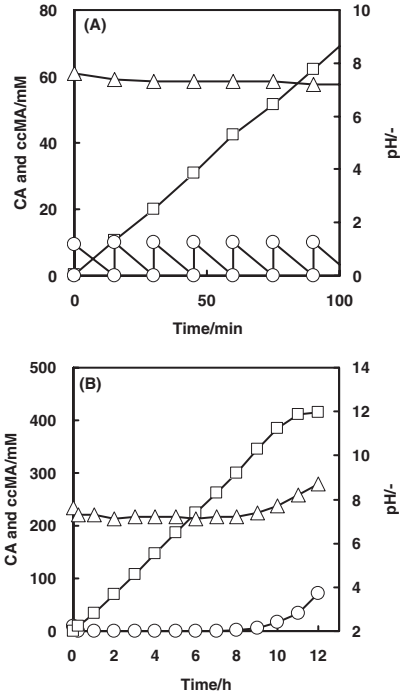

Figure 3. ccMA production through fed-batch process by whole-cell reaction using recombinant *E. coli* BL21 (DE3)/pEcatA cells under the optimal conditions with 0.1 mM $FeCl_3$. (A) Initial stage (0–90 min) of the reaction. (B) The reaction for 12 h. Both of the reactions, (A) and (B), were performed at 20 °C with 0.1 mM $FeCl_3$ with successive feeding of CA. The fed-batch process was initiated by addition of 10 mM CA and continued with successive additions of 0.25 mL of 4 M CA (final concn: 10 mM) as the substrates and 0.15 mL of 12.7 M NaOH solution for adjustment of the pH at 15-min intervals. A 0.4 mL portion of the reaction mixture was collected at 15-min intervals for measurement of CA and ccMA. Symbols; square, ccMA; circle, CA; triangle, pH.

terminal point of successive CA addition was prolonged for more than 1 h relative to reaction carried out without the additive. The effects of the concentration of $FeCl_3$ on continuous CA degradation through the fed-batch process with successive additions of CA were the same in the range of 0.1–5.0 mM (data not shown). Based on these results, further ccMA production through the fed-batch process was performed under the conditions with the addition of 0.1 mM $FeCl_3$.

The fed-batch process using whole cells with successive additions of CA was performed at 20 °C with addition of 0.1 mM $FeCl_3$. Solutions of 10 mM CA and NaOH were added at 15-min intervals. The accumulation of undegraded CA was not detected until 8 h, and ccMA production reached 300 mM (42.6 g L^{-1}) at 8 h. After 48 additions of CA over 12 h, the final concentration of ccMA reached 415 mM (59.0 g L^{-1}) without by-product as shown in Figure 3. The molar conversion yields based on the amount of consumed CA were almost the theoretical value of 100% (mol mol^{-1}), until 12 h.

In conclusion, we have demonstrated that high-yield and rapid ccMA production can be achieved using whole cells of *E. coli* expressing *catA* as a biocatalyst in aqueous solution under environmentally benign conditions. Through the direct 1-

step conversion from CA, we succeeded in producing 415 mM (59.0 g L^{-1}) ccMA without generation of by-product in 12 h. The molar conversion yields based on the amount of consumed CA were almost the theoretical value of 100% (mol mol^{-1}) during 12 h of the whole period of reactions. These results demonstrate that this biocatalytic method enables us to produce ccMA with high yield in a short time. In comparison to other bioprocesses for ccMA production,[6–8] the fed-batch process for ccMA production from CA described in this report is advantageous from the viewpoints of practical production, since ccMA produced without by-product in the reaction mixture is easily separated from CA as a substrate through an ion-exchange column. Therefore, the method described in this report represents a significant model process for the biocatalytic production of ccMA from CA by a whole-cell reaction.

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