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Integrated biooxidation and acid dehydration process for monohydroxylation of aromatics

Shiho Yamashita [a], Yuka Sameshima [a], Masaaki Konishi [a], Junichi Kato [b], Michimasa Kishimoto [a], Kohsuke Honda [a,*], Takeshi Omasa [a], Hisao Ohtake [a]

[a] *Department of Biotechnology, Osaka University, Osaka 565-0871, Japan*
[b] *Department of Molecular Biotechnology, Hiroshima University, Hiroshima 739-8530, Japan*

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Abstract

We examined the performance of an integrated biooxidation and acid dehydration process for aromatic monohydroxylation using the production of *o*-cresol from toluene as the model conversion. A toluene *cis*-glycol dehydrogenase gene (*todD*)-disrupted mutant of *Pseudomonas putida* T-57 was employed as the whole cell biocatalyst for oxidizing toluene to toluene *cis*-glycol (TCG). After bioconversion of toluene to TCG, *o*-cresol was produced by adding HCl to the culture medium, since it was found that TCG became unstable at a low pH and underwent spontaneous dehydration. When the *todD*-disrupted mutant cells were grown in a two-liquid-phase culture system with oleyl alcohol as the organic solvent, this integrated process produced 40 g l^{-1} of *o*-cresol in the organic solvent phase at 30 h. The total concentration of *o*-cresol in the two-liquid-phase culture reached 6.6 g l^{-1} at 30 h, which was approximately four-fold greater than that in the single-liquid-phase culture.

Keywords: Biocatalysis; *o*-Cresol; Monohydroxylation; *Pseudomonas putida*; Toluene dioxygenase; Two-liquid-phase culture

1. Introduction

There is a growing appreciation that the integration of biotechnology into chemical manufacturing has the potential to reduce materials and energy consumption as well as the emission of substances hazardous to health and the environment [1]. The hydroxylation of monoaromatics benzene, toluene, ethylbenzene, and xylene, collectively called BTEX, is a requirement for the synthesis of specialty chemicals and polymers and of some bulk chemicals [2]. However, since the chemical oxidation of aromatic hydrocarbons generates a variety of oxidation byproducts, their selective hydroxylation is often difficult in chemical processes. Additionally, the chemical oxidation processes are very energy intensive and generate hazardous explosive intermediates as well as toxic wastes [3]. Hence, the hydroxylation of aromatic hydrocarbons has been considered as one of realistic targets to replace chemical process steps to biocatalytic production steps [4].

Toluene monooxygenases (TMOs) are able to catalyze the hydroxylation of aromatic ring with consumption of NADH [5]. Among them, T2MO from *Pseudomonas* sp. strain JS150 [6], T3MO from *Burkholderia pickettii* strain PKO1 [7], and T4MO from *Pseudomonas mendocina* strain KR1 [8] have been particularly studied. These enzymes display a broad range of substrates and catalyze direct hydroxylation of monoaromatics. The NADH-dependent TMOs may be used in whole cells, because it is generally easier and less expensive to regenerate NADH in metabolically active cells [4,9]. However, the direct monoaromatic hydroxylation is problematic in whole cells, because the hydroxylation products are extremely toxic to metabolically active cells, which would decrease their biocatalytic activity [3].

One possible route to overcome this problem is to use toluene dioxygenase (TDO) as the biocatalyst [10]. TDO has an extraordinarily broad substrate specificity and yet operates regiospecifically. TDO catalyzes the incorporation of molecular oxygen (O_2) into aromatic ring with consumption of NADH and thus converts aromatics to corresponding *cis*-dihydrodiols (*cis*-glycols) (Fig. 1). The further metabolism of *cis*-glycols involves an NAD^+-dependent dehydrogenation reaction to form diols [10]. TDO reactions offer significant advantages in

* Corresponding author. Tel.: +81 6 6879 7437; fax: +81 6 6879 7439.
E-mail address: honda@bio.eng.osaka-u.ac.jp (K. Honda).

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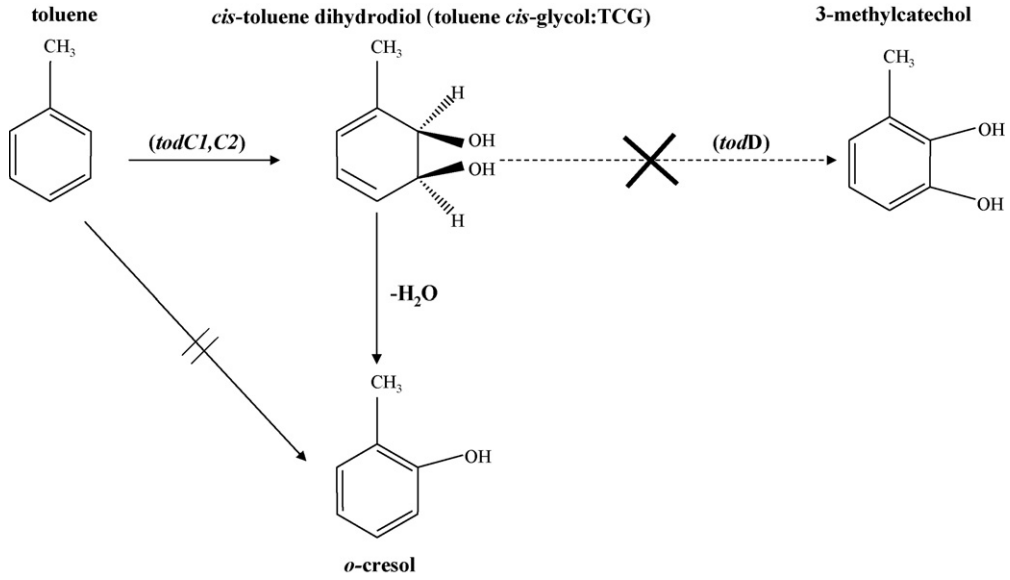

Fig. 1. The pathway for oxidizing toluene to 3-methylcatechol by *Pseudomonas putida* T-57. Although strain T-57 is able to utilize toluene as a sole source of carbon and energy, it is unable to directly oxidize toluene to *o*-cresol, probably because of its toxicity to metabolically active cells. The *todC1* and *todC2* genes encode α and β subunits of toluene dioxygenase, respectively, while the *todD* gene encodes toluene *cis*-glycol (TCG) dehydrogenase. The *todD* gene was disrupted by the direct gene replacement technique as described previously [11]. TCG is unstable at a low pH and undergoes spontaneous dehydration, resulting in the formation of *o*-cresol.

the initial oxidation of aromatics over TMO reactions, because *cis*-glycols are less toxic than monohydroxylated aromatics.

In the present study, we investigated an integrated biooxidation and acid dehydration process for aromatic monohydroxylation using the production of *o*-cresol from toluene as the model conversion. A toluene *cis*-glycol dehydrogenase gene (*todD*)-disrupted mutant of *Pseudomonas putida* T-57 was employed as the whole cell biocatalyst for oxidizing toluene to toluene *cis*-glycol (TCG). An additional strategy to produce *o*-cresol was to use spontaneous dehydration of TCG at a low pH. We found that TCG was unstable at a low pH and readily underwent spontaneous dehydration, resulting in the formation of *o*-cresol. This integrated process was also applied to the hydroxylation of other monoaromatics including benzene, ethylbenzene, and *p*-xylene.

2. Materials and methods

2.1. Bacterial strains and precultures

P. putida strain T-57 was isolated from an activated sludge sample by the method described previously [11]. The *todD*-knockout mutant strain T-57:todDd5 (Km^r) was constructed by a double-crossover event. A 2.6-kb *Not*I-*Sal*I fragment containing the *todD* gene was cloned into pBluescript II KS+ (Stratagene, La Jolla, CA) to construct pBS-ADE. A *Hinc*II-flanked *kan* (conferring kanamaycin resistance [Km^r]) from pUC4K [12] was inserted into the *Stu*I site of pBS-ADE. *P. putida* strain T-57 was then transformed with the resulting plasmid pBS-ADEK by electroporation. The *todD* gene disruption was confirmed by Southern blot analysis.

The modified salts basal (MSB) medium consisted of 4.3 g of KH_2PO_4, 3.4 g of K_2HPO_4, 2.0 g of $(NH_4)_2SO_4$, 0.34 g of $MgCl_2{\cdot}6H_2O$, 0.001 g of $MnCl_2{\cdot}4H_2O$, 0.006 g of $FeSO_4{\cdot}7H_2O$, 0.026 g of $CaCl_2{\cdot}2H_2O$, 0.02 mg of $Na_2MoO_4{\cdot}2H_2O$, 0.01 mg of $ZnCl_2{\cdot}7H_2O$, 0.01 mg of $CoCl_2{\cdot}6H_2O$, 0.01 mg of $CuSO_4$, 0.001 mg of $NiSO_4{\cdot}6H_2O$, and 0.001 mg of Na_2SeO_4 per liter of deionized water. A frozen T-57:todDd5 cell suspension, which had been stored at −80 °C, was inoculated into 100 ml of LB medium in a 500-ml baffled Erlenmeyer flask. The preculture was grown aerobically at 30 °C for 10 h.

2.2. Monoaromatic hydroxylation assays

For monoaromatic hydroxylation assays, the reaction vessel was a screw-capped cylindrical bottle (65 mm × 145 mm) containing 48 ml of fresh MSB and 12 ml of preculture. Two empty test tubes were placed in the screw-capped bottle. Benzene, toluene, ethylbenzene, and *p*-xylene were added to one of the test tubes to allow their vapor to diffuse into the medium and the headspace. Since T-57:todDd5 cells were unable to utilize toluene as a carbon and energy source, butanol was added to another test tube and supplied in a vapor. For two-liquid-phase cultures, 6 ml of oleyl alcohol was added to the culture. The bottles were incubated with a horizontal shaking (150 rpm) at 30 °C.

2.3. Batch cultures

Batch cultures were performed with a 5-l bioreactor (MDL-500; Marubishi Bioeng, Tokyo). Toluene was supplied in the vapor phase to the cultures by sparging air (0.1 l min^{-1}) through a glass sinter into an Erlenmeyer flask containing a mixture of toluene and butanol (2:1, v/v) (Fig. 2). The sparging set-up allowed the continuous introduction of toluene and butanol, while minimizing their toxicity to *P. putida* cells. Approximately 200 ml of preculture was inoculated into 800 ml MSB medium with or without 100 ml of oleyl alcohol as the organic solvent. The initial culture pH was set at pH 7.0. The culture pH was maintained in the range of 6.8 to 7.1 throughout the cultivation without pH control. The bioreactor was continuously agitated at 300 rpm and aerated at 0.1 vvm.

2.4. Analytical methods

Oxygen (O_2) uptake measurements were performed by a biological O_2 monitor equipped with a Clark-style O_2 electrode (YSI Model 5300, Yellow Springs, OH). The O_2 electrode was mounted in a glass water-jacketed reaction chamber (3 ml) maintained at 30 °C. The reaction chamber was filled with 50 mM phosphate buffer (pH 7.2) and continuously mixed by using a magnetic stirrer. The calibration of the O_2 electrode was performed with the air-saturated phosphate buffer at 30 °C. Bacterial cells, which were grown in MSB medium