Journal of Environmental Biotechnology
Vol. 7, No. 1, 39–44, 2007

Original paper (regular paper)

Bioproduction of 3-Methylcatechol from Toluene in a Two-Phase (Organic-Aqueous) System by a Genetically Modified Solvent-Tolerant *Pseudomonas putida* Strain T-57

Irvan Faizal[1], Masaki Ohba[1], Akio Kuroda[1], Noboru Takiguchi[1], Hisao Ohtake[2], Kosuke Honda[2] and Junichi Kato[1]*

[1] *Department of Molecular Biotechnology, Hiroshima University, Higashi-Hiroshima, Hiroshima 739–8530, Japan*
[2] *Department of Biotechnology, Osaka University, Suita, Osaka 565–0871, Japan*
* TEL: +81–(0)82–424–7757 FAX: +81–(0)82–424–7047
* E-mail: jun@hiroshima-u.ac.jp

(Received; 14 December, 2006/Accepted; 20 January, 2007)

Pseudomonas putida strain T-57 is a solvent-tolerant toluene-oxidizing bacterium, which utilizes toluene via toluene dioxygenase (TDO) pathway. In this study, we investigated bioproduction of 3-methylcatechol from toluene by using a 3-methylcatechol accumulating mutant derived from *P. putida* strain T-57. TDO assays revealed that TDO expression in *P. putida* strain T-57 is inducible and toluene is a good inducer. It was shown that TDO induction by toluene was under the control of catabolite repression by glucose. Glucose repressed TDO induction by toluene in a concentration-dependent manner. TDO induction by toluene was also repressed when cells were grown in LB medium. An deletion-insertion mutant of the 3-methylcatechol 2,3-dioxygense gene (*todE*) was constructed by inserting a kanamycin resistant gene cassette into the chromosomal *todE* gene of *P. putida* strain T-57 and the resulting mutant, designated *P. putida* strain TODE1, was used for bioconversion of 3-methylcatechol from toluene. To avoid catabolite repression, TODE1 cells were cultivated in a mineral salts basal medium supplemented with 2 g l^{-1} of glucose+toluene vapor or *n*-butanol+toluene vapor. It was found that 3-methylcatechol was accumulated in culture supernatants at 6 and 12 mM, respectively. Introduction of the second organic phase improved 3-methylcatechol production by TODE1. When TODE1 was grown in a two-liquid-phase system with oleyl alcohol as the second liquid extraction phase for 3-methylcatechol, and glucose and *n*-butanol as additional carbon and energy sources, 48 and 107 mM 3-methylcatehcol was accumulated in the organic solvent phase, respectively. The overall concentrations of 3-methylcatechol in the two-phase culture were twice higher than those in the single-phase culture.

Key words: bioproduction, 3-methylcatechol, solvent-tolerant bacterium, *Pseudomonas putida* strain T-57

1. Introduction

Pseudomonas putida strain T-57, which was isolated from activated sludge at a wastewater treatment facility of a fermentation plant in Kumamoto, Japan, is a solvent-tolerant aerobic bacterium[3]. It can grow even when organic solvents such as toluene, ethylbenzene, xylenes, styrene, *n*-octane, and *n*-decane are provided in a two-phase (organic-aqueous) system. This bacterium utilizes toluene, *p*-xylene, ethylbenzene, and *n*-butanol as sole sources of carbon and energy. Genetic and biochemical analysis revealed that *P. putida* strain T-57 catabolizes aromatic hydrocarbons via the toluene dioxygenase (TDO) pathway (Fig. 1). From these characteristics, *P. putida* strain T-57 seems very promising as a biocatalyst for the bioproduction of value added chemicals from hydrophobic substrates.

In the previous studies, we investigated the bioproduction of cresol from toluene by using a mutant derived from *P. putida* T-57[3,20]. In the initial step of the TDO pathway, toluene is oxidized to toluene *cis*-glycol, which is further oxidized to 3-methylcatechol by toluene *cis*-glycol dehydrogenase (the *todD* product). It is known that this first intermediate, toluene *cis*-glycol, is nonenzymatically dehydrated to cresol[12]. Therefore, we expected that a *todD* mutant strain of T-57 accumulates cresol from toluene. In fact, when the *todD* mutant cells were exposed to toluene during growing with *n*-butanol, *o*-cresol was accumulated in the medium[3]. When the *todD* mutant was grown in a two-liquid-phase system with oleyl alcohol as a second liquid extraction phase for *o*-cresol, 40 g l^{-1} of *o*-cresol was accumulated in the organic solvent phase and the overall concentration of *o*-cresol in the two-phase culture reached 6.6 g l^{-1} [20].

Substituted catechols are important in pharmaceutical production process[2,5] and catechol and its derivatives are valuable precursors in the production of stains and synthetic flavors[15]. Multi-step and energy-consuming process is employed for chemical synthesis of 3-substituted catechols, resulting in mixtures of different catechols and a low overall

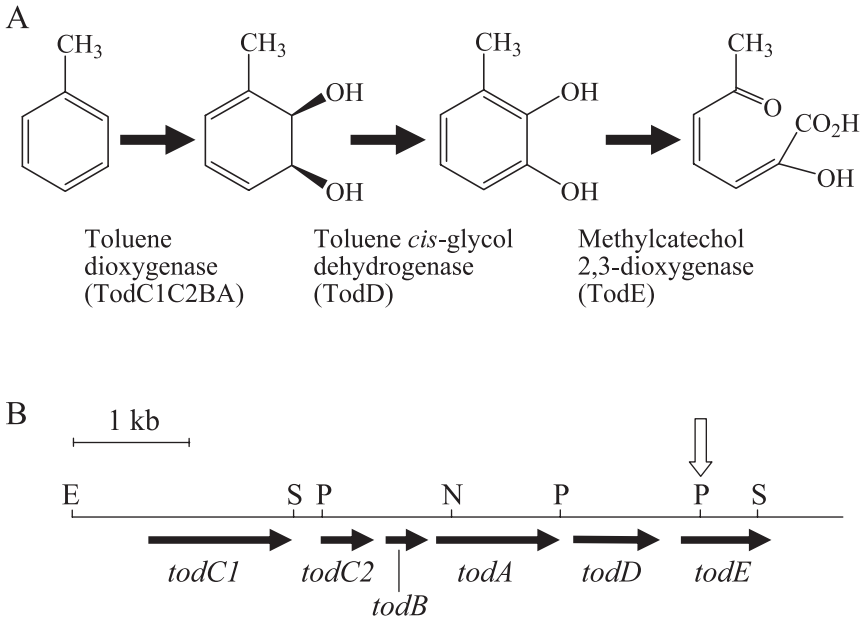

Fig. 1. Catabolic pathway for toluene metabolism by *P. putida* T-57 (A) and restriction map of the *tod* operon (B). The locations and orientations of the *todC1C2BADE* genes are indicated by horizontal arrows. The vertical arrow indicates the insertion position of a kanamycin resistant gene cassette in *P. putida* TODE1. Restriction sites: E, *Eco*RI; P, *Pst*I; N, *Not*I; S, *Sal*I.

product yield[7]. It is known that various bacteria utilize aromatic compounds via metabolic pathways with catechols as their intermediates. If these bacterial cells can be used as biocatalysts, catechols can be produced at the atmospheric pressure and moderate temperature. There have been several investigations to attempt bioproduction of catechols by using a monooxygenase[6] and dioxygenases[7,13,19]. However, toxicity of substrates and products limited these bioproduction levels.

In this study, we used solvent-tolerant *P. putida* strain T-57 as the parental strain to construct a biocatalyst for bioproduction of 3-methylcatechol from toluene, and evaluated the resulting biocatalyst for 3-methylcatechol production in the single- and two-liquid-phase cultivation systems.

2. Materials and Methods

2.1. Bacterial strains and plasmids

The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* MV1184 was used for plasmid construction and DNA manipulation. *P. putida* strains were grown at 28°C with shaking in mineral salts basal (MSB) medium[3] with appropriate carbon sources. Luria-Bertani (LB) medium[14] was used as complete medium. For solid media, 2% agar was added to MSB medium and LB medium. *E. coli* was grown at 37°C with shaking in 2xYT medium[14] supplemented with appropriate antibiotics.

2.2. DNA manipulation and electroporation

Standard procedures were used for plasmid DNA preparations, restriction enzyme digestions, ligations, transformations and agarose gel electrophoresis[14]. *P. putida* strain T-57 was transformed by electroporation as described previously[3].

Table 1. Bacterial strains and plasmids used in this study.

Strain/plasmid	Relevant characteristics[a]	Source or reference
Strains		
E. coli		
MV1184	*ara*Δ(*lac-proAB*) *rpsL thi* (ϕ80 *lacZ*ΔM15) Δ(*srl-recA*)*306*::Tn*10*(Tc^r) F'[*traD36 proAB*$^+$*lacI*^{q} *lacZ*ΔM15]	17
P. putida		
T-57	Solvent-tolerant toluene-oxidizing bacterium	3
TODE1	T-57 derivative, *todE*::*kan*	This study
Plasmids		
pUC4K	pUC4 containing a 1.3-kb *kan* gene cassette; Ap^r, Km^r	16
pT57Tod01	pUC118[17] containing *todC1C2BADE*, Ap^r	3
pT57Tod01EK	pT57Tod01 derivative containing *todE* disrupted by insertion of *kan* cassette, Ap^r, Km^r	This study

[a] Ap, ampicillin; Km, kanamycin; Tc, tetracycline.

2.3. Construction of *todE* mutant of *P. putida* strain T-57

The *todE* deletion-insertion mutant of *P. putida* strain T-57 was constructed by the direct gene replacement technique[9]. Plasmid pT57Tod01 containing *todC1C2BADE* was partially digested with *Pst*I and ligated with a *Pst*I-flanked *kan* (conferring kanamycin resistance) cartridge from pUC4K to disrupt *todE* encoding methylcatechol 2,3-dioxygenase. *P. putida* strain T-57 was transformed with the resulting plasmid pT57Tod01EK by electroporation to transfer the mutation into the genome of *P. putida* strain

Table 2. Effects of growth substrates on TDO expression in *P. putida* strain T-57.

Growth substrate[a]	Medium	TDO specific activity[b] (μg indigo min^{-1} OD_{600}^{-1})
Glucose	MSB	0
n-Butanol	MSB	0
Oleyl alcohol	MSB	0
Ethylbenzene	MSB	1.39±0.03
Toluene	MSB	4.52±0.39
NA[c]	LB	0

[a] Toluene, ethylbenzene, and *n*-butanol were added in the vapor phase. Liquid oleyl alcohol (9.1% [vol/vol]) was directly added to MSB medium.

[b] Mean±standard deviation for four experiments.

[c] Not applicable.

T-57. The deletion-insertion was confirmed by Southern blot analysis.

2.4. Toluene dioxygenase assay

P. putida strains were inoculated into 10 ml MSB medium with appropriate carbon sources in 50-ml screw-capped vials. Hydrocarbons for carbon sources were provided in the vapor phase by putting small tubes with 150 μl of hydrocarbons inside the screw-capped vials, or directly added to MSB medium at 9.1% (vol/vol). *P. putida* was grown with reciprocal shaking (120 rpm) at 28°C to a optical density at 600 nm (OD_{600}) of 1, and cells were harvested by centrifugation (10,000×*g*, 5 min, 4°C). Pelleted cells were resuspended in 50 mM phosphate buffer (pH 7.2), washed once with the same buffer, and resuspended in an original volume of phosphate buffer. Toluene dioxygenase activity was evaluated by formation of indigo from indole. A cuvette contained 1 ml of cell suspension and 100 μl of 100 mM indole in *N*,*N*-dimethylformamide and maintained at 28°C. In this enzyme assay, toluene dioxygenase oxidizes indole to indole-*cis*-2,3-dihydrodiol. Indole-*cis*-dihydrodiol is spontaneously oxidized to indoxyl and indoxyl is dimerized to the dark blue pigment, indigo, by air oxidation. Formation of indigo was recorded at 600 nm.

2.5. Batch cultivation for 3-methylcatechol production

P. putida cells grown in LB medium overnight at 28°C with reciprocal shaking (120 rpm) were inoculated into 10 ml MSB medium with 2 g l^{-1} glucose (a 1% inoculum). Bacterial cells were grown at 28°C with shaking to OD_{600} of 1 and harvested by centrifugation. Cells were suspended in 10 ml MSB medium in the 50 ml screw-capped vials. Growth substrates and toluene were added to the vials. Glucose was added to MSB medium at a concentration of 2 g l^{-1}, liquid oleylalcohol was directly added to MSB medium at 9.1% (vol/vol), and toluene and *n*-butanol were provided in the vapor phase by putting small tubes with 150 μl of toluene or *n*-butanol inside the vials. The screw-capped vials were then incubated with reciprocal shaking (120 rpm) at 28°C. After 22 h incubation, the aqueous supernatant and the organic phase were subjected to measurement of 3-methylcatechol.

2.6. HPLC

Metabolites in culture supernatants and organic phase were determined by HPLC on a TSK-GEL ODS-80TM column (260×4.6 mm; TOSOH, Tokyo) at a flow rate of 0.5 ml min^{-1}. The mobile phase consisted of isocratic composition of water:acetonitrile (30 : 70, vol/vol). The elution was monitored at 220 nm.

3. Results and Discussion

3.1. Expression of toluene dioxygenase is inducible

P. putida strain T-57 oxidizes toluene to 3-methylcatechol through 2-step oxidation in the TDO pathway (Fig. 1A). When 3-methylcatechol is produced from toluene by using strains derived from *P. putida* strain T-57 as biocatalysts, high expression of TDO, which initially oxidizes toluene to toluene *cis*-glycol, is important. Therefore, we first investigated effects of growth substrates on TDO expression in T-57. Strain T-57 cells grown in MSB medium containing each of growth substrates were examined for TDO activity. When strain T-57 was grown on glucose as a sole carbon source, no TDO activity was detected (Table 2). The highest TDO activity (4.52 μg indigo min^{-1} OD_{600}^{-1}) was detected in cells grown on toluene followed by cells grown ethylbenzene (1.39 μg indigo min^{-1} OD_{600}^{-1}). But cells grown in MSB with *n*-butanol and oleyl alcohol and in LB medium showed no TDO activity. Thus, these results suggest that TDO expression in *P. putida* T-57 is inducible and that toluene and ethylbenzene are good inducers.

3.2. TDO expression is under the control of catabolite repression

In the TDO pathway, toluene is initially oxidized to toluene *cis*-glycol, which is further oxidized to 3-methylcatechol (Fig. 1A). This product undergoes meta-cleavage fission catalyzed by methylcatechol 2,3-dioxygenase encoded by *todE*. For production of 3-methylcatechol, the *todE* gene should be inactivated to prevent further oxidation of 3-methylcatechol. It is expected that the *todE* mutant of strain T-57 is not able to utilize toluene as a growth substrate. Therefore, additional carbon and energy sources are required for production of 3-methylcatechol by the *todE* mutant in the growing conditions. We then investigated effects of additional growth substrates on TDO induction by toluene in T-57. Strain T-57 was grown in MSB medium with additional growth substrates or in LB medium supplemented with toluene in the vapor phase and TDO activity in cells was determined. LB medium supported the highest growth rate with a cell doubling time of 1.5 h, while cell doubling times were approximately 2.1–2.4 h when grown in MSB medium with carbon sources. TDO activity decreased with increase of glucose concentrations (Table 3). Strain T-57 cells grown in LB medium showed only a trace level of

Table 3. Effects of additional carbon and energy sources on TDO induction by toluene in *P. putida* strain T-57[a].

Co-substrate[b]	Medium	TDO specific activity[c] (μg indigo min^{-1} OD_{600}^{-1})
None	MSB	4.52±0.39
Glucose (2 g l^{-1})	MSB	2.73±0.15
Glucose (5 g l^{-1})	MSB	2.37±0.16
Glucose (10 g l^{-1})	MSB	1.25±0.21
Glucose (15 g l^{-1})	MSB	0.16±0.12
Glucose (20 g l^{-1})	MSB	0.07±0.02
n-Butanol	MSB	2.64±0.30
n-Octanol	MSB	1.83±0.49
Oleyl alcohol	MSB	3.71±0.54
n-Butanol+ oleyl alcohol	MSB	2.77±0.67
—	LB	0.06±0.05

[a] *P. putida* strain T-57 was grown in MSB with additional carbon sources or in LB supplemented with toluene in the vapor phase.

[b] Toluene, *n*-butanol, and *n*-octanol were added in the vapor phase. Liquid oleyl alcohol (9.1% [vol/vol]) was directly added to MSB medium.

[c] Mean±standard deviation for four experiments.

Table 4. Bioproduction of 3-methylcatechol from toluene by *P. putida* strain TODE1 grown in MSB medium with toluene and co-substrates[a].

Co-substrate	3-Methylcatechol (mM)[c]		
	Aqueous phase	Organic phase	Overall[d]
None	1.1±0.3	—	1.1±0.3
Oleyl alcohol	8.9±1.0	71.8±4.8	14.6±1.4
Glucose	5.7±0.6	—	5.7±0.6
Glucose+oleyl alcohol	7.2±2.3	47.6±4.3	10.9±2.4
n-Butanol[b]	12.1±1.3	—	12.1±1.3
n-Butanol[b]+oleyl alcohol	16.1±2.3	107.3±7.9	24.4±2.7

[a] Toluene was supplemented in the vapor phase.

[b] *n*-Butanol was supplemented in the vapor phase.

[c] Mean±standard deviation for four experiments.

[d] Overall concentration=(concentration in aqueous phase×volume of aqueous phase+concentration in organic phase×volume of organic phase)/(volume of aqueous phase+volume of organic phase).

TDO activity even in the presence of toluene. These results suggest that TDO expression in strain T-57 is subject to catabolite repression by glucose and a component(s) in LB medium. When cells were cultivated in MSB medium containing liquid oleyl alcohol (9.1% [vol/vol]) or supplemented with *n*-butanol in the vapor phase, TDO levels were more than 60% of the fully-induced level. Thus, oleyl alcohol and *n*-butanol were indicated to be suitable for additional carbon and energy sources for 3-methylcatechol production by the *todE* mutant.

3.3. Construction of a *todE* mutant of T-57

We constructed a *todE* (encoding catechol 2,3-dioxygenase) mutant of T-57 to produce 3-methylcatechol from toluene. The *todE* gene was inactivated by inserting a *kan* cassette into the chromosomal *todE* gene of T-57 and the resulting mutant was designated *P. putida* strain TODE1. The rapid spot test described by Pankhurst[11] was carried out to assess methylcatechol 2,3-dioxygenase activity in TODE1. Methylcatechol dissolved in acetone was sprayed onto TODE1 and T-57 colonies grown on MSB agar plates supplemented with *n*-butanol and toluene vapor. Colonies of T-57 turned yellow, but not those of TODE1 (data not shown), indicating that methylcatechol 2,3-dioxygenase was not produced in TODE1. TODE1 was not able to grow in MSB medium with toluene as a sole source of carbon and energy. Since TODE1 showed the expected mutant phenotype, it was further used for bioproduction of 3-methylcatechol from toluene.

3.4. Bioproduction of 3-methylcaetchol from toluene

We conducted bioproduction of 3-methylcatechol from toluene by using *P. putida* strain TODE1. Since TDO assays in T-57 revealed that *n*-butanol and 2 g l^{-1} of glucose did not greatly affect TDO induction by toluene (Table 3), glucose and *n*-butanol were used as carbon and energy sources. We first examined bioproduction of 3-methylcatechol in a single-liquid-phase culture. Strains T-57 and TODE1 were grown in MSB medium supplemented with 2 g l^{-1} of glucose+toluene vapor or *n*-butanol+toluene vapor for 22 h and 3-methylcatechol in culture supernatants was measured. In these culture conditions, both strains showed similar growth with cell doubling times of approximately 2.1–2.5 h. In supernatant of the T-57 culture, 3-methylcatechol was not detected (data not shown), while 5.7 and 12.1 mM 3-methylcatechol was accumulated in culture supernatants when TODE1 was cultivated in MSB medium with 2 g l^{-1} of glucose+toluene and *n*-butanol+toluene, respectively (Table 4).

In the previous study, we demonstrated that production levels of *o*-cresol by the *todD* (encoding toluene *cis*-glycol dehydrogenase [Fig. 1]) mutant of *P. putida* strain T-57 increased by addition of liquid oleyl alcohol to medium as the second, organic phase[20]. Therefore, we examined effects of the second, organic phase on 3-mthylcatechol production by TODE1. Addition of 9.1% (vol/vol) oleyl alcohol significantly increased 3-methylcatechol production by TODE1 (Table 4). When *n*-butanol or glucose were added to medium as an additional carbon and energy source, overall concentrations of 3-methyl catechol in the two-liquid-phase culture were twice as high as those in the single-liquid-phase culture. In the two-liquid-phase culture with *n*-butanol and oleyl alcohol (*n*-butanol+oleyl alcohol) as the additional carbon and energy sources, the highest overall product concentration of 24.4 mM was obtained, consisting of 107.3 mM in the oleyl alcohol phase and 16.1 mM in the aqueous phase. Increased volume of oleyl alcohol in the two-liquid-phase culture did not improve 3-methylcatehcol production (data not shown). Since oleyl alcohol can be used as a source of carbon and energy by *P. putida* strain T-57 and it did not

greatly affect TDO induction by toluene (Table 3), bioproduction of 3-methylcatechol was conducted in MSB medium with 9.1% (vol/vol) oleyl alcohol+toluene vapor. An overall concentration of 3-methycatechol reached 14.6 mM, which was about 60% of that in the two-phase-liquid culture with *n*-butanol as the additional carbon and energy source. The result suggests that *n*-butanol has a positive effect on 3-methylcatechol production by TODE1 in the two-liquid-phase culture. In consistent with it, we previously found that *n*-butanol increased the oxygen consumption rate of *P. putida* strain T-57 cells in the two-liquid-phase culture[20].

Although *P. putida* strain T-57 and its derivative *P. putida* strain TODE1 are tolerant to organic solvents, 3-methylcatechol may still have a detrimental effect on these bacterial cells. The second organic phase scavenges the toxic product from the aqueous phase. Probably, it is the reason why introduction of oleyl alcohol improves 3-methylcatechol production from toluene by *P. putida* strain TODE1. The color of medium was white in the start of the single-liquid-phase cultivation and it changed to dark reddish brown after 22 h cultivation. It is due to *o*-benzoquinone and polymerized compounds produced by the oxidation of 3-methylcatechol with dissolved oxygen[10,15]. Enzymatic reactions may be also involved in this coloration of culture broth. Wang *et al.* proposed that oxidative enzymes such as peroxidase catalyze a one-electron oxidation reaction, generating a free radical from catechols and initializing the formation of quinones and their subsequent polymerization, which is detected as a black precipitate[18]. Consistent with their proposition, cells harvested by centrifugation were also dark reddish brown, suggesting that the color compounds deposited on cells. No TDO activity was detected with these deep-colored cells (data not shown). Deposition of color compounds around the cell may interfere with access of a substrate (toluene) to TDO inside the cell, resulting in loss of TDO activity. In the two-liquid-phase culture, the color of the organic and aqueous phases turned pale brown after 22 h incubation even though higher concentrations of 3-methylcatechol accumulated in the culture medium. This result suggests that removal of 3-methylcatechol from the aqueous phase by oleyl alcohol decreased further oxidation of 3-methylcatechol by bacterial cells, resulting in decreased polymerization. Thus, it is likely that the second organic phase in the two-liquid-phase cultivation contributes to improvement of 3-methylcatechol production by protecting TDO activity in bacterial cells from interference by deposition of polymerized compounds on cells.

P. putida F1 is a toluene-oxidizing bacterium, which degrades toluene via the TDO pathway[21], and *P. putida* F107 is a 3-methylcatechol accumulating mutant (probably *todE* mutant) of *P. putida* F1[4]. Hüsken *et al.*[7] constructed 3-methylcatechol hyperaccumulating *P. putida* MC2 from *P. putida* F107 by integrating the extra copy of the *todC1C2BAD* genes into the *P. putida* F107 genome, and investigated bioproduction of 3-methylcatechol from toluene by using MC2 cells as a biocatalyst[8]. When MC2 cells were grown in the medium containing 50% (vol/vol) of 1-octanol as a second liquid extraction phase, the overall concentration of 3-methylcatechol reached 25 mM. In this cultivation, it took more than 50 h to reach the maximum overall concentrations of 3-methylcatechol. In this study, production levels of 3-methylcatechol by TODE1 were almost same to that by *P. putida* MC2, however, the time to attain the maximum product concentration (22 h) was shorter than that of *P. putida* MC2, suggesting that *P. putida* strain TODE1 showed the higher production rate of 3-methylcatechol than that of *P. putida* MC2. Thus, *P. putida* strain TODE1 is thought to be a good candidate as a biocatalyst for bioproduction of 3-substituted catechols from aromatic compounds.

Acknowledgments

This work was carried out as part of the Project for Development of a Technological Infrastructure for Industrial Bioprocess on R&D of New Industrial Science and Technology Frontiers by the Ministry of Economy, Trade, and Industry (METI), which was entrusted by New Energy and Industrial Technology Development Organization (NEDO).

References

1) Cruden, D.L., J.H. Wolfram, R.D. Rogers, and D.T. Gibson. 1992. Physiological properties of a *Pseudomonas* strain which grows with *p*-xylene in a two-phase (organic-aqueous) medium. Appl. Environ. Microbiol. 58: 2723–2729.
2) Ennis, M.D., and N.B. Ghazal. 1992. The synthesis of (+)- and (–)-flesinoxan: application of enzymatic resolution methodology. Tetrahedron Lett. 33: 6287–6290.
3) Faizal, I., K. Dozen, C.S. Hong, A. Kuroda, N. Takiguchi, H. Ohtake, K. Takeda, H. Tsunekawa, and J. Kato. 2005. Isolation and characterization of solvent-tolerant *Pseudomonas putida* strain T-57, and its application to biotransformation of toluene to cresol in a two-phase (organic-aqueous) system. J. Ind. Microbiol. Biotechnol. 32: 542–547.
4) Finette, B.A., and D.T. Gibson. 1988. Initial studies on the regulation of toluene degradation by *Pseudomonas putida* F1. Biocatalysis 2: 29–37.
5) Hartog, J., and W. Wouters. 1988. Flesinoxan hydrochloride. Drugs Future 13: 31–33.
6) Held, M., W. Suske, A. Schmid, K.-H. Engesser, H.-P.E. Kohler, B. Witholt, and M.G. Wubbolts. 1998. Preparative scale production of 3-substituted catechols using a novel monooxygenase from *Pseudomonas azelaica* HBP1. J. Mol. Catal. B Enzym. 5: 87–93.
7) Hüsken, L.E., R. Beeftink, J.A.M. de Bont, and J. Wery. 2001. High-rate 3-methylcatechol production in *Pseudomonas putida* strains by means of a novel expression system. Appl. Microbiol. Biotechnol. 55: 571–577.
8) Hüsken, L.E., M.C.F. Dalm, J. Tramper, J. Wery, J.A.M. de Bont, and R. Beeftink. 2001. Integrated bioproduction and extraction of 3-methylcatechol. J. Biotechnol. 88: 11–19.
9) Kato, J., Y. Sakai, T. Nikata, and H. Ohtake. 1994. Cloning and characterization of a *Pseudomonas aeruginosa* gene involved in the negative regulation of phosphate taxis. J. Bacteriol. 176: 5874–5877.
10) Mijangos, F., F. Varona, and N. Villota. 2006. Changes in solution color during phenol oxidation by Fenton reagent. Environ. Sci. Technol. 40: 5538–5543.

11) Pankhurst, E.S. 1965. A spot test for catechol 2,3-dioxygenase in bacteria. J. Appl. Bacteriol. 28: 309–315.
12) Robertson, J.B., J.C. Spain, J.D. Hadock, and D.T. Gibson. 1992. Oxidation of nitrotoluenes by toluene dioxygenase: evidence for a monooxygenase reaction. Appl. Environ. Microbiol. 58: 2643–2648.
13) Robinson, G.K., G.M. Stephens, H. Dalton, and P.J. Geary. 1992. The production of catechols from benzene and toluene by *Pseudomonas putida* in glucose fed-batch culture. Biocatalysis 6: 81–100.
14) Sambrook, J., E.F. Fritsch, and T. Maniatis. 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N. Y.
15) Shirai, K. 1986. Screening of microorganisms for catechol production from benzene. Agric. Biol. Chem. 50: 2875–2880.
16) Vieira, J., and J. Messing. 1982. The pUC plasmids, an M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. Gene 19: 259–268.
17) Vieira, J., and J. Messing. 1987. Production of single-stranded plasmid DNA. Methods Enzymol. 153: 3–11.
18) Wang, C.-J., S. Thiele, and J.-M. Bollag. 2002. Interaction of 2,4,6-trinitrotoluene and 4-amino-2,6-dinitrotoluene with humic monomers in the presence of oxidative enzymes. Arch. Environ. Contam. Toxicol. 42: 1–8.
19) Wery, J., D.I. Mendes da Silva, and J.A.M. de Bont. 2000. A genetically modified solvent-tolerant bacterium for optimized production of a toxic fine chemicals. Appl. Microbiol. Bitechnol. 54: 180–185.
20) Yamashita, S., Y. Sameshima, M. Konishi, J. Kato, M. Kishimoto, K. Honda, T. Omasa, and H. Ohtake. 2007. Integrated biooxidation and acid dehydration process for monohydroxylation of aromatics. Process Biochem. 42: 46–51.
21) Zylstra, G.J., and D.T. Gibson. 1989. Toluene degradation by *Pseudomonas putida* F1. Nucleotide sequence of the *todC1C2BADE* genes and their expression in *Escherichia coli*. J. Biol. Chem. 264: 14940–14946.