COMMUNICATION TO THE EDITOR

Production of L-DOPA(3,4-Dihydroxyphenyl-L-alanine) from Benzene by Using A Hybrid Pathway

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Abstract: As an alternative approach to the production of L-DOPA from a cheap raw material, we constructed a hybrid pathway consisting of toluene dioxygenase, toluene *cis*-glycol dehydrogenase, and tyrosine phenol-lyase. In this pathway, catechol is formed from benzene through the sequential action of toluene dioxygenase and toluene *cis*-glycol dehydrogenase, and L-DOPA is synthesized from the resulting catechol in the presence of pyruvate and ammonia by tyrosine phenol-lyase cloned from *Citrobacter freundii.* When the hybrid pathway was expressed in *E. coli,* production of L-DOPA was as low as 3 m*M* in 4 h due to the toxic effect of benzene on the cells. In order to reduce lysis of cells, *Pseudomonas aeruginosa* was employed as an alternative, which resulted in accumulation of about 14 m*M* L-DOPA in 9 h, showing a stronger resistance to benzene. *Biotechnol Bioeng* **58:** 339–343, 1998.

Keywords: L-DOPA(3,4-dihydroxyphenyl-L-alanine); hybrid pathway; tyrosine phenol lyase; toluene dioxygenase; benzene

INTRODUCTION

L-DOPA (3,4-Dihydroxylphenyl-L-alanine) is the drug of choice for treatment of Parkinson's disease, a degenerative brain disorder that causes tremors, rigidity, slowness of speech, and eventually, dementia (Dougan et al., 1975).

Recently, an enzymatic process for L-DOPA production has been commercialized using tyrosine phenol-lyase (TPL; E.C.4.1.99.2) from *Erwinia herbicola* or *Citrobacter freundii.* This enzyme normally catalyzes the degradation of tyrosine to pyruvate, phenol, and ammonia (Yamada and Kumagai, 1975). However, this reaction is reversible, and if catechol is substituted for phenol, L-DOPA is produced (Enei et al., 1973).

Catechol used as a starting material in the TPL-catalyzed process is known to be very susceptible to autooxidation. Although Enei et el. (1973) demonstrated that intermittent feeding of catechol and antioxidant could, to some extent, reduce the autooxidation, instability of catechol still remains a major drawback of the process. In addition, catechol at high concentration is toxic to microorganisms. One of the possible alternatives can be combining TPL and the catechol-producing enzyme(s), so that catechol is consumed as soon as it is produced, thus minimizing the autooxidation of catechol and reducing the toxicity to microorganisms.

Biological production of catechol by two-step reactions of toluene dioxygenase (TDO) and *cis*-toluene dihydrodiol dehydrogenase (TCGDH) has been reported (Johnston and Renganathan, 1987). Robinson et al. (1992) demonstrated the production of catechol from benzene to the levels of 30 m*M* by using the whole cells of *Pseudomonas putida.*

Benzene is one of the toxic wastes discharged in large amounts from many industries, and classified as a cheap bulk chemical. If TDO/TCGDH were successfully linked to TPL for L-DOPA synthesis, it would provide not only an economical alternative to the current process of L-DOPA synthesis but also an efficient way of reusing the toxic waste.

In this work, we constructed a strain harboring a hybrid pathway which can produce L-DOPA from benzene. Genes for TPL, TDO, and TCGDH were recruited to make a hybrid pathway for L-DOPA production. Through the hybrid pathway, L-DOPA was produced from benzene under optimal reaction conditions in the host of *E. coli* and *Pseudomonas aeruginosa.* The inhibitory effect of benzene and catechol on the enzymes and the expression level of the enzymes in *E. coli* and *P. aeruginosa* were investigated to determine the factors affecting the whole conversion.

MATERIALS AND METHODS

Chemicals

Benzene, pyruvate, catechol, pyridoxal phosphate (PLP), and L-DOPA were obtained from Sigma (St. Louis, MO). Methanol and acetic acid were obtained from Merck (Darmstadt, Germany). All other chemicals were of analytical grade.

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Bacterial Strains and Plasmids

Bacterial strains used in this work were *E. coli* JM105, *E. coli* JM109, and *P. aeruginosa* NCTC 50182. Plasmids used were pDTG602 carrying the genes for toluene dioxygenase and toluene *cis*-glycol dehydrogenase (Zylstra and Gibson, 1989), pTPL carrying the gene for tyrosine phenol lyase cloned from *Citrobacter freundii* (Lee et al., 1996), and RSF1010 a broad-host-range vector for gram negative bacteria (Scholtz et al., 1989). pHS004 and pHS404 were constructed in this work.

Media and Culture Conditions

Luria Bertani (LB) was used for cultivation of *E. coli.* For antibiotic selection, 100 μg/mL ampicillin was used for *E. coli* harboring pTPL, pDTG602, and pHS004. Streptomycine was added to the final concentration of 50 μg/mL for both *E. coli* and *P. aeruginosa* carrying RSF1010 and pHS404. When the culture was grown to a cell density of A_{600} = 0.5 at 37°C, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 m*M* for enzyme induction and the cultivation was continued for 6 h. Cells were centrifuged at 10,000 g for 5 min at 4°C and washed with an equal volume of 50 m*M* sodium/potassium phosphate buffer (pH 7.2).

DNA Manipulation and Construction of pHS004 and pHS404

All DNA manipulations were performed as described by Sambrook et al. (1989). A schematic diagram for the construction of pHS004 and pHS404 is shown in Figure 1.

Assays to Determine Optimal Reaction Condition

Conversion of benzene to catechol: *E. coli* JM109 containing pDTG602 was used for the biotransformation. Harvested cells were resuspended in 50 m*M* sodium/potassium phosphate buffer (pH 7.2) supplemented with 20 m*M* glucose. Benzene was added to a final concentration of 3 m*M*, and the cells were incubated at 30°C. After 1–2 h, catechol in the reaction mixture was determined by HPLC.

Conversion of catechol to L-DOPA: *E. coli* JM105 containing pTPL was used for the biotransformation. The reaction mixture for L-DOPA production contained 50 m*M* pyruvate, 50 m*M* catechol, 450 m*M* ammonium acetate, 0.15% (w/v) sodium sulfite, 2.5 m*M* disodium EDTA. The pH was adjusted to 8.2 with ammonium hydroxide at 30°C. After 1–2 h, 2 N HCl was added to an equal volume of sample to stop the reaction, and catechol and L-DOPA were determined by HPLC.

Analytical Methods

Gas chromatography was used to determine the concentration of benzene (Choi et al., 1992). Catechol and L-DOPA were analyzed by HPLC (Lee et al., 1996). Pyruvate was determined according to the method of Berntsson (1955).

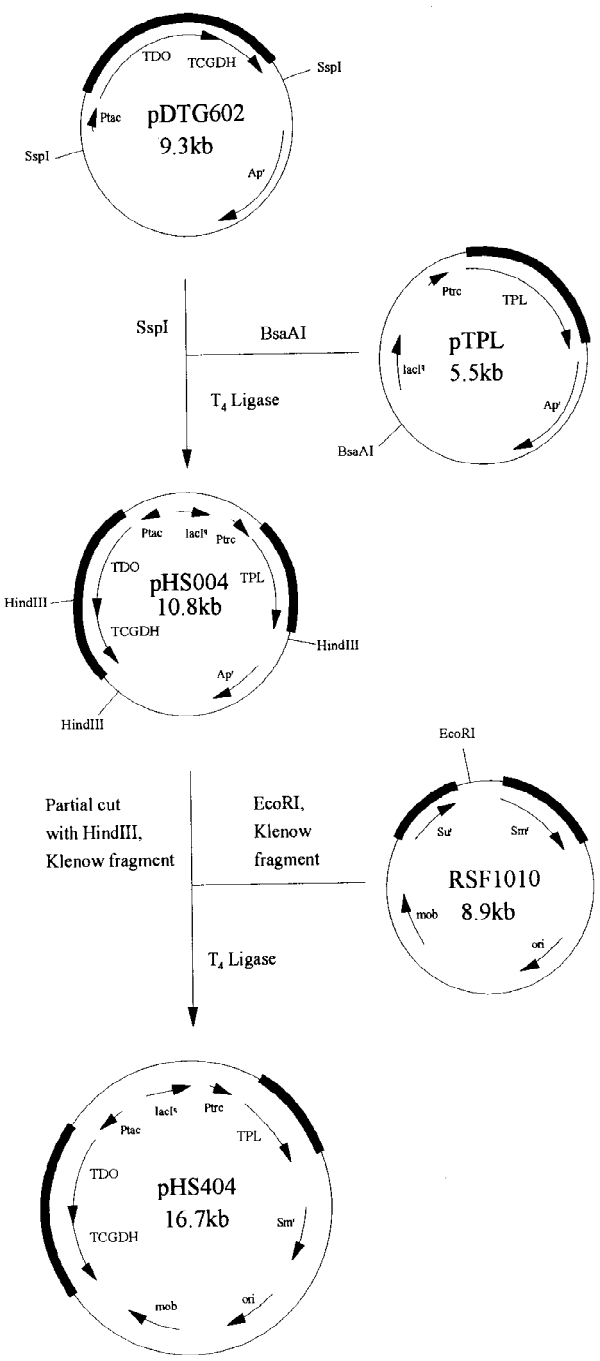

Figure 1. Schematic diagram for the construction of pHS004 and pHS404.

RESULTS

Optimal Reaction Condition

Our scheme for L-DOPA production basically consists of two independent reactions as shown in Figure 2: the forma-

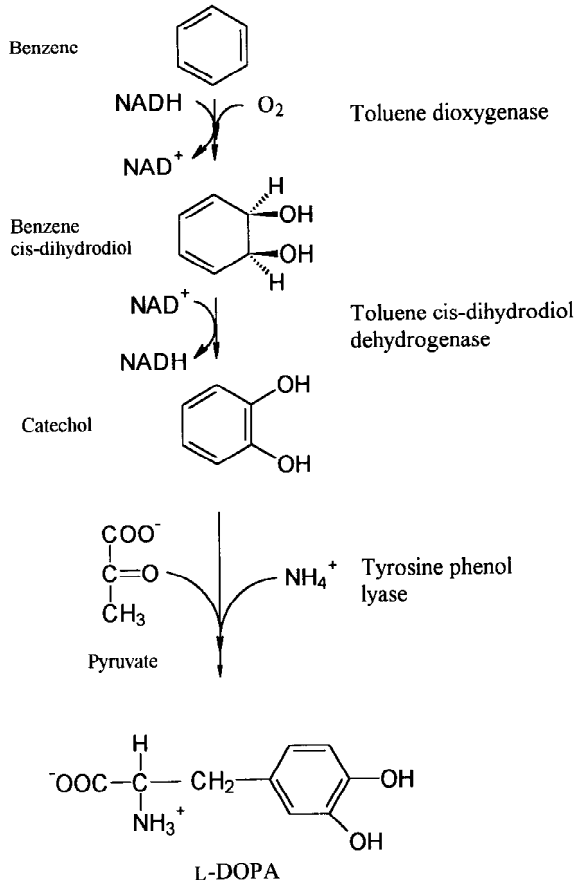

Figure 2. Hybrid pathway for the production of L-DOPA from benzene.

tion of catechol from benzene by TDO/TCGDH and the production of L-DOPA from catechol by TPL. Each reaction was studied separately, and then the optimal conditions for the whole process were determined by compromising the optimal conditions for each individual reaction.

TDO/TCGDH reaction showed a broad pH optimum which ranged from pH 7.0 to 8.4, while TPL had a sharp pH profile at around pH 8.2. Both reactions were dependent on the choice of buffer. TDO/TCGDH favored phosphate buffer, and TPL favored Tris-HCl buffer. Because the TPL reaction is reversible, high concentration of NH_4^+ would shift the equilibrium of the reaction towards L-DOPA production. On the contrary, the activity of TDO/TCGDH was inhibited by high concentration of NH_4^+. Temperature optimum for TDO/TCGDH was found to be 20–30°C, and for TPL about 20°C. At temperatures higher than 20°C, condensation between L-DOPA and pyruvate became predominant, which significantly reduced the yield of L-DOPA.

Based on the above results and the information found elsewhere (Yamada and Kumagai, 1975), the optimal condition for L-DOPA production from benzene was determined as: 50 m*M* pyruvate, 50 μ*M* pyridoxal phosphate as a cofactor for TPL, 2.5 m*M* EDTA, 20 m*M* glucose as a source of maintenance energy in 100 m*M* Tris-buffer titrated with NH_4OH and HCl to pH 8.2 at 20°C. Unless otherwise stated, L-DOPA production was performed under this condition.

Production of L-DOPA from Benzene by Recombinant *E. coli* Strain

A hybrid pathway for the production of L-DOPA from benzene was designed in pHS004 as described in Materials and Methods, and pHS004 was introduced to *E. coli* JM105. Because the expression of TDO and TCGDH was under the control of Ptac and that of TPL under Ptrc, all three enzymes could be induced at the same time by IPTG.

IPTG-induced cells of JM105/pHS004 were incubated with 3 m*M* benzene under the optimized reaction condition. At 3 h, additional 3 m*M* of benzene was added. As shown in Figure 3, about 50% of added benzene was converted to L-DOPA in 5 h. Catechol first accumulated in the reaction mixture and then rapidly consumed as L-DOPA accumulated. When another 3 m*M* of benzene was added to the reaction mixture to keep the reaction in progress, no consumption of benzene was observed, nor were the productions of catechol and L-DOPA. Residual activities of TDO/TCGDH and TPL were measured separately after cells were harvested. The activity of TPL remained almost the same compared with that at the beginning of the reaction. On the contrary, significant loss in the activities of TDO/TCGDH was observed.

It has been reported that benzene and catechol are inhibitory to TDO and TCGDH (Robinson et al., 1992). In order to investigate the inhibitory effect of benzene and catechol, cells were incubated with different concentrations of benzene and catechol, respectively, for 6 h, and the residual activities of TDO/TCGDH were measured. Both benzene

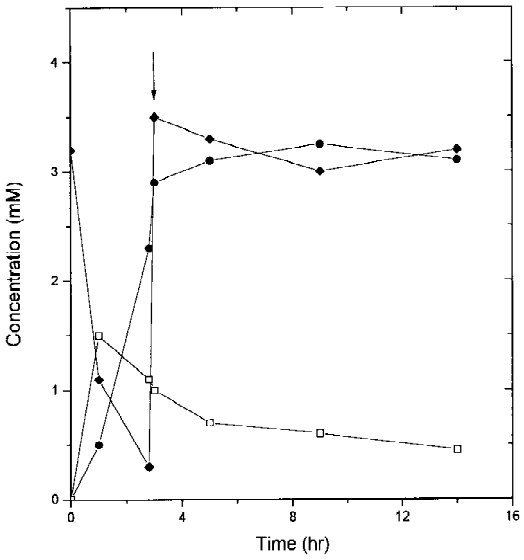

Figure 3. Production of L-DOPA from benzene by *E. coli* JM105/pHS004. Benzene was fed at 0, 3 h to a final concentration of 3m*M*. Arrow indicates the addition of benzene. Symbols are: L-DOPA (●), catechol (□), benzene (♦).

and catechol inhibited TDO/TCGDH irreversibly (data not shown). Catechol at high concentration is known to inhibit toluene dioxygenase, and benzene as a permeabilizing agent is reported to cause a severe damage to the cell membrane components, accelerating the lysis of cells (De Smet et al., 1978). Inhibition by benzene was apparently much stronger than that by catechol. In addition, benzene at concentrations as low as 1 m*M* was found to induce the lysis of cells. Considering that concentration of catechol was less than 1 m*M* during the reaction, deactivation of TDO/TCGDH seemed to mainly result from the toxic effect of benzene to the cells.

Production of L-DOPA by Recombinant *Pseudomonas aeruginosa*

Organic solvents usually result in the increase in membrane fluidity, which often leads to the lysis of the cell if the concentration of solvent exceeds a certain threshold value. Some *Pseudomonas* strains, however, have been reported to show resistance to organic solvents, in part, because of their unique composition and structure of cell membrane (Inoue and Horikoshi, 1989). If an appropriate *Pseudomonas* strain is used as a host in our system, the toxic effect of benzene would be reduced to some extent.

Several *Pseudomonas* strains were tested for their resistance to benzene. *Pseudomonas aeruginosa* NCTC 50182 showed the strongest resistance and was selected as a host strain. This strain was found not to have any catalytic activity towards benzene, catechol, or L-DOPA.

pHS404 was introduced into *P. aeruginosa.* To test the resistance of this strain to benzene, IPTG-induced cells were incubated with various concentrations of benzene in the reaction mixture. After 4 h of incubation, cells were harvested and residual activities of TDO/TCGDH and TPL were measured (data not shown). As in the case of *E. coli* strain, effect of benzene on the activity of TPL was negligible up to the concentration of 10 m*M.* TDO/TCGDH activities were still negatively affected by benzene, but the inhibitory effect was much less than that observed in *E. coli.* About 70% of the activity was retained at benzene concentration of 2.5 m*M,* compared with that in the absence of benzene.

Specific production rates of catechol and L-DOPA by *P. aeruginosa* harboring pHS404 were compared with those by *E. coli.* As can be seen in Table I, *P. aeruginosa* produced catechol at a similar rate to that by *E. coli,* whereas the production rate of L-DOPA by *P. aeruginosa* was about 50% of that by *E. coli.* This seems to be due to the fact that the expression level of TPL in *P. aeruginosa* was relatively low compared with that in *E. coli.*

IPTG-induced cells of *P. aeruginosa*/pHS404 were suspended in the reaction mixture, and benzene and pyruvate were added intermittently to the final concentrations of 2 and 40 m*M,* respectively. A typical reaction profile is shown in Figure 4. Catechol and L-DOPA accumulated concurrently for the first 5 h. Concentration of catechol remained almost constant since then, while L-DOPA continued to accumulate until 9 h. Although about 10 m*M* of catechol was present in the reaction mixture at 9 h, accumulation of L-DOPA ceased. The final concentration of L-DOPA reached 14 m*M.* Concentration of catechol then dropped sharply, probably due to autooxidation.

Table I. Specific production rates of catechol and L-DOPA by different expression systems.

Strain/plasmid	Production rate of catechol (m*M*/g DCW/h)[a]	Production rate of L-DOPA (m*M*/g DCW/h)[b]
E. coli JM109/pDTG602	150	
E. coli JM105/pTPL		405
E. coli JM105/pHS004	120	214
P. aeruginosa/pHS404	105	125

[a]Conversion rate of benzene to catechol by TDO/TCGDH.

[b]Production rate of L-DOPA from catechol, ammonia, and pyruvate by TPL (Detailed reaction conditions are described in Materials and Methods.).

Because a significant drop of optical density was not detected, low conversion was not due to the loss of viability of cells caused by benzene, but probably due to the inhibitory effect of catechol and its autooxidized polymer (Robinson et al., 1992). As the expression level of TPL was low in *P. aeruginosa,* the activity of TPL was too low to convert all catechol formed from benzene to L-DOPA. As a result, catechol accumulated in the course of reaction to some extent. Catechol and its polymer inhibited not only toluene dioxygenase, benzene-attacking enzyme, but also tyrosine phenol lyase, the catechol-converting enzyme irreversibly. Once the oxidized polymer of catechol appeared in the reaction mixture, oxidation of benzene and conversion of cat-

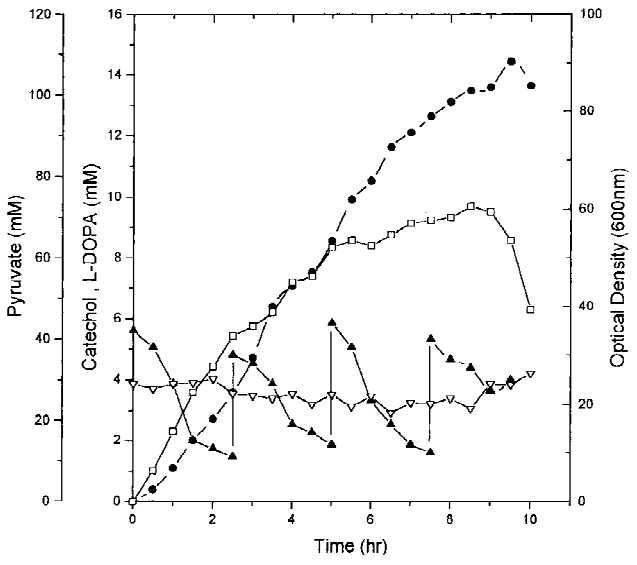

Figure 4. Production of L-DOPA from benzene by *P. aeruginosa*/pHS404. Benzene and pyruvate were intermittently added to the final concentrations of 2 and 40 m*M*, respectively. Symbols are: L-DOPA (●), catechol (□), pyruvate (▲), optical density (▽).

echol decreased gradually, and both reactions stopped eventually.

DISCUSSION

Benzene is one of the most toxic components in petroleum and its release into the environment leads to a serious contamination problem. Most treatment processes for benzene have focused mainly on the mineralization rather than its reuse after discharge. The results presented in this work, however, suggest that benzene could possibly be reused as a starting material for L-DOPA synthesis by combining TDO/TCGDH and TPL using metabolic engineering techniques. The major drawback of the TPL catalyzed process for L-DOPA synthesis is the chemical instability of catechol and its toxicity to microorganism. Although loss of catechol by autooxidation could be avoided to some extent in our system, toxicity of benzene to the microorganism was so serious that benzene at concentration higher than 1 m*M* could not be used when *E. coli* was selected as a host. *Pseudomonas* strain showed a stronger resistance to benzene, but low expression level of TPL resulted in accumulation of catechol, which caused deactivation of TPL as well as TDO/TCGDH.

The yield of L-DOPA obtained in this work was much lower than those reported elsewhere mainly because of the low expression level of genes encoding the enzymes and the toxicity of benzene. Choi et al. (1992) reported an efficient method of supplying aromatic hydrocarbons into the aerobic process, minimizing loss of the compounds. The toxic effect of benzene might be greatly reduced by using such a reactor for L-DOPA synthesis. There may also be a better expression system designed for *Pseudomonas* strains than that used in this work. We are currently testing various reactor systems, and constructing a new expression system which could result in a comparable expression level to that in *E. coli* strains.

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