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Production of *cis,cis*-Muconic Acid from Benzoic Acid *via* Microbial Transformation

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For the production of *cis,cis*-muconic acid *via* biocatalytic conversion reactions from a toxic cosubstrate, benzoic acid, a fed-batch process using computer-controlled DO-stat feeding was developed. The mutant strain of *Pseudomonas putida* BM014 produced *cis,cis*-muconic acid from benzoic acid with high conversion yield. More than 32 g/L of *cis,cis*-muconic acid was accumulated in 42 h and a productivity of 1.4 g/(L · h) was achieved.

Key words: *cis,cis*-muconic acid, fed-batch process, *Pseudomonas putida*

INTRODUCTION

cis,cis-Muconic acid is an unsaturated dicarboxylic acid with six carbon atoms. This compound is suggested to be potentially useful as a raw material for new functional resins, pharmaceuticals and agrochemicals. At least, it can be easily converted to adipic acid, a commodity chemical for the production of nylon-6,6. Because it contains a dicarboxylic group as well as conjugated double bonds, it has a diverse reactivity. So far, however, there have been no secure and economical methods to synthesize *cis,cis*-muconic acid, so the industrial applications have not been developed, althogh it was such an attractive compound. *cis,cis*-Muconic acid is an intermediate in the β-ketoadipate pathway and can be produced from benzene, toluene, benzoic acid, or catechol (Fig. 1).

Several studies have been performed to produce *cis,cis*-muconic acid from toluene, benzoic acid and catechol *via* microbial transformation. In U. S. patents, productions of *cis,cis*-muconic acid from toluene using a mutant strain of *Pseudomonas putida* lacking *cis,cis*-muconic acid degrading enzymes were reported [1, 2]. Production of *cis,cis*-muconic acid from catechol was attempted by Gomi but the product yield and stability of the strain was rather low [3]. Two groups have reported on the production of *cis,cis*-muconic acid from benzoic acid [4, 5]. Kuwahara *et al.* used a mutant stain of *Corynebacterium glutamicum*, but its productivity and yield were rather low [4]. Mizuno *et al.* produced 44.1 g/L of *cis,cis*-muconic acid using an *Arthrobacter* sp., a kind of coryneform bacteria for 48 h by succesive feeding of small amounts of benzoate [5]. These starting materials are fairly cheap, but have strong toxic effects on microbes. Moreover, most of the enzymes catalyzing the reaction are unstable oxygenases. Thus, it is necessary to overcome these problems for the microbial production of *cis,cis*-muconic acid.

In this paper, the mutant strain of *Pseudomonas putida* BM014 was developed for the production of *cis,cis*-muconic acid and the DO-stat fed-batch culture was attempted to enhance *cis,cis*-muconic acid productivity.

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MATERIALS AND METHODS

Microorganisms and culture conditions

The wild-type *Pseudomonas putida* HL06-3 was isolated from soil samples which obtained around the storage tanks of crude oil and aromatic compounds. The mutant strain, *Pseudomonas putida* BM014, was originated from the wild-type HL06-3 strain. Stock cultures of HL06-3 and BM014 were maintained on nutrient Y medium agar plates, consisting of (per litre of water) bacto-peptone 10 g, yeast extract 5 g, NaCl 10 g, and agar 15 g, and the pH was adjusted to 7.0.

P. putida BM014 was cultured in the NO3 mineral salt medium of the following compositions: $Na_2HPO_4 \cdot 12H_2O$ 18 g/L, KH_2PO_4 13.6 g/L, $(NH_4)_2SO_4$ 2.25 g/L, $MgSO_4 \cdot 7H_2O$ 3 mM, $CaCl_2 \cdot 2H_2O$ 0.3 mM, and $FeSO_4 \cdot 7H_2O$ 0.03 mM, and, if necessary, the NO3 mineral salt medium was supplemented with appropriate amount of sodium benzoate and/or glucose.

Authentic *cis,cis*-muconic acid was prepared from catechol by chemical synthesis as described by Elvidge *et*

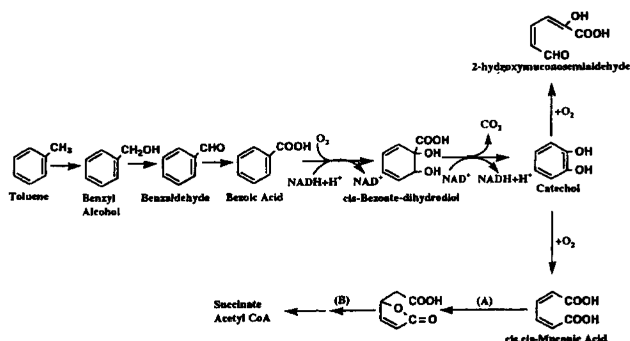

Fig. 1. Metabolic pathways of benzoic acid transformation in *Pseudomonas* sp.; (A) Muconate lactonizing enzyme, (B) Muconolactone isomerase.

al. [6]. The *trans,trans*-muconic acid was purchased from Fisher Chemical Co. Ltd., and benzoic acid and all other chemicals were of analytical grade.

Analytical methods

Bacterial concentrations were determined by measuring optical density of diluted samples at 670 nm and expressed as g dry weight/L.

Catechol 1,2-dioxygenase activity was assayed at 30°C as previously described [7].

The protein contents of crude cell extracts were measured by the Folin-Lowry method using bovine serum albumin as a standard [8].

cis,cis-Muconic acid and benzoic acid were determined by high performance liquid chromatography (HPLC) and gas chromatography (GC). For HPLC, C_{18} μ-Bondapack column was used and a mixture of water, methanol and phosphoric acid in the ratio of 700:300: 0.2 or of water and acetonitrile in the ratio of 60:40 was used as a eluent at a flow rate of 0.6 ml/min. For GC, the sample was treated by acid-catalyzed esterification with methanol. To the 2.0 ml of methanol containing 5-10%(w/v) concentrated H_2SO_4, 0.2 ml of sample and 0.2 ml of internal standard (10 g/L of sodium octanoate) were added. After vigorous mixing, the mixture was incubated at 100°C for 1 h and cooled down to room temperature and 1 ml of chloroform was added to the mixture, followed by vortexing to extract the esterified samples. The organic phase containing the esterified samples was separated by removing the supernatant aqueous phase and 0.5 g of Na_2SO_4 was added to the remaining organic phase to eliminate water completely. These esterified samples were dissolved in chloroform and analyzed by GC.

Screening and mutations

The soil samples obtained around the stroage tanks of crude oils and aromatic compounds were suspended in sterilized saline solution and filtered by cotton filter. The filtrates were inoculated to the Y medium and cultured in shaking incubator at 30°C for 12 h. The cultured broths were spread on the NO3 minimal medium agar plates containing 1~1.5% sodium benzoate as a sole carbon source and incubated for 48 h. The isolated colonies were tested for its activities of benzoate assimilation and accumulation of *cis,cis*-muconic acid. Among the isolated colonies, strains could assimilate benzoate *via* ortho ring cleavage of catechol were selected by the method previously described [9].

For the mutation, we used the chemical mutagen N-methyl-N'-nitro-N-nitrosoguanidine (NTG) at a final concentration of 150 μg/ml. The mutants were enriched on the medium containing p-hydroxybenzoate and antibiotics (Penicillin G and D-cycloserine) to improve the selection efficiency [10]. The isolated colonies from the enriched mutant culture broth were tested for the blockage of assimilation pathway of benzoate and accumulation of *cis,cis*-muconic acid using minimal medium supplemented with sodium benzoate or succinate.

DO-stat fed-batch culture

Cultures were carried out in the 2.5 L volume reactor and the system enables to monitor the temperature, pH, dissolved oxygen tension (DOT), oxygen uptake rate (OUR), carbon dioxide evolution rate (CER) and rpm with real time and to control the temperature, pH, DOT and feed rate. The medium was fed by the peristaltic pump connected to the DO channel. Especially, when the membrane and electrolyte of DO electrode used for the fed-batch operation were replaced, it was calibrated in the buffer solution with nitrogen and air.

RESULTS AND DISCUSSION

Screening of benzoate-assimilating strains and development of *cis,cis*-muconic acid producing strain

Among the benzoate-assimilating strains isolated from soil samples, strain HL06-3 showed a high p-hydroxybenzoate-assimilating activity and degraded benzoate *via* ortho ring cleavage of catechol. Thus, HL06-3 identified as *Pseudomonas putida* was selected as a wild-type strain for *cis,cis*-muconic acid producing mutant. The metabolic pathways of D-mandelate and p-hydroxy-D-mandelate in *Pseudomonas putida* are shown in Fig. 2 [11]. Benzoate and p-hydroxybenzoate are intermediates in both pathways and finally metabolized to succinate and acetyl-CoA *via* enol-lactone [12]. The mutants lacking in muconate lactonizing enzyme (A) or muconolactone isomerase (B) obtained by NTG treatment were incubated in p-hydroxybenzoate minimal medium and transferred to benzoate minimal medium containing 0.1 mg/ml of D-cycloserine and penicillin G (10^4 units/ml). This enrichment process was repeated 4-6 times to enhance the selection efficiency for *cis,cis*-muconic acid accumulating mutant. Four lytic cycles of enrichment process enhanced the occurrence ratio of benzoate non-assimilating mutants up to 10^{-2} and six lytic cycles to 1.3×10^{-1}. Among the mutant colonies which could not grow in benzoate minimal medium, 70% of them accumulated *cis,cis*-muconate. Although some part of these strains lost *cis,cis*-muconate accumulating activity during the repeated cultures, the majority produced *cis,cis*-muconate with stable conversion activity. The mutants capable of accumulating *cis,cis*-muconate showed large differences

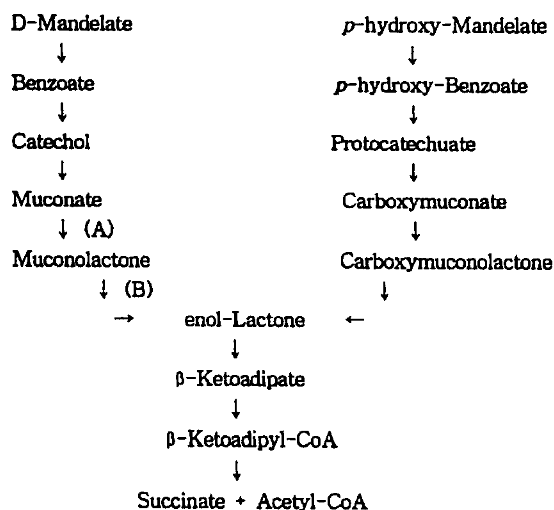

Fig. 2. Metabolic pathways for the transformation of D-mandelate and p-hydroxy-D-mandelate; (A) Muconate lactonizing enzyme, (B) Muconolactone isomerase.

Table 1. Accumulated amounts of *cis,cis*-muconic acid when the isolated mutant colonies were cultured in Y medium supplemented with 10 mM of sodium benzoate for 24 h

Colony NO.	Concentration of *cis,cis*-muconic acid (mM)
BM 011	9.01
BM 012	4.33
BM 013	8.72
BM 014	9.78
BM 015	6.21
BM 016	9.36
BM 017	8.39
BM 018	9.15
BM 019	0.84
BM 020	ND

ND: not detected

in their conversion activity (Table 1). The mutant strains with higher conversion activities were considered to be deleted in muconate lactonizing enzyme (A) and muconolactone isomerase (B) simultaneously but the others seemed to have deletion in muconolactone isomerase (B) alone, resulting in further assimilation of muconate to muconolactone.

From the 120 mutant colonies obtained, strain BM 014 was finally selected as the production strain due to its higher and stable conversion activity as well as rapid growth rate.

Batch culture of *P. putida* BM014

Pseudomonas putida BM014 has shown the typical exponential growth pattern when cultured in NO3 medium supplemented with glucose as a sole carbon source. After 3 h of lag period, exponential growth continued for 10 h. However, when the culture was performed in the NO3 medium supplemented with 10 g/L of glucose, 5 g/L of yeast extract and 10 mM of benzoate, growth inhibition by benzoate caused the extension of the lag period up to 5-6 h as shown in Fig. 3. Before the exponential growth phase, all the benzoate were converted to *cis,cis*-muconic acid and accumulated muconate remained constant in the reactor.

Induction of metabolic enzymes

It is known that the first two enzymes in the *cis,cis*-muconate biosynthetic pathway, benzoate dioxygenase and dihydrodihydroxybenzoate dehydrogenase, are induced by benzoate [9]. The key enzyme is the third enzyme, catechol 1,2-dioxygenase, converting catechol to *cis,cis*-muconic acid [9]. In *Pseudomonas* species, catechol 1,2-dioxygenase is inducible by the product, *cis,cis*-muconic acid [13]. Because the wild-type strains of Pseudomonad are generally non-permeable to dicarboxylic acid, such as *cis,cis*-muconic acid, they can not grow on *cis,cis*-muconate minimal medium [10]. *Pseudomonas putida* HL06-3 strain was also shown to be non-permeable to *cis,cis*-muconic acid and catechol 1,2-dioxygenase was not induced by the externally added *cis,cis*-muconic acid (data not shown).

Fig. 4 shows the specific activities of catechol 1,2-dioxygenase at various initial concentration of benzoate. At benzoate concentrations up to 10 mM, specific activities were increased but rapidly decreased above 10 mM of benzoate. The inducing effect of benzoate may be an apparent phenomenon caused by the

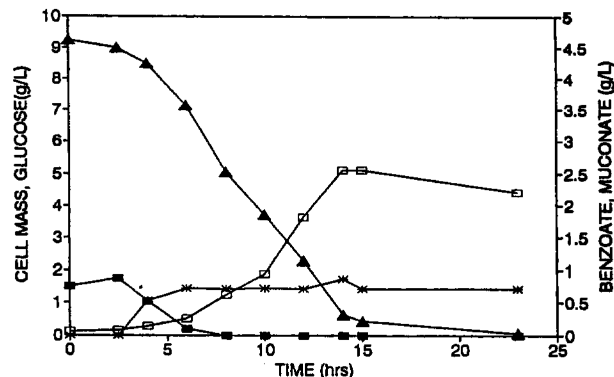

Fig. 3. Time course curve of batch culture in NO3 medium containing 10 g/L of glucose, 5 g/L of yeast extract and 10 mM of sodium benzoate; □ Cell mass, ▲ Glucose, ■ benzoate, * *cis,cis*-muconate.

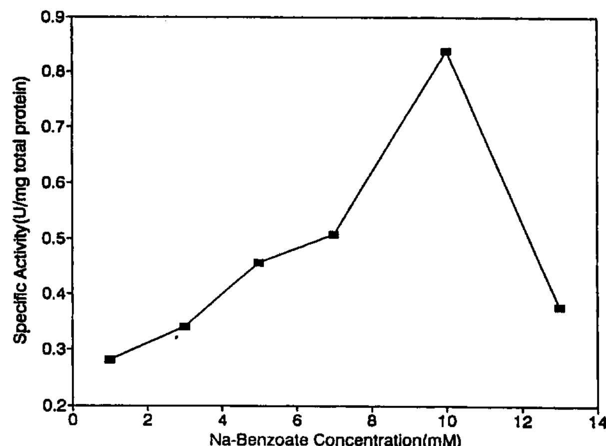

Fig. 4. Catechol 1,2-dioxygenase activities vs. various initial benzoate concentrations; one unit of catechol 1,2-dioxygenase is defined as 1 mole of catechol oxidized per mg of protein for 1 min.

muconic acid intracellularly generated from benzoate.

Cell growth inhibition by benzoate

Although benzoic acid is rather mild as compared with other aromatic bulk chemicals such as toluene, it still has the inhibition effect on cell growth. The inhibition effect is a expected phenomenon, considering that it has been used as the antiseptic agents for foods and drinks. Pirt *et al.* have explained the cell growth inhibition by chemicals in terms of the competitive and non-competitive enzyme kinetics [14]. Generally, the cell growth inhibition by non metabolizable chemicals was regarded as non-competitive inhibition.

Following such a view, the inhibition effect of benzoate on cell growth in the current study was assumed as non-competitive inhibition. The model equation of cell growth was taken as

$$\mu = \frac{\mu_{max} \cdot S}{K_s + S} \cdot \frac{1}{\alpha} \qquad \alpha = 1 + i/K_i$$

, where K_i, K_S, and i are inhibition constant, saturation constant for glucose, and benzoate concentration, respectively. μ_{max} and K_i were estimated to be 0.632 h^{-1} and 1.471 g/L, respectively. Considering the effects of benzoate on enzyme induction and cell growth in-

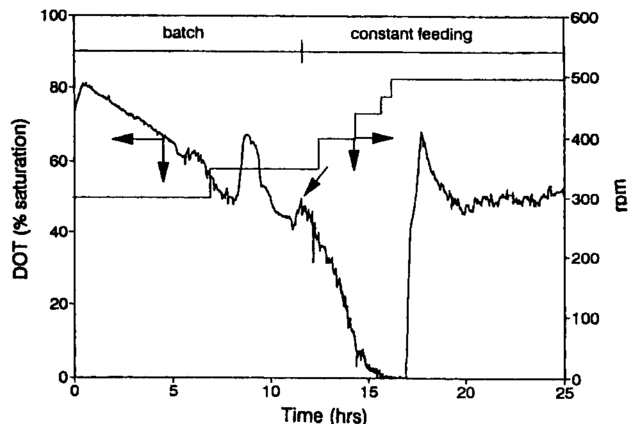

Fig. 5. Time course DOT profile of batch culture and constant feeding fed-batch culture. The arrow at 12 h indicates the starting point of constant feeding.

hibition, the initial concentration of benzoate in fed-batch culture was controlled at a value between 7 mM and 10 mM.

Fed-batch production *via* DO-stat feeding

DO-stat fed-batch culture is commonly performed when the growth inhibitory substrate is used or when the high cell density culture is carried out [15-17]. Based upon the fact that the biotransformation from benzoic acid to *cis,cis*-muconic acid, in addition to the assimilation of glucose as a main carbon source for cell growth, consumes much oxygen for its oxidative pathway, the DO-stat method for feeding benzoic acid was employed in order to control the benzoic acid concentration with a view to its inhibitory effect on cell growth and enzyme activity.

In the fermentation run shown in Fig. 5, earlier part of the run was carried out in batch mode and later culture was changed to a constant feeding mode. The feeding medium was NO3 medium supplemented with sodium benzoate and glucose at concentration of 50 g/L and 30 g/L, respectively. The flow rate during constant feeding period was 7.5 ml/h. The DOT profile in Fig. 5 can be interpreted in terms of the balance between oxygen supply and demand, and intracellular assimilation of benzoate, glucose, and their metabolic intermediates. Based upon observing the sensitive response of DOT profile to substrate concentrations, DOT was selected as a parameter upon which to base the feed control method [18].

Fig. 6A shows the changes in cell mass, substrate and product concentrations during the DO-stat fed-batch operation and the corresponding change in DOT profile is shown in Fig. 6B. The batch operation was started with the NO3 medium supplemented with 10 g/L of glucose and 1 g/L of sodium benzoate. When the initially added benzoate was completely consumed, feeding solution (Glucose 60 g/L, benzoate 100 g/L, $(NH_4)_2SO_4$ 9 g/L) was fed at the constant rate of 7.7 ml/h. The switchover from constant feeding operation to DO-stat feeding operation was dictated by the rapid increase in DOT value as shown in Fig. 6B. During the DO-stat feeding operation, when the DOT value exceeded the upper deviation bound from the set point (50% of air saturation) by 5%, 2 ml of feeding solution (Glucose 60 g/L, benzoate 100 g/L, $(NH_4)_2SO_4$ 9 g/L) was fed within 30

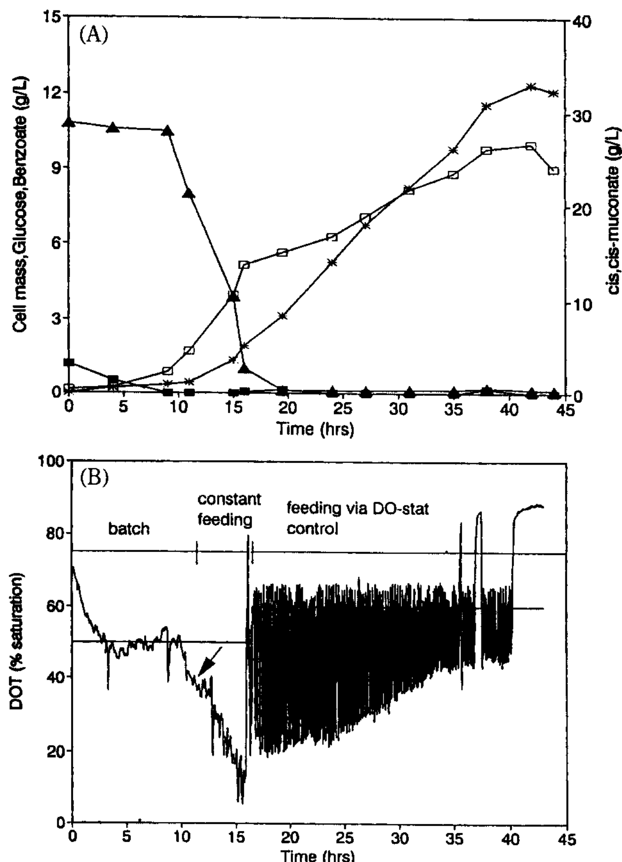

Fig. 6. Fed-batch production of *cis,cis*-muconic acid *via* DO-stat feeding; (A) □ Cell mass, ▲ Glucose, ■ benzoate, * *cis, cis*-muconate; (B) DOT profile. The arrow at 12 h indicates the starting point of constant feeding.

seconds. The concentration of *cis,cis*-muconate reached 32.4 g/L at 40 h. Considering the amount of benzoate supplied, the conversion yield reached almost the theoretical value of 100%. The productivity of fed-batch operation excluding initial induction time was 1. 16 g/(L·h). Considering the DO-stat feeding period only, productivity was calculated to be 1.4 g/(L·h).

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