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Enhancement of *cis,cis*-Muconate Productivity by Overexpression of Catechol 1,2-Dioxygenase in *Pseudomonas putida* BCM114

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For enhancement of *cis,cis*-muconate productivity from benzoate, catechol 1,2-dioxygenase (C12O) which catalyzes the rate-limiting step (catechol conversion to *cis,cis*-muconate) was cloned and expressed in recombinant *Pseudomonas putida* BCM114. At higher benzoate concentrations (more than 15 mM), *cis,cis*-muconate productivity gradually decreased and unconverted catechol was accumulated up to 10 mM in the case of wild-type *P. putida* BM014, whereas *cis,cis*-muconate productivity continuously increased and catechol was completely transformed to *cis,cis*-muconate for *P. putida* BCM114. Specific C12O activity of *P. putida* BCM114 was about three times higher than that of *P. putida* BM014, and productivity was enhanced more than two times.

Key words: *Pseudomonas putida* BCM114, *cis,cis*-muconate, *cat*A cloning, catechol 1,2-dioxygenase

cis,cis-Muconic acid is potentially useful as a precursor for new functional resins, pharmaceuticals, and agrochemicals. Several attempts to produce *cis, cis*-muconic acid from toluene, benzoic acid or catechol by microorganisms have been reported [1-3]. More recently *cis,cis*-muconate was produced from glucose *via* a novel biosynthetic pathway reconstructed by metabolic engineering [4]. In our previous work, production of *cis,cis*-muconate from benzoate by *Pseudomonas putida* BM014 was performed in a DO-stat fed-batch culture [5], immobilized bioreactor and a cell-recycle bioreactor [6] with a maximum productivity of 5.5 g/(l·h), the highest productivity to the best of our knowledge.

For the further enhancement of the *cis,cis*-muconate productivity, it is essential to develop a better strain as well as a biotransformation process. In the benzoate degradation pathway, the conversion step of catechol to *cis,cis*-muconate catalyzed by catechol 1,2-dioxygenase (C12O) is the rate-limiting step [7]. Overexpression of this unstable enzyme is indispensable for a higher *cis,cis*-muconate production rate. In this work, recombinant *P. putida* BCM114 was developed to overexpress C12O for enhancing *cis,cis*-muconate productivity.

P. putida BM014, *E. coli* S17-1 and cosmid vector pVK100 were used to develop the recombinant strain, *P. putida* BCM114. *CatA* gene fragment originating from *P. aeruginosa* PAO [8] was employed as a probe DNA for hybridization. All strains and plasmids used in this study are shown in Table 1.

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P. putida BM014 was cultivated in Y medium [11] and all genetic manipulations were routinely performed according to manuals [12]. The genomic DNA from *P. putida* BM014 was digested with *Eco*RI and ligated with pVK100. This ligation mixture was treated with *in vitro* packaging kit (STRATAGENE Gigapack III Gold Packaging Extract) and then transferred into *E. coli* S17-1 grown in LB supplemented with 0.2% maltose and 10 mM $MgSO_4$. All restriction enzymes were purchased from Promega, and calf intestinal alkaline phosphatase and T4 DNA ligase were purchased from New England Biolabs Inc.

The membrane used in hybridization was Hybond TM-N+ (Amersham). Probe DNA was labeled by biotinylated dUTP with a nick translation kit (Gibco BRL Nick Translation System or Promega Nick T ranslation System) or by [^{32}P]dCTP (5,000 Ci/mmol,

Table 1. Strains and plasmids

Strains and plasmids	Characteristics	Reference
P. putida BM014	conversion of benzoate to *cis,cis*-muconate	5
E. coli S17-1	*tra*-gene of RP4 plasmid chromosomally integrated	9
pVK100	23 kb, cosmid, Km^r, Tc^r, mob^+, tra^-	10
pGEM3Z : *Pst*I-6	3.9 kb *Pst*I *cat*A-containing fragment from pRO2337 in pGEM3Z	8

Dupont) with a random primer extension kit (Promega Prime-a-Gene Labeling System).

For mating of *E. coli* S17-1 harboring the recombinant plasmid with a recipient, *P. putida* BM014, the spot agar mating technique was applied [13]. *P. putida* BM014, grown in a nutrient broth, was mixed with a equal volume of *E. coli* S17-1. This mixture (0.5 *ml*) was spotted on a nutrient broth agar plate and incubated at 30℃ overnight. The transconjugants were selected on NO3 [11] agar plates supplemented with 0.3 M 1,3-butanediol and 12.5 mg tetracycline/*l*.

The recombinant *P. putida* BCM114 was cultured for the determination of *cis,cis*-muconate productivity and specific C12O activity. Flask cultures (50 *ml*) were performed using NO3 medium supplemented with 25 μg tetracycline/*ml*. Benzoate was added at different concentrations (5-20 mM). Cells were harvested, resuspended in 100 mM KH_2PO_4 buffer (pH 7.5), and disintegrated by ultrasonicater. Specific activity of C12O was determined by the method previously described [6].

To identify the *catA* gene of *P. putida* BM014, total genomic DNA was isolated and digested with *Eco*RI, *Hind*III, *Sal*I, *Xho*I, or *Bam*HI. The DNA fragments were transferred to a nylon membrane and hybridized with a ^{32}P-labeled *catA* fragment of *P. aeruginosa* PAO [8]. Hybridization signals occurred at one 20 kbp *Eco*RI-fragment, two small *Hind*III-fragments, and several small *Sal*I and *Xho*I-fragments. To clone the 20 kbp *Eco*RI-fragment, a genomic library was constructed in *E. coli* S17-1. The genomic DNA was partially digested with *Eco*RI and ligated with *Eco*RI-restricted cosmid vector, pVK100. The ligation mixture was *in vitro* packaged and infected into *E. coli* S17-1. Four positive clones were identified from 6,000 candidates by colony hybridization with the ^{32}P-labeled probe of 3.9 kbp *Pst*I-fragment of C12O locus from *P. aeruginosa* PAO. The recombinant plasmids were isolated from positive candidates, digested with *Eco*RI, and then hybridized with the ^{32}P-labeld probe. Hybridization signals clearly occurred in the 20 kbp *Eco*RI-fragment and the resulting recombinant plasmid was named pVKcat239.

The hybrid plasmid pVKcat239 was conjugatively transferred from *E. coli* S17-1 to *P. putida* BM014 on NO3 agar plates containing 0.3 M 1,3-butanediol as the sole carbon source and 12.5 mg/*l* tetracycline. Because *E. coli* S17-1 can't grow on 1,3-butanediol and plasmid pVKcat239 is resistant to tetracycline, *P. putida* BM014 harboring pVKcat239 can only grow on this agar plate. After several days incubation at 30℃, twenty-five transconjugants appeared. These transconjugants were cultured in nutrient Y medium supplemented with 7 mM benzoate and tetracycline, and specific activities of C12O were measured to identify whether C12O was overexpressed or not. The recombinant *P. putida* BCM114 had the highest specific C12O activity of 1.94 U/mg protein, which was two times higher than that of wild-type *P. putida* BM014. The overexpression of C12O was also confirmed in SDS-PAGE analysis of crude extracts for both strains. As shown in Fig. 1, there was no marked difference in any protein band for crude extracts of *P. putida* BM014 cultured at various benzoate concentrations (5-20 mM). In the case of *P. putida* BCM114, however, overexpression of C12O was clearly observed at a molecular weight of around 35 kDa. Based on the well known fact that the molecular weight of C12O is about 34 kDa [14-16], we concluded that cloning of *catA* encoding C12O was successfully expressed in recombinant *P. putida* BCM014. It is of interest that another protein whose molecular weight is approximately 41 kDa also seemed to be overproduced. Since other genes, e.g. genes for benzoate dioxygenase [17, 18],

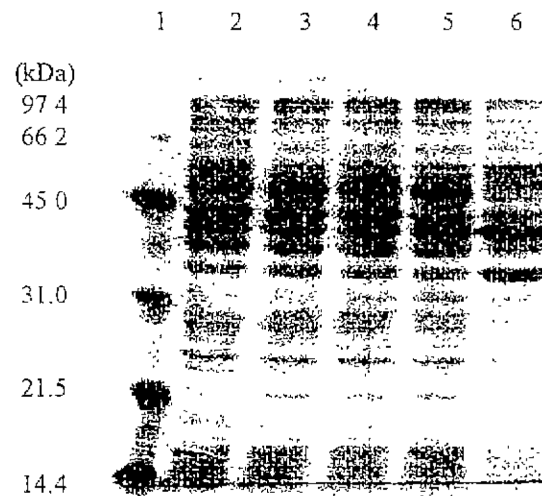

Fig. 1. SDS-PAGE analysis of crude extracts for *P. putida* BM014 and recombinant *P. putida* BCM114.Lane 1, marker; lane 2-5, crude extracts of *P. putida* BM014 cultured at various benzoate concentrations (5-20 mM); lane 6, crude extracts of *P. putida* BCM114 cultured at 5 mM benzoate.

Table 2. Production of *cis,cis*-muconate by *P. putida* BM014 and recombinant *P. putida* BCM114.

Benzoate (mM)	Final cell conc. (g dry weight/*l*)		Catechol (mM)		Productivity (mg/*l* · h)		Specific activity of C12O (U/mg protein)	
	P. putida BM014	*P. putida* BCM114	*P. putida* BM014	*P. putida* BCM114	*P. putida* BM014	*P. putida* BCM114	*P. putida* BM014	*P. putida* BCM114
5	1.8	0.82	0	0	16	16	1.2	2.7
10	1.3	0.40	0	0	27	36	1.3	3.6
15	0.96	0.35	1.3	0	22	46	0.9	3.7
20	0.79	0.32	1.1	0	20	45	1.0	3.1

closely related to *catA* in the benzoate conversion pathway are also clustered in plasmid pVKcat239, this gene product might be overexpressed simultaneously with C12O.

P. putida BM014 was cultivated at 30℃ for 26 h in NO3 medium containing 10 g glucose/*l* as the growth carbon source and benzoate at concentrations ranging from 5 to 20 mM. Recombinant *P. putida* BCM114 was also cultured in the same medium supplemented with 25 μg tetracycline/*ml*. As shown in Table 2, benzoate inhibited cell growth for both *P. putida* BM014 and *P. putida* BCM114, and the final cell concentration of *P. putida* BCM114 was lower than that of *P. putida* BM014 due to the growth inhibition caused by tetracycline as well as benzoate. However, *cis,cis*-muconate productivity gradually decreased and unconverted catechol accumulated up to 10 mM in case of *P. putida* BM014, whereas *cis,cis*-muconate productivity continuously increased and catechol was completely transformed to *cis,cis*-muconate even at higher benzoate concentrations for *P. putida* BCM114. From the values of specific activities of C12O for both strains, the enhancement of productivity is mainly caused by the overexpression of C12O in recombinant *P. putida* BCM114. Specific C12O activity of *P. putida* BCM 114 was about three times higher than that of *P. putida* BM014, and productivity was enhanced more than two times. The biotransformation process employing recombinant *P. putida* BCM114 is being developed and volumetric productivity is expected to be further enhanced more than 5.5 (g/*l* · h).

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