Production of (*R*)-3-Chloro-1,2-Propanediol from Prochiral 1,3-Dichloro-2-Propanol by *Corynebacterium* sp. Strain N-1074

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The production of (*R*)-3-chloro-1,2-propanediol [(*R*)-MCP] from prochiral 1,3-dichloro-2-propanol (DCP) was examined with a bacterial strain identified as a *Corynebacterium* strain. The addition of glycerol as a carbon source or some chlorinated alcohols to a medium was effective for the induction of activity catalyzing the transformation of DCP into MCP. The optimum pH for (*R*)-MCP production by the resting cell reaction was around 8.0. The optical purity of (*R*)-MCP formed was improved by keeping the level of DCP in the reaction mixture at a low concentration. (*R*)-MCP was obtained from 77.5 mM DCP with a 97.3% molar conversion yield and an 83.8% enantiomeric excess of its optical purity by periodic feeding of the substrate.

Optically active glycerol derivatives, e.g., optically active 3-chloro-1,2-propanediol (MCP) and epichlorohydrin, are very important starting materials for the synthesis of various optically active compounds (1, 9, 11). Although optically active MCP and epichlorohydrin can be synthesized from D-mannitol or its derivative by chemical reactions (1, 6, 13), these methods are so complicated as to be inapplicable to industrial production. Some microbial methods for the production of optically active MCP from racemic MCP or 2,3-dichloro-1-propanol have been reported previously (14, 20). However, such optical resolution methods of racemic substrates have the disadvantage that the resulting yield of the desired enantiomer is less than 50% unless the residual substrates are racemized. From an industrial point of view, we have attempted the manufacture of optically active compounds by the method of enantioselective microbial transformations of prochiral starting materials, expecting a quantitative yield of the desired enantiomer. Recently, we found that several bacterial strains isolated from soil samples were able to transform prochiral 1,3-dichloro-2-propanol (DCP) into optically active MCP (16).

In this article, we describe the production of (*R*)-MCP from DCP by one of these strains, *Corynebacterium* sp. strain N-1074.

MATERIALS AND METHODS

Chemicals. DCP, (*R,S*)-MCP, (*R*)-MCP, and Extract Ehlrich were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Polypeptone and yeast extract were purchased from Oriental Yeast (Tokyo, Japan). All other chemicals were obtained from commercial sources.

Microorganism and culture conditions. Bacterial strain N-1074, which was previously isolated from a soil sample (16), was used in this study. This strain was identified according to the classification described in *Bergey's Manual of Systematic Bacteriology* (12). The amino acid composition of peptidoglycan was determined by the method of Schleifer and Kandler (19).

Subcultivation was carried out at 30°C for 24 h with reciprocal shaking in a test tube containing 10 ml of a

medium (pH 7.0) consisting of 10 g of glucose, 5 g of polypeptone, 3 g of yeast extract, and 3 g of Extract Ehlrich per liter of tap water. In culture condition experiments, 0.1 ml of the subculture was inoculated into a test tube containing 10 ml of a basal medium (pH 7.2) supplemented with 1% (wt/vol) carbon source and 0.2% (wt/vol) inducer. The basal medium consisted of 2 g of (NH₄)₂SO₄, 2.5 g of KH₂PO₄, 5 g of Na₂HPO₄, 0.1 g of MgSO₄ · 7H₂O, and 2 g of yeast extract per liter of tap water. The cultivation was carried out at 30°C for 46 h with reciprocal shaking. In the other experiments, 1 ml of the subculture was inoculated into a 500-ml Erlenmeyer flask containing 100 ml of the basal medium supplemented with 1% (wt/vol) glycerol and 0.2% (wt/vol) MCP. The cultivation was carried out at 30°C for 48 h with shaking.

Preparation of resting cells. Cells were harvested by centrifugation at 10,000 × *g* for 20 min and washed once with 100 mM Tris-HCl buffer (pH 8.0). Then the cells were suspended in 1 M Tris-HCl buffer. This cell suspension was used for the resting cell reaction. The cell concentration was estimated turbidimetrically by means of a dry cell calibration curve of the *A*₆₃₀: 0.461 mg (dry weight) of cells per ml was equivalent to an optical density at 630 nm of 1.0.

Assay for enzyme activity. In culture condition experiments, the MCP-producing activity was assayed in a standard reaction mixture (2 ml) containing 77.5 mM DCP, 500 mM Tris-HCl buffer (pH 8.0), and an appropriate amount of resting cells at 20°C for 3 h. In other cases, the reaction conditions were as described below. All data were expressed as the means of triplicate experiments and were consistent within ±5% or less.

Analytical methods. The amounts of DCP and MCP were measured by gas-liquid chromatography. Gas-liquid chromatography was performed with a Shimadzu GC-4CM system (Kyoto, Japan) equipped with a flame ionization detector with a glass column (3 mm by 1 m) packed with OV-225 on 80- to 100-mesh Chromosorb W (Gasukurokogyo, Inc., Tokyo, Japan). The injection port and column temperatures were set at 250 and 160°C, respectively. N₂ was used as the carrier gas at a flow rate of 50 cm³/min.

The optical purity of the MCP formed was determined by high-performance liquid chromatographic (HPLC) analysis of its *p*-toluenesulfonate derivative (2-hydroxy-3-chloropro-

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TABLE 1. Effects of carbon sources and chlorinated compounds added to the medium on formation of activity catalyzing the transformation of DCP into MCP^a

Carbon source	Chlorinated compound	Growth ^b	Activity ^c
Glycerol	None	5.45	2.45
	NaCl	5.15	1.96
	MCP	5.81	3.14
	DCP	3.33	2.96
	2-Chloroethanol	4.85	3.15
	1-Chloro-2-propanol	1.11	2.83
Sucrose	None	8.03	0.581
	NaCl	7.47	0.581
	MCP	7.12	1.81
	DCP	2.12	0.614
	2-Chloroethanol	6.62	1.39
	1-Chloro-2-propanol	1.52	2.43
Glucose	None	6.06	0.233
	NaCl	6.06	0.233
	MCP	3.94	1.62
	DCP	1.11	2.74
	2-Chloroethanol	5.81	0.821
	1-Chloro-2-propanol	1.52	0.928

^a Cultivation was carried out at 30°C for 46 h with shaking as described in Materials and Methods.

^b Shown as the optical density at 630 nm.

^c Using resting cells, the formation rate of MCP from DCP was measured by the method described in Materials and Methods. Activity is expressed as micromoles per hour per milligram (dry weight) of cells.

pyl *p*-toluenesulfonate) (9). MCP was extracted from a reaction mixture with ethyl acetate. After drying over anhydrous sodium sulfate, the extract was evaporated to remove the solvent and residual DCP. The *p*-toluenesulfonate derivative of MCP was prepared by the reaction with *p*-toluenesulfonyl chloride in pyridine (6). HPLC was performed with a JASCO 800 series system (Tokyo, Japan) equipped with a Chiralcel OC column (4.6 by 250 mm; Daicel Industries Ltd., Tokyo, Japan) by using hexane-2-propanol (98.5:1.5 [vol/vol]) as the eluent at a flow rate of 2.5 ml/min. The A_{235} was measured. Optical rotations were measured with a JASCO DIP-360 apparatus.

¹H and ¹³C nuclear magnetic resonance (NMR) spectra were recorded in CDCl₃ by using tetramethylsilane as an internal standard with a Nihondenshi JNM GX-270 (Tokyo, Japan) apparatus. Infrared and mass spectra were recorded with a Perkin-Elmer 1710 FT-IR (Norwalk, Conn.) and a Hitachi M-80 (Tokyo, Japan) apparatus, respectively.

RESULTS

Identification of the microorganism. Previously, bacterial strains which transform DCP into optically active MCP were isolated from soil samples. We have used one of these bacteria, strain N-1074, for further experiments. This bacterium was a gram-positive, aerobic, non-spore-forming, and irregular rod-shaped (0.3 to 0.5 by 0.6 to 1.5 μ m) organism. Oxidase and urease were negative. Catalase was positive. The cell wall peptidoglycan is based on diaminobutyric acid, and *meso*-diaminopimelic acid was not observed in the cell hydrolysate. On the basis of these results, strain N-1074 was identified as a plant-pathogenic species of *Corynebacterium*.

Culture conditions for *Corynebacterium* sp. strain N-1074. The effects of carbon sources and chlorinated compounds as inducers on the formation of activity transforming DCP into MCP were examined with basal medium (Table 1). The activity was effectively formed with glycerol as a carbon

FIG. 1. Effects of pH on MCP formation and optical purity. The reaction was carried out at 20°C for 3 h in a reaction mixture (50 ml) consisting of 77.5 mM DCP, an appropriate amount of cells, and 1 M potassium phosphate (○), Tris-HCl (●), or Tris-H₂SO₄ (△). The formation rate of MCP with Tris-HCl buffer (pH 8.0), corresponding to 3.06 μ mol/h/mg (dry weight) of cells, was taken to be 100%.

source without any inducers. The activity was barely formed with glucose or sucrose as a carbon source without inducers, and the addition of MCP resulted in an increase of activity. The addition of some chlorinated alcohols also gave similar results. Considering the cell growth and the specific activity, we used the basal medium supplemented with 1% (wt/vol) glycerol and 0.2% (wt/vol) MCP for further experiments.

Reaction conditions for production of (R)-MCP by resting cells. The effects of pH on the formation of MCP and on its optical purity were examined (Fig. 1). The formation rate of MCP was elevated at high pHs. The optical purity of (R)-MCP formed was affected by pHs and kinds of buffer for the reaction, and using Tris-HCl buffer (pH 8.0) gave the highest optical purity of (R)-MCP. The reaction at a pH above 8.0 caused a decrease in the yield of (R)-MCP formed, since DCP was unstable at high pHs (data not shown). The effect of the substrate concentration on the formation rate of MCP was also examined (Fig. 2). The formation rate was highest at about 5 to 200 mM DCP, and a high concentration of DCP, more than 400 mM, caused a significant decrease in MCP-producing activity. This marked decrease in the activity seemed to be a result of an inactivation by a high concentration of DCP, because the cells lost about 90% of the activity when incubated at 20°C for 30 min in 1 M Tris-HCl buffer (pH 8.0) containing 400 mM DCP. The optical purity of (R)-MCP was also affected by the DCP concentration in the reaction mixture, and the reaction at a low concentration of the substrate gave a higher optical purity of the product (Table 2).

Production of (R)-MCP from DCP. Figure 3A shows the course of the formation of (R)-MCP from DCP by the resting cell reaction. The reaction was carried out at 20°C in a reaction mixture (30 ml) consisting of 77.5 mM DCP, 1 M Tris-HCl buffer (pH 8.0), and 153 mg (dry weight) of cells. The formation of MCP from DCP was almost stoichiometric. The product was extracted from the reaction mixture with ethyl acetate (50 ml three times) after the cells were removed by centrifugation for 20 min at 10,000 $\times$ g. After drying over anhydrous sodium sulfate, the extract was evaporated to remove the solvent and residual DCP. The infrared spectrum

FIG. 2. Effect of the concentration of DCP on the formation of MCP. The MCP-producing activity was measured in a reaction mixture (10 ml) containing DCP, 1 M Tris-HCl buffer (pH 8.0), and an appropriate amount of cells at 20°C for 3 h. The maximum activity corresponding to 3.06 $\mu\text{mol/h/mg}$ (dry weight) of cells at 77.5 mM DCP was taken to be 100%.

of the product thus obtained agreed well with that of authentic MCP (data not shown). Identification by ^1H NMR, ^{13}C NMR, and mass spectroscopy was also performed. Data were as follows: ^1H NMR (CDCl_3) δ 3.5 to 3.8 (4H, m), 3.9 (1H, m), 4.2 (2H, s); ^{13}C NMR (CDCl_3) δ 45.6, 63.6, 71.7; EI-MS m/z 79, 81 ($\text{M}^+ - \text{CH}_2\text{OH}$), 61 ($\text{M}^+ - \text{CH}_2\text{Cl}$). The optical purity of resulting (R)-MCP was determined to be 72.2% enantiomeric excess by HPLC analysis of its *p*-toluenesulfonate derivative. The resulting MCP gave a $[\alpha]_D^{25}$ of -5.56° (concentration = 1.78, H_2O).

A periodic substrate feeding system was attempted for the production of (R)-MCP to keep the level of DCP at a low concentration, since the optical purity of the product was expected to improve (Fig. 3B). During the course of the reaction, about 15.5 mM DCP was fed at intervals. With five feedings of DCP during a 6-h reaction, (R)-MCP was formed from 77.5 mM DCP with a 97.3% molar conversion yield, and its optical purity was determined to be 83.8% enantiomeric excess by the HPLC analysis.

DISCUSSION

There have been many studies of the application of the stereospecificity of enzymes for the synthesis of chiral compounds from symmetrical substrates. For example, oxidations of some symmetrical diols by horse liver alcohol dehydrogenase proceed with enantioselectivity (2, 17, 18), and some *meso*- and prochiral diesters are transformed into

FIG. 3. Course of the formation of (R)-MCP from DCP by resting cells. The reaction was carried out at 20°C in 30 ml of 1 M Tris-HCl buffer (pH 8.0) containing 153 mg (dry weight) of resting cells by one addition of 77.5 mM DCP (A) or five feedings of 15.5 mM DCP (B). Symbols: ●, MCP; ▲, DCP.

chiral half esters by hydrolytic enzymes such as chymotrypsin or pig liver esterase (5, 7, 10).

Here, we have investigated the production of (R)-MCP from prochiral DCP by using *Corynebacterium* sp. strain N-1074. The activity transforming DCP into MCP was formed by the addition of some chlorinated alcohols such as MCP and chloroethanol to a medium. The use of glycerol as a carbon source was effective for the induction of the activity, even if no chlorinated alcohols were added. These facts suggest that some metabolites of the chlorinated alcohols also act as inducers for the formation of the enzyme activity. The optical purity of (R)-MCP was improved to a certain extent by altering the reaction conditions; about 84% enantiomeric excess of (R)-MCP was obtained by periodic feeding of the substrate. However, this value may not be yet sufficient for the use of (R)-MCP as a chiral building reagent. Many attempts to improve the enantioselectivity for enzymatic production of chiral compounds have been reported, and it was supposed that some of these improvements depended on conformational changes of enzymes by additives to the reaction mixture (3, 8). Therefore, further enhancement of the optical yield of (R)-MCP also might be possible with some additives in our case.

Recently, both activities interconverting DCP to epichlorohydrin and those transforming epichlorohydrin into MCP were found in *Corynebacterium* sp. strain N-1074, and each of these activities was separated (unpublished data). This fact indicates that the transformation of DCP into MCP by *Corynebacterium* sp. strain N-1074 proceeds via epichlorohydrin. Similar pathways have been also reported by Castro and Bartnicki (4), Kasai et al. (14), and van den Wijngaard et al. (21), although enantioselectivity for the transformation of DCP into epichlorohydrin was not mentioned. We already purified one of the enzymes, halohydrin hydrogen-halide-lyase, catalyzing the interconversion of halohydrins to epoxides from a recombinant *Escherichia coli* carrying its gene from *Corynebacterium* sp. strain N-1074, and then characterized the enzyme (15). The enzymatic properties were similar to those of haloalcohol dehalogenase purified from *Arthrobacter* sp. strain AD2 (22). Attempts to improve the

TABLE 2. Effect of DCP concentration on the optical purity of (R)-MCP formed^a

DCP concn (mM)	Reaction time (h)	MCP formed (mM)	Optical purity of (R)-MCP (% e. e.)
150	5.5	85.5	43.3
77.5	5.5	68.6	72.0
38.8	2.75	40.3	80.8

^a The reaction was carried out at 20°C in a reaction mixture (50 ml) consisting of 1 M Tris-HCl (pH 8.0), 265 mg (dry weight) of cells, and DCP. Optical purity is shown as the percent enantiomeric excess (e. e.).

optical purity of (*R*)-MCP formed are currently in progress on the basis of the information from studies of each enzyme involved in the transformation of DCP into (*R*)-MCP.

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