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Resolution of L-Carnitine from DL-Carnitine by Resting Cells of the *Enterobacter* sp. NH-104

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Abstract For the resolution of L-carnitine from DL-carnitine, resting cells of *Enterobacter* sp. NH-104, which had a higher capacity of D-carnitine decomposition, were harvested at maximal specific activity of D-carnitine decomposition of 47.05 unit/mg cell. The cells were frozen at -80°C to assess functions as enzyme sources. Optimal concentration of cells and DL-carnitine were 17 g/l and 20 g/l, respectively, and reaction buffer was best at 75 mM of Tris · HCl. Optimal temperature and pH were 36°C and 8.2, respectively. When the reaction at optimal conditions was carried out for 14 h, the optical purity was 98.21%, and the quantity and yield of remaining L-carnitine were 4.432 g/l and 44.32%, respectively.

Key words: L-Carnitine, resting cells, *Enterobacter* sp., resolution

Carnitine (β-hydroxy-γ-trimethylammonium butyrate), which stimulates the oxidation of long-chain fatty acids, has been safely used for a number of years for different purposes. Specifically, it has been reported that the compound has an effect on heart failure and obesity [10]. However, the D-carnitine present in the racemic mixture caused unexpected side effects [5] and showed competitive inhibition for carnitine acetyltransferase and carnitine palmitoyltransferase, the enzymes specific for L-carnitine *in vivo* [1, 3, 14]. Therefore, it was necessary to resolute the natural isomer, L-carnitine, efficiently.

Thus far, L-carnitine has been produced by four different methods; (i) extraction from animal tissues or yeasts [8], (ii) synthesis from chiral substances by multiple steps and asymmetric synthesis from intermediates of chiral substances by reduction [2, 6, 15, 17], (iii) synthesis from achiral precursors using stereospecific enzymes [16], and (iv) optical resolution of the racemic mixture, DL-carnitine or its derivatives [4, 12].

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In a previous study, we attempted the isolation and development of the bacterium which preferentially assimilated D-carnitine over L-carnitine when DL-carnitine was the sole carbon and energy source [7]. Also, we optimized the culture conditions for cells capable of decomposing D-carnitine.

In the present study, we describe a new mode for resolution of L-carnitine, namely, preferential degradation of D-carnitine by resting cells of *Enterobacter* sp. NH-104 in a reaction mixture containing DL-carnitine.

MATERIALS AND METHODS

Microorganism and Chemicals

Enterobacter sp. NH-104, isolated from a soil sample from Jin-ju (Korea), was a mutant strain with a lessened L-carnitine decomposition rate after the treatment with a nitrosoguanidine mutagen [7]. DL-Carnitine, L-carnitine, D-carnitine, nicotinamideadenine dinucleotide, acetyl coenzyme A, 5,5'-dithiobis-(-2-nitrobenzoate), and carnitine acetyltransferase were purchased from Sigma Chemical Co., U.S.A. The other chemicals were commercial preparations of analytical reagent grade.

Media and Culture Conditions

Enterobacter sp. NH-104 was grown in the fermentation medium (pH 6.4) containing 20 g of DL-carnitine, 3.40 g of $NH_4H_2PO_4$, 0.5 g of yeast extract, 1.5 mg of $CaCl_2$, 0.06 mg of $Fe_2(SO_4)_3$, 0.02 mg of $ZnSO_4 \cdot 7H_2O$, 0.02 mg of $CuSO_4 \cdot 5H_2O$, and 0.02 mg of $MnSO_4$ H_2O in 1 l of distilled water [7]. Cultivation was carried out in 500-ml Erlenmeyer flasks containing 120 ml of the fermentation medium agitated at 130 rpm. The culture temperature was 33°C and the inoculum was used with 1% (v/v) broth cultured overnight in Luria-Bertani medium (tryptone 10 g, yeast extract 5 g, and NaCl 5 g per liter).

Preparation of Resting Cells and Reaction Conditions

Enterobacter sp. NH-104 was cultivated in the fermentation medium described above until maximal degradation of

D-carnitine was achieved. This corresponded to the early logarithmic phase. It was then harvested by centrifugation (8,000×g for 30 min) and washed twice by resuspension in 50 mM phosphate buffer (pH 7.2). Resting cells were prepared by subjecting cells to several cycles of freezing and thawing. Cell-free extracts were obtained from equivalent amounts of whole cells used by sonication (Model 450 Sonifier, Branson, U.S.A.) for 5 min. Also, equivalent cells were treated for 30 min at 30°C in 50 mM phosphate buffer containing 10% (v/v) organic solvents and washed twice by resuspension in 50 mM phosphate buffer. The standard reaction mixture consisted of 10 g/l of DL-carnitine, 10 g/l (wet weight) of cells, and 75 mM phosphate buffer (pH 7.2) in a final volume of 10 ml. The reactions were carried out for 6 h at 30°C with shaking (50 rpm).

Analytical Methods

Analyses of DL-carnitine were performed by spectrophotometry and thin layer chromatography [11]. The analysis of L-carnitine was carried out by the high performance liquid chromatography with a Shodex C_{18} column (Shoko Co., Japan) and UV detector at 220 nm [7] and enzymatic method using carnitine acetyltransferase (EC 2.3.1.7) specific to L-carnitine [13]. The degrading activity of D-carnitine was measured by using the cells harvested at each growth phase and carrying out the reaction in the standard reaction mixture above. One unit of D-carnitine degrading activity was defined as the quantity of cells required to degrade 1 μmole D-carnitine at 30°C for 10 min. Optical rotations were obtained in a 10-cm cell at 589 nm (sodium line) and 25°C using a JASCO Model DIP-370 polarimeter (Japan Spectroscopic Co.). Optical purity (%) was defined as weight/weight percentage concentration of L-carnitine to total DL-carnitine in the reaction mixtures. The growth of strains was determined by measuring the optical density of the culture broths at 660 nm. The cell concentration was determined by measuring the absorbance at 660 nm and comparing to a standard curve.

Results and Discussion

Optimal D-Carnitine Degrading Activity in the Cultivation of *Enterobacter* sp. NH-104

We had isolated a bacterium, partially identified as *Enterobacter* sp., which had a higher capability of assimilating D-carnitine than L-carnitine from the racemic mixture [7]. Also, L-carnitine decomposition by *Enterobacter* sp. NH-104 was reduced by decreasing the acitivities of L-carnitine dehydrogenase and β-hydroxybutyrate dehydrogenase with *N*-methyl-*N'*-nitrosoguanidine (NTG) mutagenesis [7]. Culture conditions were optimized to obtain cells having a higher capacity for assimilating D-carnitine than L-carnitine.

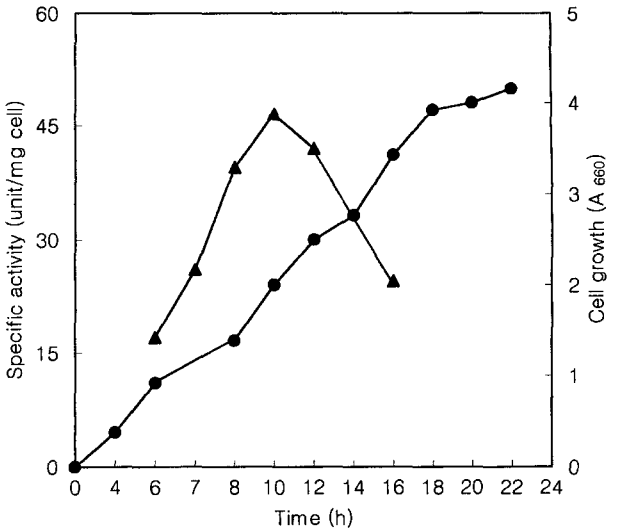

Fig. 1. Time course of the D-carnitine degrading activity in *Enterobacter* sp. NH-104.

Cultivation was carried out at 33°C in the fermentation medium (pH 6.4) containing 20 g/l of DL-carnitine, 3.40 g/l of $NH_4H_2PO_4$, 0.5 g/l yeast extract, 400 μg/l of Ca^{2+} and the other components. Working volume was 120 ml per 500-ml Erlenmeyer flask. ▲---▲, D-Carnitine degrading actvity; ●---●, Cell growth.

For the resolution of L-carnitine from DL-carnitine by a resting cell system, changes in D-carnitine degradation and cell weight were investigated under optimal culture conditions. The specific activity of D-carnitine degradation was increased up to the midpoint of the logarithmic phase of growth, as shown in Fig. 1. Afterwards, the specific activity dramatically decreased, indicating that D-carnitine degrading activity was induced by D-carnitne and inhibited by an increase of L-carnitine ratio in the medium. Thus, cells harvested after 10 h of cultivation were used for the enzyme sources in the production of L-carnitine from DL-carnitine by preferential degradation of D-carnitine.

Effect of Cell Treatment on the Resolution of DL-Carnitine

To assess the reactions between the substrate and enzyme systems related to the degradation of D-carnitine in reaction mixtures, the harvested cells were treated with various conditions. As shown in Table 1, the cell-free extract and cells treated with organic solvents had no effect on the resolution when compared to the control, whole cells. These observations suggested that the enzyme systems responsible for the degradation of D-carnitine were more affected by the complete destruction of cell membrane. Meanwhile, the cells treated with freezing at -80°C showed that D-carnitine degradation and optical purity increased by 48% and 10%, repectively, over the control (immediately harvested cells). Thus, the cells frozen at

Table 1. Effect of cell treatment on the resolution of DL-carnitine.

Cell treatment	Residual L-carnitine (g/l)	L-Carnitine ratio (%)*
Whole cell	4.721	63.52
Frozen cell	3.854	72.77
Cell-free extract	4.827	54.23
10% Acetone	4.853	52.72
10% Toluene	4.925	51.93

Reactions were carried out for 6 h at 30°C in a reaction mixture containing 10 g/l (wet weight) of cell concentration, 10 g/l of DL-carnitine and 75 mM of phosphate buffer (pH 7.2).

*L-Carnitine ratio (%) = $\frac{\text{Residual L-carnitine}}{\text{Residual DL-carnitine}} \times 100$.

-80°C were used as the enzyme source for the degradation of D-carnitine from a DL-carnitine mixture.

Optimal Conditions for the Optical Resolution of DL-Carnitine

To investigate the optimal cell concentration for enzyme sources for reactions to degrade D-carnitine from DL-carnitine, the cell concentrations were varied in the standard reaction mixture as described in Materials and Methods. As shown in Fig. 2, the degradation of D-carnitine increased linearly with respect to cell concentration to 17 g/l (wet weight) and the optical purity of L-carnitine also increased in a linear fashion. At higher cell concentrations, the degradation of D-carnitine was saturated, and showed the almost same optical purity as L-carnitine. This corresponded to 80.24% of the L-carnitine ratio and 3.647 g/l of L-carnitine remaining. Thus, the optimal cell concentration of 17 g/l was used for further experiments.

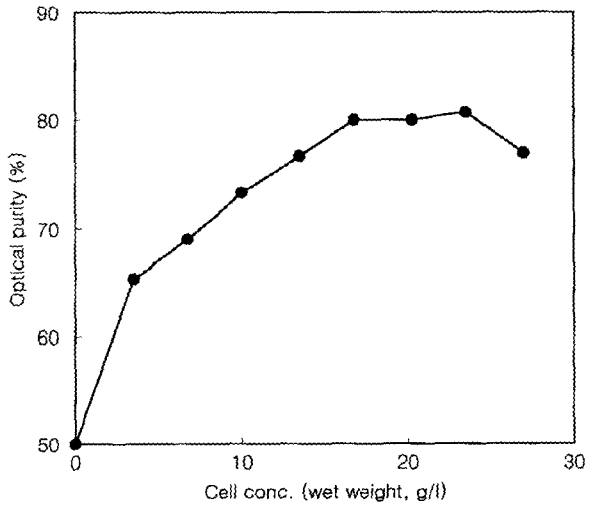

Fig. 2. Effect of cell concentration on L-carnitine resolution with resting cells.

Reactions were carried out for 6 h at 30°C in reaction mixtures containing various concentrations of frozen cells, 20 g/l of DL-carnitine, and 75 mM of phosphate buffer (pH 7.2).

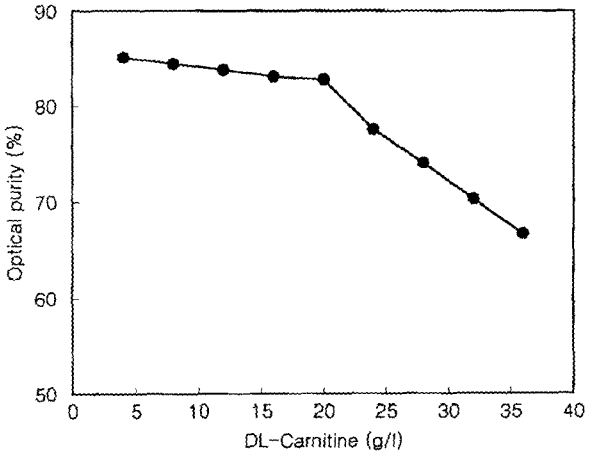

Fig. 3. Effect of DL-carnitine concentration on L-carnitine resolution with resting cells.

Reactions were carried out 6 h at 30°C in reaction mixture containing various concentrations of DL-carnitine, 17 g/l (wet weight) of frozen cells, and 75 mM of phosphate buffer (pH 7.2).

To study the effect of DL-carnitine concentration on L-carnitine production by resting cells, the substrate concentration was changed while the cell concentration was kept at 17 g/l in the standard reaction mixture. As shown in Fig. 3, the degradation of D-carnitine was linear up to 20 g/l of DL-carnitine. At higher concentrations, the degradation of D-carnitine was decreased with increasing DL-carnitine concentration, suggesting that the effect of the substrate in the reaction was a hyperbolic dependence. A concentration of 20 g/l of DL-carnitine gave the high value for L-carnitine remaining of 7.056 g/l with the optical purity of 83.17%. This was the optimal concentration of DL-carnitine. These indicated that relative increase of residual L-carnitine in reaction mixtures might inhibit the activities of enzyme systems responsible for the degradation of D-carnitine.

Table 2. Effect of different buffers on L-carnitine production of DL-carnitine with resting cells.

Buffers	Concentration (mM)	Residual L-carnitine (g/l)	L-Carnitine ratio (%)*
	25	7.235	75.04
	50	7.304	79.65
Tris-HCl	75	7.532	83.16
	100	7.347	80.31
	125	7.271	77.24
KH_2PO_4/Na_2HPO_4	75	7.123	83.12
KH_2PO_4/NaOH	75	7.307	81.02
Imidazole-HCl	75	6.688	80.54

Reactions were carried out for 6 h at 30°C in a reaction mixture containing 17 g/l (wet weight) of frozen cells, 20 g/l of DL-carnitine. All buffers were adjusted into pH 7.2.

*L-Carnitine ratio (%) = $\frac{\text{Residual L-carnitine}}{\text{Residual DL-carnitine}} \times 100$.

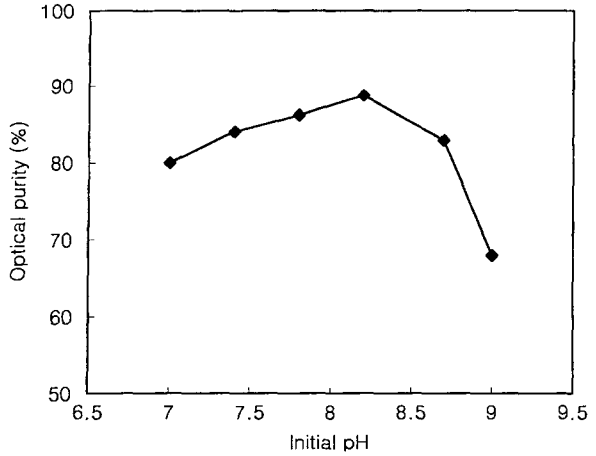

Fig. 4. Effect of initial pH on L-carnitine resolution with resting cells.

Reactions were carried out 6 h at 30°C in reaction mixture containing 17 g/l (wet weight) of frozen cells, 20 g/l of DL-carnitine, and 75 mM of Tris-HCl buffer.

To investigate the effect of reaction buffers, different buffering systems at 75 mM were used, as shown in Table 2. Optical purity was not affected by the difference between phosphate and Tris-HCl buffer but the L-carnitine remaining was increased by 5% in Tris-HCl buffer. Also, the optimal pH of the reaction mixture was examined within the buffering ranges of Tris-HCl buffer, pH 7.0 to 9.0. As shown in Fig. 4, the optical purity of L-carnitine was gradually increased up to pH 8.2 but over pH range 8.2, the optical purity was dramatically decreased. This observation indicated that the optimal pH of the enzyme systems related to the degradation of D-carnitine was different from that of L-carnitine dehydrogenase specific L-carnitine degradation, which has a pH optimum at 9.1

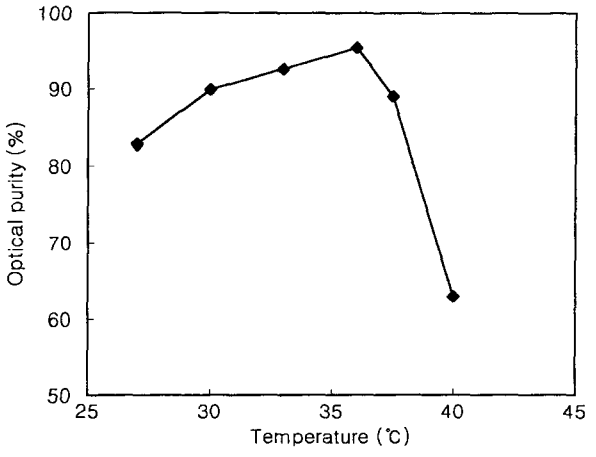

Fig. 5. Effect of temperature on L-carnitine resolution with resting cells.

Reactions were carried out 6 h at various temperatures in reaction mixture containing 17 g/l (wet weight) of frozen cells, 20 g/l of DL-carnitine, and 75 mM of Tris-HCl buffer (pH 8.2).

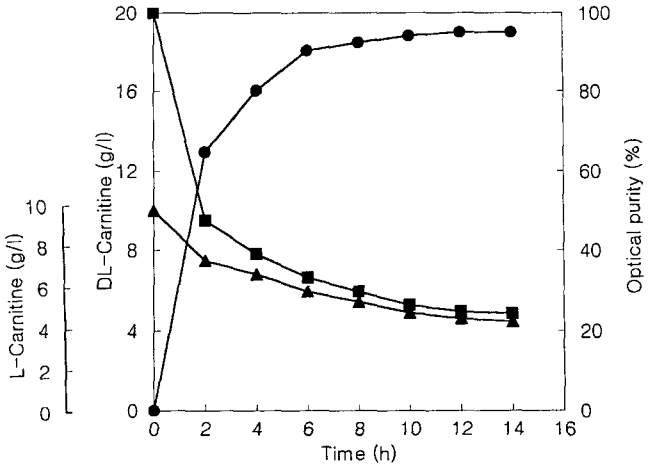

Fig. 6. Time course of L-carnitine resolution with resting cells.

Reactions was carried out at 36°C in reaction mixture containing 17 g/l (wet weight) of frozen cells, 20 g/l of DL-carnitine, and 75 mM of Tris-HCl buffer (pH 8.2). ●---●, Optical purity; ■---■, DL-Carnitine; ▲---▲, L-Carnitine.

[9]. To examine the optimal reaction temperature, the reaction temperature was varied from 27 to 40°C (Fig. 5). The optical purity of L-carnitine was the highest at 36°C, but higher temperatures exhibited dramatic decrease in the optical purity of L-carnitine, suggesting that enzyme systems responsible for the degradation of D-carnitine possessed weak thermal stability. This result was consistent with that obtained in the cultivation of *Enterobacter* sp. NH-104 [7]. Under the above optimal reaction conditions, a 16 h time course of the optical resolution of DL-carnitine was studied (Fig. 6). The degradation of D-carnitine was gradually increased up to 8 h. After that, the degradation ratio of L-carnitine was greater than D-carnitine. For high levels of optical purity of L-carnitine, the reaction continued to 14 h. The terminal yield of L-carnitine was 44.3%, which corresponded to 4.43 g/l of L-carnitine remaining and a 98.21% optical purity.

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