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Conversion of γ-butyrobetaine to L-carnitine by *Achromobacter cycloclast*

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L-Carnitine is an ubiquitous substance that plays a major role in the transportation of long-chain fatty acids. We investigated crucial factors that influence microbial conversion of γ-butyrobetaine to L-carnitine using an *Achromobacter cycloclast* strain. Two-stage culture results showed that γ-butyrobetaine induced enzymes essential for the conversion, which suggests that the precursor should be present in the initial cell growth stage. The addition of yeast extract enhanced L-carnitine production whereas inorganic nitrogen sources inhibited it. Under nitrogen-limiting conditions, the cells accumulated poly-β-hydroxybutyrate instead of L-carnitine. Among the trace elements tested, nickel addition enhanced L-carnitine production by almost twice that of the control and copper strongly inhibited the conversion. L-Carnitine production was reduced when the medium contained inorganic salts of sodium, potassium, and calcium at a concentration greater than 2 g l^{-1}. A higher L-carnitine yield was achieved when cells were incubated in a lower culture volume. The optimal pH for L-carnitine production was 5 to 5.5, whereas that of growth was 7.0, indicating that a pH shift was required. Under optimal conditions, L-carnitine concentrations as high as 15 g l^{-1} were obtained in 62 h with a 45% molar conversion yield. *Journal of Industrial Microbiology & Biotechnology* (2001) **26,** 309–315.

Keywords: L-Carnitine; γ-Butyrobetaine; production; *Achromobacter cycloclast*

Introduction

L-Carnitine ((*R*)-3-hydroxy-4-*N*-trimethyl aminobutyrate) belongs to a class of quasi-vitamins found in higher animals. The important function of L-carnitine is transportation of long-chain fatty acids into the mitochondrial matrix [6,22]. L-Carnitine has a wide range of applications in pharmaceuticals, food products and feed additives. A deficiency of L-carnitine leads to cardiac infarction, ketonemia, and skeletal myopathy [21,24]. It is used in replacement therapy for hemodialysis patients [5] and also in the production of both monoclonal antibodies and murine hybridoma cells [2,25]. Due to the increase in demand for L-carnitine its production has drawn great attention. The chemical synthesis of L-carnitine produces a racemic mixture and complicated separation strategies are necessary to obtain the biologically active L-compound. Brass *et al* [3] reported that biotechnological processes produce nearly 50% less total organic waste, 25% less waste water and 90% less waste for incineration than the chemical process, which prompted us to investigate the microbial production of L-carnitine.

Microbial production of L-carnitine from achiral precursors such as γ-butyrobetaine, crotonobetaine, and dehydrocarnitine has been reported. Among these, the methods using γ-butyrobetaine have been preferred as it can be readily synthesised from 4-amino-butyrate. Since the 1980s, Lonza, Switzerland has played a pioneering role in developing a microbial process for the production of L-carnitine from γ-butyrobetaine, and recently holds 75% of the total world market [7–11,14]. They screened a microorganism that taxonomically lies between *Agrobacterium* and *Rhizobium* and derived the mutant strain HK 13, which lacks L-carnitine dehydrogenase. With this strain, they were able to produce 60 g l^{-1} of L-carnitine with a productivity of 5.4 g l^{-1} h^{-1} by a cell-recycling system. Only a few other microorganisms that are capable of converting γ-butyrobetaine to L-carnitine have been reported [9,17]. Eukaryotic cells such as *Saccharomyces* and other fungal strains of *Penicillium*, *Rhizopus*, *Aspergillus*, *Neurospora* and *Mucor* accumulate L-carnitine in their cells, but only in a low yield (less than 200 mg g^{-1} cells [18,23,26]. Kyowa Hakko, Tokyo, Japan reported that other strains of the *Pseudomonas* species and *Achromobacter cycloclast* can produce L-carnitine from γ-butyrobetaine [12]. When both strains were cultivated in a defined medium containing 1% γ-butyrobetaine for 72 h at 26°C, *Pseudomonas* sp. FERM BP-1397 produced only a small amount of L-carnitine (0.37 g l^{-1}) after recovery, whereas the *A. cycloclast* ATCC 21921 strain yielded 1.79 g l^{-1} of L-carnitine. The *A. cycloclast* strain would be a strain of choice for microbial production of L-carnitine with a higher commercial potential. However, no experiments designed to develop an efficient L-carnitine production process using *A. cycloclast* strains have been reported.

We previously reported a higher production of L-carnitine (4.6 g l^{-1}) with the same *A. cycloclast* strain by coaddition of glycerol into a γ-butyrobetaine-containing medium [13]. This report describes crucial factors influencing the microbial conversion of γ-butyrobetaine to L-carnitine with the *A. cycloclast* strain. For this, we examined the effect of nitrogen sources, trace elements, inorganic salts, pH, and dissolved oxygen levels on the conversion of γ-butyrobetaine to L-carnitine. In addition, we also attempted to maximize the L-carnitine production in a jar fermentor under optimal conditions.

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Materials and methods

Chemicals

The achiral precursor (γ-butyrobetaine), product (L-carnitine), acetyl-CoA, 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) and carnitine acetyltransferase were purchased from Sigma (St. Louis, MO). The glycerol assay kit was purchased from Boehringer Mannheim, Germany. The other chemicals were of analytical grade from Junsei Chemical, Tokyo, Japan.

Microorganism and culture media

A. cycloclast ATCC 21921 was used throughout this work. The organism was maintained in Luria broth (LB) that contained (per litre) 20 g of glucose, 10 g of yeast extract, 10 g of tryptone, and 10 g of NaCl. The organism was subcultured every 2–3 weeks.

The basal fermentation medium (BT) had the composition (per litre) 20 g glycerol, 0.5 g yeast extract, 0.5 g $MgSO_4 \cdot 7H_2O$, 10 g choline chloride, 0.5 g KH_2PO_4, 20 g γ-butyrobetaine, 3 g $CaCO_3$ and 0.15 g $FeSO_4 \cdot 7H_2O$. The production medium consisted of the BT medium supplemented with 3 g l^{-1} of yeast extract, and 0.5 mM each of $FeSO_4 \cdot 7H_2O$ and $NiCl_2 \cdot 6H_2O$. The pH of the medium was adjusted to 7.0 with 3 N NaOH prior to sterilization.

Flask cultures

For flask cultures, a loop of the microorganism was inoculated into 25 ml of the LB medium held in a 250-ml baffled flask and cultivated with shaking at 140 rpm for 24 h at 27°C on a rotary shaker. A 2.5-ml suspension of the cells grown in the LB was used to inoculate 25-ml of BT medium or production medium in 250-ml baffled flasks, which were incubated as described above. To examine the effects of dissolved oxygen on the L-carnitine conversion yields, different volumes of media were used in both round and baffled 500-ml flasks.

A two-stage culture technique was also used to examine the inducibility of enzymes involved in L-carnitine synthesis and the effects of trace elements on L-carnitine production. To examine inducibility, the microorganism was first grown at 27°C for 72 h in 25 ml of BT medium containing L-lysine, L-methionine, choline, betaine, trimethylamine, or γ-butyrobetaine. Then the cells (75 mg dry weight), harvested by centrifugation at 8000×*g* for 10 min, were suspended in 25 ml of BT medium containing 100 μg ml^{-1} of chloramphenicol to suppress further cell growth. Similar experiments were performed to examine the effects of trace elements of L-carnitine production. The cells (75 mg dry weight), grown at 27°C for 48 h in 25 ml of BT medium, were transferred to 25 ml of BT medium containing 100 μg ml^{-1} of chloramphenicol and 0.5 mM of different trace elements (Mn^{2+}, Co^{2+}, Zn^{2+}, Cu^{2+} and Ni^{2+}).

All flask cultures were prepared and analysed in triplicate. The results are expressed as mean values of three determinations within a deviation of not more than 15%.

Jar fermentor operation

Cultivations of *A. cycloclast* were done in a 2.5-l jar fermentor (Korea fermentor, Incheon, Korea) equipped with an Ingold dissolved oxygen analyzer and a pH controller. A seed culture (100 ml) grown in LB medium at 27°C overnight in shake flasks was used to inoculate the fermentor containing 900 ml of the production medium. To examine the effect of pH on L-carnitine production, pH was initially controlled at pH 7 during cell growth for 12 h, and then shifted to the desired values by turning off the pH controller. The culture temperature was maintained at 27°C. The agitation speed was maintained at 500–700 rpm, and the aeration rate was 1 vvm.

A fed-batch operation was also performed to obtain higher L-carnitine production. The feed medium consisting of (per litre) 20 g glycerol, 3 g yeast extract, 20 g γ-butyrobetaine and 0.5 g KH_2PO_4 was supplied intermittently to maintain the γ-butyrobetaine concentration of 5–20 g l^{-1} in the culture broth. The initial pH 7.0 decreased to 5.5 during cell growth, and then maintained at 5.5. The other culture conditions were the same as with the batch fermentations.

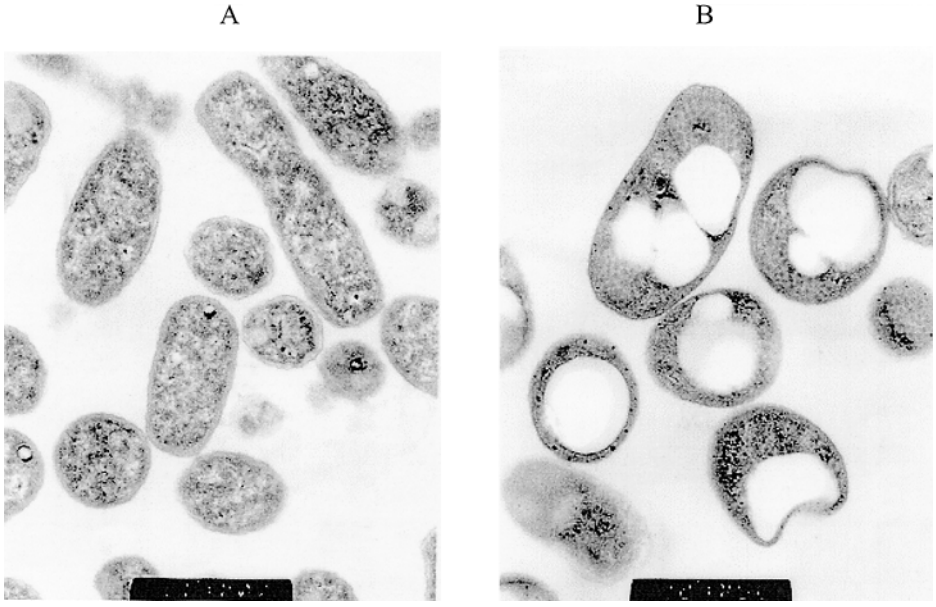

Figure 1 Photomicrographs of *A. cycloclast* cells (A) incubated under nitrogen-rich conditions (BT medium) and (B) under nitrogen-limiting conditions. The incubation was carried out at 27°C for 48 h.

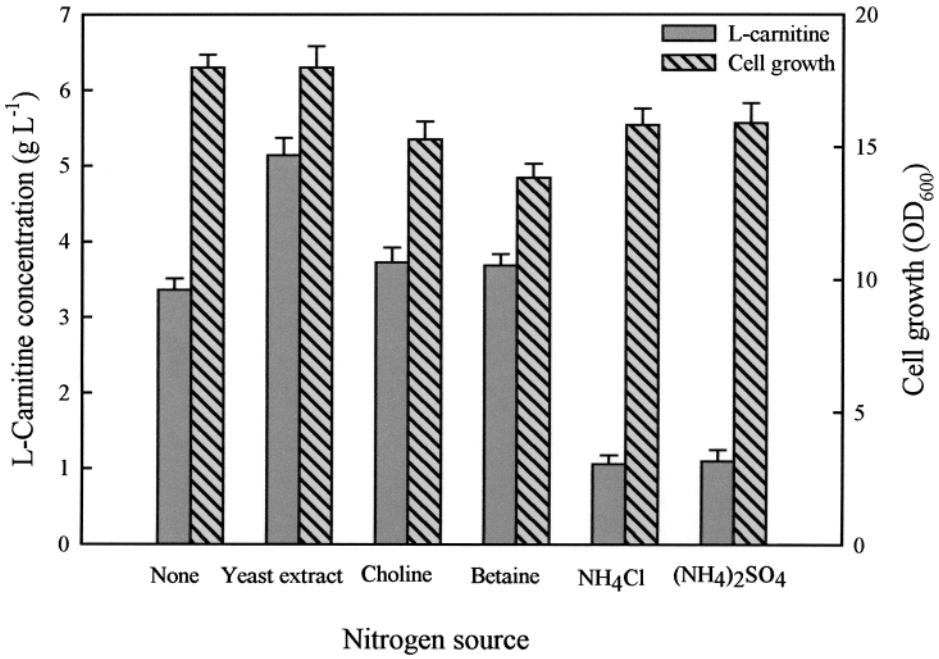

Figure 2 Effect of nitrogen source on L-carnitine production by *A. cycloclast*. Flask cultures were carried out for 48 h in 25 ml BT medium supplemented with 10 g of different nitrogen sources.

Analytical methods

Cell growth was monitored by measuring the optical density of the culture broth at 600 nm. Glycerol concentrations were determined with a glycerol assay kit (Boehringer Mannheim, Indianapolis, IN). The $PO4^{3-}$ concentrations were determined according to the method described by Chen *et al* [4]. L-Carnitine was analysed by the L-carnitine acetyltransferase method, reacting it with DTNB [15]. One millilitre of the reaction mixture contained 0.5 mM acetyl CoA, 10 mM DTNB in Tris–HCl buffer at pH 7.5 and 1 unit of L-carnitine acetyl transferase. The reaction mixture was incubated at 37°C for 12 min and the absorbance was read at 412 nm against a reagent blank. γ-Butyrobetaine was analysed by HPLC (Tosho, Tokyo, Japan) with an Asahipak NH_2P column. The mobile phase consisted of 75:25 volume ratio of acetonitrile and 20 mM KH_2PO_4 adjusted to pH 3.5 with phosphoric acid, and eluted at a flow rate of 1 ml min^{-1} [16].

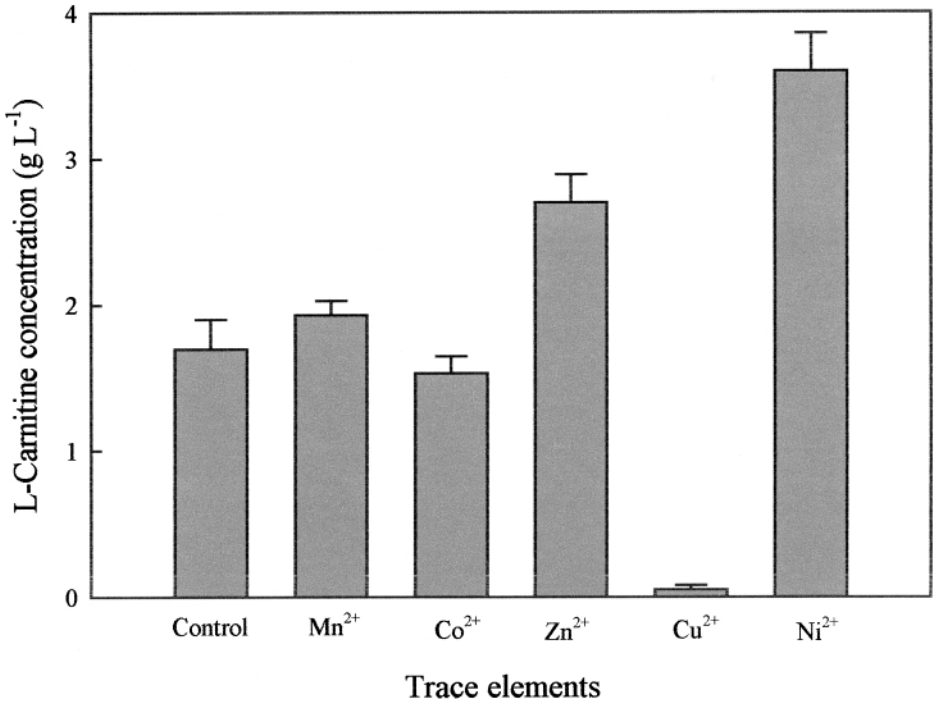

Figure 3 Effect of trace elements on L-carnitine production. A two-stage culture technique was employed as described in Materials and Methods. The L-carnitine concentrations are the values obtained in the second stage. The control did not contain any trace element.

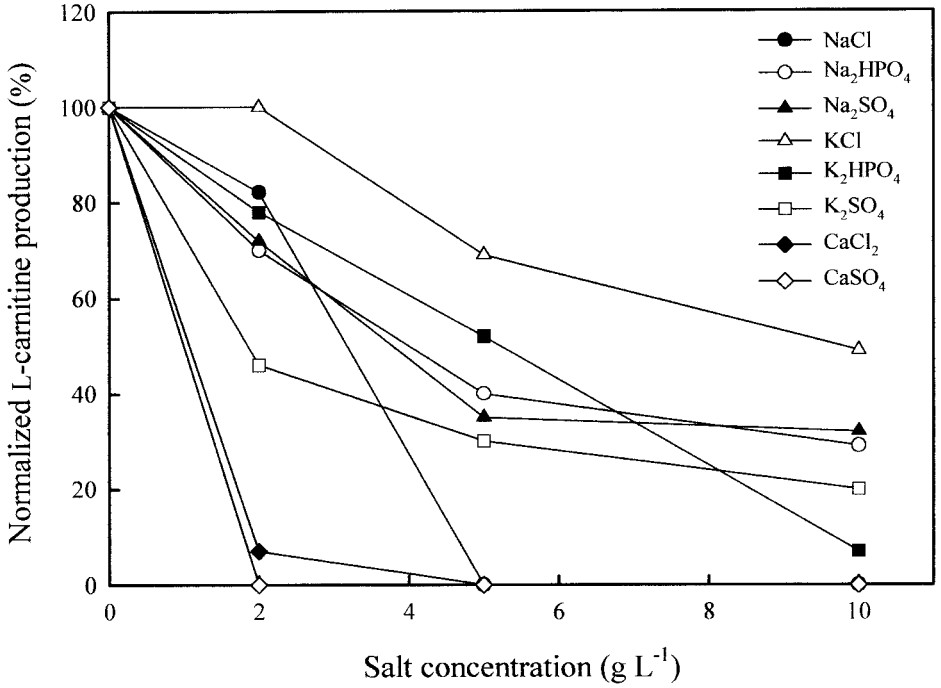

Figure 4 Effect of inorganic salts on L-carnitine production. Flask cultures were carried out for 48 h in 25 ml production medium supplemented by varying the concentrations of different inorganic salts.

Results

Selection of a possible inducer for L-carnitine synthesis

Microbial conversion of γ-butyrobetaine can be increased by the proper induction of enzymes that are responsible for the L-carnitine metabolic pathway. A two-stage culture technique was employed to select compounds that enhance L-carnitine synthesis. Quaternary ammonium compounds such as choline, betaine, trimethylamine and γ-butyrobetaine, and amino acids L-lysine and L-methionine were tested because they are either structurally analogous to the product or metabolic precursors of it. Only cells grown in medium containing γ-butyrobetaine at the first stage produced L-carnitine in the second stage, whereas no conversion was observed with the others. This result suggests that it is necessary to add the precursor γ-butyrobetaine during the cell-growth step to support the induction of enzymes responsible for L-carnitine synthesis.

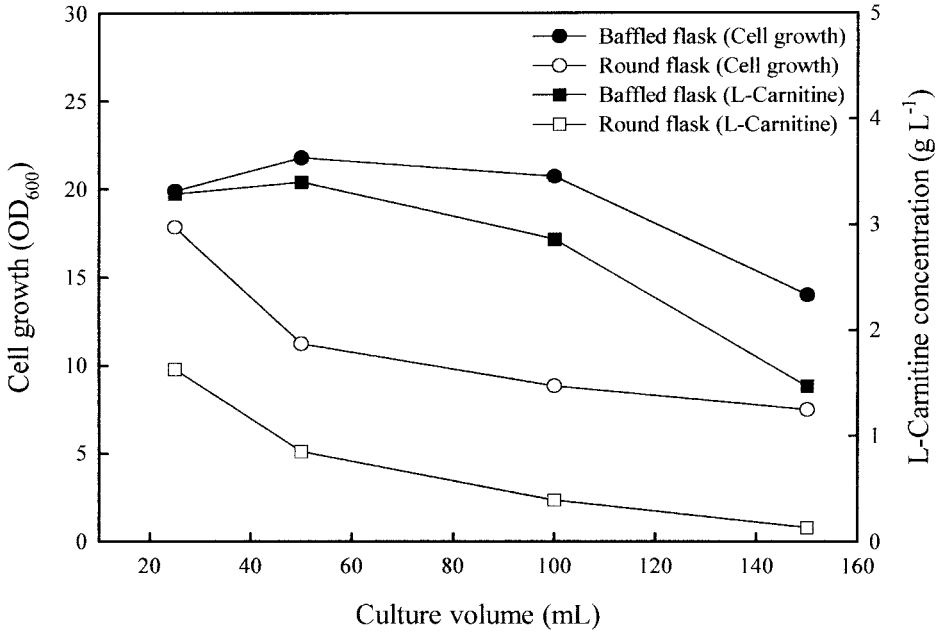

Figure 5 Effect of culture volume in 500-ml baffled flasks on L-carnitine production by *A. cycloclast*. Flask cultures were carried out in different volumes of production medium for 48 h.

Effect of nitrogen source on L-carnitine production

Initially, in order to examine the conversion efficacy of γ-butyrobetaine to L-carnitine, cells grown in BT medium were transferred to either a nitrogen-rich or a nitrogen-limited BT medium. The nitrogen-rich medium contained 3 g l^{-1} yeast extract whereas the nitrogen-limited medium contained only γ-butyrobetaine. Under nitrogen-rich conditions, the cells produced 4.8 g l^{-1} of L-carnitine in 48 h, but a low yield (0.73 g l^{-1}) was obtained under nitrogen-limiting conditions. The microscopic photographs of the cells showed that cells grown under nitrogen-limiting conditions accumulated granules inside the cells that were not present in cells grown under nitrogen-rich conditions (Figure 1). On analysis it was revealed that the inclusion bodies were poly-β-hydroxybutyrate, suggesting that under nitrogen-limited conditions, *A. cycloclast* switches over to a different metabolic pathway. This result indicates that a nitrogen source should be maintained at a level that is not limiting.

To find suitable nitrogen sources that produce a high L-carnitine yield, we cultivated the cells for 72 h in BT medium supplemented with either organic or inorganic nitrogen sources. The cell concentrations were not very much different from one another with the different nitrogen sources (Figure 2). However, L-carnitine production was the highest (5.2 g l^{-1}) in media containing yeast extract, but was very low (less than 1.5 g l^{-1}) in media containing inorganic nitrogen sources such as ammonium chloride and ammonium sulphate.

Effect of trace elements on L-carnitine production

A two-stage culture technique was employed to examine the effect of trace elements on L-carnitine production. Cells were first grown for 48 h in BT medium to allow for induction of enzymes by γ-butyrobetaine. Subsequently, an equal amount of induced cells (75 mg dry weight) was transferred to BT medium containing 0.5 mM of the different trace elements. The addition of Ni increased L-carnitine production more than twice (3.6 g l^{-1}) that of the control (Figure 3). However, Cu was a strong inhibitor of L-carnitine synthesis.

Effect of inorganic salts on L-carnitine synthesis

We examined the effect on L-carnitine synthesis of the inorganic salts, e.g., of Na^+, K^+, Ca^{2+}, PO_4^{3-}, and SO_4^{2-} as these contain essential ions present in the cell. As shown in Figure 4, L-carnitine synthesis decreased significantly as the concentrations of any inorganic salt was increased. The detrimental effect was profound with inorganic salts containing calcium ions. From this result, it can be concluded that low concentrations of inorganic salts are required to achieve a high conversion of γ-butyrobetaine to L-carnitine.

Effect of dissolved oxygen on L-carnitine production

To examine the effect of dissolved oxygen on both cell growth and L-carnitine production, *A. cycloclast* was cultivated in different volumes of BT medium held in 500 ml of either round or baffled flasks. Both cell growth and L-carnitine synthesis were higher in baffled flasks than in round flasks (Figure 5). In addition both the cell concentration and L-carnitine production decreased as the culture volume increased. The highest L-carnitine production was attained when the cells were grown in 50 ml BT medium in a 500-ml baffled flask. From these results it was found that the dissolved oxygen level has significant effect on L-carnitine production.

Effect of pH on L-carnitine production

The study on the effect of pH was carried out in a jar fermentor because pH control in shake flasks is difficult. The pH was controlled at the optimal pH for cell growth (7.0) for 12 h, and then shifted to the different pH: 5.0, 5.5, 6.0, 6.5 and 7.0. L-Carnitine production was very low (0.2 g l^{-1}) when the pH was set at 7.0 (Figure 6). At lower pH values L-carnitine production increased. When the pH was maintained at 5.5, L-carnitine production (2 g l^{-1}) was the highest with the highest molar conversion yield (22.5%).

Fed-batch fermentation for L-carnitine production under optimal conditions

We attempted a high production of L-carnitine in fed-batch fermentations by employing optimal conditions determined from the above studies. The results in Figure 7 show the time courses for cell growth, pH, and the concentrations of γ-butyrobetaine and L-

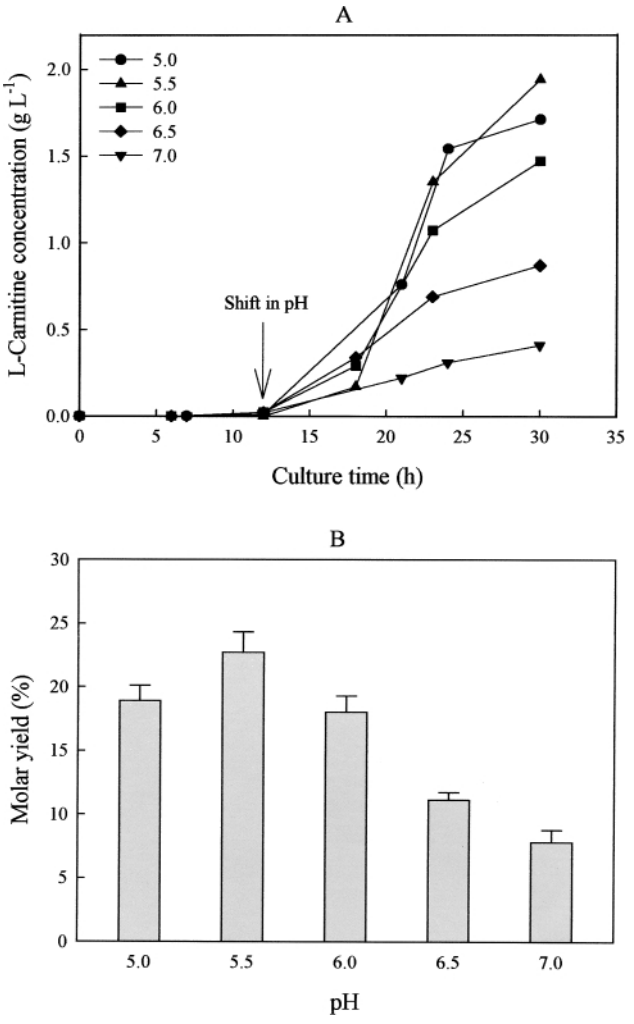

Figure 6 Effect of pH on production of L-carnitine by *A. cycloclast*. (A) L-Carnitine concentration and (B) molar conversion yield. Details are described in Materials and Methods.

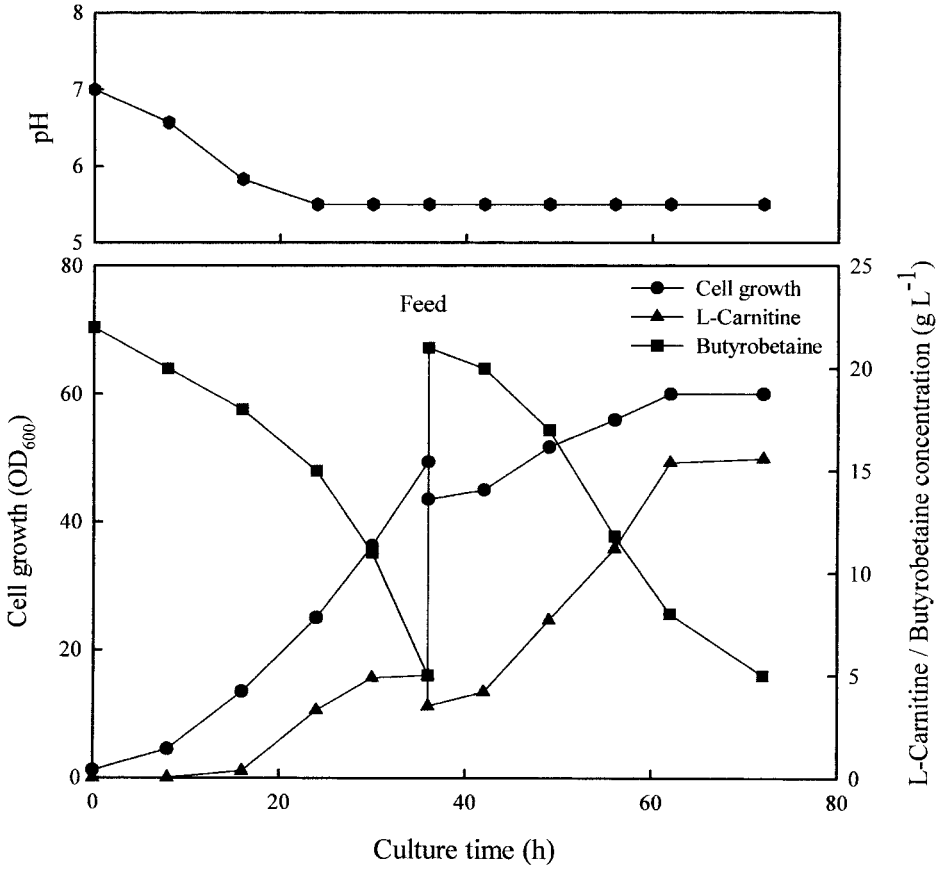

Figure 7 Time profiles of L-carnitine, γ-butyrobetaine, cell growth, and pH during fed-batch fermentation under optimal conditions. Details are described in Materials and Methods.

carnitine during the fed-batch fermentation. *A. cycloclast* cells were grown in the production medium containing 20 g l^{-1} of butyrobetaine, and the feed medium was supplied when the γ-butyrobetaine concentration was less than 5 g l^{-1}. Initially, fermentation was begun at pH 7.0 for the cells to grow. The pH was then left uncontrolled until it reached 5.5, where it was maintained with a mixture of 3 N KOH and 3 N NaOH. L-Carnitine concentrations continued to increase from the start and a maximum production of L-carnitine (15 g l^{-1}) was obtained in 62 h. The molar conversion yield of L-carnitine from γ-butyrobetaine was 45%.

Discussion

Precise details of the pathway involved in the conversion of γ-butyrobetaine to L-carnitine are yet unknown. However, it is likely that the conversion of γ-butyrobetaine to L-carnitine involves β-oxidation because γ-butyrobetaine hydroxylase was not detected in *A. cycloclast* in our preliminary study. Thus, we believe that γ-butyrobetaine is first converted to γ-butyrobetaine-CoA by an acyl-CoA synthetase (EC 6.2.1.3), followed by conversion to crotonobetainyl-CoA by acyl-CoA dehyrogenase (EC 1.3.99.10). Subsequent hydration to L-carnitinyl-CoA by an enoyl-CoA hydratase (EC 4.2.1.17) and hydrolysis would yield L-carnitine.

To enhance the metabolic flux of γ-butyrobetaine into L-carnitine, the induction of key enzymes involved in L-carnitine synthesis from γ-butyrobetaine is of importance. In this study, we found that γ-butyrobetaine is the one that induces the necessary enzymes for the conversion of γ-butyrobetaine to L-carnitine, although neither choline or betaine, which have a similar structure to L-carnitine, did not enhance L-carnitine production. It has been reported in many L-carnitine production systems that the starting materials induce the key enzymes. Obon *et al* [20] and Yokozeki *et al* [27] revealed that crotonobetaine, a precursor of L-carnitine, induces the L-carnitine metabolic pathway in both *Escherichia coli* and *Proteus mirabilis*. On the other hand, Nobile and Deshusses [19] stated that betaine is a poor inducer for γ-butyrobetaine transport in the *Agrobacterium* species.

Transport of γ-butyrobetaine appears to be another key factor in the conversion of γ-butyrobetaine to L-carnitine. Beradi *et al* [1] showed γ-butyrobetaine transport is strongly dependent upon both cations and anions. The results showing reduced yields of L-carnitine with inorganic salts (Figures 2 and 4) are in good agreement with the previous report. It is likely that high concentrations of NH_4^+, Na^+, K^+ and Ca^{2+} inhibit the transport of γ-butyrobetaine into the cell, consequently reducing L-carnitine production. A lower L-carnitine production at a higher pH (Figure 6) supports this since a greater amount of alkaline solution was necessary to control a higher pH set value. A strategy to reduce the use of inorganic ions would result in a much greater conversion of γ-butyrobetaine to L-carnitine.

In this study, an L-carnitine concentration of 15 g l^{-1} was obtained in 0.45 molar conversion yield by a fed-batch

fermentation of an *A. cycloclast* strain. This is a much higher production titre than reported previously [13], and is attributed to the pH shift, a lower concentration of inorganic salts, and the addition of both Ni and yeast extract. This work demonstrates the feasibility of producing L-carnitine by the biocatalytic transformation of γ-butyrobetaine.

Acknowledgements

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