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Enzymatic Production of Pyrimidine Nucleotides Using *Corynebacterium ammoniagenes* Cells and Recombinant *Escherichia coli* Cells: Enzymatic Production of CDP-Choline from Orotic Acid and Choline Chloride (Part I)

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Enzymatic production of cytidine diphosphate choline (CDP-choline) using orotic acid and choline chloride as substrates was investigated using a 200-ml beaker as a reaction vessel. When *Corynebacterium ammoniagenes* KY13505 cells were used as the enzyme source, UMP was accumulated up to 28.6 g/liter (77.6 mM) from orotic acid after 26 h of reaction. In this reaction, UDP and UTP were also accumulated, but CTP, a direct precursor of CDP-choline, was not accumulated sufficiently. *Escherichia coli* JF646/pMW6 cells, which overproduce CTP synthetase by selfcloning of the *pyrG* gene, were used together with cells of KY13505 for the enzymatic reaction using orotic acid as a substrate. CTP was produced at 8.95 g/liter (15.1 mM) after 23 h of this reaction. To produce CDP-choline, two additional enzyme activities were needed. *E. coli* MM294/pUCK3 and MM294/pCC41 cells, which express a choline kinase from *Saccharomyces cerevisiae* (CKIase; encoded by the *CKI* gene) and a cholinephosphate cytidylyltransferase from *S. cerevisiae* (CCTase; encoded by the *CCT* gene) respectively, were added to this CTP-producing reaction system. After 23 h of the reaction using orotic acid and choline chloride as substrates, 7.7 g/liter (15.1 mM) of CDP-choline was accumulated without addition of ATP or phosphoribosylpyrophosphate (PRPP). ATP and PRPP required in the CDP-choline forming reaction system are biosynthesized by those cells using glucose as a substrate.

Key words: CDP-choline; orotic acid; enzymatic production; ATP regeneration; PRPP supply

Cytidine diphosphate choline (CDP-choline) is an important intermediate in the biosynthesis of phospholipids, such as lecithin,[1,2] and widely used as an effective drug for various kinds of brain injuries.[3] This compound has been made by chemical[4] enzymatic[5,6] methods from cytosine nucleotide or its precursors, such as CMP and cytidine. We attempted to construct another enzymatic system for CDP-choline production using cells of microorganisms as enzyme sources from orotic acid and choline chloride as substrates. Orotic acid was obtained at a high yield by culturing a mutant of *Corynebacterium glutamicum*.[7] On the other hand, choline chloride is a cheap material and commercially available. As shown in Fig. 1, CDP-choline is synthesized through six successive enzyme reactions from orotic acid. After being phosphorylated by choline kinase, choline is incorporated into CDP-choline. Therefore, seven enzyme activities in total are required for the production of CDP-choline from orotic acid and choline chloride. Four activities among the seven require ATP and another one needs PRPP (phosphoribosylpyrophosphate). Usually six such successive and one branch reactions are expected to be too complex to use for industrial production. To use such complex reactions, we decided to use *Corynebacterium ammoniagenes* (formerly called *Brevibacterium ammoniagenes*[8]) cells, which were expected to have strong ATP regeneration activity and a sufficient PRPP supply. Because, this bacterium has been used industrially for the production of nucleotides such as GMP[9] and ATP.[10] Namely, in the production of GMP, the conversion reaction of XMP to GMP needs ATP regeneration, and in the production of ATP, the conversion reaction of adenine to ATP needs not only ATP regeneration but also a sufficient PRPP supply.

In this study, we demonstrate the possibility of the enzymatic production of CDP-choline in a one-batch mixed reaction, using *C. ammoniagenes* KY13505 cells and cells of three kinds of recombinant *Escherichia coli* strains as the enzyme sources.

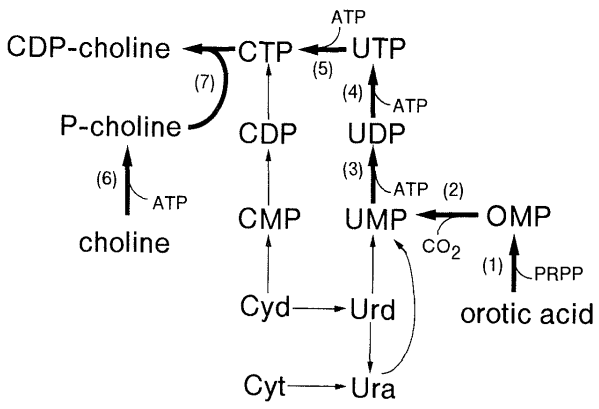

Fig. 1. Scheme of CDP-Choline Production from Orotic Acid and Choline.

The individual reactions are catalyzed by (1) orotate phosphoribosyltransferase, (2) orotate 5'-monophosphate decarboxylase, (3) nucleoside monophosphate kinase, (4) nucleoside diphosphate kinase, (5) CTP synthetase, (6) choline kinase, and (7) cholinephosphate cytidylyltransferase

Abbreviations: Ura, uracil; Urd, uridine; Cyt, cytosine; Cyd, cytidine; P-choline, phosphorylcholine; PRPP, phosphoribosylpyrophosphate.

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Materials and Methods

Bacterial strains and plasmids. Strain KY13505, a mutant of *C. ammoniagenes* (decoyinine-resistant, nucleotidase-weak, and temperature-sensitive for growth) is our laboratory strain. *E. coli* strain JF646 (*PyrE, pyrG, cdd, argE, his4, proA, thr1, thi1, recA*) and plasmid pMW6 were provided by Howard Zalkin *et al.*, Purdue University.[11] Plasmid pMW6 was constructed by ligating the 2.4-kb *Nru*I–*Pst*I segment of the *E. coli pyrG* gene, which encodes CTP synthetase into the *Sma*I–*Pst*I polylinker sites in pUC8. Plasmids pCC41 and pUCK3 were donated by Satoshi Yamashita *et al.*, Gunma University.[12,13] Plasmid pCC41 was constructed by ligating the 1.3-kb *Dra*I fragment of the *S. cerevisiae CCT* gene encoding cholinephosphate cytidylyltransferase into the *Sma*I site of pUC18. Plasmid pUCK3 was constructed by ligating the 2.3-kb blunt ended *Hpa*I–*Hin*dIII fragment of *S. cerevisiae CKI* gene encoding choline kinase into the *Hin*cII site of pUC19. Another host strain is *E. coli* strain MM294 (F^-, *supE44, thi1, endA1, hsdR17*).[14]

Media and cultivation. In the cultivation of *C. ammoniagenes* KY13505, following media were used:

Seed medium A; 50 g/liter glucose, 10 g/liter polypeptone, 10 g/liter yeast extract, 5 g/liter urea, 5 g/liter $(NH_4)_2SO_4$, 1 g/liter KH_2PO_4, 3 g/liter K_2HPO_4, 1 g/liter $MgSO_4 \cdot 7H_2O$, 0.1 g/liter $CaCl_2 \cdot 2H_2O$, 10 mg/liter $FeSO_4 \cdot 7H_2O$, 10 mg/liter $ZnSO_4 \cdot 7H_2O$, 20 mg/liter $MnSO_4 \cdot 4$–$6H_2O$, 20 mg/liter cysteine, 10 mg/liter calcium D-pantothenate, 5 mg/liter vitamin $B_1 \cdot HCl$, 5 mg/liter nicotinic acid, and 30 μg/liter biotin, the pH adjusted to 7.2 with NaOH.

Seed medium B; 100 g/liter glucose, 10 g/liter meat extract, 10 g/liter polypeptone, 1 g/liter KH_2PO_4, 1 g/liter KH_2PO_4, 1 g/liter $MgSO_4 \cdot 7H_2O$, 0.1 g/liter $CaCl_2 \cdot 2H_2O$, 20 mg/liter $FeSO_4 \cdot 7H_2O$, 10 mg/liter $ZnSO_4 \cdot 7$-H_2O, 20 mg/liter $MnSO_4 \cdot 4$–$6H_2O$, 15 mg/liter β-alanine, 20 mg/liter cysteine, 100 μg/liter biotin, 2 g/liter urea (separately sterilized), and 5 mg/liter vitamin $B_1 \cdot HCl$ (separately sterilized), adjusted to pH 7.2 with NaOH.

Fermentation medium; 180 g/liter glucose, 10 g/liter KH_2PO_4, 10 g/liter K_2HPO_4, 10 g/liter $MgSO_4 \cdot 7H_2O$, 0.1 g/liter $CaCl_2 \cdot 2H_2O$, 20 mg/liter $FeSO_4 \cdot 7H_2O$, 10 mg/liter $ZnSO_4 \cdot 7H_2O$, 20 mg/liter $MnSO_4 \cdot 4$–$6H_2O$ (separately sterilized), 15 mg/liter β-alanine, 20 mg/liter cysteine, 1 g/liter sodium glutamate, 100 μg/liter biotin, 2 g/liter urea (separately sterilized), and 5 mg/liter vitamin $B_1 \cdot HCl$ (separately sterilized), and being adjusted to pH 7.2 with NaOH.

A loopful cells of *C. ammoniagenes* KY13505 was inoculated into test tubes (20 cm × 2 cm) containing 10 ml of the seed medium A and incubated at 28°C for 24 h on a reciprocal shaker (300 rpm). Twenty ml of the seed culture was transferred into a 2-liter Erlenmeyer flask with baffles, which contained 230 ml of the seed medium A, and was incubated at 28°C for 24 h on a rotary shaker (190 rpm). The culture (250 ml) was then inoculated into 2.5 liter of the seed medium B in a 5-liter jar fermentor (KMJ-5, Mitsuwa Bio Systems Co., Ltd.). This seed cultivation was done at 32°C with aeration (1 vvm) and agitation (600 rpm). The pH of the culture was kept at 6.8 with 28% NH_4OH. When the glucose in the supernatant of the seed culture was completely consumed, a 350 ml portion of the culture was transferred into 2.5 liters of the fermentation medium in a 5-liter jar fermentor. The cultivation was done at 32°C with aeration (1 vvm) and agaitation (600 rpm). The pH of the culture was kept at pH 6.8 with 28% NH_4OH. The cultivation was stopped when the glucose in the supernatant of the culture was completely consumed. The cultured broth was centrifuged at 16,000 × *g* for 40 min at 4°C and harvested cells were frozen and kept at −20°C.

LB medium (1% Bacto tryptone, 0.5% yeast extract, 0.5% NaCl) was adjusted to pH 7.2 with NaOH to cultivate *E. coli* strains. After sterilization, an ampicillin solution was added to media (50 mg/liter) through a membrane filter. Ten ml of the seed culture of *E. coli* cultivated in a test tube (20 cm × 2 cm) at 28°C overnight was inoculated into 400 ml of LB medium in a 1-liter Erlenmeyer flask, and cultivated at 35°C for 17 h on a reciprocal shaker (75 rpm). Cells were harvested from the culture broth by centrifugation at 16,000 × *g* for 30 min at 4°C and were frozen and kept at −20°C. These stored cells of *C. ammoniagenes* KY13505 and *E. coli* strains were used in the enzymatic reaction described in the next paragraph.

Enzymatic reaction. The reaction mixture contained the stored cells, substrates, glucose, and other required ingredients. Precise compositions are described in the legends to Fig. 1. Twenty ml of the reaction mixture was poured into a 200-ml beaker and incubated at 32°C in a water bath for 23–26 h, with agitation at 900 rpm by a magnetic stirrer. The pH of the reaction mixture was maintained at pH 7.2 with 7% NH_4OH. During the reaction, an appropriate amount of glucose, KH_2PO_4, and $MgSO_4 \cdot 7H_2O$ were added to the reaction mixture, if necessary.

Analyses. The concentration of CDP-choline, UMP, and uracil in the reaction were measured by high performance liquid chromatography (HPLC) under these conditions: column, Partisil 10 SAX 4.6 mm × 250 mm (Whatmann); detection, 254 nm; mobile phase, 0.05 M KH_2PO_4 (pH 3.5); column temperature, 50°C; and flow rate, 1.5 ml/min. UTP and CTP were also measured by HPLC. The conditions for HPLC were as follows: column, Partisil 10 SAX 4.6 mm × 150 mm; detection, 254 nm; mobile phase, 0.4 M KH_2PO_4 (pH 5.0); column temperature, 50°C; and flow rate, 1.4 ml/min. Glucose, inorganic phosphate, and magnesium ion were measured using assay kits, GlucoseB-Test Wako, PhosphorB-Test Wako, and MagnesiumB-Test Wako respectively, purchased from Wako Pure Chemical Industry Ltd. (Osaka).

Chemicals. Orotic acid used was the product of Kyowa Hakko Kogyo Co., Ltd. The surfactant, Nymeen S-215 (polyoxyethylene stearylamine), was purchased from Nippon Oil and Fats Co., Ltd. (Tokyo). All other chemicals used here were commercially available and of analytical grade.

Results and Discussion

Production of UMP

Nakayama *et al.* reported that 4.3 g/liter of UMP was accumulated fermentatively in the culture broth when *C. ammoniagenes* ATCC 6872 was incubated in a medium containing orotic acid.[15] Based on the report, we examined the enzymatic reaction using *C. ammoniagenes* KY13505 cells whether UMP was accumulated to a higher level or not in the presence of orotic acid. We expected to accumulate a larger amount of UMP because the pH was maintained at an optimal level and cells were treated with Nymeen S-215 and xylene, which enable the substrate and products to permeate well across the cell membrane. A typical time course of UMP production is shown in Fig. 2. A large amount of UMP, 28.6 g/liter (77.6 mM), was accumulated in 26 h, while UDP and UTP were co-produced at far lower level than UMP. In this reaction, phytic acid was added as a weak chelating agent to prevent magnesium ion from forming insoluble salts such as magnesium ammonium phosphate. The reason for the high accumulation of UMP was that cells used for this reaction were treated with the surfactant, Nymeen S-215 and xylene,

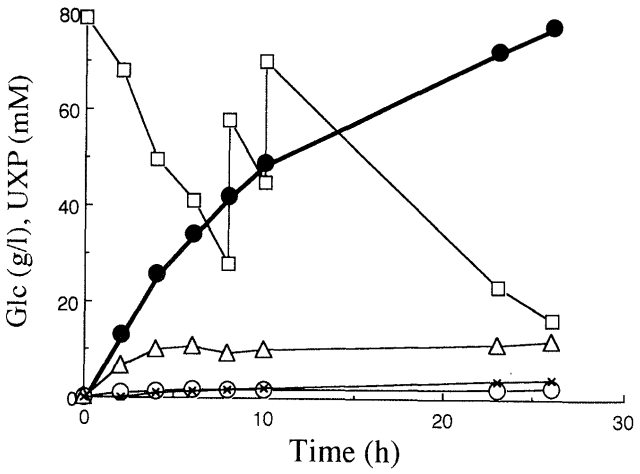

Fig. 2. Time Course of UMP Production.

The initial reaction mixture consisted of 20 g/liter orotic acid, 80 g/liter glucose, 5 g/liter phytic acid, 10 g/liter KH_2PO_4, 5 g/liter $MgSO_4 \cdot 7H_2O$, 4 g/liter Nymeen S-215, 10 ml/liter xylene and 200 g/liter *C. ammoniagenes* KY13505 cells (wet weight). Glucose was fed 30 g/liter (at 8 h) and 20 g/liter (at 10 h), respectively.
Symbols: ●, UMP; ○, UDP; △, UTP; ×, uracil; □, glucose.

resulting in higher permeability of the cell membrane to UMP accumulated in the cells. Recently, Doi *et al.* reported high production of uridine (65 g/liter) from glucose by a mutant of *Bacillus subtilis*.[16] Our result indicates the possibility that UMP can be produced from orotic acid at a low cost. This is the first report on the accumulation of uridine nucleotides at an industrially applicable level. UMP is one of the important products as food additives and medicine or its precursor.

We further investigated the possibility of CTP production using KY13505 cells, but CTP was not accumulated sufficiently compared to UTP even after optimization of several reaction conditions (data not shown). As shown in Fig. 3, the accumulation of CTP did not depend on the presence of orotic acid in contrast to that of UTP. It appeared that the CTP accumulated in the reaction mixture was synthesized from precursors in the cell such as CMP from RNA decomposition, but not converted from orotic acid added from outside. Very low activity of CTP synthetase or tight regulation of CTP synthetase by CTP might be the reason for such lower CTP accumulation.

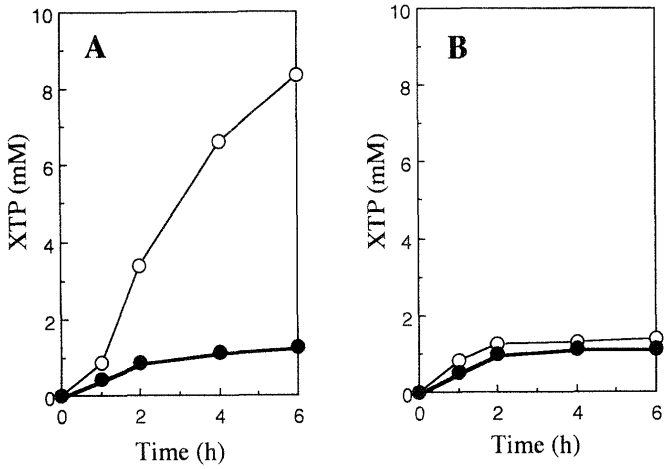

Fig. 3. Production of CTP and UTP in the Presence (A) or Absence (B) of Orotic Acid.

The reaction mixture consisted of 10 g/liter or 0 g/liter orotic acid, 80 g/liter glucose, 5 g/liter phytic acid, 20 g/liter KH_2PO_4, 5 g/liter $MgSO_4 \cdot 7H_2O$, 4 g/liter Nymeen S-215, 10 ml/liter xylene and 200 g/liter *C. ammoniagenes* KY13505 cells (wet weight). Symbols: ●, CTP; ○, UTP.

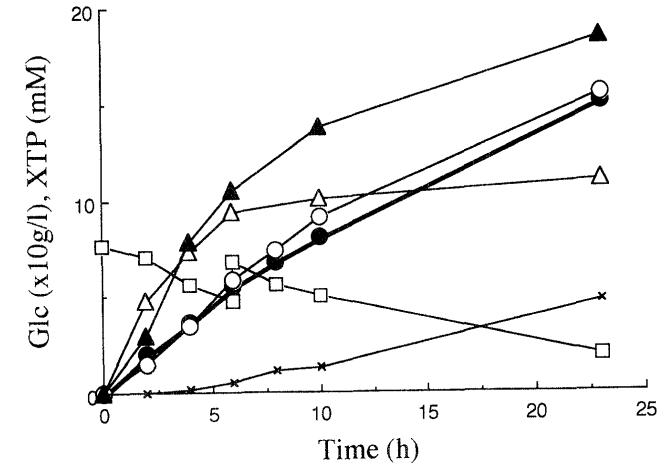

Fig. 4. Time Course of CTP Production.

The initial reaction mixture consisted of 15 g/liter orotic acid, 80 g/liter glucose, 5 g/liter phytic acid, 20 g/liter KH_2PO_4, 5 g/liter $MgSO_4 \cdot 7H_2O$, 1.5 g/liter ATP·$Na_2 \cdot 3H_2O$, 0.3 g/liter guanine, 4 g/liter Nymeen S-215, 10 ml/liter xylene, 20 g/liter *E. coli* JF646/pMW6 cells (wet weight), and 150 g/liter *C. ammoniagenes* KY13510 cells (wet weight). Glucose was fed 20 g/liter at 6 h of the reaction.
Symbols: ●, CTP; ○, UTP; △, UDP; ▲, UMP; ×, uracil; □, glucose.

Production of CTP

Recently, Asahi *et al.* reported that 14.2 g/liter of cytidine was accumulated by a mutant of *B. subtilis*. In this case, the CTP synthetase of this mutant strain had been changed to be free from feedback regulation by CTP.[17] Therefore, to accumulate CTP at a higher level, the increase of CTP synthetase activity was supposed to be important. The *E. coli pyrG* gene, which encodes CTP synthetase, has been cloned and highly expressed in *E. coli*.[11] We decided to use *E. coli* JF646/pMW6 cells, which overexpressed CTP synthetase as an enzyme source. Figure 4 shows a typical time course of CTP accumulation in the mixed reaction of *C. ammoniagenes* KY13505 and *E. coli* JF646/pMW6. After 23 h of incubation, 8.95 g/liter (15.1 mM) of CTP was accumulated, which was much higher than the case using *C. ammoniagenes* cells alone. UMP, UDP, and UTP were as well accumulated to roughly the same level as CTP. The accumulated CTP was from orotic acid (data not shown). If the reaction conditions were optimized, the production level of CTP might become still higher. The fact that a significant amount of nucleotides such as CTP, UTP, UDP, and UMP was accumulated indicates the existence of low nucleotidase activity in this reaction mixture. Namely, *C. ammoniagenes* KY13505 cells are genetically nucleotidase weak (see Materials and Methods), and *E. coli* JF646/pMW6 cells also have very low nucleotidase activity under these reaction conditions. Therefore, this experiment represents the possibility that CTP can be industrially produced from orotic acid at a low cost.

Production of CDP-choline

To produce CDP-choline from orotic acid and choline chloride, another two enzymes were required. The genes of these two enzymes originated from *S. cerevisiae* were cloned and expressed in *E. coli*.[12,13] Therefore, we decided to use the enzymes of *S. cerevisiae*. The cholinephosphate cytidylyltransferase (encoded by the *CCT* gene) and choline kinase (encoded by the *CKI* gene) were expressed in *E. coli* strains, MM294/pCC41 and MM294/pUCK3, respectively. After these strains were independently cultured as described in Materials and Methods, the cells of each strain were collected and added to the CTP producing reaction mixture described above. Since the preliminary experiment shown that Nymeen S-215 evidently inhibited CCTase activity (data not shown), we used only xylene as a reagent to make the cells permeable.

Figure 5 shows a typical time course of the reaction. After 23 h of reaction, 7.7 g/liter (15.1 mM) of CDP-choline was accumulated. The soluble phosphate concentration was maintained in the range from 70 mM to 140 mM. During this reaction, CTP was accumulated at a low level (maximum 2.7 mM), suggesting that CCTase and CKIase activities rather than CTP synthetase activity were rate-limiting. The nucleotidase (or phosphatase) activity of *E. coli* cells was also negligible under these reaction conditions, in spite of the large amount of *E. coli* cells that were used.

This report proves that CDP-choline can be produced from orotic acid and choline chloride in a one-vessel enzymatic reaction. However, it is another question whether

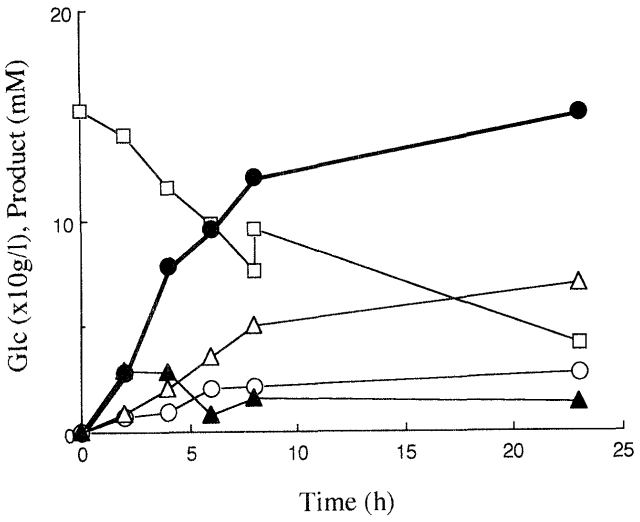

Fig. 5. Time Course of CDP-Choline Production.

The initial reaction mixture consisted of 10 g/liter orotic acid, 11.2 g/liter choline chloride, 150 g/liter glucose, 5 g/liter phytic acid, 20 g/liter KH_2PO_4, 5 g/liter $MgSO_4 \cdot 7H_2O$, 1.5 g/liter ATP·$Na_2 \cdot 3H_2O$, 0.3 g/liter guanine, 10 ml/liter xylene, 20 g/liter *E. coli* JF646/pMW6 cells (wet weight), 20 g/liter *E. coli* MM294/pUCK3 cells (wet weight), 50 g/liter *E. coli* MM294/pCC41 cells (wet weight) and 150 g/liter *C ammoniagenes* KY13505 cells (wet weight). Glucose was fed 20 g/liter at 8 h of the reaction.
Symbols: ●, CDP-choline; ○, CTP; △, UTP; ▲, UMP; ×, uracil; □, glucose.

such reactions could be conducted on a commercial basis because handling concomitantly four kinds of strains, three of which requires high cell concentrations, is difficult. Additionally, collected and freeze-thawed cells were used in this experiment. To use cells in such a condition were far from the industrially applicable stage. It is required that the cultured brothes must be used directly for the industrial production. To give a solution for such a problem, improved processes will be described in the accompanying paper.

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