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Effects of Several Factors on CDP-choline Production by Yeast*

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Favorable reaction conditions were sought for CDP-choline production from CMP and choline compounds by *Candida* sp. N-25-2. CDP-choline production from CMP and choline was achieved by the addition of toluol to intact cells or dried cells. An examination of the effects of choline compounds on CDP-choline production revealed that phosphoryl choline and choline chloride gave comparably high yields of CDP-choline, but the addition of choline phosphate in high concentration decreased the yield. The effects of other factors, for example, the concentration of glucose, phosphate, Mg^{2+} and intact cells, on CDP-choline production were also studied. Under optimal conditions, a high rate of conversion of CMP to CDP-choline of 80~87% was obtained.

Further, the effect of ATP on CMP phosphorylation and CDP-choline production was investigated in several yeast strains. ATP was found to enhance CTP production but not CDP-choline production.

CDP-choline is an important intermediate in the biosynthetic pathway of phospholipid, and an effective drug for various brain injuries. Hitherto it has been prepared by chemical methods.[1] Recently, Tochikura *et al.*,[2] Kimura *et al.*,[3] Kariya *et al.*[4] and Miyauchi *et al.*[5] reported the formation of CDP-choline by microorganisms.

In the previous paper,[6] we reported the preparation of yeast cells for CDP-choline production. This paper deal with the optimal reaction conditions for CDP-choline production by *Candida* sp. N-25-2.

Materials and Methods

Microorganisms The yeasts used were from stock cultures in our laboratory.

Culture method Strains were cultured at 30°C for 24—48 hr with shaking in 500-ml flasks containing 100 ml of the medium shown in Table 1.

Preparations of yeast cells Cultured yeast cells were collected by centrifugation at 3,000 rpm for 10 min, washed twice with distilled water, and resuspended in 200 mM phosphate buffer (pH 7.0). Washed cells were also dried by lyophilization or in air after acetone treatment.

Enzyme reaction The standard reaction mixture is shown in Table 2. The mixture was incubated

Table 1. Composition of medium.

Cane molasses (as sugar)	3.0 (%)
$(NH_4)_2HPO_4$	0.2
KH_2PO_4	0.2
$MgSO_4 \cdot 7H_2O$	0.1
Peptone	0.5
Yeast extract	0.2

pH 6.0

Table 2. Standard reaction mixture.

Glucose	400 mM
$Na_2CMP \cdot 4H_2O$	20 〃
Choline compound	50 〃
$MgSO_4 \cdot 7H_2O$	15 〃
Potassium phosphate buffer (pH 7.0)	200 〃
Cells (as dry cell weight)	5% (w/v)
Toluol	5% (v/v)

pH 7.0

* Enzymatic Studies on Nucleic Acid Related Compounds of Microorganisms (V)

Abbreviation. CR, cytidine; CMP, cytidine-5′-monophosphate; CDP, cytidine-5′-diphosphate; CTP, cytidine-5′-triphosphate; AMP, adenosine-5′-monophosphate; ADP, adenosine-5′-diphosphate; ATP, adenosine-5′-triphosphate; CDP-choline, cytidine diphosphate choline.

under continuous reciprocal shaking at 30°C, and the reaction was stopped by heating the mixture in boiling water for 5 min.

Analytical methods Cytidine, CMP, CDP, CTP and CDP-choline were determined by paper chromatography as reported previously,[6] and this identity was confirmed by high voltage paper electrophoresis.

Results and Discussion

Effect of toluol and surfactants on CDP-choline production Kimura *et al.*[8] reported that water content of yeast cells greatly affected the formation of CDP-choline. Nevertheless, we sought an economical process for producing CDP-choline with intact cells of *Candida* sp. N-25-2. In this experiment, both intact and dried cells were used.

As shown in Table 3, in the absence of toluol, CDP-choline was formed by acetone-dried cells, but not by intact or lyophilized cells, which instead produced cytidine from CMP. In the presence of toluol, however, the decomposition of CMP to cytidine was repressed and the formation of CDP-choline was strikingly accelerated. These effects were observed in both lyophilized cells and intact cells. Two surfactants tested with intact cells were ineffective for CDP-choline production.

Based on these results, the CDP-choline productivity of intact cells in the presence of toluol was examined further.

Effect of choline compounds on CDP-choline production CDP-choline biosynthesis is known to involve the phosphorylation of CMP to CTP, and the addition of phosphoryl choline to CTP to form CDP-choline. In addition phosphoryl choline is formed by the phosphorylation of choline.

The effects of choline compounds on CDP-choline production by *Candida* sp. N-25-2 were examined, and the results are shown in Fig. 1. With choline chloride and phosphoryl choline, the formation of CDP-choline increased proportion to the concentration of the choline compound, and the yields of CDP-choline from these compound were similar. On the other hand, a high concentration of choline phosphate repressed the formation of CDP-choline. This mechanism was studied in detail and will be reported elsewhere.

Table 3. Effect of additives on CDP-choline production by *Candida* sp. N-25-2.

Enzyme source	Additive		Yield	
			CDP-choline	Cytidine
Acetone-dried cells	none		9.6 (mM)	2.0 (mM)
Lyophilized cells	none		0.8	6.2
//	Toluol	1% (v/v)	9.8	1.8
//	//	3	14.2	trace
//	//	5	15.0	//
Intact cells	none		trace	7.6
//	Toluol	1% (v/v)	8.2	2.8
//	//	3	14.4	trace
//	//	5	14.8	//
//	SLS*	0.2% (w/v)	1.2	6.4
//	//	1.0	0.8	6.8
//	CTAB**	0.1% (w/v)	1.0	5.9
//	//	0.5	0.6	6.0

* Sodium lauryl sulfate
** Cetyltrimethyl ammonium bromide
The reaction mixture was as described in Table 2 except that toluol was replaced by the additives shown above. Phosphoryl choline was used as choline compound, and incubation was carried out with shaking at 30°C for 16 hr.

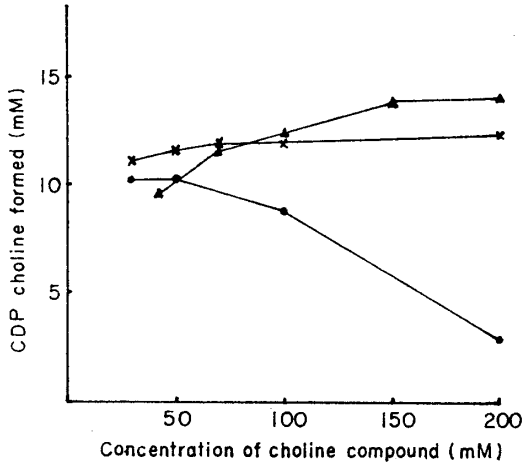

Fig. 1. Effect of choline compounds on CDP-choline production by *Candida* sp. N-25-2.
The reaction mixture was as described in Table 2 except for the concentrations of choline compounds. Intact cells were used, and incubation was carried out with shaking at 30°C for 16 hr.
●, choline phosphate; ▲, choline chloride; ×, Ca. phosphoryl choline chloride.

Effect of other several factors on CDP-choline production To establish the optimal conditions for CDP-choline production by *Candida* sp. N-25-2, the effects of several factors were studied.

The effect of phosphate concentration is shown in Fig. 2, which reveals an optimum of at least 120 mM.

Glucose is important as an energy source for phosphorylation of nucleotide and choline.

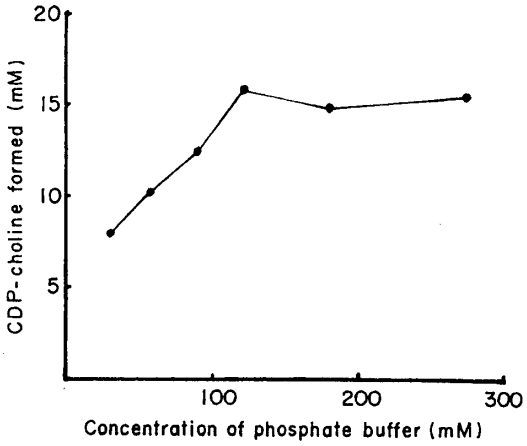

Fig. 2. Effect of phosphate concentration on CDP-choline production.
The reaction mixture was as described in Table 2 except for phosphate buffer concentration. Choline chloride and intact cells were used, and incubation was carried out with shaking at 30°C for 16 hr.

As shown in Fig. 3, the optimal concentration of glucose lay above 400 mM.

The effect of Mg^{2+} concentration was also investigated. As shown in Fig. 4, the addition of Mg^{2+} enhanced CDP-choline production, and 15 mM was a sufficient concentration. Even without addition of Mg^{2+}, however, CDP-choline was formed, probably due to the presence of endogenous Mg^{2+} in cells.

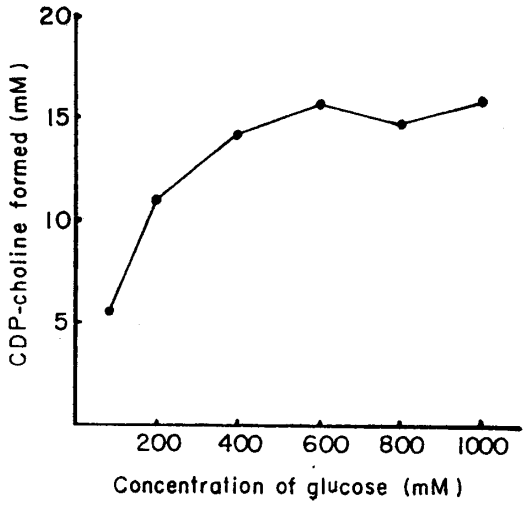

Fig. 3. Effect of glucose concentration on CDP-choline production.
The reaction mixture was as described in Table 2 except for glucose concentration. Choline chloride and intact cells were used, and incubation was carried out with shaking at 30°C for 16 hr.

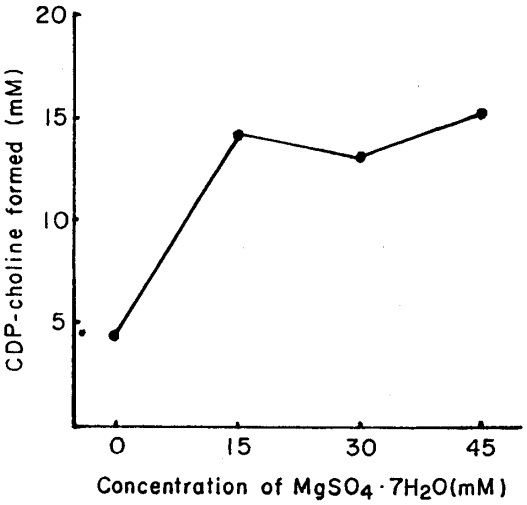

Fig. 4. Effect of Mg^{2+} concentration on CDP-choline production.
The reaction mixture was as described in Table 2 except for $MgSO_4 \cdot 7H_2O$ concentration. Choline chloride and intact cells were used, and incubation was carried out with shaking at 30°C for 16 hr.

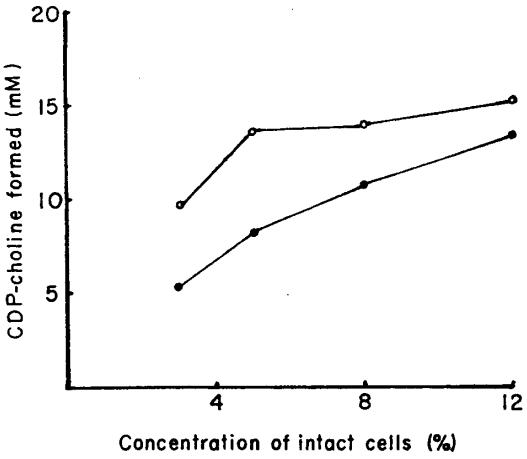

Fig. 5. Effect of concentration of intact cells on CDP-choline production.
The reaction mixture was as described in Table 2 except for cell concentration. Choline chloride was used, and incubation was carried out with shaking at 30°C.
●, 8 hr reaction, ○, 16 hr reaction.

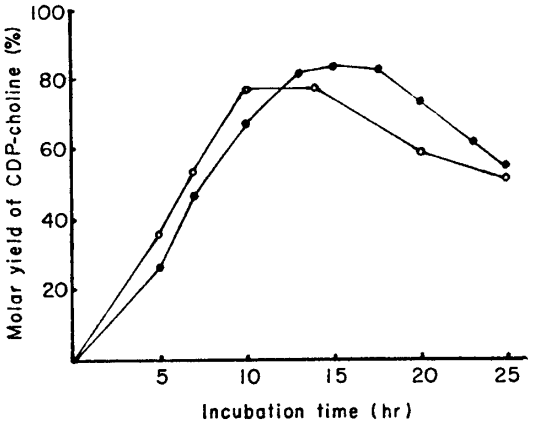

Fig. 6. Time course of CDP-choline production.
The reaction mixture was as described in Table 2 except for the concentration of choline compound. Intact cells were used, and incubation was carried out with shaking at 30°C.
○, 50 mM of phosphoryl choline;
●, 200 mM of choline chloride.

Figure 5 shows the relationship between the concentration of cells and CDP-choline production. At the early stage of the reaction (8 hr), the yield of CDP-choline increased in proportion to the concentration of cells, though the reaction seemed not to have gone to completion. However, if the reaction was prolonged (16 hr), the yield of CDP-choline reached its maximal level at the higher than 5% of cells.

The time course of CDP-choline production in the optimal reaction system was examined, and the results are shown in Fig. 6. In the early stage of the reaction, phosphoryl choline gave slightly higher CDP-choline production than choline chloride. The final yield of CDP-choline was higher from choline chloride than phosphoryl choline, however and the maximal yields were respectively 80% and 87% (molar yield).

Effect of ATP addition on CMP phosphorylation and CDP-choline production

We have previously reported that such

Table 4. Effect of ATP addition on CDP-choline production.

Strain	ATP not added		ATP added	
	Yield (mM)		Yield (mM)	
	CDP-choline	CDP, CTP	CDP-choline	CDP, CTP
Saccharomyces lactis	0.8	9.0	1.0	14.2
Debaryomyces hansenii	3.8	1.6	4.0	12.8
Hansenula anomala	0	0.8	0	1.2
Rhodotorula glutinis	0	4.5	0	9.2
Trigonopsis variabilis	0	8.9	0	13.2
Kloeckera apiculata	0	1.2	0	1.0
Endomycopsis fibuligera	0.9	0.8	0.8	1.0
Sporobolomyces salmonicolor	0	1.0	0	1.2
Brettanomyces petrophilum	14.2	3.8	14.8	3.7
Candida sp. N-25-2	14.8	3.6	15.1	3.8

The culture medium was as described in Table 1. Cultivation time was 48 hr. The reaction mixture was as described in Table 2, except when 5 mM ATP was added, as shown above. Phosphoryl choline and intact cells were used, and incubation was carried out with shaking at 30°C for 18 hr.

adenine nucleotides as ATP, ADP and AMP enhanced CTP production by yeasts.[7] In the previous paper, we observed that several kinds of yeast could not produce CDP-choline from CMP and phosphoryl choline because they lack the ability to produce CTP from CMP. Therefore, we assumed that the CTP productivity of these strains could be promoted by ATP, they might also produce CDP-choline from CMP and phosphoryl choline. The effects of ATP on CDP-choline production by yeasts with various CDP-choline productivities were therefore studied.

The results are summarized in Table 4. Of the 10 strains, *Hansenula anomala, Kloeckera apiculata, Endomycopsis fibuligera* and *Sporobolomyces salmonicolor* produced cytidine from CMP, as shown in previous report. In these strains, ATP did not promote even for the phosphorylation of CMP. In contrast, *Saccharomyces lactis, Debaryomyces hansenii, Rhodotorula glutinis* and *Trigonopsis variabilis* showed enhanced productivity of CDP and CTP in the presence of ATP, but not of CDP-choline. This suggested that these strains lack choline phosphate cytidyltransferase.

Lastly, in the strains capable of producing CDP-choline, *Brettanomyces petrophilum* and *Candida* sp. N-25-2, the addition of ATP had no marked effect on CDP-choline production.

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