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REGENERATION OF ATP BY IMMOBILIZED MICROBIAL CELLS AND ITS UTILIZATION FOR THE SYNTHESIS OF ATP AND CDP-CHOLINE

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A number of fermentative methods for the production of various important nucleotides from their precursors have been developed in Japan, and some of these nucleotides have been industrially produced for more than a decade. For the production of these nucleotides, required ATP is usually supplied to the fermentation media either exogenously or by the naturally occurring ATP regenerating system involved in microbial cells. In this paper, the authors describe an attempt to immobilize ATP-regenerating microbial cells and then utilize such immobilized cells for the continuous production of various nucleotides.

INTRODUCTION

In the present paper two examples were chosen as model reactions for the biosynthesis of nucleotides. The first one was the production of ATP from AMP by *Saccharomyces cerevisiae* cells, developed by Tochikura et al. (1). As shown in Fig. 1, 2 mol of ATP must be regenerated from equimolar ADP to produce 1 mol of ATP from equimolar AMP, and the glycolytic process of the yeast cells acts as an ATP regenerator. The second example was the production of cytidine diphosphate-choline (CDP-choline) from CMP and choline chloride by *S. cerevisiae*, reported by Nakayama and Hagino (2) and also by Tochikura et al. (3).

CDP-choline is known as a precursor of a phospholipid, lectithin, and is used as a therapeutic agent for brain nerve injury. As shown in Fig. 2, for the synthesis of CDP-choline from CMP and choline chloride, three enzymes, choline kinase, CMP kinase, and CDP-choline pyrophosphorylase, are involved, and 3 mol of ATP must be regenerated from equimolar ADP to synthesize 1 mol of CDP-choline.

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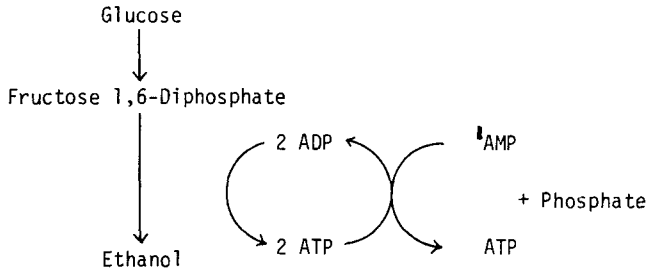

FIG. 1. Multiple enzyme system for the synthesis of ATP in *S. cerevisiae*.

MATERIALS AND METHOD

Culture of Microorganism

S. cerevisiae KY-5697 was used throughout the present study, both as the producer of the nucleotides and as an ATP regenerator. The yeasts were grown in a 15-l medium of pH 5.3 in a 30-l jar fermentor containing 3.0% glucose, 0.5% ammonium sulfate, 0.1% KH_2PO_4, 0.05% magnesium sulfate, 0.01% sodium chloride, 0.01% calcium chloride, and a few kinds of metallic ions, with aeration of 1.0 vvm and agitation of 300 rpm for 36 h at 30°C. The cells were collected by centrifugation, washed two times with 0.01 M sodium acetate buffer (pH 6.0), lyophilized, and used for immobilization.

Analysis

High-performance liquid chromatography was conveniently applied for the analysis of the substrates and the products. For the analysis of

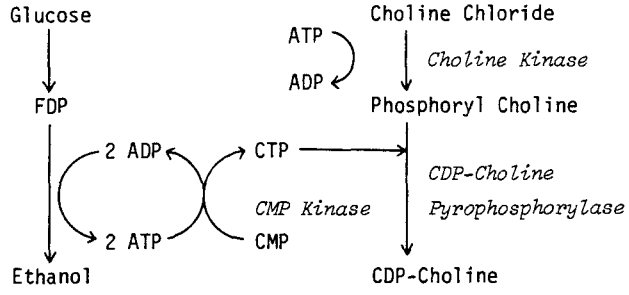

FIG. 2. Multiple enzyme system for the synthesis of CDP-choline in *S. cerevisiae*.

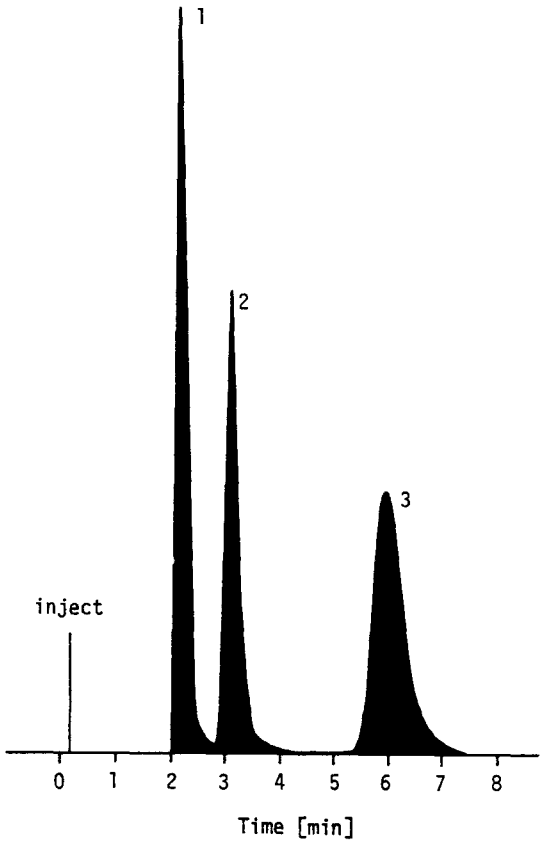

FIG. 3. HPLC standard curve of adenosine derivatives: AMP (1), ADP (2), and ATP (3). Conditions: gel, Lichrosorb-NH_2; column, 2.6 mm (ID) × 250 mm (RT); solvent, 0.5-M KH_2PO_4; flow rate, 1.0 ml/min (75 kg/cm^2); detector, UV 254 nm 0.32 AUFS.

adenosine derivatives, Lichrosorb-NH_2 was used as a packing material for the column and the products charged were eluted with a 0.5-M KH_2PO_4 solution of pH 4.0 at a speed of 0.5 ml per min, then detected by UV sensor at 254 nm. For the analysis of cytidine derivatives, Microbondapak-NH_2 was packed in the column, the products were charged and a linear gradient elution was carried out at the rate of 1 ml per min by a mixture of 0.01-M $NH_4H_2PO_4$ of pH 5 and 0.25-M $NH_4H_2PO_4$ of pH 3.5. The substances eluted were detected similarly. The chromatographic patterns of the reference substances are shown in Fig. 3 and 4.

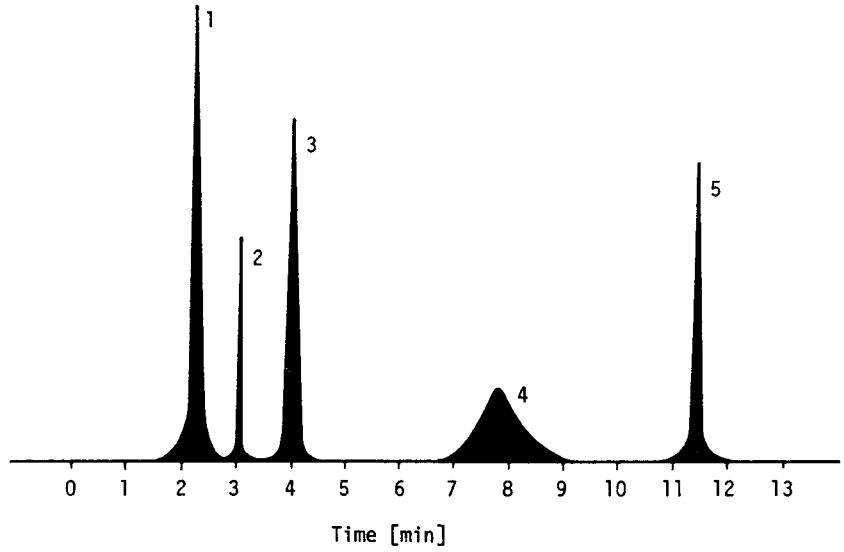

FIG. 4. HPLC standard curve of cytidine derivatives (0.2 g/l): cytidine (1), CDP-choline (2), CMP (3), CDP (4), and CTP (5).

Immobilization Method of Microbial Cells

Two different immobilization methods were employed. The first method was microencapsulation of microbial cells in ethylcellulose with or without chitosan pretreatment. Ten grams of ethylcellulose powder, 145 g of benzene (as dispersion medium), 55 g of *n*-hexane (as dispersion aid) and 4 g of sorbitan monolaurate (as dispersing agent) were mixed and stirred vigorously at 5°C for 10 min.

To this mixture, a suspension of microbial cells in 55 ml water containing 1% NaCl (and 1% chitosan) was added and stirred vigorously for 10 min to form a water/oil (w/o) emulsion. This emulsion was then dropped into 600 ml of 1% polyethyleneglycol aqueous solution and stirred vigorously at 5°C for 30 to 40 min to form a water/oil/water (w/o/w') emulsion. Then 1,000 ml of *n*-hexane was added to the emulsion to precipitate the ethylcellulose-encapsulated cells. The encapsulated cells were recovered by filtration and washed with 5 times volume of 0.1-M phosphate buffer (pH 7.5). The preparations were spherical beads of diameters between 100 and 200 μm.

The second method was microcapsulation in the same way as the previous method, but cellulose acetate butyrate was used instead of ethylcellulose. The former cellulose derivative is available as a material for the

ultrafiltration membrane and can hopefully prevent the leakage of low molecular weight coenzymes from the encapsulated cells.

Three grams of cellulose acetate butyrate powder, 27 g of isobutyl acetate (as dispersing agent) and 1.8 g of detergent were mixed and stirred vigorously at 5°C for 10 min. To this mixture, a suspension of microbial cells in 13 ml of distilled water containing 1% sodium chloride and 1% chitosan was added and stirred vigorously for 10 min to form a water/oil (w/o) emulsion. Then, this emulsion was dropped into 120 ml of an aqueous solution containing 1.44 g Emulgen-985 (nonionic surfactant, the product of Kao Soap Co., Ltd.) and 0.6 g Celogen-PR (dispersing agent, the product of Daiichikogyo Co., Ltd.) and stirred vigorously at 5°C for 30 to 40 min to form a water/oil/water (w/o/w') emulsion.

The Apparatus for the Long-Run Enzyme Reaction

The typical apparatus used in the continuous column operation of immbilized cells is shown in Fig. 5. The reactor was equipped with a gas purge tube on the top to eliminate CO_2 gas evolved during the reaction. The substrate solutions were sterilized either by autoclaving or by millipore

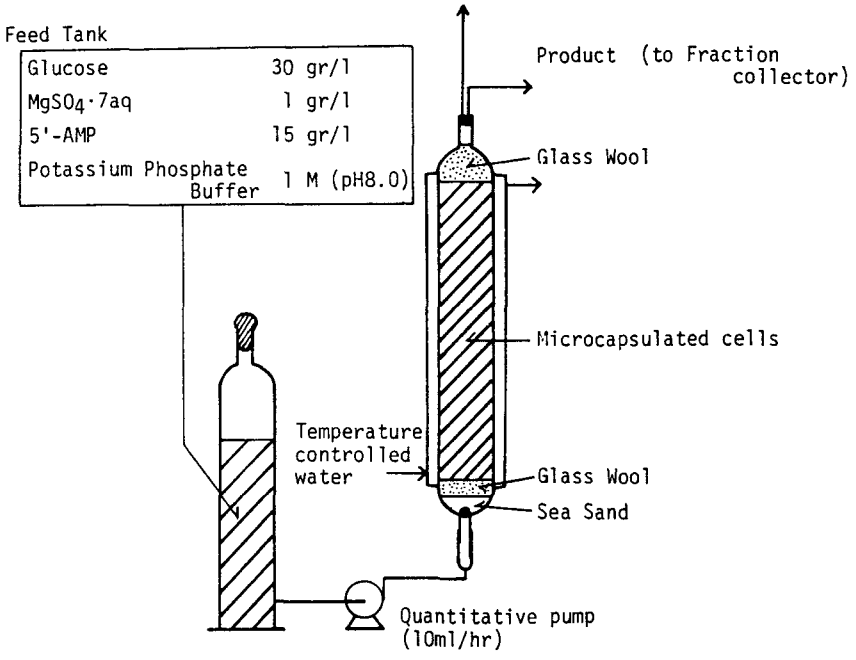

FIG. 5. Packed column reactor for continuous reaction.

filtration. The substrate solutions were sent to the reactor at a constant flow rate by a rate-controlled pump as ascending flow. When sterile conditions were needed, the entire line of the apparatus was sterilized by flowing lysozyme solution, or immobilized lysozyme columns were placed before and after the main reactor.

RESULTS

Comparison of Immobilization Methods of Microbial Cells

Some characteristics of immobilized cells prepared as described above were examined and results are summarized in Table 1. In comparison with ethylcellulose and chitosan, microcapsulation with cellulose acetate butyrate and chitosan was not able to entrap as many microbial cells per unit volume of the final gel, but the retention of activity was almost comparable to that of the former. Concerning mechanical strength, the latter was superior to the ethylcellulose and chitosan microcapsules.

On the other hand, ethylcellulose microcapsulation without chitosan treatment produced much more fragile microcapsules than the other two entrapment methods, and the activity of such a preparation showed a gradual but unambiguous decrease in a comparatively short-run operation.

Production of ATP from AMP by S. cerevisiae

Properties of the Immobilized Cells and Native Cells. The pH-activity relationship of the immobilized cells and the native cells for the production of ATP was examined and the results are shown in Fig. 6. The two curves of activity vs. pH are almost similar and show a constant activity in the pH region from 7.5 to 8.0. The effect of temperature on the activity of the cells

TABLE 1. **Comparison of Immobilization Methods of Microbial Cells**

	AMP → ATP			CMP → CDP-choline		
Entrapped method	A[a]	B	C	A	B	C
Ethylcellulose microcapsulation	200	62	10	200	62	—
Ethylcellulose and chitosan microcapsulation	300	71	15	300	62	10
Cellulose acetate butyrate and chitosan microcapsulation	150	70	—	150	70	10

[a] Column headings are defined as follows: A, entrapped cells (my·cell/ml gel); B, retention of activity (%); C, stability in continuous operation (days).

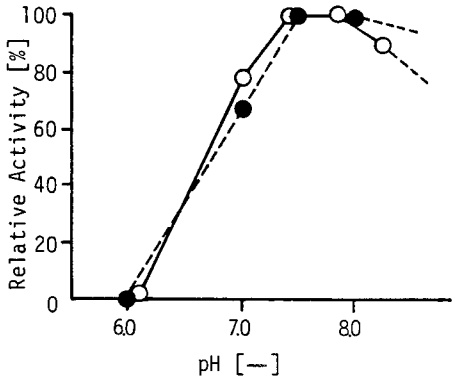

FIG. 6. Effect of pH on the activity of immobilized cells (open circles) and native cells (solid circles).

entrapped with ethylcellulose and chitosan and that of the native cells was examined and the results are shown in Fig. 7. The pH stability of the immobilized and the native cells was studied in the presence of the phosphate buffers. Both cells were exposed to various pH values for 5 h, and the residual activities were assayed at pH 8.0. The results are shown in Fig. 8. Both the immobilized and the native cells were found to be stable in the pH region from 7.5 to 8.0 The heat stability of the immobilized and native cells was examined in the presence of the substrates. Both cells were exposed to different temperatures for 5 h, and then assayed at 35°C. The results are shown in Fig. 9. The immobilized cells are more stable at higher temperatures than the native cells.

Long-Run Operation of the Column of the Immobilized Cells. The substrate solution was composed of 166-mM glucose, 43-mM 5'-AMP

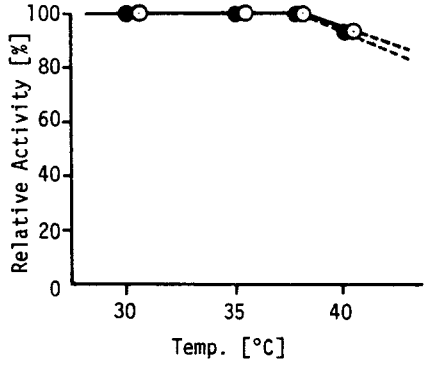

FIG. 7. Effect of temperature on the activity of immobilized cells (open circles) and native cells (solid circles).

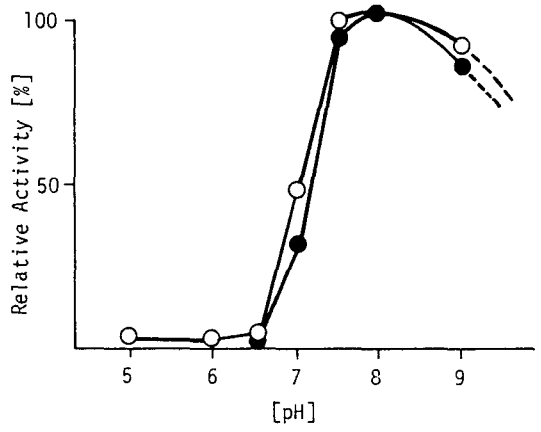

FIG. 8. Effect of pH on stability of activity of immobilized cells (open circles) and native cells (solid circles).

(15 mg/ml), 4-mM $MgSO_4$, and 1-M phosphate buffer (pH 8.0) and passed through the column reactor at s.v. 0.2 and at 35°C. At the beginning, the molar conversion rate from AMP to ATP was more than 70%. Although the enzyme activity decreased gradually, a conversion rate of more than 50% was maintained during 10 days of operation (Fig. 10).

Production of CDP-Choline from CMP and Choline Chloride

Properties of the Immobilized and Native Cells. The pH-activity relationship of the cells entrapped with cellulose acetate butyrate and

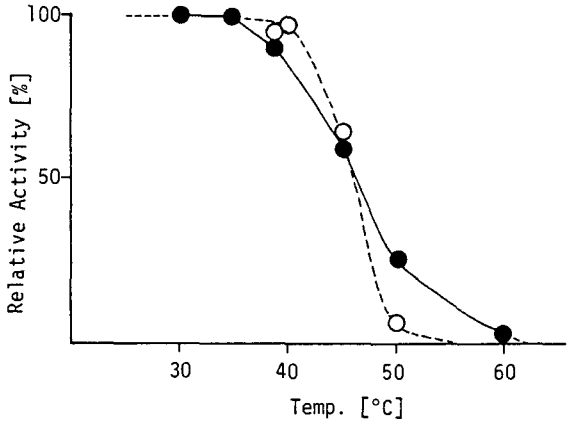

FIG. 9. Effect of temperature on stability of activity of immobilized cells (open circles) and native cells (solid circles).

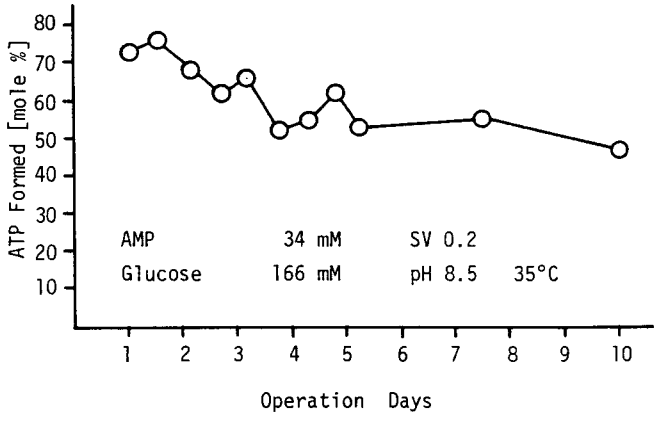

FIG. 10. Continuous operation of column of immobilized cells for production of ATP.

chitosan and that of the native cells were examined in the pH region from 6.0 to 8.5 (Fig. 11). The native cells show the maximum activity at two pH values (7.5 and 8.0), presumably due to the different stability of CDP-choline pyrophosphorylase in different salt solutions. The optimum pH for the activity of the immobilized cells is 7.8. The effect of temperature on the stability of the immobilized cells was examined, and the results are shown in Fig. 12. We find that the optimum temperature of the native cells is 35°C, but that of the immobilized cells broadens from 30° to 40°C. The pH stability was examined in the presence of acetate buffers (from 6.0 to 7.0) and phosphate buffers (from 7.0 to 9.0). The results are shown in Fig. 13, where

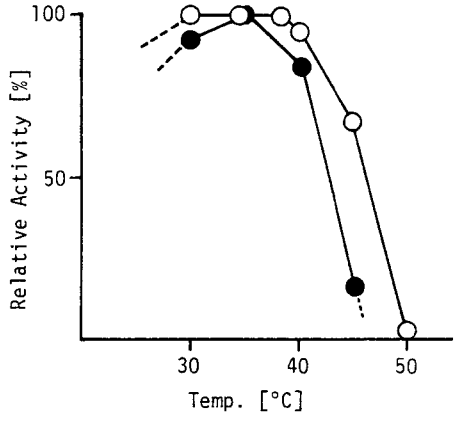

FIG. 11. Effect of pH on the activity of immobilized cells (open circles) and native cells (solid circles).

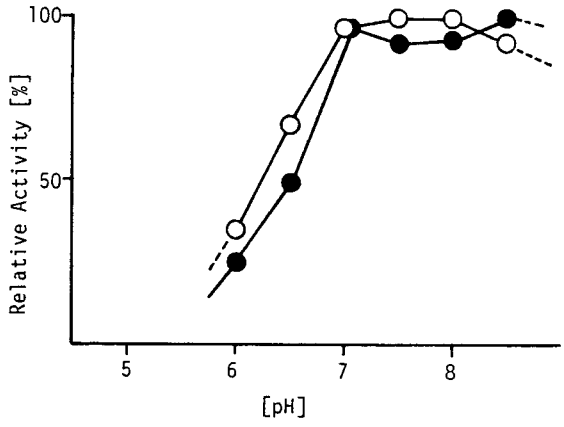

FIG. 12. Effect of temperature on the activity of immobilized cells (open circles) and native cells (solid circles).

we see that the immobilized cell is more stable in a wider pH region than the native cells. The results of the effect of temperature on stability of activity are shown in Fig. 14. Both the immobilized and the native cells were stable at lower temperatures than 38°C.

Continuous Production of CDP-Choline with the Column of the Immobilized Cells. The optimum conditions of the regeneration of ATP were determined from the results of the previous examination of the production of ATP from AMP. A long-run operation was carried out with an apparatus similar to that shown in Fig. 5. The substrate solution was composed of 400 mM glucose, 20-mM 5'-CMP (6.5 mg/ml), 100-mM choline chloride, 50-mM $MgSO_4$,

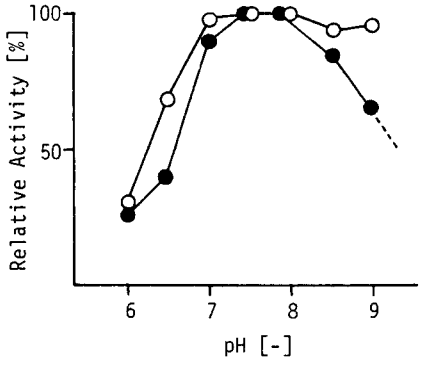

FIG. 13. Effect of pH on stability of activity of immobilized cells (open circles) and native cells (solid circles).

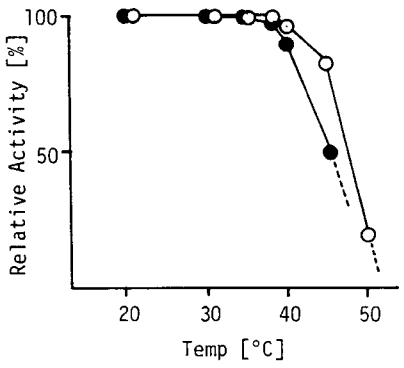

FIG. 14. Effect of temperature on stability of activity of cells (open circles) immobilized and native cells (solid circles).

and 0.4-M phosphate buffer (pH 7.5), and was passed through the reactor column of the immobilized cells at s.v. 0.25 and at 35°C. During the first eight days, the molar conversion rate from 5'-CMP to CDP-choline was maintained at 50% to 60% (Fig. 15).

DISCUSSION

As hitherto mentioned, the naturally occurring ATP regeneration system in microbial cells seems a very useful tool of ATP regeneration required for the synthesis of energy-rich compounds by immobilized enzyme or cell systems because such natural ATP regenerating systems are

FIG. 15. Yield from continuous operation of column of immobilized cells for production of CDP-choline.

TABLE 2. **Turnover Number of ATP**

Conditions	ATP	CDP-choline
Key substrate	AMP, 43 mM	CMP, 20 mM
Product	ATP, 30.1	CDP-choline 10
Conversion rate	70%	50%
ADP/ATP (mole/mole product)	2	3
Endogenous ATP	4×10^{-3} mM	4.3×10^{-3} mM
Reaction Time (hrs)	5	5
Turnover number of ATP[a] (cycles/h)	3010	ca. 1400

[a] Turnover number of ATP $= \dfrac{\text{mM product} \times \text{mM ATP required}}{\text{Conc. endo. ATP (mM)} \times \text{reaction time (h)}}$.

usually rather cheap and stable. Also, inexpensive energy donors like glucose can be used in such systems.

Furthermore, from the experimental data described in Figs. 10 and 15, the approximate turnover numbers of endogeneous ATP in immobilized yeast cells were calculated in both cases of ATP and CDP-choline production, and are given in Table 2. Concentrations of endogeneous ATP in the immobilized yeast cells used for ATP and CDP-choline production were determined to be 4 μM and 4.3 μM, respectively.

Conversion rates from AMP to ATP and from CMP to CDP-choline in the early stages were assumed to be 70% and 50%, respectively. Under such assumptions, the turnover numbers of endogenous ATP for ATP production and CDP-choline production were calculated to be 3,010 and about 1,400 cycles/h, respectively. These turnover numbers of ATP suggest that yeast cells themselves might be practically useful tools for ATP regeneration.

Whitesides et al. (6) reported that the immobilized adenyl kinase and actate kinase system is the most economical ATP regeneration system now available. However, the authors believe that naturally assembled ATP regenerating systems must be more economical in some cases if suitable targerts and combinations are selected.

REFERENCES

1. TOCHIKURA, T., KUWAHARA, M., YAGI, S., OKAMOTO, H., TOMINAGA, Y., KONO, T., and OGATA, K. (1967) J. Ferment. Technol. 45 : 511.
2. NAKAYAMA, K., and HAGINO, H. (1973) Japanese Patent 48–40757.
3. TOCHIKURA, T., KIMURA, A., KAWAI, H., TACHIKI, T., and GOTAN, T. (1970) J. Ferment. Technol. 48 : 763.

4. SHIMIZU, S., MORIOKA, H., TANI, Y., and OGATA, K. (1975) J. Ferment. Technol. 53 : 77.
5. CHIBATA, I., KAKIMOTO, T., NISHIMURA, N., and NABE, K. (1975) Japanese Open Patent 50-126884.
6. WHITESIDES, G. M., LAMOTTE, A., ADALSTEINSSON, O., and COLTON, C. K. (1976) Methods in Enzymology 44 : 887.