ATP Production by a Methanol Yeast, *Candida boidinii* (*Kloeckera* sp.) No. 2201: Effects of Sorbitol Treatment and Zinc on Cell Structure as to ATP Production

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Received August 31, 1987

The effect of sorbitol treatment on the cell structure of *Candida boidinii* No. 2201 was studied by transmission electron microscopy (TEM). The high ATP-producing activity of the cells was proved to be due to the plasmolysis without destruction of essential organelles for the ATP-producing system.

To prepare active cells constantly, the essential elements of the yeast extract in the culture medium were identified: biotin was an essential growth factor; Fe^{2+} and thiamine stimulated more or less the growth; and Zn^{2+} strongly stimulated the growth. Limitation of Zn^{2+} in the culture medium (0.5 mg/l of $ZnCl_2$) improved the ATP yield from AMP, the conversion rate being 92%, possibly due to repression of AMP deaminase, for which Zn^{2+} was a cofactor and which affected the ATP-producing activity.

A Zn^{2+}-supplemented culture provided fully-active cells as to ATP production. TEM revealed that Zn^{2+} was a necessary factor for the formation of peroxisomes in which alcohol oxidase and catalase were localized.

So far we have investigated the ATP production system[1~6] in sorbitol-treated cells of a methanol yeast, *Candida boidinii* (*Kloeckera* sp.) No. 2201, in which the salvage synthesis of ADP and the oxidative phosphorylation of ADP through the oxidation of methanol are involved. A highly efficient system was established, 198 mM (100 g/l as free acid) being produced from adenosine at a conversion rate of 77.4 mol%.[6] In the system, the treatment of yeast cells with sorbitol was essential for the preparation of cells to premeate and secrete nucleotidic compounds.[2,5] The supplementation of yeast extract to the methanol medium was also essential for maximum growth of the yeast.

In this study, we analyzed the essential structure of the cells for ATP-producing activity by means of transmission electron microscopy (TEM) in order to constantly prepare fully-active yeast cells.

MATERIALS AND METHODS

Strain and materials. *C. boidinii* No. 2201 was used. Epoxy resin (Luveak® 812) was purchased from Nakarai Chemicals, Ltd. All other chemicals were purchased from usual commercial sources and used without further purification.

Culture conditions. The yeast was cultivated in a synthetic methanol (SM) medium or yeast extract-methanol (YM) medium. SM medium consisted of 20 ml methanol, 4 g NH_4Cl, 1 g KH_2PO_4, 1 g K_2HPO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 10 ml vitamin mixture (100 μg thiamine·HCl and 10 μg biotin) and 10 ml metal salt mixture (20 mg $CaCl_2 \cdot 2H_2O$, 20 mg $FeSO_4 \cdot 7H_2O$, 5 mg $ZnCl_2$, 2 mg $MnCl_2 \cdot 4H_2O$ and 0.5 mg $CuSO_4 \cdot 5H_2O$) in 1 l of deionized water, pH 6.0. YM medium was the same as SM medium except that the vitamin and metal salt mixtures were replaced with 2 g yeast extract. Other conditions were described previously.[3] The cell amount was determined turbidimetrically.

Preparation of sorbitol-treated cells. Washed and sorbitol-treated cells were prepared as described previously.[2]

Assay for ATP-producing activity. The standard reaction mixture for ATP production from AMP[3] contained 100 μmol potassium phosphate buffer, pH 6.5, 5 μmol NAD^+, 5 μmol reduced glutathione, 300 μmol sorbitol, 500 μmol methanol, 10 mg cells, as dry weight, and 20 μmol AMP in a total volume of 0.5 ml. The reaction was aerobically carried out at 25°C in a 10-ml Erlenmeyer flask with the addition of 0.2 ml cell suspension containing 10 mg, as dry weight, of sorbitol-treated cells in 1.5 M sorbitol. Nucleotidic compounds were analyzed by high performance liquid chromatography.[5]

Preparation of yeast cells for TEM. Immobilization: the washed cell paste (0.1 ~ 0.5 g as wet weight) was washed twice with deionized water by centrifugation and then suspended in 4.6 ml of 0.1 M potassium phosphate buffer, pH 7.2. The cells in suspension were pre-fixed by adding 0.4 ml of a 25% aqueous glutaraldehyde (GA) solution, followed by vigorous shaking and then keeping at 4°C for 2 hr. The resultant cells were washed 5 times with the same buffer. The cells were then post-fixed with the addition of 10 ml of a 1.5% $KMnO_4$ solution. After keeping for 16 hr at 4°C, the cells were thoroughly washed with deionized water until the brown color of the supernatant disappeared.

In the case of sorbitol-treated cells, an alternative method was also used. The cell paste was pre-fixed with GA as described above except for the use of the buffer containing 0.6 M sorbitol. The washing of cells was performed thoroughly with the same buffer containing sorbitol. The cells were then post-fixed with the addition of 5 ml of 2% OsO_4 in the same buffer containing sorbitol. After keeping for 2 hr at 4°C, the cells were washed twice with deionized water.

Dehydration: to the immobilized cell paste, 3 ml of a 1.5% aqueous uranyl acetate solution was added. After keeping for 2 hr at 4°C, the cells were collected by centrifugation, and then dehydrated for 5 min by the addition of 5 ml of 50, 70, 90 and 95% acetone, respectively. After subsequent dehydration with 100% acetone twice for 5 min, the cells were washed with 5 ml of propylene oxide twice for 10 min. In each step, the cells were separated by centrifugation.

Infiltration: for the dehydrated cells, a 4-ml mixture of epoxy resin (Luveak® 812) and propylene oxide, in the ratio of 1 : 1, 3 : 1 and 7 : 1, respectively, was infiltrated. The epoxy resin was prepared by the Luft method.[7] Each infiltration was performed for 2 hr at room temperature, followed by centrifugation. Then they were infiltrated with 1 ml of 100% epoxy resin overnight and then cured in fresh resin for 4 hr twice.

Embedding: after infiltration, the paste was transferred to a gelatin capsule and then embedded in the same resin. After keeping for 12 hr at room temperature, the capsule was heated for 12 hr at 45°C and then for more than 24 hr at 60°C for hardening.

Sectioning: after hardening, a number of thin sections were cut with an ultramicrotome (LKB Ultrotome® type 4801A) and then placed on a fine copper mesh. The post-staining of thin sections post-fixed with OsO_4 was performed with saturated uranyl acetate and lead citrate[8] for 10 min each.

TEM analysis. All samples were observed under a Hitachi H-300 transmission electron microscope.

RESULTS AND DISCUSSION

Ultrastructure of sorbitol-treated cells

The addition of a high concentration of sorbitol to a cell suspension of *C. boidinii* No. 2201 yielded active cells as to ATP production.[2] Some other osmoregulatory compounds were also effective for the preparation of ATP-producing cells.[2] The activity was enhanced by treatment at 37°C and/or storage at −80°C.[5] These findings showed that active cells might be altered as to structure through a kind of plasmolysis but still keep the membrane structures of organelles such as mitochondria and peroxisomes.

Figure 1 shows typical TEM images of methanol-grown cells with or without sorbitol treatment and with GA-$KMnO_4$ immobilization. Mitochondria and peroxisomes, whose membrane structures are essential for the ATP-producing system, were not damaged by the sorbitol treatment (Fig. 1A). No clear signs of plasmolysis were recognized but swelling of the cell wall was observed with the sorbitol-treated cells. This phenomenon might be due to the post-fixation with $KMnO_4$, which caused the swelling of phospholipid membranes.[9]

Subsequently, the same sorbitol-treated cells were post-fixed with OsO_4 instead of $KMnO_4$, followed by staining with uranyl acetate and lead citrate. As shown in Fig. 2, clear images showing a gap between the cell wall and cytoplasmic membrane of sorbitol-treated cells were observed. This phenomenon is regarded as a kind of plasmolysis.

Thus, it was proved that the sorbitol treatment plasmolyzed the yeast cells so that the cell became permeable for nucleotidic compounds without destruction of cell organelles

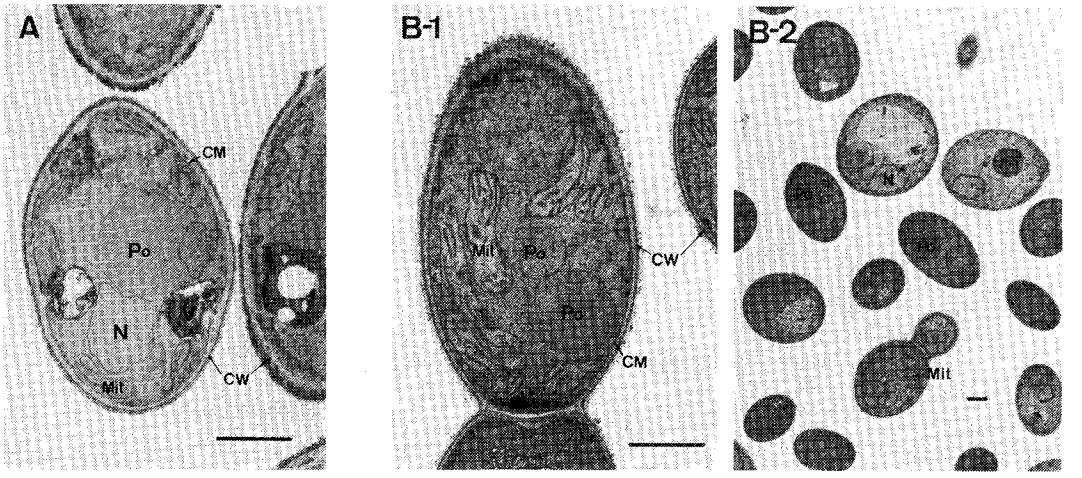

FIG. 1. Electron Micrographs of *C. boidinii* No. 2201 Cells.

The cells were cultivated in YM medium for 24 hr and then immobilized with GA-$KMnO_4$ as described in the text. A: Sorbitol-treated cells, B-1 and B-2: Intact cells. N, nucleus; V, vacuole; CW, cell wall; CM, cytoplasmic membrane; Mit, mitochondrion; Po, peroxisome. Bars, 1 μm.

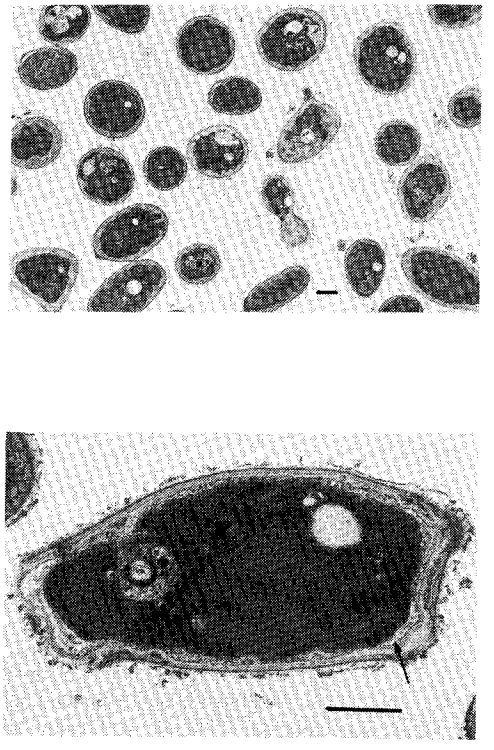

FIG. 2. Electron Micrographs of Sorbitol-treated Cells of *C. boidinii* No. 2201.

Cells cultivated in YM medium for 24 hr were treated with sorbitol, immobilized with GA-OsO_4 and then stained by the method described in the text. Bars, 1 μm.

whose membrane structures were indispensable for the methanol oxidation and oxidative phosphorylation.

Construction of a chemically-defined medium for preparation of ATP-producing cells

The ATP-producing activity of sorbitol-treated cells varied with different cultivations with YM medium. Yeast extract was added as the sole natural component in the YM medium to prepare active cells. A vitamin mixture[10] could be replaced by yeast extract for growth but the resultant cells showed no ATP-producing activity. On the other hand, it was reported that trace metal elements were indispensable for the full growth of methanol yeasts.[11] SM medium, which contained the vitamin and metal salt mixtures instead of yeast extract, allowed good growth of the yeast (Fig. 3). When the vitamin mixture or metal salt mixture was removed from the SM medium, the yeast showed no or only low growth. Therefore, the effects of biotin, thiamine and metal salts on the growth were studied.

Effect of biotin. C. boidinii No. 2201 was characterized as a biotin-required yeast[12] but the effect of the biotin concentration on growth has not been required. Figure 4 shows

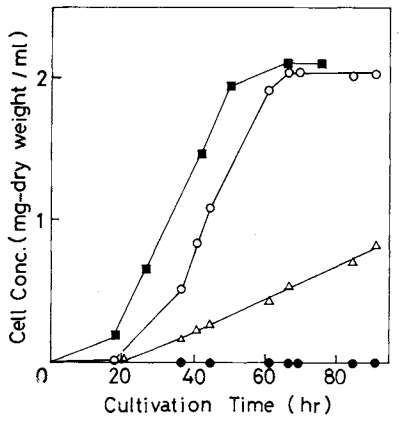

FIG. 3. Cultivation Patterns of *C. boidinii* No. 2201.

YM medium, —■—; SM medium, —○—; vitamin mixture-omitted SM medium, —●—; metal mixture-omitted SM medium, —△—.

Cultivation was carried out in a 500-ml shaking flask containing 100 ml of medium at 30°C.

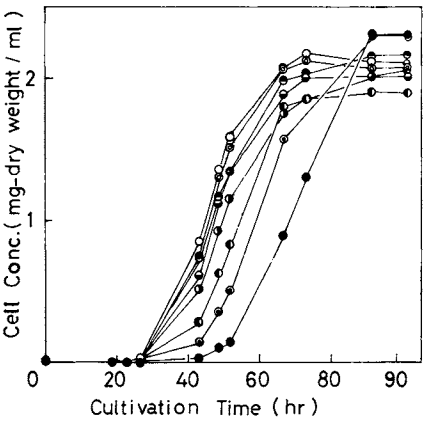

FIG. 5. Effect of Thiamine·HCl on the Growth of *C. boidinii* No. 2201.

SM medium was used with various concentrations of thiamine·HCl: —●—, 0 μg/l; —⊙—, 2.5 μg/l; —◐—, 5.0 μg/l; —◑—, 10 μg/l; —◒—, 25 μg/l; —◓—, 50 μg/l; —◎—, 75 μg/l; —○—, 100 μg/l.

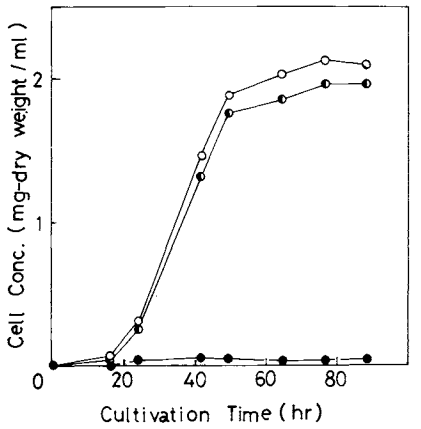

FIG. 4. Effect of Biotin on the Growth of *C. boidinii* No. 2201.

SM medium was used with various concentrations of biotin: —●—, 0 μg/l; —◐—, 0.1 μg/l; —○—, 10 μg/l.

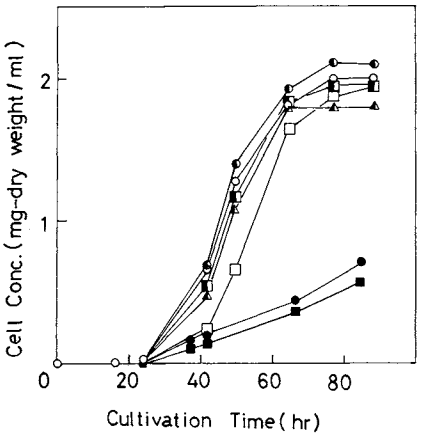

FIG. 6. Effects of Trace Metal Ions on the Growth of *C. boidinii* No. 2201.

SM medium was used as the basal medium: —○—, complete; —■—, minus Zn^{2+}; —◧—, minus Mn^{2+}; —◐—, minus Cu^{2+}; —▲—, minus Ca^{2+}; —□—, minus Fe^{2+}; —●—, minus all trace metals.

that the yeast required biotin at a concentration of 0.1 μg/l for full growth.

Effect of thiamine. Thiamine was reported as a growth-stimulative factor for *C. boidinii* No. 2201.[10] Figure 5 shows that thiamine was not essential for growth but accelerated the growth rate of the yeast. The minimal concentration of thiamine·HCl for good growth was 10 μg/l.

Effects of metal salts. The metal sources in the SM medium consisted of Ca^{2+}, Cu^{2+}, Fe^{2+}, Mn^{2+} and Zn^{2+} as chloride or sulfate forms. The results of growth experiments with each metal salt omitted showed that Zn^{2+} and Fe^{2+} were effective for the growth of *C. boidinii* No. 2201 (Fig. 6). The omission of Ca^{2+}, Cu^{2+} and Mn^{2+} hardly affected the cell growth. Zn^{2+} showed a clear positive effect on the growth and so was considered to be an

essential growth factor for the yeast. The low growth on Zn^{2+}-omitted medium might be due to Zn^{2+} contaminating components of the SM medium. The concentration of $ZnCl_2$ required for good growth was determined to be more than 0.5 mg/l (Fig. 7). Fe^{2+} was less effective for the growth than Zn^{2+} but is known as a co-factor for an enzyme essential for methanol oxidation, catalase.

Based on the results described above, a chemically-defined medium for the yeast was developed, as shown in Table I. When the yeast was cultivated in the medium, sorbitol-treated cells constantly exhibited sufficient ATP-producing activity in a reaction mixture containing AMP or adenosine as a substrate.

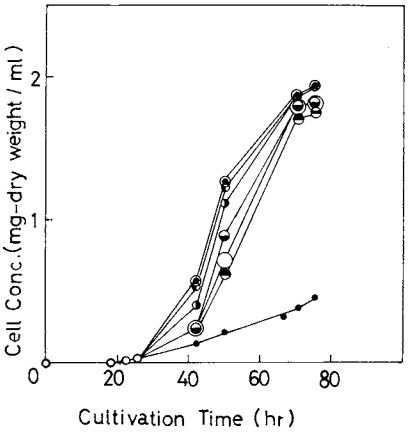

FIG. 7. Effect of Zn^{2+} on the Growth of *C. boidinii* No. 2201.

SM medium was used with various concentrations of $ZnCl_2$: —●—, 0 mg/l; —⊙—, 0.5 mg/l; —◐—, 1.0 mg/l; —◑—, 2.0 mg/l; —◒—, 5.0 mg/l; —◓—, 10 mg/l; —○—, 20 mg/l.

Effect of the Zn^{2+} concentration in the culture medium on ATP production

Zn^{2+} was reported to be a potent allosteric inhibitor and also an essential co-factor for yeast AMP deaminase.[13] Deamination of AMP to IMP by the enzyme decreased the ATP yield in the present yeast cell system.[5] The addition of Zn^{2+} to the reaction mixture at any concentration inhibited both the deamination of AMP and the production of ATP.[5] Therefore, the effect of Zn^{2+} in the culture medium on the ATP-producing activity of cells was investigated with a system containing AMP as the substrate. Table II shows that Zn^{2+} limitation in the culture medium improved the ATP yield from AMP. The highest conversion rate, 92 mol% from AMP, was obtained when 0.5 mg/l of $ZnCl_2$

TABLE I. COMPOSITION OF THE MINIMAL SYNTHETIC CULTURE MEDIUM

NH_4Cl	4.0 g/l
KH_2PO_4	1.0 g/l
K_2HPO_4	1.0 g/l
$MgSO_4 \cdot 7H_2O$	0.5 g/l
Biotin	0.1 μg/l
Thiamine·HCl	10.0 μg/l
$ZnCl_2$	0.5 mg/l
$FeSO_4 \cdot 7H_2O$	0.5 mg/l
Methanol	10.0 or 20.0 ml/l

TABLE II. EFFECT OF THE ZINC CONCENTRATION IN THE CULTURE MEDIUM ON ATP-PRODUCING ACTIVITY

The reaction was carried out with the assay system for ATP production from AMP.

$ZnCl_2$ (mg/l)	Yeast extract (mg/l)	AMP (mM)		Production (mM) of:			
		Residual	Consumed	ATP	ADP	IMP	Mol% ATP[a]
in culture medium		in reaction mixture					
0	0	33.4	6.6	0.4	0.3	6.0	1.0
0.5	0	1.2	38.8	36.8	trace	2.0	92.0
1.0	0	0.4	39.6	34.4	trace	3.8	86.0
10.0	0	1.0	39.0	31.0	1.0	7.0	77.5
0	2000	0	40.0	24.0	1.1	14.8	60.0

[a] (Moles of ATP produced/moles of AMP added) × 100.

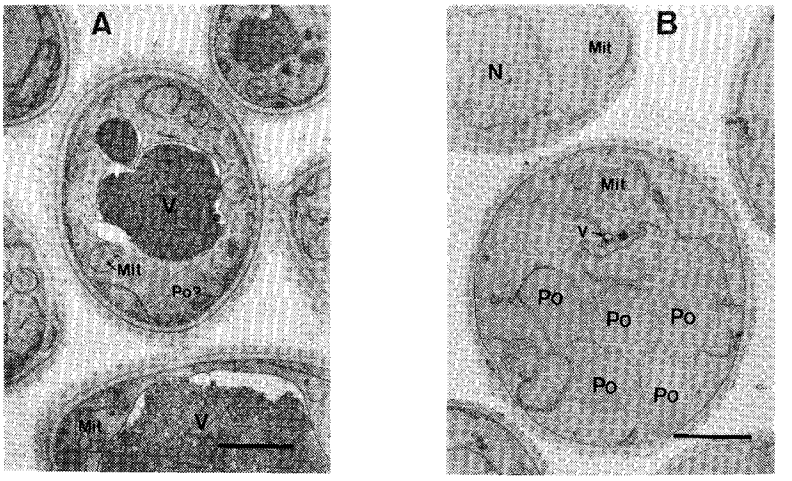

FIG. 8. TEM Images of *C. boidinii* No. 2201 Grown on the Medium with or without Zn^{2+}.

The cells were cultivated in the chemically-defined medium in Table I (B) or that without Zn^{2+} (A) for 40 hr, and then immobilized with GA-$KMnO_4$, as described in the text. Cell organelles are abbreviated as in Fig. 1. Bars, 1 μm.

was added to the culture medium.

These results show that variation in the Zn^{2+} concentration may be one of the reasons for the instability of the ATP-producing activity of cells grown on YM medium containing 0.2% yeast extract.

Effect of Zn^{2+} on the ultrastructure of cells

The effect of Zn^{2+} in the culture medium on the ultrastructure of yeast cells was studied. Figure 8 shows TEM images of cells grown on the medium supplemented with or without Zn^{2+}. Large vacuoles and immature peroxisomes were observed in the cells grown on the medium without Zn^{2+} (Fig. 8A). On the other hand, large mature peroxisomes and small vacuoles were observed in the cells grown on the medium with Zn^{2+} (Fig. 8B). Mitochondria were detected in both types of cells.

Thus, Zn^{2+} was determined to be indispensable for the formation of peroxisomes, which are responsible for the methanol oxidation essential for the ATP production.

Acknowledgment. The authors wish to thank Mr. M. Okada, Plastics Laboratory, Toray Industries, Inc., for his help in the TEM.

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