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Production of Formaldehyde by Detergent-Treated Cells of a Methanol Yeast, *Candida boidinii* S2 Mutant Strain AOU-1

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Treatment of cells of a methanol yeast, *Candida boidinii*, with the cationic detergent cetyldimethylbenzylammonium chloride (Cation M2) improved the production of formaldehyde. Formaldehyde production was improved twofold with respect to the initial amount of formaldehyde and 1.61-fold with respect to the final amount of formaldehyde after a 12-h reaction under optimized detergent treatment conditions. The treatment caused formaldehyde and formate dehydrogenases to leak out of the cells more rapidly than catalase, but there was no leakage of alcohol oxidase. The improvement in formaldehyde production was considered to be due to the increased permeability of yeast cell membranes and to lower activities of formaldehyde and formate dehydrogenases in Cation M2-treated cells than in intact cells. Changes in the ultrastructure of the cells were observed upon Cation M2 treatment. Several developed peroxisomes were observed in intact cells. After Cation M2 treatment, the cells were obviously damaged, and several peroxisomes seemed to have fused with each other.

In methanol yeasts, methanol is oxidized to CO_2 through the action of methanol-dissimilating enzymes, i.e., alcohol oxidase (EC 1.1.3.13), catalase (EC 1.11.1.6), formaldehyde dehydrogenase (EC 1.2.1.1), and formate dehydrogenase (EC 1.2.1.2). The former two enzymes are localized in special organelles, peroxisomes, and the latter two are cytosolic enzymes (1, 15). We previously studied formaldehyde production in intact cells of an alcohol oxidase-enhanced mutant strain, AOU-1, of *Candida boidinii* S2 (11, 17–19). Cells grown in a methanol-limited chemostat culture at a low dilution rate contained the highest amount of alcohol oxidase and showed the highest formaldehyde production (11).

Detergents are known to change the permeability of cellular membranes. Obayashi (8) used cetyltrimethylammonium chloride to extract NAD^+ from cells of *Saccharomyces cerevisiae*. Although immobilized cells of a methanol yeast, *Hansenula polymorpha*, exhibiting high alcohol oxidase activity were activated by cetyltrimethylammonium chloride (4), the reason for this activation was not determined.

In this study, the improvement of formaldehyde production through treatment with a cationic detergent, cetyldimethylbenzylammonium chloride (Cation M2), is described. The leakage of catalase and formaldehyde and formate dehydrogenases but not alcohol oxidase from cells and changes in the ultrastructure of the cells due to the treatment are also described.

MATERIALS AND METHODS

Chemicals. Cation M2 was a product of Nippon Oil & Fats Co., Ltd., Tokyo, Japan. Luveak-812 and glutaraldehyde (25% in water) were purchased from Nakarai Chemicals Ltd., Kyoto, Japan. All other chemicals were from the usual commercial sources.

Organism and cultivation. *C. boidinii* S2 mutant strain AOU-1 (17) was used throughout this study. This strain is preserved in the AKU Type Culture Collection (Faculty of Agriculture, Kyoto University, Kyoto, Japan). The yeast was grown in a methanol-limited chemostat culture at a dilution rate of 0.075 h^{-1}, at which the cells exhibit maximum alcohol oxidase activity, as described previously (11).

Cell mass. Cell mass was determined as described previously (11). The amount of Cation M2-treated cells was expressed as the initial amount of intact cells.

Cation M2 treatment. Chemostat-grown cells were washed with 0.1 M potassium phosphate buffer (KPB) (pH 7.5), suspended in KPB containing 0.1% cation M2 to a concentration of 30 mg (dry cell weight [DCW])/ml, and incubated at 37°C for 2 h under static conditions. Cation M2 was then removed, and the cells were washed with KPB three times by centrifugation at 10,000 × *g* and used for formaldehyde production.

Heat-treated cells were prepared by incubating the intact cell suspension (90 mg [DCW]/ml of 0.1 M KPB [pH 7.5]) at 37°C for 24 h.

Assay of formaldehyde production. The formaldehyde production assay was performed at 4°C under pure oxygen in 0.33 M KPB at pH 6.0 for intact cells and heat-treated cells and at pH 7.0 for Cation M2-treated cells. The cell concentration was 30 mg (DCW)/ml, and the initial methanol concentration was 3 M. The other reaction conditions were the same as those described previously (18). The reaction was performed for 20 h unless otherwise stated and was stopped by adding 0.2 N HCl to the reaction mixture. The amount of formaldehyde produced was measured by the method of Nash (7). The increase in formaldehyde production was also confirmed with a Shimadzu GC-7A gas-liquid chromatograph equipped with a temperature conductivity detector as described previously (18). The concentration of methanol was estimated as described previously (18).

Assay of methanol-dissimilating enzymes. A cell extract was prepared by sonification, and alcohol oxidase activity was assayed as described previously (17). Formaldehyde and formate dehydrogenase activities were determined as

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TABLE 1. Formaldehyde production in cation M2-treated cells[a]

Cells	Treatment time	Formaldehyde production		Remaining methanol (mmol)	Reaction time (h)
		Initial	Final		
Cation M2 treated (% [wt/vol])					
0.03	15 min	301 (137)	1,015 (107)		20
	1 h	283 (129)	1,020 (107)		20
	24 h	253 (115)	941 (99)		20
0.10	15 min	480 (218)	1,058 (111)		20
	1 h	460 (209)	1,140 (120)		20
	2 h	432 (196)	1,160 (122)	1,780	7
	2 h		1,380 (145)	1,520	20
	2 h[b]	340 (155)	1,530 (161)	1,380	12
	24 h	209 (95)	760 (80)		20
0.30	15 min	277 (126)	987 (104)		20
	1 h	251 (114)	999 (105)		20
	24 h	25 (11)	90 (9)		20
1.00	15 min	25 (11)	134 (14)		20
	1 h	24 (11)	144 (15)		20
Heat treated		330 (150)	1,090 (115)	1,830	9
Intact		220 (100)	950 (100)	1,980	9

[a] Cells (30 mg [DCW]/ml) were treated with Cation M2 in 0.1 M KPB (pH 7.5) under the indicated conditions. Other treatment conditions and the conditions for heat treatment and formaldehyde production are given in the text. Initial values are reported in millimoles per hour, and final values are reported in millimoles. Values in parentheses are relative values expressed as a percentage of that observed in intact cells.

[b] Glycerol (10% [wt/vol]) was added to the reaction mixture for formaldehyde production.

described by Schütte et al. (13), and catalase activity was determined by the method of Bergmeyer (2). Protein was determined by the method of Bradford (3) with a protein assay kit (Bio-Rad Laboratories, Richmond, Calif.).

Electron microscopy. Cation M2-treated and intact cells in 0.1 M KPB (pH 7.5) were fixed by adding 25% (wt/vol) glutaraldehyde to a final concentration of 2.0% (wt/vol). After standing for 2 h at 4°C, the cells were washed with KPB by centrifugation and postfixed sequentially with 1.5% (wt/vol) $KMnO_4$ and 1.5% (wt/vol) aqueous uranyl acetate. The material was dehydrated in a graded acetone series and then embedded in Luveak-812 epoxy resin. Ultrathin sections in gelatin capsules (lot 00; Nakarai Chemicals Ltd., Kyoto, Japan) were cut with a diamond knife and then observed under a Hitachi H-300 transmission electron microscope.

RESULTS AND DISCUSSION

Improved formaldehyde production in Cation M2-treated cells. After testing cell treatments with various chemicals, we found that treatment with Cation M2 improved formaldehyde production under appropriate treatment conditions (Table 1). For high-yield formaldehyde production, treatment of cells (20 to 40 mg [DCW]/ml) with 0.1% Cation M2 at 37°C for 2 h was the optimum treatment conditions. With lower cell concentrations or higher Cation M2 concentrations, formaldehyde production decreased (Table 1). Cells treated at 37°C showed higher formaldehyde production than did those treated at 4°C.

In addition to treatment with Cation M2, heat treatment (the cells were incubated at 37°C in 0.1 M KPB [pH 7.5]) was also effective in increasing formaldehyde production (Table 1).

The effect of the initial pH of the formaldehyde production assay mixture on formaldehyde production was investigated with 0.33 M KPB. The optimum pH for the intact or heat-treated cells was 6.0, with a shoulder on the alkaline side (pHs 6.5 to 7.5). On the other hand, Cation M2-treated cells needed a sharp optimum pH of 7.0. The amount of formaldehyde produced by Cation M2-treated cells at pH 7.0 was 1.35-fold higher than that produced by intact cells at pH 6.0. The maximum amount of formaldehyde was obtained with cell concentrations above 30 mg (DCW)/ml in the formaldehyde production assay mixture.

With Cation M2 treatment, formaldehyde production increased by 45% with respect to the final amount and by about 100% with respect to the initial amount (Table 1). Cation M2-treated cells produced 1,380 mmol of formaldehyde during a 20-h reaction, with an initial rate of 432 mmol/h. When 10% glycerol was added to the formaldehyde production assay mixture, the cells produced 1,530 mmol of formaldehyde in 12 h from 3 mol of methanol (Table 1). After the reaction, neither formic acid nor methyl formate was detected in the reaction mixture. Control experiments showed that the stoichiometrical loss relative to the total amount of formaldehyde plus methanol was due to vaporization from the reaction mixture.

Leakage of methanol-dissimilating enzymes during Cation M2 treatment. To investigate the reason for the improved formaldehyde production in Cation M2-treated cells, we compared the activities of methanol-dissimilating enzymes in heat-treated cells, Cation M2-treated cells, and intact cells (Table 2). It was found that heat treatment did not affect the enzyme activities very much, although the catalase activity of heat-treated cells was slightly lower than that of intact cells. Intact cells and Cation M2-treated cells exhibited nearly the same level of alcohol oxidase activity, but the activities of other methanol-dissimilating enzymes were lower in Cation M2-treated cells. The activities of catalase, formaldehyde dehydrogenase, and formate dehydrogenase

TABLE 2. Activities of methanol-dissimilating enzymes in cells after heat treatment or Cation M2 treatment[a]

Cells [mg of protein/ml]	Activity (U/mg of DCW) of:			
	Alcohol oxidase	Catalase	Formaldehyde dehydrogenase	Formate dehydrogenase
Intact [6.24 (100)]	0.690 (100)	337 (100)	0.0820 (100)	0.0131 (100)
Heat treated [6.20 (99)]	0.651 (94)	314 (93)	0.0795 (97)	0.0133 (102)
Cation M2 treated [3.39 (54)]	0.685 (99)	285 (85)	0.0344 (42)	0.0059 (45)

[a] A cell extract was prepared by sonification of heat-treated cells, Cation M2-treated cells, or intact cells, and the activities of methanol-dissimilating enzymes were determined as described in the text. Values in parentheses are relative values expressed as a percentage of that observed in intact cells.

in Cation M2-treated cells were 85, 42, and 45% of the activities in intact cells, respectively.

To determine whether Cation M2 acted directly on and thus inhibited these enzymes, we added 0.1% Cation M2 to a cell extract, incubated the solution at 37°C for 2 h, and then measured the enzyme activities. None of these enzymes was inhibited by Cation M2, although some precipitate appeared.

The protein content of the cells after Cation M2 treatment was 54% that of intact cells. Figure 1 shows the leakage of methanol-dissimilating enzymes into the supernatant during Cation M2 treatment. Alcohol oxidase did not leak out at all from the cells up to 8 h of incubation, whereas the other three enzymes started to leak out immediately after the addition of 0.1% Cation M2. The degree of leakage during treatment for each enzyme was expressed as the *Kl* value (in hours), i.e., the time required for half of the final activity to appear in the supernatant. The *Kl* values for formaldehyde dehydrogenase, formate dehydrogenase, catalase, and alcohol oxidase were about 2, 2, 5, and more than 15 h, respectively. Thus, the former two cytosolic enzymes leaked out faster than the latter two peroxisomal enzymes.

With a simple Cation M2 treatment, cells with reduced formaldehyde and formate dehydrogenase activities but with a normal level of alcohol oxidase activity were obtained. The optimum pH, 6.0, for formaldehyde production by intact or heat-treated cells was rather acidic, as compared with the optimum pH of purified alcohol oxidase activity, to repress the activities of formaldehyde and formate dehydrogenases (18). As the pH optimum of purified alcohol oxidase is 7.0 to 9.0 (18), the increase in the optimum pH from 6.0 to 7.0 for formaldehyde production with Cation M2 treatment suggests the possibility of an increased contribution of alcohol oxidase to formaldehyde production, owing to the leakage of formaldehyde and formate dehydrogenases. The activities of these two dehydrogenases in cells after an 8-h treatment with Cation M2 were lower than those after a 2-h treatment (Fig. 1). However, the highest formaldehyde production was obtained with a treatment time of 2 h. This result might have been due to the amount of catalase activity in the cells. Cells treated for 2 h exhibited higher catalase activity than did those treated for 8 h. Catalase plays a positive role in formaldehyde production through its peroxidative action (14, 20). Without catalase, alcohol oxidase is rapidly inactivated by one of the reaction products, H_2O_2 (6, 16). Also, cells that had been chemostat grown on H_2O_2-containing media exhibited high catalase activity and formaldehyde production (10). Thus, maximum formaldehyde production was obtained as the combined result of a decrease in the activities of formaldehyde and formate dehydrogenases and the remaining activities of alcohol oxidase and catalase.

Electron microscopic observations. Because the *Kl* values of the two cytosolic enzymes were lower than those of the two peroxisomal enzymes, it was interesting to see how Cation M2 acted on the ultrastructure of the yeast cells, especially on the peroxisomes. Cation M2-treated cells and intact cells were fixed with glutaraldehyde-$KMnO_4$ and then subjected to transmission electron microscopy (Fig. 2). When cells were chemostat grown on methanol at a dilution rate of 0.075 h^{-1}, they contained several developed peroxisomes, which were almost cubical in shape and had clear membranes. A continuous plasma membrane, mitochondrial cristae, and a nuclear membrane were also clearly observed (Fig. 2A). These cellular structures were obviously damaged by the Cation M2 treatment (Fig. 2B). The nucleus and mitochondria could not be observed clearly, and the plasma membrane was discontinuous after the treatment. The peroxisomes had fused with each other and did not have a definite membrane. Thus, Cation M2 was found to act not only on the plasma membrane but also on the structures of organelles and intracellular membranes.

Formaldehyde and formate dehydrogenases had lower *Kl* values than alcohol oxidase. This difference in *Kl* values could be ascribed to several factors. (i) Cytosolic enzymes would leak out at an earlier stage than would peroxisomal

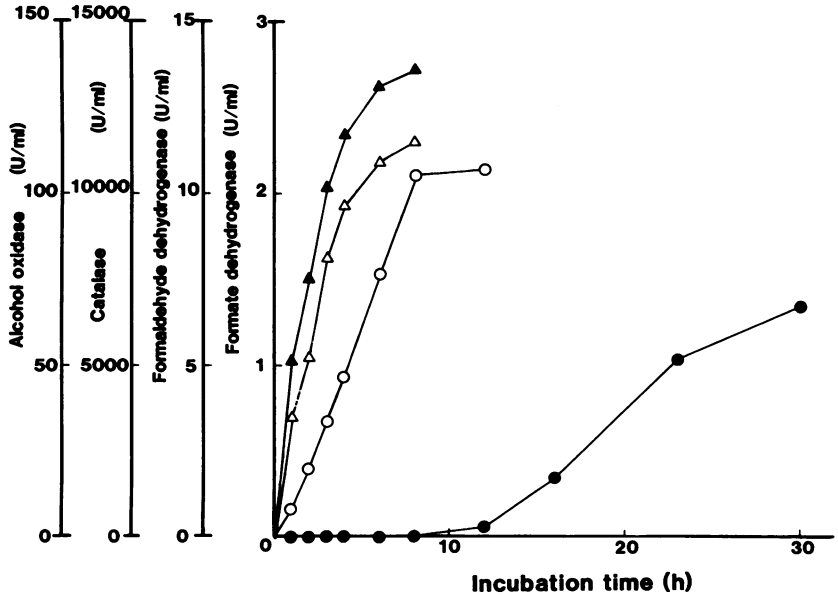

FIG. 1. Leakage of methanol-dissimilating enzymes following Cation M2 treatment. Cells were treated with 0.1% Cation M2 for various times and then collected by centrifugation at 10,000 × *g* for 10 min. The activities of methanol-dissimilating enzymes in the supernatant were assayed. Because Cation M2 interfered with the protein assay, the protein concentration in the Cation M2 solution was not determined. The enzyme assay methods and the conditions for Cation M2 treatment are described in the text. Symbols: ●, alcohol oxidase; ○, catalase; ▲, formaldehyde dehydrogenase; △, formate dehydrogenase.

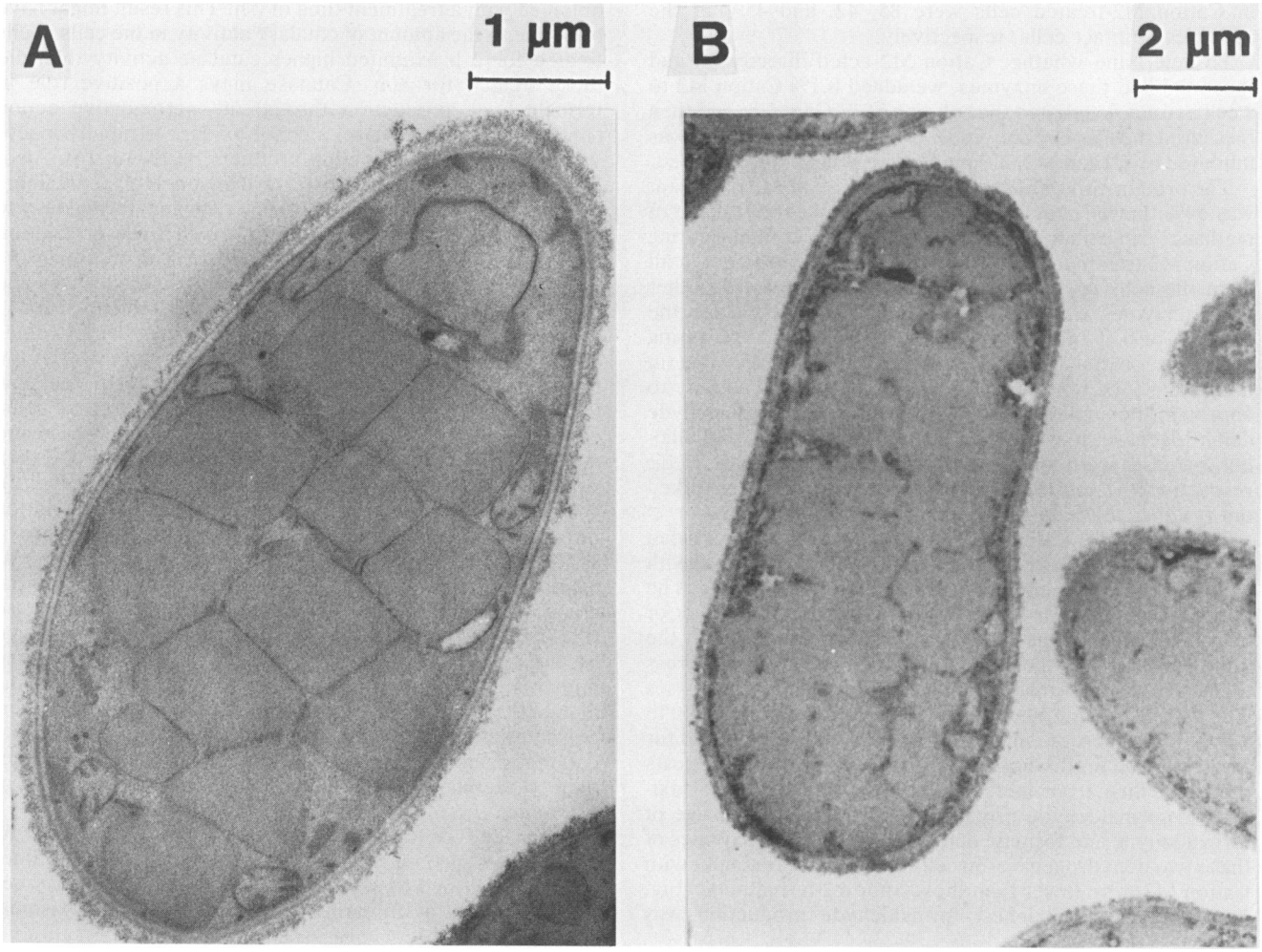

FIG. 2. Transmission electron microscopic photographs of intact (A) and Cation M2-treated (B) cells. Specimens were prepared as described in the text.

enzymes as Cation M2 penetrated into the cells. (ii) The molecular weights of alcohol oxidase, catalase, formaldehyde dehydrogenase, and formate dehydrogenase are ca. 600,000, 240,000, 80,000, and 70,000, respectively (1, 15). Smaller molecules would leak out more easily than larger ones. (iii) By means of sucrose density gradient ultracentrifugation, Goodman (5) showed that catalase has a bimodal localization, i.e., in the cytosol and peroxisomes, whereas alcohol oxidase is located only in the peroxisomes. The existence of two separate compartments for catalase or the preferential leakiness of peroxisomes for catalase possibly resulted in catalase having a lower *Kl* value than alcohol oxidase. (iv) A crystalloid structure of alcohol oxidase in peroxisomes was observed in glutaraldehyde-OsO_4-fixed cells (but not in glutaraldehyde-$KMnO_4$-fixed cells) of methanol-grown *C. boidinii* 2201 (9) and *H. polymorpha* (21). The large crystalloids formed upon fusion of the peroxisomes in Cation M2-treated cells (Fig. 2B) might decrease the leakage of alcohol oxidase.

Formaldehyde production by heat-treated cells was described elsewhere (12), and Cation M2 treatment was conveniently used for the development of a simple purification method for alcohol oxidase (12a).

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