Production of Catalytic Cells for Formaldehyde Production and Alcohol Oxidase by a Catabolite Repression-Insensitive Mutant of a Methanol Yeast, *Candida boidinii* A5

A catabolite repression-insensitive mutant of *Candida boidinii* A5, strain ADU-15, was investigated as to alcohol

Yoshiki Tani and Tohru Sawai*
Research Center for Cell and Tissue Culture, Kyoto University, Kyoto 606, Japan

Yasuyoshi Sakai
Department of Agricultural Chemistry, Faculty of Agriculture, Kyoto University, Kyoto 606, Japan

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A catabolite repression-insensitive mutant of *Candida boidinii* A5, strain ADU-15, was investigated as to alcohol oxidase production and the production of cells exhibiting the maximum catalytic activity for formaldehyde production. The mutant strain ADU-15 showed higher cell productivity and higher alcohol oxidase activity when grown on mixed substrates (glucose–methanol), especially with a high concentration of glucose in the medium. Thus, even under substrate (glucose–methanol)-limited chemostat conditions, where the glucose concentration was low, partial derepression of alcohol oxidase by glucose in mutant strain ADU-15 was detected. The chemostat culture conditions with the glucose–methanol medium were optimized for alcohol oxidase production and the production of cells exhibiting the maximum catalytic activity for formaldehyde production, respectively. By means of chemostat culturing on mixed substrates, we improved the alcohol oxidase productivity 5.0-fold and the productivity of cells exhibiting the maximum catalytic activity for formaldehyde production 3.8-fold, in comparison with the parent strain chemostat cultured with methanol as the single substrate.

INTRODUCTION

Recently, alcohol oxidase from methanol yeast has attracted increasing attention as to the preparation of alcohol electrodes[1,2] and the enzymatic analysis of alcohol or hydrogen-peroxide-producing systems.[3] The alcohol oxidase-catalyzed conversion of ethanol to acetaldehyde as an ethanol recovery system was also proposed.[4] Baratti et al. and we have investigated the formaldehyde production from methanol with an immobilized enzyme[5] or catalytic yeast cells exhibiting high alcohol oxidase activity.[6–9] And we reported the production of other useful aldehydes, i.e., acetaldehyde, propionaldehyde, and acrolein, with the catalytic cells.[10] Although cells grown on methanol have high alcohol oxidase activity, the cell productivity with methanol is rather low when compared with that with glucose. An increase in methanol concentration to improve the cell productivity decreased the cell growth and the growth yield. On the other hand, alcohol oxidase activity is decreased by glucose through catabolite repression[11] and catabolite unactivation.[12,13]

In a previous study, we derived a catabolite repression-insensitive mutant, strain ADU-15, from a methanol yeast, *Candida boidinii* A5, which produces alcohol oxidase in the presence of glucose in batch cultures.[14] In the present work, mutant strain ADU-15 was chemostat grown on mixed substrates, and the cultural conditions were optimized for alcohol oxidase production and for the production of a biocatalyst for formaldehyde production, respectively. The derepression of alcohol oxidase in mutant strain ADU-15 under chemostat culture conditions is discussed.

MATERIALS AND METHODS

Organisms

The parent strain, *Candida boidinii* A5, and mutant strain ADU-15 were used throughout this study. Mutant strain ADU-15 was derived in a previous study[14] as a catabolite repression-insensitive mutant that produces alcohol oxidase in the presence of glucose.

Media and Culture Conditions

The parent strain or mutant strain ADU-15 of *C. boidinii* was grown on a synthetic medium developed by Egli and

* On leave from the Bio Technology Research Laboratory, Mitsubishi Heavy Industries Ltd., Takasago, Hyogo 676, Japan.

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Fiechter,[15] except that *meso*-inositol, Ca–pantothenate, and pyridoxine–HCl were omitted, with methanol or a methanol–glucose mixture as the sole source of carbon and energy in carbon-limited chemostat cultures. Glucose was replaced by glycerol, xylose, or ethanol in one experiment (Table I). The concentration of a growth substrate, added after autoclaving, was expressed as a percentage (w/v).

A flow-controlled continuous cultivation was carried out at 28°C in a 2-L fermentor, KMJ (Mitsuwa Rikagaku Kogyo Co., Ltd., Osaka). The working volume was 1.2 L, which was kept constant with an overflow siphon. Cultures were stirred at 400 rpm, and the aeration rate was 1 vvm. The pH was kept constant at pH 4.0 by automatic adjustment with 4*N* NaOH. Samples for analysis were taken after at least three and a half volume changes.

Assays

Cell Mass

Cells from 10 mL culture broth were collected and then washed twice with distilled and deionized water by centrifugation for 10 min at $6 \times 10^3 g$ and finally dried at 105°C. The cell mass was expressed as dry cell weight.

Alcohol Oxidase

Preparation of cell-free extracts by sonication and alcohol oxidase assay were carried out as described previously.[7] Alcohol oxidase activity was expressed as units per milligram of cells because this rather than specific activity (U/mg protein) reflected the formaldehyde productivity.[9]

Formaldehyde Production

The reaction for formaldehyde production was performed at 4°C and pH 6.0, under pure oxygen, as described previously,[8] except that a 3-mL reaction mixture containing 90 mg chemostat-grown cells was used and the reaction time was 10 h. An Erlenmeyer flask (50 mL) equipped with a rubber balloon for a continuous oxygen supply was used as the reaction vessel. The concentration of formaldehyde was determined by the method of Nash.[16]

Definition of Productivities

Formaldehyde productivity refers to the maximum catalytic activity (expressed as the final concentration of formaldehyde) determined under the conditions described in the preceding. On the other hand, cell productivity (mg/mL h) or alcohol oxidase productivity (U/mL h) is defined as the amount of cells or alcohol oxidase produced per unit time from a unit volume of the culture.

RESULTS

Alcohol Oxidase Activity and Formaldehyde Productivity during Growth on Single or Mixed Substrates in Parent and Mutant Strain ADU-15

Each growth substrate supplemented to the methanol medium was tested as to improvement of the growth yield with maintenance of the maximum alcohol oxidase activity and formaldehyde production. The parent strain and mutant strain ADU-15 were grown at a dilution rate of 0.075 h^{-1} on glucose, ethanol, glycerol, or xylose as a substrate, with or without methanol, and alcohol oxidase activity and formaldehyde productivity were compared between the parent and mutant strain ADU-15 (Table I). Grown on methanol as a single substrate, both the parent and mutant strains showed the maximum alcohol oxidase activity and formaldehyde productivity at a dilution rate of 0.075 h^{-1}. Formaldehyde productivity completely paralleled alcohol oxidase activity in all cases tested. The alcohol oxidase in both the parent and mutant strains were derepressed during growth on glucose, glycerol, or xylose. Also, cells of both strains exhib-

Table I. Alcohol oxidase activity and formaldehyde productivity of parent strain *C. boidinii* A5 and mutant strain ADU-15 grown at a dilution rate of 0.075 h^{-1} on single substrates and on the same substrates together with methanol.[a]

	Parent strain			Mutant strain ADU-15		
Growth substrate	Cell concentration (mg/mL)	Alcohol oxidase activity (U/mg cells)	Formaldehyde productivity (m*M*)	Cell concentration (mg/mL)	Alcohol oxidase activity (U/mg cells)	Formaldehyde productivity (m*M*)
0.3% Methanol	0.78	0.58	900	0.80	0.58	900
0.3% Glucose	1.50	0.02	23	1.61	0.10	155
0.3% Glucose, 0.3% methanol	2.25	0.54	840	2.40	0.58	900
0.3% Ethanol	1.60	0	0	1.60	0	0
0.3% Ethanol, 0.3% methanol	2.40	0	0	2.39	0	0
0.3% Glycerol	0.80	0.34	520	0.80	0.35	540
0.3% Glycerol, 0.3% methanol	1.39	0.54	835	1.40	0.55	853
0.3% Xylose	1.30	0.20	310	1.32	0.22	320
0.3% Xylose, 0.3% methanol	2.08	0.52	807	2.14	0.52	807

[a] Alcohol oxidase activity and formaldehyde productivity assayed as described in Materials and Methods.

ited higher alcohol oxidase activity when grown on these substrates with methanol than without methanol. Although the extent of alcohol oxidase derepression, when grown on glycerol or xylose, was almost the same in the parent and mutant strain ADU-15, cells of the mutant grown on glucose had almost 5-fold higher alcohol oxidase activity than those of the parent strain. On the other hand, the synthesis of alcohol oxidase was completely repressed during growth on ethanol, and the repressive effect of ethanol on alcohol oxidase synthesis could not be abolished by the addition of methanol in both the parent and mutant strains.

When chemostat grown on glucose–methanol, glycerol–methanol, or xylose–methanol medium at a dilution rate of 0.075 h^{-1}, cells of both the parent strain and mutant strain ADU-15 exhibited relatively high alcohol oxidase activity (at least 89% of the maximum level). Although the mutant grown on the glucose–methanol medium showed the maximum alcohol oxidase activity, the activity in the parent strain was decreased by 7%. In the case of growth on the glycerol–methanol or xylose–methanol medium, alcohol oxidase activity was almost the same in the parent and mutant strains. Thus, insensitivity to catabolite repression of mutant strain ADU-15 could only be observed in the case of growth on glucose or the glucose–methanol medium.

At a dilution rate of 0.075 h^{-1} for both strains, the biomass produced was found to be virtually the same as the sum of the individual biomasses with each substrate. Because mutant strain ADU-15 grown on the glucose–methanol medium showed the highest cell productivity and retained the maximum alcohol oxidase activity and formaldehyde productivity, from among the growth substrates tested, glucose was selected as the additional carbon source to improve the cell productivity.

Optimization of Culture Conditions with Glucose–Methanol Medium for Alcohol Oxidase Production and for Production of Catalytic Cells for Formaldehyde Production

Effect of Concentration of Additional Glucose in Methanol Feed Medium

Various concentrations of glucose were added to the 0.4% methanol medium, and then cells of the parent strain and mutant strain ADU-15 were chemostat grown at a dilution rate of 0.10 h^{-1}. The growth yield of the mutant with the glucose–methanol medium was slightly higher than that of the parent strain (Fig. 1). Although the alcohol oxidase activity and formaldehyde productivity in the parent strain decreased gradually above 0.4% glucose, the mutant exhibited the maximum activities up to 0.6% glucose (Figs. 1 and 2). Thus, the lower sensitivity to catabolite repression of mutant strain ADU-15 was observed in chemostat cultures on the glucose–methanol medium. Consequently, alcohol oxidase productivity with the mixed substrates was improved in mutant strain ADU-15, as shown in Figure 2, when compared with the parent strain.

Effect of Dilution Rate

Cells were grown on a medium containing a mixture of 0.6% glucose–0.4% methanol as a carbon source at various dilution rates. The growth yield of mutant strain ADU-15 was slightly higher at each dilution rate than that of the parent strain (Fig. 3). Although the alcohol oxidase activity and formaldehyde productivity in the parent strain gradually decreased above a dilution rate of 0.075 h^{-1}, in mutant strain ADU-15 they remained at the maximum levels up to a dilu-

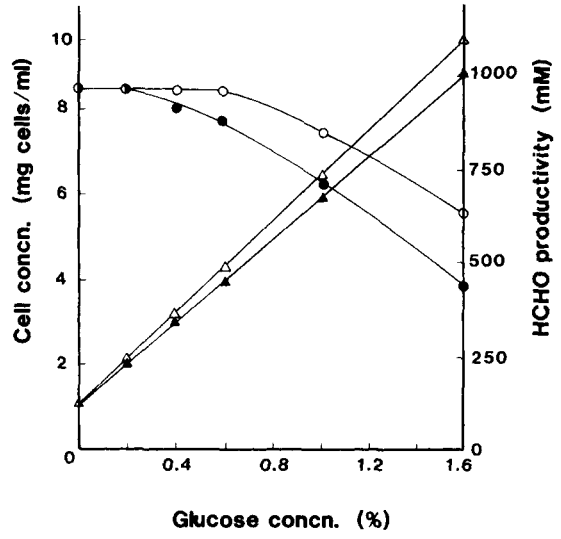

Figure 1. Effect of concentration of additional glucose in feed medium on cell growth and formaldehyde productivity of parent strain *C. boidinii* A5 and mutant strain ADU-15 chemostat grown on glucose–methanol medium. Dilution rate fixed at 0.10 h^{-1}. Various concentrations of glucose added to 0.4% methanol medium. Formaldehyde productivity of parent strain (●) and mutant strain ADU-15 (○) and cell growth of parent strain (▲) and mutant strain ADU-15 (△) assayed as described in Materials and Methods.

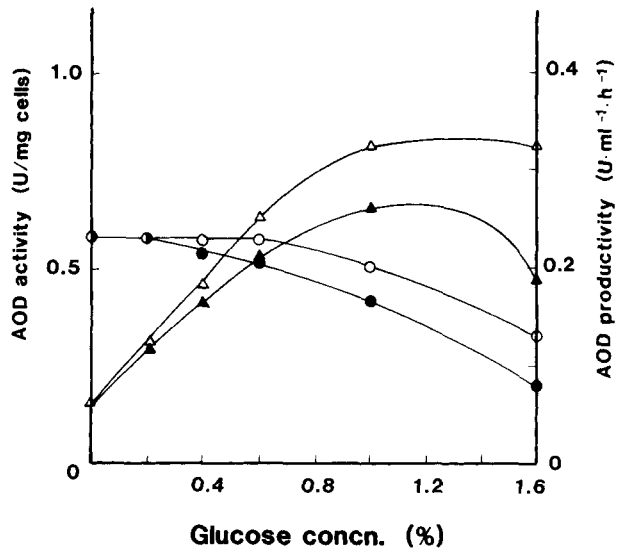

Figure 2. Effect of concentration of additional glucose in feed medium on alcohol oxidase activity and alcohol oxidase productivity of parent strain *C. boidinii* A5 and mutant strain ADU-15 chemostat grown on glucose–methanol medium. Dilution rate fixed at 0.10 h^{-1}. Various concentrations of glucose added to 0.4% methanol medium. Alcohol oxidase activity of parent strain (●) and mutant strain ADU-15 (○) and alcohol oxidase productivity of parent strain (▲) and mutant strain ADU-15 (△) assayed as described in Materials and Methods.

tion rate of 0.10 h^{-1} (Fig. 3 and 4). The alcohol oxidase productivity with the mixed substrate mixture was improved at each dilution rate in the mutant, as shown in Figure 4, when compared with the parent.

Optimum Chemostat Culture Conditions

After statistical analysis of data obtained for each dilution rate or glucose concentration, we obtained the maximum alcohol oxidase productivity of 0.33 U/mL h in a culture of mutant strain ADU-15 on a 1.0% glucose–0.4% methanol medium at a dilution rate of 0.10 h^{-1} (Table II). This value was about 1.3-fold higher than that of the parent strain under the same culture conditions. When the dilution rate was raised above 0.10 h^{-1} or the glucose concentration in the feed medium was higher than 0.6%, the alcohol oxidase productivity was the same or decreased. The highest alcohol oxidase productivity in mutant strain ADU-15 with the glucose–methanol medium was 4.93-fold higher than that in the mutant grown on methanol as a single substrate and 5.1-fold higher than that in the parent strain grown on methanol. However, cells exhibiting the maximum catalytic activity could not be obtained with the parent strain or mutant strain ADU-15 under the optimum culture conditions for alcohol oxidase production (Table II).

The maximum cell yield with retention of the maximum catalytic activity for formaldehyde production (or the maximum alcohol oxidase activity) was obtained with cells of mutant strain ADU-15 grown on the 0.6% glucose–0.4% methanol medium at a dilution rate of 0.10 h^{-1}. At glucose concentrations above 0.6% or a dilution rate of 0.10 h^{-1}, both the alcohol oxidase activity (Figs. 2 and 4) and form-

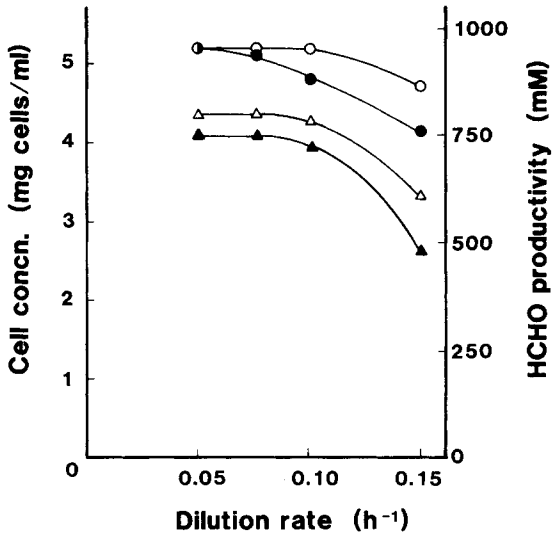

Figure 3. Effect of dilution rate on cell growth and formaldehyde productivity of parent strain and mutant strain ADU-15, of *C. boidinii* A5 chemostat-grown on the glucose–methanol mixture. The concentration of glucose and methanol in the feed medium was 0.6 and 0.4%, respectively. Symbols are the same as Figure 1.

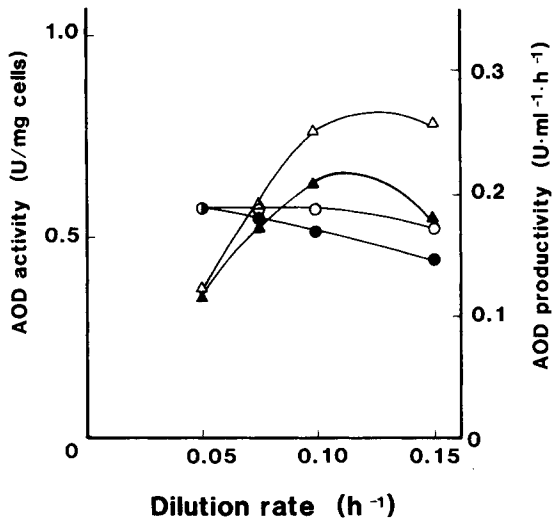

Figure 4. Effect of dilution rate on alcohol oxidase activity of parent strain and mutant strain ADU-15 of *C. boidinii* A5 chemostat-grown on the glucose–methanol mixture. The concentration of glucose and methanol in the feed medium was 0.6 and 0.4%, respectively. Symbols are the same as Figure 2.

Table II. Optimization of chemostat culture conditions with glucose–methanol medium for alcohol oxidase production and production of catalytic cells as to formaldehyde production with a mutant strain, ADU-15, of *C. boidinii* A5.[a]

Growth substrate	Dilution rate (h^{-1})	Strain	Cell concentration (mg/mL)	Productivities of: Cells (mg/mL h)	Productivities of: Formaldehyde (m*M*)	Alcohol oxidase: Activity (U/mg cells)	Alcohol oxidase: Productivity (U/mL h)
0.3% Methanol	0.075	parent	1.50	0.112	900	0.58	0.065
		ADU-15	1.53	0.115	900	0.58	0.067
	0.10	parent	1.35	0.135	770	0.50	0.067
		ADU-15	1.36	0.136	780	0.50	0.068
0.6% Glucose,	0.10	parent	3.97	0.397	820	0.53	0.21
0.4% methanol		ADU-15	4.30	0.430	900	0.58	0.25
1.0% Glucose,	0.10	parent	5.93	0.593	660	0.43	0.25
0.4% methanol		ADU-15	6.40	0.640	790	0.51	0.33

[a] Alcohol oxidase activity and formaldehyde productivity assayed as described in Materials and Methods.

aldehyde productivity (Figs. 1 and 3) decreased. Cells of the parent strain grown under the same culture conditions did not exhibit the maximum levels of formaldehyde productivity and alcohol oxidase activity, and the cell yield was lower than in the case of the mutant. The maximum cell yield with the maximum catalytic activity of the mutant grown on the glucose–methanol medium was about 3.8-fold higher than that of the methanol-grown parent strain or the mutant grown with a single substrate.

DISCUSSION

In a previous paper,[14] we reported the insensitivity to catabolite repression of mutant strain ADU-15. The parent and mutant strains differed in growth characteristics and in the sensitivity to glucose, especially at the early stage of enzyme induction in batch cultures. Under substrate-limited chemostat conditions, it is assumed to be difficult to distinguish clearly between the parent and the catabolite repression-insensitive mutant because the glucose concentration in chemostat cultures is lower than that in batch cultures. However, the lower sensitivity to alcohol oxidase repression by glucose of mutant strain ADU-15 was confirmed under the carbon-limited chemostat culture conditions. In cells grown on mixed substrates, the maximum alcohol oxidase activity could be maintained up to a higher dilution rate in mutant strain ADU-15 than in the parent strain. Consequently, both the alcohol oxidase productivity and the production of a biocatalyst for formaldehyde production were improved. In order to overcome catabolite repression, although a chemostat culture on mixed substrates is a powerful tool, the derivation of catabolite repression-insensitive mutants would be an alternative.

The difference in sensitivity to alcohol oxidase repression by glucose between the parent strain and mutant strain ADU-15 was very similar to that between *Hansenula polymorpha* and *C. boidinii (Kloeckera)* sp. 2201 reported by Egli et al.[17–19] Therefore, the differences in alcohol oxidase activity between *H. polymorpha* and *C. boidinii* sp. 2201 under glucose-limited chemostat conditions[17] or with the glucose–methanol medium[17,18] can be considered to be due only to the difference in the sensitivity to alcohol oxidase repression by glucose between two strains. The severe alcohol oxidase repression by glucose in *Candida* species could be relieved by means of mutation, as shown in this article. In both the parent and mutant strains, the alcohol oxidase activity tended to decrease as the ratio of glucose to methanol or the dilution rate increased, as in *H. polymorpha* and *C. boidinii* sp. 2201.[17,18]

Repression by ethanol in both the parent and mutant strains could not be overcome by the addition of methanol to the feed medium in chemostat cultures, as reported by Eggeling and Sahm.[20] So the repression of alcohol oxidase by ethanol must be very severe. However, the oversynthesis of alcohol oxidase reported by them in both the parent and mutant strains was not observed in the present study.

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