Original Papers

Isolation of Stable Mutants of *Candida guilliermondii* Producing One Type of ADH

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ABSTRACT

*Mutants of **Candida guilliermondii** partially deficient in alcohol dehydrogenase (ADH) were isolated using allyl alcohol as a selective agent. On glucose medium containing allyl alcohol produced mutants deficient in ADH1, while mutants free of ADH2 were isolated on ethanol medium containing allyl alcohol. An addition of 1% of yeast extract to the isolation medium resulted in a stability of the mutants against a high concentration of allyl alcohol. On this medium the cells resistant up to 80 mM of allyl alcohol compared to those with a resistance of 10 mM allyl alcohol on basal salt medium without addition of yeast extract. Furthermore, this resistance to allyl alcohol seems to be related to a stability of the mutants to produce only one type of ADH for a long incubation period (more than 2 years). Cells which were resistant only up to 10 mM of allyl alcohol started to produce two kind of ADHs after six months of incubation. However, this resistance to a high allyl alcohol resulted in a decrease of specific activity of ADHs. Mutant ADH1s had activities only 23 – 50%, where as mutant ADH2s had only 1 – 3% of the parent strain activity.*

INTRODUCTION

Candida guilliermondii is a *Candida* strain used in the production of beverages, such as "kanji" from North India and Israel, or "tea cider" from Asia, Europe, and the Soviet (Chambell-Platt 1987; Kozaki et al. 1972). *Candida guilliermondii* Y4 was isolated from traditionally fermented Indonesian tea cider (Indrati and Ohta 1990). Production of alcohol dehydrogenase, an enzyme responsible for the conversion of sugars to ethanol in the fermentation of alcoholic beverages, from this strain was described in a previous report (Indrati and Ohta 1991). It had two alcohol dehydrogenases, ADH1 and ADH2, which were observed on gel electrophoresis stained for ADH activity. ADH1 prossessed a functional role mainly in the production of ethanol from acetaldehyde, while ADH2 functioned mainly in the oxidation of ethanol to acetaldehyde. Even so both enzymes were capable of carrying out the reverse of good flavor of alcoholic beverages (Ohbuchi et al. 1991), mutants partially deficient in ADH, have been produced in several laboratories using an allyl alcohol as a selective agent (Megnet 1967, Lutstorf and Megnet 1968, Wills and Phelps 1975, Wills 1976). This paper deals with the isolation of stable mutants of *Candida guilliermondii*.

MATERIALS AND METHODS

Microorganism

The strains used throughout these studies were: the wild type strain of *Candida guilliermondii* Y4 isolated from the traditionally fermented Indonesian tea cider (Indrati and Ohta 1990); mutant strains A80-03 and A80-10, deficient in ADH1; mutant strain B10-01, B10-05, B10-10, deficient in ADH2 (Indrati et al. 1991); and mutant strains B80-012 and B80-103, deficient in ADH2.

Isolation of ADH Deficient Mutants

Isolation of ADH2-deficient mutants was carried

out using a complete EYSM agar medium, containing 1% (v/v) ethanol, 1% (w/v) yeast extract, 0.1% (v/v) soybean extract, 1 mM $MnCl_2$, and 2% agar, adjusted to pH 7.0, and supplemented with allyl alcohol. The cells were precultured for 6 h at 30°C in the corresponding media lacking allyl alcohol and agar. The concentration of allyl alcohol was increased stepwise throughout the selection procedure. Mutant colonies appearing after 10 days of incubation at 30°C on medium containing 1 mM allyl alcohol were picked up and plated on the same medium containing 5 mM allyl alcohol. The procedure was repeated using increasing amounts of allyl alcohol through 10, 20, 40, and 80 mM. Above 80 mM concentration, the growth was inhibited. Thus, 80 mM allyl alcohol was decided as the maximum concentration.

Isolation of ADH2-deficient mutants was also performed using the Singer and Finnerly method (1985), as described in a previous report (Indrati et al. 1991). The mutants selected were able to grow on basal salts medium containing up to 10 mM allyl alcohol. The method for isolation of ADH1-deficient mutants was the same as that for ADH2-deficient mutant on GYSM medium (same as the EYSM medium containing 1% (w/v) of glucose instead of ethanol), as described by Indrati et al. 1991. These mutants were resistant to 80 mM allyl alcohol.

Medium and Cultivation

The cells used for enzyme extraction were cultured on GYSM or EYSM medium at 30°C, in 500 ml Sakaguchi flasks on a reciprocal shaker. Growth was monitored with a spectrophotometer (U 2000, Hitachi).

Enzyme Extraction

Cells were harvested by centrifugation at 11,000 G for 15 min and washed twice with 50 mM sodium phosphate buffer (pH 8.0). The cell paste was suspended in the same buffer containing 115 mM β-mercaptoethanol (5 – 10 g wet weight of cells/10 ml buffer) and broken using a sonicator (Kubota 201 M at 160 W and 1.5 A). Intact cells and cell debris were removed by centrifugation at 11,000 G for 10 min. The resulting supernatant was used for electrophoresis and enzymic analysis.

Enzyme Assay

ADH activity was measured by monitoring the formation of NADH at 340 nm, 37°C, using a spectrophotometer (U-2000, Hitachi). The reaction mixture (3 ml total volume) for the oxidation assay was composed of 32 mM sodium phosphate buffer (pH 8.0, preheated to 37°C), 5 mM NAD^+, 200 mM ethanol, and an adequate amount of enzyme solution. The reaction mixture of 3 ml total volume in the reductive assay (where indicated) consisted of 4.7 mM acetaldehyde, 0.27 mM of NADH, 46.7 mM phosphate buffer pH 8.0, and an amount of enzyme at 37°C. One unit activity (U) was defined as the amount of enzyme producing 1 μmole of NADH per min under these assay conditions. Specific activity (U/mg) was the enzymatic activity per amount of protein.

Protein Determination

Protein content was determined with the method of Lowry et al. (1951) using bovine serum albumin as the standard.

Electrophoresis

Analytical slab gel electrophoresis under nondenaturing conditions was performed according to the method of Davis (1964), using 12% polyacrylamide gel. The ADH activity bands were located by incubating the gels for 30 min at 37°C in the dark, in a 50 mM sodium phosphate buffer (pH 8.0), containing ethanol (12 ml/l), NAD^+ (200 mg/l), *p*-nitroblue tetrazolium chloride (200 mg/l) and phenazine methosulphate (70 mg/l).

RESULTS AND DISCUSSION

Isolation of mutants partially deficient in ADH using allyl alcohol as a selective agent, is based on a reaction of allyl alcohol to acrolein due to the activity of ADH enzyme. This acrolein is toxic to yeast cells, so that cells exhibited ADH activity will be killed (Megnet 1967). Mechanism of its toxicity, however, was unknown (Wills 1976).

In a previous report (Indrati et al. 1991), was shown that ADH1 was active in glucose medium, whereas ADH2 was active in an ethanol medium. Thus, isolation of yeast cells on glucose medium, where

ADH1 was active, would resulted in mutants deficient in ADH1, while isolation using ethanol medium, where ADH2 is active, would resulted in mutants deficient in ADH2. In this study several mutants lacking ADH1 and ADH2 were produced on glucose and ethanol medium, respectively. The mutants isolated were tested for their activity pattern on gel electrophoresis and the migration rate of the ADHs was compared with that of the wild type strain. Cell free extracts were prepared from cells grown on GYSM medium for 30 h. As shown in Fig. 1, all five mutants isolated on ethanol medium (B80-012, B80-103, B10-01, B10-05, B10-10) had only ADH1 activity which electrophoretically were indistinguishable from that of the parent strains, while mutants isolated on glucose medium (A80-03 and A80-10) had only ADH2 activity which electrophoretically were indistinguishable from that of the parent strains. ADH1 mutants of B80-012 and B80-103 were resistant up to 80 mM allyl alcohol on medium containing ethanol and yeast extract. This differed from ADH1 mutants of B10-01, B10-05 and B10-10, which were resistant only up to 10 mM of allyl alcohol on basal salt medium without addition of yeast extract. Moreover, ADH2 mutants of A80-03 and A80-10 were the same as mutant B80-012 and B80-103, namely resistant up to 80 mM allyl alcohol on medium containing yeast extract. These results indicate that the resistance of mutant strains against allyl alcohol seems to be dependent on the medium composition for their isolation, where enriched media (containing 1% yeast extract) produced more stable mutants against a high allyl alcohol than basal media containing no yeast extract.

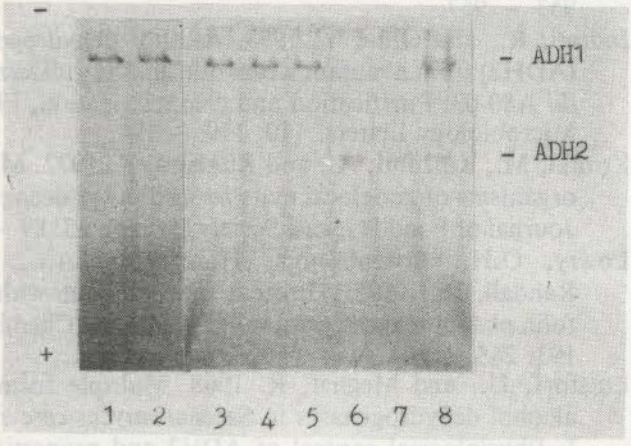

Fig. 1. **Alcohol dehydrogenase patterns of mutants grown on GYSM medium with glucose as carbon source. Incubation was at 30°C for 30 h. Lane 1, strain B80-012; lane 2, strain B80-103; lane 3, strain B10-01; lane 4, strain B10-05; lane 5, strain B10-10; lane 6; strain A80-03; lane 7, strain A80-10; lane 8, strain Y4 (parent)**

All the mutants isolated were monitored for their consistency to produce only one kind of ADH. The ADH1-deficient mutants of A80-03 and A80-10 were stable for more than two years, consistently producing ADH2 only. Furthermore, the mutants lacking ADH2 of B80-012 and B80-103 were also stable for more than two years and consistently producing ADH1 only. However, the mutants deficient in ADH2 of B10-01, B10-05, and B10-10 started to produce ADH2 after six months of incubation. The fact that the later mutants were resistant only up to 10 mM of allyl alcohol compared to the other mutants which were resistant up to 80 mM of allyl alcohol, suggest that the stability of mutants to produce only one kind of ADH seems to be related to the resistance of the wild type strain to a high concentration of allyl alcohol. This resistance may be dependent on the medium composition. These results exhibit that the method for isolation of mutant partially deficient in ADH of Singer and Finnerly (1985) using basal salt medium produced unstable mutants, rather spontaneous revertant strains after a short time. These results also prove that the method of Megnet (1967) is useful for selection of stable mutants producing only one kind of ADH even after a long time period. However, the yeast strain studied was much more resistant to a higher concentration of allyl alcohol than those studied by Megnet (1967).

Specific ADH activity of mutant and parent strains were calculated and compared. Cells were grown on EYSM medium for 30 h at 30°C by reciprocal shaking. As shown in Table 1, strain A80-03 and A80-10 had lower specific activities than that of the parents or other mutant strains. Their reductive assays showed a decrease to approximately 0.9 – 1.2%, while oxidative assays indicated a decrease to 2.3 – 30% of the parent strain. The mutant strains of B10-01, B10-05, and B10-10 had activities as high as that of the parent strains, both for reductive and oxidative assays, while strains B80-012 and B80-103 had activities approximately 23 – 50% of the parent strain. This difference in specific activities of these two kind ADH1 mutants, may be resulted from the mutation, that much more mutation may have occurred in the production of B80 mutants than B10 ones. This conclusion appeared from the fact that the selection was done stepwise throughout a series of increasing concentration of allyl alcohol. Because much more steps of increasing the concentration of allyl alcohol have

been done in strain B80 than B10, thus much more mutation could have occurred in the mutants B80 than that mutants B10. In addition, these mutations may have influenced the gene for controlling ADH activity, though the nature of the mutation had not yet been obtained. These may also explain the low ADH activities of mutants A80-03 and A80-10.

The ratio of reductive to oxidative assay of the mutant strains are high for mutants strain B and low for mutants strain A (Table 1), which is an evidence that ADH1 is a reductive enzyme responsible for the production of ethanol from acetaldehyde. This conclusion was also proven from kinetic characteristics of purified ADH1 (Indrati and Ohta 1992). On the other hand, ADH2 is possible an oxidative enzyme participating in the conversion of ethanol to acetaldehyde, although this has not yet be proved kinetically (Indrati and Ohta 1993).

Table 1. **Variation in the specific activity of alcohol dehydrogenase of parent and mutant strains grown on EYSM medium for 30 h**

Strain	Specific Activity (U/mg)		Ratio*)
	Reductive Assay	Oxidative Assay	
Y4	10.93	5.55	1.97
A80-03	0.13	0.17	0.76
A80-10	0.10	0.13	0.77
B10-01	12.45	5.71	2.18
B10-05	9.75	5.70	1.71
B10-10	11.35	5.38	2.11
B80-012	5.05	2.85	1.77
B80-103	2.56	1.28	2.00

*)Specific activity in reductive assay/specific activity in oxidative assay.

CONCLUSION

Stable mutants which produce consistently only one type of ADH after a long period of time could be isolated using enriched media containing 1% yeast extract and supplemented with allyl alcohol. On glucose and ethanol media produced mutants lacking ADH1 and ADH2, respectively. The addition of yeast extract to the isolation medium resulted in a stability of the mutants against a high concentration of allyl alcohol, and this seems to be related to their stability to produce consistently only one kind of ADH after a long period of time. However, this caused in a decrease of their specific activities in which mutant ADH1s had activities of 23 – 50% of the parent strain while mutant ADH2s only 1 – 3%.

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