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THE SUBCELLULAR LOCALIZATION OF ALCOHOL DEHYDROGENASE ACTIVITY IN BAKER'S YEAST

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SUMMARY

The subcellular location of the NAD-dependent alcohol dehydrogenase activity in baker's yeast has been studied. The alcohol dehydrogenase activities in the particulate fraction sedimenting between 3000 and 15 000 × *g* and in the 100 000 × *g* supernatant obtained from protoplasts were examined spectrophotometrically and by electrophoresis on polyacrylamide gels. During the exponential and stationary phases of growth, glucose-grown cultures contain alcohol dehydrogenase activity with low reactivity to cinnamyl alcohol in both the particulate fraction and the 100 000 × *g* supernatant. In each case the particulate fraction contains one band on electrophoresis, whereas two bands are observed in the 100 000 × *g* supernatant of the stationary phase cultures. The second band, which is not present in freshly prepared 100 000 × *g* fractions of exponential phase cultures, appears upon storage of the preparations at 4°. In ethanol-grown cultures, the electrophoretic patterns in both the particulate and 100 000 × *g* supernatant fractions are similar to those seen in glucose-grown cultures, but the activity with cinnamyl alcohol as substrate relative to the activity with ethanol is high in the 100 000 × *g* supernatant as compared with the other fractions. It appears that baker's yeast can produce at least three NAD-dependent alcohol dehydrogenases, one of which is associated with a particulate fraction and two with the soluble portion of the cytoplasm.

INTRODUCTION

Two NAD-dependent alcohol dehydrogenases (EC 1.1.1.1) which differ as to their substrate specificities and kinetic constants have been characterized in varieties of *Saccharomyces cerevesiae*[1,2]. More recently, it has been demonstrated that log phase cultures of Saccharomyces contained only one alcohol dehydrogenase. During further growth of the culture, a second enzyme appeared, which was distinguished from the

Abbreviations: C/E ratio, ratio of alcohol dehydrogenase activity with cinnamyl alcohol as substrate relative to that obtained with ethanol; MTT-tetrazolium, 3-(4,5-dimethyl thiazotyl-2)-2,5-diphenyl tetrazolium bromide.

first enzyme by both its heat sensitivity and its electrophoretic mobility[3,4]. The first enzyme was considered to be the fermentative enzyme and to correspond to the enzyme isolated according to RACKER[1], while the second enzyme was considered to be responsible for the oxidation of ethanol and to correspond to the enzyme isolated according to EBISUZAKI AND BARRON[2]. Different genes were shown to control the appearance of the two enzymes[4,*].

Our interest in the metabolism of yeasts led us to consider this pattern of alcohol dehydrogenase activity in terms of a possible spatial separation of the isozyme forms.

MATERIALS AND METHODS

Source of reagents. Reagent grade chemicals obtained from commercial sources were used. Yeast alcohol dehydrogenase was obtained from the Sigma Chemical Corporation, St. Louis, Mo. and from Boehringer Mannheim, New York, N.Y. Snail enzyme was obtained from the Industrie Biologique Française, Gennevilliers, France. Difco yeast nitrogen base was used to prepare the culture media. The acrylamide gels were prepared from grout AM-9, 95% acrylamide–5% bis-acrylamide, obtained from American Cyanamid Corporation, Bound Brook, New Jersey.

Growth of yeast cells. Two media each containing 6.7 g yeast nitrogen base were used. The carbon source was either 15 g of glucose or 15 ml of 99% ethanol per l.

Yeast strain. The yeast strain was obtained from a single cell isolate from commercial (Fleischman's) baker's yeast. The strain was maintained by serial transplantation on glucose medium containing 3% agar.

Preliminary cultures were obtained by transferring cells from an agar slope to 50 ml of the glucose medium in a 500-ml erlenmeyer flask. The cultures were incubated in an Eberbach water shaking bath for 12–18 h at 27°. The cultures attained an absorbance at 540 mμ of 1.5–2.

Working cultures were obtained by 2 methods: (1) A volume of the preliminary culture equivalent 150 absorbance units was transfered to 12 l of media in the fermentor described by BLUM AND PADILLA[5]. The gas phase was 95% air, 5% CO_2 and the temperature was maintained at 27°. (2) 10 absorbance units were transferred to 800 ml of fresh medium in a 4-l erlenmeyer flask, and the flask was incubated in an Eberbach water shaking bath at 27°. After the growth period the cells were harvested by centrifugation and washed twice with water before further use.

Manometric techniques. The O_2 consumption and CO_2 production were measured by standard manometric techniques with a Gilson respirometer. The cells were suspended in 0.05 M potassium phosphate buffer, pH 6.2. The substrate concentration was 25 mM.

Preparation of homogenates of yeast. The yeast cells were suspended in Tris–HCl buffer, pH 6.7 and broken by decompression from 20 000 lb./inch^2 in a French pressure

* EBISUZAKI AND BARRON[2] referred to the enzyme which they isolated as alcohol dehydrogenase II. By inference alcohol dehydrogenase I should be the enzyme isolated according to RACKER[1]. LUTSTORF AND MEGNET[4] referred to this latter enzyme as alcohol dehydrogenase 4 on the basis of its electrophoretic mobility. In view of the variations in electrophoretic mobility of alcohol dehydrogenase observed in Saccharomyces, it would be preferable to designate the isozymes in the order of their characterization. In this paper alcohol dehydrogenase I is used to designate the enzyme form having the properties of the enzyme isolated according to RACKER[1].

cell. Two passes through the press were employed. The homogenates were centrifuged at 100 000 × *g* for 30 min before being used for electrophoresis or spectrophotometric assays.

Preparation of mitochondria. Mitochondria were prepared by a modification of a method described previously[6]. The cells were preincubated in 2-aminoethanethiol[7]. The concentration of mannitol was 12.5% and Tris buffer was substituted for tricine. The washed mitochondria were suspended in dilute Tris buffer, sonicated for 1.5 min at maximum intensity with a model BP2 Fisher ultrasonic probe, and then centrifuged at 100 000 × *g* for 30 min.

Spectrophotometry. Alcohol dehydrogenase activity was assayed essentially by the method of BONNICHSEN[8] with 1.0 M ethanol and 25 mM NAD^+ at 25° with a Gilson model 2000 recording spectrophotometer (Gilford Instruments Inc., Oberlin, Ohio). The specific activity was expressed as μmoles NADH produced per h per mg protein.

Electrophoresis. Polyacrylamide disc gel electrophoresis was done at 5° using the pH-9.5 system of DAVIS[9] with a gel concentration of 7%. Before staining for alcohol dehydrogenase activity, the gels were washed with pyrophosphate–semicarbazide–glycine buffer[8]. They were incubated in fresh buffer containing 1 M ethanol, 2.5 mM NAD^+, 0.5 mg/ml 3-(4,5-dimethyl thiazotyl-2)-2,5-diphenyl tetrazolium bromide (MTT-tetrazolium) and 5 μg/ml phenazine methosulfate. The reaction was allowed to proceed at room temperature in the dark. Controls were run in which ethanol was not included.

RESULTS

In Fig. 1a are shown the polyacrylamide gel electrophoretic patterns of alcohol dehydrogenase obtained from cells grown on glucose in the fermentor[5]. In the 12-h culture (Fig. 1a (1)) only one band (s) having a slow electrophoretic mobility was visible. As growth proceeded a fast band (f) and a band of intermediate (i) mobility appeared. However, the (f) and (i) bands appeared also when the homogenate from the 12-h culture was stored at 4° (Fig. 1b). When the homogenates, corresponding to the samples used in Fig. 1a, were lyophilized immediately after preparation and then examined by electrophoresis, the patterns of the samples were virtually identical (Fig. 1c). The appearance of the (f) and (i) bands in extracts of cells containing initially only the (s) band was observed also when whole lyophilized yeast was stored at −15°, but the process required several weeks.

The pattern of alcohol dehydrogenase activity was then studied under three different conditions of growth: (1) 12 h on glucose; (2) 5 days on glucose; (3) 2 days on ethanol. The cultures were grown under Condition 2, described in MATERIAL AND METHODS. Under these conditions of cultivation the cultures represent respectively: (1) the end of the exponential phase of growth; (2) the stationary phase and (3) the stage in ethanol cultures of declining growth between exponential and stationary phases.

The electrophoretic patterns of alcohol dehydrogenase obtained from the three cultures are shown in Fig. 2a. Towards the end of the exponential phase of growth only the (s) form was seen in freshly prepared homogenates. Occasionally, if the culture had progressed a little further, the (f) band could be detected in small amounts.

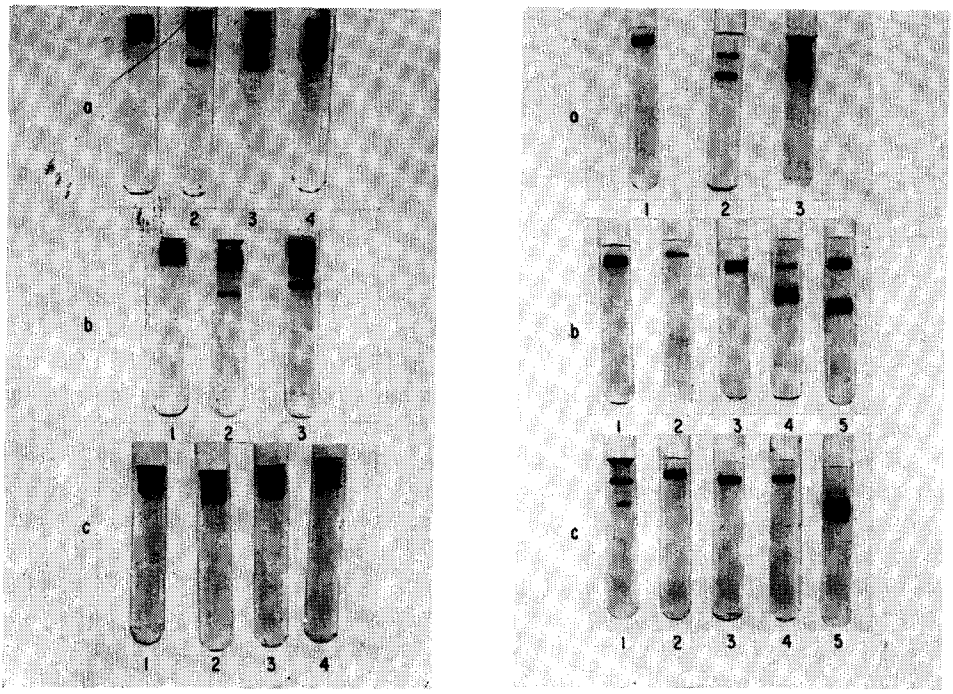

Fig. 1. a. Change in alcohol dehydrogenase pattern during growth on glucose. (1) 12 h; (2) 36 h; (3) 60 h; (4) 84 h. b. Change in alcohol dehydrogenase pattern in homogenates of yeast grown 12 h on glucose during storage at 4°. (1) control; (2) storage 24 h; (3) storage 72 h. c. Effect of lyophylization on alcohol dehydrogenase pattern. Samples the same as (a) but lyophilized immediately after preparation.

Fig. 2. Alcohol dehydrogenase pattern in mitochondrial and soluble supernatant fractions. a. (1) Yeast grown 12 h on glucose in water shaking bath, (2) yeast grown 5 days on glucose in water shaking bath, (3) yeast grown 2 days on ethanol in water shaking bath. b. (1) Mitochondria, 12 h on glucose: (2) mitochondria, 5 days on glucose; (3) mitochondria, 11 h on glucose–11 h on ethanol; (4) soluble supernatant, 5 days on glucose; (5) soluble supernatant, 11 h on glucose–11 h on ethanol. c. Effect of heating and storage on mitochondrial and soluble supernatant fraction from 12 h glucose-grown cultures. (1) Soluble supernatant, 12 h on glucose, stored 24 h; (2) mitochondria, 12 h on glucose and stored 24 h; (3) supernatant 12 h on glucose, heated at 60° for 10 min and stored 24 h; (4) supernatant 12 h on glucose, stored 24 h and heated at 60° for 10 min; (5) Sigma yeast alcohol dehydrogenase.

In stationary phase cultures, both the (s) and (f) bands and occasionally traces of the (i) band were seen. The ethanol-grown cultures contain all three bands. The alcohol dehydrogenase of the ethanol-grown cultures is distinguished from that of either glucose culture by its high activity with cinnamyl alcohol relative to that with ethanol (Table I). The cinnamyl alcohol dehydrogenase activity can not be assigned to any one particular band, since on electrophoresis all bands show some activity with this alcohol.

Location of alcohol dehydrogenase activity

Cells grown on glucose. In both the 2-day and 5-day glucose-grown cultures only the (s) band was seen on electrophoresis of the particulate fraction sedimenting

TABLE I

RELATIVE ALCOHOL DEHYDROGENASE ACTIVITIES WITH CINNAMYL ALCOHOL AND ETHANOL AS SUBSTRATES IN CELL FRACTIONS OF YEAST GROWN UNDER VARIOUS CONDITIONS

Growth condition	*C/E ratio in fractions derived from protoplasts (%)*		
	Whole yeast	*Particulate fraction*	*100 000 × g supernatant*
Glucose, 12 h	11	12	11
Glucose, 5 days	20	18	18
Ethanol, 2 days	90	—	—

between 3000 and 15 000 × *g* (Fig. 2b, 1 and 2), even though the (f) band was the predominent band in the 100 000 × *g* supernatant of the 5-day glucose-grown cultures (Fig. 2b, 4). However, on storage no (f) band appeared in the particulate fraction (Fig. 2c, 2). The (f) band appeared in the 100 000 × *g* supernatant fraction of the 12-h cultures upon storage overnight at 4° (Fig. 2c, 1). The appearance of this band could be prevented by heating the freshly prepared homogenates for 10 min at 60° (Fig. 2c, 3). This band once formed could be destroyed also by heating the stored homogenate (Fi.g 2c, 4).

In spite of preincubation with thiol compounds, the formation of protoplasts by the stationary phase cultures was poor. Many cells disintegrated, and the snail enzyme was without visible effect on others. Therefore, some selection of cells definitely occurred. However, the ratio of alcohol dehydrogenase activity with cinnamyl alcohol as substrate relative to that obtained with ethanol. (C/E ratio) obtained from homogenates of protoplasts differed very little from those obtained from the whole cells (Table I). In the cells selected there was a difference in the electrophoretic properties of the alcohol dehydrogenase from the particulate and 100 000 × *g* supernatant fractions. The C/E ratio for the alcohol dehydrogenase activity of the various cell fractions is shown also in Table I. In the 12-h and 5-days cultures the C/E ratio for the 100 000 × *g* supernatant fraction was low and approximately equal to that of the particulate fraction. However, the activities of the supernatant and particulate alcohol dehydrogenase were distinguishable using propanol as substrate. The P/E ratio for the supernatant fraction was 52% but only 33% for the particulate fraction. In the exponential phase cultures the total activity isolated with the particulate fraction was 0.5–1.0% of that isolated in the supernatant fraction.

Cells grown on ethanol. The C/E ratio of the cultures grown on ethanol varied between 70 and 90% and was considerably higher than that found in any of the fractions obtained from the glucose-grown cultures (Table I). It was impossible to make a satisfactory protoplast preparation with the cells grown for two days on ethanol, in spite of preincubation with thiols.

An attempt was made to obtain cells with a high C/E ratio suitable for the production of protoplasts by incubating washed exponential phase cells from glucose cultures in the ethanol medium. The changes observed in the specific activity of the alcohol dehydrogenase, the C/E ratio, and the absorbance of the preparation are shown in Table II. After 5 h in ethanol little growth had occurred, but there had

TABLE II

INCREASE IN ALCOHOL DEHYDROGENASE ACTIVITY ON INCUBATION IN ETHANOL MEDIUM OF A GLUCOSE-GROWN CULTURE

The values were obtained from ruptured whole cells.

Time in ethanol (h)	$A_{540\ m\mu}$	*Specific alcohol dehydrogenase activity*	*C/E (%)*
0	1.6	65	15
2	1.6	82	13
5	1.8	200	40
24	3.2	386	45

been an increase in the specific activity of alcohol dehydrogenase and in the C/E ratio. The increase continued during the 24-h incubation period. The absorbance of the culture started to increase at about 5 h. During the 24-h incubation period, the wet weight of the cells doubled, and the C/E ratio rose from 15 to 45%. The C/E ratio for the new enzyme activity was 50%.

For the production of protoplasts, cells were grown 11 h on glucose. They were washed under sterile conditions, transferred to a volume of ethanol medium equal to twice the original volume of the glucose medium, and reincubated for 11 h. The cells in the culture varied greatly in size and appearance and tended to agglutinate. Protoplast formation was less satisfactory than with the 12-h glucose cultures but better than with the stationary phase glucose-grown cells. The C/E ratios for the various fractions are shown in Table III. The C/E ratio for the 100 000 × *g* supernatant from the protoplasts was a little less than that of the ruptured whole cells, which is consistent with some selection from the cell population during protoplast formation. However, the C/E ratio for the particulate fraction was only about one-half that of the 100 000 × *g* supernatant, and only one (s) band was seen in this fraction, while the (f) band predominated in the whole yeast and 100 000 × *g* supernatant from the protoplasts (Fig. 2b, 3 and 5).

Commercial alcohol dehydrogenase. The commercial alcohol dehydrogenase isolated according to the method of RACKER[1] moves electrophoretically at the same velocity as the (i) and (f) bands (Fig. 2c, 5), but the (f) band predominates. The C/E ratio was found to be 27%.

TABLE III

SPECIFIC ACTIVITIES AND C/E RATIO OF ALCOHOL DEHYDROGENASE IN FRACTIONS DERIVED FROM CULTURES GROWN 11 h IN GLUCOSE FOLLOWED BY 11 h IN ETHANOL

Sample prepared from	*Specific activity*		*C/E (%)*
	Ethanol	*Cinnamyl alcohol*	
Whole yeast	308	132	43
100 000 × *g* supernatant from protoplasts	229	87	37
Particulate fraction from protoplasts	66	13	19

TABLE IV

O_2 CONSUMPTION AND CO_2 PRODUCTION BY INTACT BAKER'S YEAST AND ALCOHOL DEHYDROGENASE ACTIVITIES AND C/E RATIOS OF THE HOMOGENATES

Yeast grown	*air* Q_{O_2}		*air* Q_{CO_2} *Glucose*	*R.Q.*	*Specific activity*	*C/E (%)*
	Ethanol	*Glucose*				
Glucose, 12 h	12	61	300	5	76	11
Glucose, 5 days	9	9	275	31	102	20
Ethanol, 2 days	137	117	189	1.4	272	90

Relationship between alcohol dehydrogenase activity and the Q_{O_2} and the Q_{CO_2} and R.Q.

In Table IV are shown the Q_{O_2} and Q_{CO_2} values of cultures grown under the indicated conditions along with the specific activities and C/E ratios of the alcohol dehydrogenase. At the end of the exponential phase of aerobic growth on glucose, the terminal oxidative pathway is developed. Considerable alcohol is being formed from glucose as indicated by the R.Q. of 5. At this time, ethanol has little effect on O_2 uptake. Since ethanol will accumulate in the medium, there is apparently no diffusion barrier, and the block in the oxidation of ethanol must be between ethanol and the terminal oxidative pathway. After 5 days of growth on glucose, the Q_{O_2} with glucose falls to the level obtained with ethanol. The Q_{CO_2} changes very little and the R.Q. rises to 31. The specific activity of the alcohol dehydrogenase has increased, and the cells are capable of fermentation but have lost most of their oxidative capacity. These cells are probably heterogeneous with respect to their metabolic capabilities.

In ethanol-grown cultures, the specific activity of the alcohol dehydrogenase rises to almost 3 times the level found in the glucose-grown cultures. A high C/E ratio characterizes this alcohol dehydrogenase activity. These cultures differ from the glucose-grown cultures in their ability to oxidize ethanol. The block in ethanol oxidation which was seen in the glucose-grown cells is absent in this case. The R.Q. with glucose as substrate approaches 1. Either little alcohol is produced or most of that which is produced is metabolized immediately.

DISCUSSION

The understanding of yeast metabolism requires a knowledge of the enzymatic pathways leading to the production and to the oxidation of ethanol, as well as a knowledge of the sites at which the reactions occur. The study of these reactions is especially interesting in baker's yeast because, under defined conditions, this yeast either produces ethanol or utilizes it for growth. In addition, it has been shown to contain at least two different alcohol dehydrogenases[1-4]. It was interesting to us to determine whether there was any separation of the alcohol dehydrogenase activity among the subcellular components which would help elucidate the roles of the different enzymes.

Various species of Saccharomyces show different electrophoretic patterns of alcohol dehydrogenase activity. The basic pattern produced on polyacrylamide gels

by the strain used in these experiments is similar to the pattern produced on starch gel by the A63 strain of LUTSTORF AND MEGNET[4]. Early glucose-grown cultures of our strain produce only one band of alcohol dehydrogenase activity, but with further growth on glucose or growth on ethanol other bands appear. These additional bands appear also when the preparations derived from early glucose-grown cultures are stored at 4°. Since the C/E ratio does not change on storage, these new bands probably represent an alteration of the original enzyme. This change in electrophoretic form is characteristic of the enzymic activity found in the cytosol which accounts for about 99% of the total activity in the exponential phase cultures. The electrophoretic mobility of the alcohol dehydrogenase in the glucose-grown cultures corresponds after storage (which would be equivalent to the preparatory procedure) to alcohol dehydrogenase I. Therefore, the alcohol dehydrogenase I appears to be located in the cytosol.

During growth on ethanol the (f) band is accentuated. There is an increase in the C/E ratio (a characteristic of alcohol dehydrogenase II) in the cytosol. Alcohol dehydrogenase II, therefore, is also located in the cytosol. It has the same electrophoretic mobility in the system used as the altered form of alcohol dehydrogenase I. LUTSTORF AND MEGNET[4] found an antigenic relationship between alcohol dehydrogenases I and II, so the two enzymes may be related forms of the same basic molecule.

During growth on glucose the metabolism of the yeast cell is geared to the breakdown of glucose. We have shown that under this growth condition an extramitochondrial alcohol dehydrogenase (alcohol dehydrogenase I) is produced which is probably responsible for ethanol production. This is further supported by the demonstration that yeast carboxylase, which catalyses the reaction immediately preceding the reaction catalyzed by alcohol dehydrogenase, is present in the cytosol of one strain of Saccharomyces[10]. Additionally, the kinetic constants of alcohol dehydrogenase I favor the production of ethanol from acetaldehyde[4].

During growth on ethanol an active gluconeogenic system functions. To provide both the carbon source and energy for this system, the yeast cell requires an efficient method for oxidizing ethanol. Alcohol dehydrogenase II activity and the total alcohol dehydrogenase activity are higher during growth on ethanol as compared to glucose. For these reasons alcohol dehydrogenase II, which is also located in the cytosol, has been considered to be responsible for the oxidation of ethanol[3,4], and a system has been proposed consisting of the extramitochondrial oxidation of ethanol to acetaldehyde and the subsequent oxidation of the resulting NADH by the mitochondria[11,12]. However, like alcohol dehydrogenase I, the affinity of alcohol dehydrogenase II is two orders of magnitude greater for acetaldehyde than for ethanol. Thus, the kinetic properties of both enzymes favor alcohol production. Under the conditions of high ethanol concentration and the efficient removal of acetaldehyde, both enzymes could function in the oxidation of ethanol. If the functions of these enzymes are strictly segregated, some form of compartmentalization within the cytosol in necessary.

Particulate fractions from the three types of cultures studied all show alcohol dehydrogenase activity. The mitochondrial alcohol dehydrogenase has a low activity with cinnamyl alcohol as substrate, irrespective of the carbon source used to grow the yeast culture. In this, and in its electrophoretic properties, it differs from alcohol dehydrogenase II. It differs also from alcohol dehydrogenase I with respect both to its activity with propanol as substrate and its storage properties. The reports of several authors indicate that the mitochondrion of Saccharomyces possesses alcohol

dehydrogenase as a constituent of the oxidative metabolic machinery[7,13,14]. These workers prepared mitochondria capable of oxidizing ethanol which exhibited respiratory control and phosphorylation with ethanol as substrate. The oxidation rates with ethanol and tricarboxylic-acid-cycle intermediates approached that achieved with NADH, the most active substrate[7].

At the present time, it is difficult to assign a specific role for the mitochondrial alcohol dehydrogenase. If it is indeed responsible for ethanol oxidation, the failure of our glucose-grown cells to oxidize ethanol, even though they have mitochondrial alcohol dehydrogenase activity, is unexplained, unless there is a barrier preventing the diffusion of ethanol between cytosol and mitochondria in these cells.

Thus, there are at least three NAD-dependent alcohol dehydrogenases in baker's yeast, two in the cytosol and one in the mitochondrion. The role of alcohol dehydrogenase I in the cytosol seems indeed to be that of ethanol production. However, the pathway or pathways of ethanol utilisation and the functions of the cytosol alcohol dehydrogenase II and the mitochondrial alcohol dehydrogenase require further elucidation.

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