Metabolism of acetaldehyde and Custers effect in the yeast *Brettanomyces abstinens*

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Brettanomyces abstinens growing on different initial glucose concentrations showed an anaerobic inhibition of fermentation. This Custers effect decreased as the initial glucose concentration in the medium increased. Two aldehyde dehydrogenases, one NAD^+-linked and the other $NADP^+$-linked were observed. The results suggest that the NAD^+-linked enzyme is involved in the production of acetic acid and is repressed by glucose. The $NADP^+$-linked enzyme seems to be a constitutive enzyme. Acetyl-CoA synthetase activity also was not greatly affected by the growth conditions.

The results support the earlier hypothesis that the Custers effect in *Brettanomyces* is provoked by the reduction of NAD^+ in the conversion of acetaldehyde to acetic acid.

INTRODUCTION

The inhibition of fermentation under anaerobic conditions has been considered as a common biochemical characteristic of the yeasts belonging to the genus *Brettanomyces* (Wikén et al., 1961; Scheffers and Wikén, 1969). For this "negative Pasteur effect", the term Custers effect was proposed (Scheffers, 1966). Minute amounts of O_2 or organic H-acceptors (e.g. acetone, acetoin, dihydroxyacetone) abolish the anaerobic inhibition of fermentation (Scheffers,

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1961, 1967). The Custers effect is attributed (Scheffers and Misset, 1974) to the strong tendency of *Brettanomyces* to produce acetic acid from glucose with the concomitant reduction of NAD^+. Under aerobic conditions the coenzyme is reoxidized by the respiratory pathway. However, under anaerobic conditions in the absence of a H-acceptor, the production of even the slightest amounts of acetic acid will result in a drop in the NAD^+/NADH ratio and consequently in stagnation of the glycolytic flux at the level of glyceraldehyde 3-phosphate dehydrogenase (Scheffers and Nanninga, 1977).

Acetic acid may be produced in yeasts by the oxidation of acetaldehyde. In baker's yeast, two aldehyde dehydrogenases have been described. One of them, active with NAD^+ and $NADP^+$, is essentially dependent on K^+ (Steinman and Jakoby, 1967) and is localized in the mitochondria of aerobically grown yeast. Another $NADP^+$-linked aldehyde dehydrogenase, described by Seegmiller (1955), is localized in the cytosol and activated by Ca^{2+} and Mg^{2+}. Llorente and Núñez de Castro (1977) have suggested that the inducible $NAD(P)^+$-linked aldehyde dehydrogenase in *Saccharomyces cerevisiae* is involved in the oxidation of ethanol to acetyl-CoA via acetaldehyde and acetate, and that the constitutive $NADP^+$-linked enzyme is operative as an alternative pathway from pyruvate to acetyl-CoA.

The present work was undertaken in order to gain information about the metabolism of acetaldehyde in the yeast *Brettanomyces abstinens* and its possible implications for the Custers effect.

Materials and Methods

Organisms, growth media and culture conditions

The yeast *Brettanomyces abstinens* CBS 6055 was maintained on malt agar slants with 2% $CaCO_3$. Working cultures of the yeast were grown in liquid medium containing 1% yeast extract supplemented with a carbon source as indicated in the text, and adjusted to pH 5.5. Conical flasks (1000 ml) containing 250 ml medium were incubated on a rotatory shaker (150 rev./min) at 30°C. *Saccharomyces cerevisiae*, isolated from baker's yeast, was grown as described elsewhere (Núñez de Castro et al., 1974). The yeasts were harvested in the initial stationary phase of growth by centrifugation at 400 × *g*, washed twice with 0.16 M NaCl and finally with 0.1 M Tris-HCl, pH 8.

Cells depleted of endogenous substrates were obtained by incubation on a shaker in 0.1 M Tris-HCl, pH 8, at 30°C. After 3 h, without addition of respiratory substrate, no oxygen uptake was detected by the method described by Chico et al. (1978).

Manometric techniques

Gas exchange was determined at 30°C by standard manometric techniques

with a Braun-Melsungen respirometer. Each vessel contained: 45 mM potassium dihydrogen phosphate of pH 4.5, 0.1 M glucose, and yeast corresponding to about 5 mg dry weight in a final volume of 2 ml. Anaerobic conditions were obtained by flushing with oxygen-free nitrogen for 30 min prior to the addition of glucose to the yeast suspension. In aerobic fermentation experiments correction for respiration was made (Wikén et al., 1961). The values for oxygen uptake were measured separately in Warburg vessels containing alkali in the center cup. To estimate dry weight, 10 ml of the cell suspension were passed through a 0.45 μm pore size filter (Millipore) which was then washed with water and dried at 60 °C till constant weight.

Analytical methods

Aldehyde dehydrogenases were assayed according to Llorente and Núñez de Castro (1977); acetyl-CoA synthetase as described by Klein en Jahnke (1968). Acetic acid was determined by the enzymatic method of Holz and Bergmeyer (1974). Protein concentration was measured as described by Lowry et al. (1951) using bovine serum albumin as a standard.

Results

Brettanomyces abstinens grown on media with different concentrations of glucose demonstrated a Custers effect (Fig. 1). The ratio between aerobic and anaerobic fermentation decreased (9.5, 6.1 and 2.5) as the initial glucose concentration in the medium increased (0.3, 2 and 10%). *Saccharomyces cerevisiae* showed a Pasteur effect, the ratio between aerobic and anaerobic fermentation being 0.66 (Fig. 1).

On the basis of cell biomass, *Br. abstinens* grown on 2% glucose showed the highest accumulation of acetic acid in the culture medium (Table 1).

The activity of the $NADP^+$-dependent aldehyde dehydrogenase did not change appreciably with the initial glucose concentration (Table 1) nor with the growth time (Fig. 2). On the other hand, the NAD^+-linked enzyme was repressed by glucose (Table 1). Its specific activity sharply increased during the latter part of the exponential phase and the early part of the stationary phase of growth; in the same period acetic acid accumulated in the culture medium (Fig. 2). The NAD^+-dependent aldehyde dehydrogenase also showed some activity with $NADP^+$, but this activity never was more than 10% of the activity with NAD^+.

As for the acetyl-CoA synthetase, the results presented in Table 1 suggest that the activity of this enzyme is not greatly affected by the carbon source.

In order to gain further information about the metabolism of acetaldehyde, the respiratory capacity of the yeasts was measured with acetaldehyde as the substrate (Fig. 3). The respiration rate of *Sacch. cerevisiae* grown on 0.5%

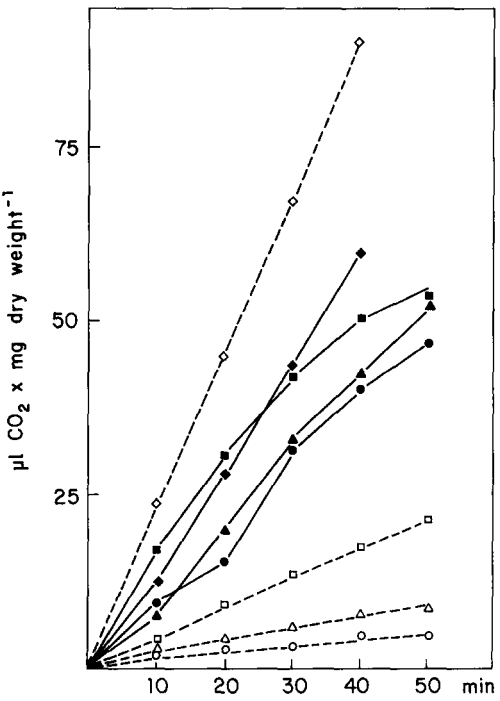

Fig. 1. Custers effect of *Brettanomyces abstinens*: (○, ●) grown on 0.3% glucose; (△, ▲) grown on 2% glucose; (□, ■) grown on 10% glucose. Pasteur effect of *Saccharomyces cerevisiae*: (◇, ◆) grown on 0.5% glucose. Open symbols are used for anaerobic fermentation, full symbols for aerobic fermentation.

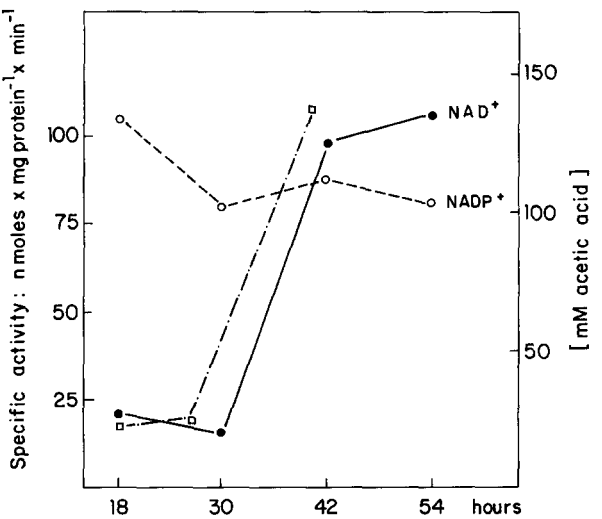

Fig. 2. Specific activity of NAD^+ and $NADP^+$-linked aldehyde dehydrogenases of *Brettanomyces abstinens* versus the growth time of cells grown on 2% glucose at 30°C. The exponential phase of growth was between 12 and 36 h. (⊡) concentration of acetic acid in the culture medium.

Table 1. Activity of aldehyde dehydrogenases (AlDH) and acetyl-CoA synthetase, and acetic acid production in the culture medium of yeasts, grown under different conditions, in the early stationary phase of growth.

Organism	Growth time (h)	Initial conc. of C-source	NAD^+-linked AlDH[1]	$NADPH^+$-linked AlDH[1]	Acetyl-CoA synthetase[1]	Acetic acid production[2]
Br. abstinens	45	0.3% glucose	105	144	176	3.2
Br. abstinens	42	2% glucose	98	88	109	10.8
Br. abstinens	42	10% glucose	14	80	86	5.9
Br. abstinens	72	2% lactate	81	54	166	4.7
Br. abstinens	72	1% ethanol	70	73	128	1.5
S. cerevisiae	24	0.5% glucose	160[3]	45[3]	162	0

[1] In nmoles × mg protein^{-1} × min^{-1}
[2] In mmoles × g wet weight^{-1}
[3] From Llorente and Núñez de Castro (1977).

glucose was about twice that of *Br. abstinens* grown at the same initial glucose concentration.

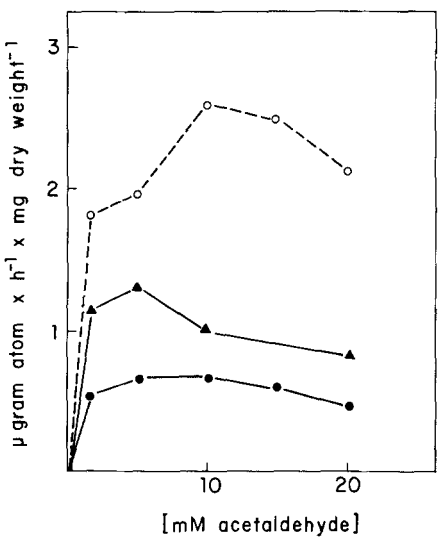

Fig. 3. O_2 uptake by *Brettanomyces abstinens* depleted of endogenous substrate: (▲) grown on 0.5% glucose; (●) grown on 2% glucose.
O_2 uptake by depleted *Saccharomyces cerevisiae* grown on 0.5% glucose: (○).
Acetaldehyde was used as the only respiratory substrate in 0.1 M Tris-HCl buffer, pH 8.

Discussion

The results presented here confirm that the Custers effect is a biochemical feature of yeast belonging to the genus *Brettanomyces*, and is not restricted to yeast grown within a narrow range of glucose concentrations. Nevertheless, the extent of the Custers effect is different for cells grown on different initial glucose concentrations. The decrease in the fermentation under anaerobic conditions is more pronounced in cells grown on 0.3% glucose, which also show maximum activity for NAD^+-linked aldehyde dehydrogenase (Table 1). This enzyme apparently is involved in the production of acetic acid (Fig. 2) in *Brettanomyces* and thus may be implicated in the Custers effect. As in *Sacch. cerevisiae* (Llorente and Núñez de Castro, 1977), the $NADP^+$-linked aldehyde dehydrogenase seems to be a constitutive enzyme in *Br. abstinens*.

The respiration rate of acetaldehyde by *Brettanomyces*, although lower than in *Saccharomyces* (Fig. 3) may well account for the production of acetic acid in *Brettanomyces*. However, in contrast to *Saccharomyces*, *Brettanomyces* excretes acetid acid in considerable quantities (see also Scheffers and Misset, 1974). Apparently, in *Brettanomyces*, acetate produced in the acetaldehyde dehydrogenase reaction, far from being completely activated to acetyl-CoA, is largely excreted into the medium. The activity of acetyl-CoA synthetase in *Brettanomyces* (Table 1) does not seem to account for this block in the oxidative pathway. At present it is not possible to establish the limiting step in the metabolism of acetate. Sanfaçon et al. (1976) have reported that in *Brettanomyces bruxellensis* the pathways leading from acetate via succinate can only operate at a limited rate.

Scheffers (1966) postulated that the Custers effect may be explained by a shortage of NAD^+, brought about by the activity of redox systems in the cell after the addition of glucose under anaerobic conditions. In the absence of O_2, the NADH produced in the aldehyde dehydrogenase reaction lowers the NAD^+/NADH ratio, with a concomitant decrease in the glycolytic flux. As was demonstrated by Scheffers and Nanninga (1977), the ratio NAD^+/NADH sharply decreases immediately after the addition of glucose to an anaerobic suspension of *Brettanomyces* cells. The hypothesis, brought forward by Scheffers and Misset (1974) that the formation of acetic acid may be linked with the reduction of NAD^+ and thus be involved in the mechanism of the Custers effect finds support in the present results.

Further studies are needed on the functioning of ethanol dehydrogenase, pyruvate dehydrogenase complex, tricarboxylic acid cycle reactions, hexose monophosphate pathway, and glycerol formation in *Brettanomyces*. Especially the kinetic relationships between branched catabolic pathways in this genus require investigation in order to further elucidate the Custers effect.

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