Biotechnology Letters Vol 10 No 1 37-42 (1988)
Received December 3

THE ROLE OF ALCOHOL DEHYDROGENASE IN THE FERMENTATION OF D-XYLOSE BY CANDIDA SHEHATAE ATCC 22984

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SUMMARY

When the oxygen supply to an aerobic chemostat culture of *Candida shehatae* growing on D-xylose was reduced to oxygen-limited and anoxic conditions, accumulation of ethanol occurred, the specific activity of alcohol dehydrogenase (ADH) increased up to four-fold and the number of ADH isozymes detected increased from one to three. ADH in crude extracts prepared from anoxically-grown cells had a lower affinity for but was more tolerant to ethanol than in extracts prepared from aerobically grown cells. ADH activity appeared to be sufficient for ethanol production by *C.* shehatae under anoxic conditions.

INTRODUCTION

The production of ethanol from D-xylose by *Candida shehatae* reflects a series of coordinated enzyme reactions where the final step to ethanol is catalyzed by ADH. This process is internally balanced and thermodynamically favourable provided the ATP and cofactors are recycled. In *Saccharomyces cerevisiae,* ADH occurs as three isozymes that catalyze the interconversion of acetaldehyde and ethanol (Van Loon and Young, 1986). The isozymes are NAD (H) specific and are important in maintaining the intracellular redox balance during fermentation. A redox imbalance in the initial oxidoreductive steps from xylose to xylulose has previously been implicated in the poor fermentation by yeasts and this imbalance can be relieved by the presence of oxygen and hydrogen acceptors (Bruinenberg et al., 1984). Little is known about the role of ADH in the fermentation of xylose to ethanol by C. *shehatae,* one of the more efficient D-xylose

+ Maintained in cooperation with the University of Wisconsin - Madison.
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fermenting yeasts (Du Preez and Van der Walt, 1983). However, the inability of fully aerobically grown *C. shehatae* to perform anaerobic fermentation may be related to the ADH isozyme present (Alexander et al., 1987b). We show that the specific activity and number of isozymes of ADH increases concomitantly with ethanol production when oxygen availability to a *C. shehatae* culture is reduced.

MATERIALS AND METHODS

Organism. *Candida shehatae* ATCC 22984 was used and maintained on yeast malt peptone glucose agar (Difco) at 32°C.

Medium. The basal medium used was described previously (Du Preez and Van der Walt, 1983). D-xylose or D-glucose were added as carbon sources at concentrations indicated in the text.

Growth conditions. The organism was grown in a 2 ℓ Multigen fermentor (New Brunswick Scientific Co., New Brunswick, NJ) with a 1 ℓ working volume. The culture mode (continuous or fed-batch), growth temperature, stirring speed and aeration are indicated in the text. Steady-state conditions were ascertained as described previously (Alexander et al., 1987).

Culture harvesting. The culture was withdrawn from the sampling port of the reactor and immediately chilled on ice. The cells were washed twice by centrifugation (10,000 x g for 10 min) with ice-cold 0.05 M potassium phosphate buffer (pH7) and the cell pellet was frozen at -70°C until required.

Cell disruption. The frozen cell pellets were thawed and disrupted by one of two methods. The glass bead method (Ciriacy, 1975) was used to disrupt approximately 1 g quantities of wet cells by adding 0.5 g glass beads (0.5 mm dia). The suspensions (used for experiments reported in Table 1 and Fig. 1) were vortexed for one min following by cooling on ice. This was repeated twice. Larger amounts of cells were disrupted by high pressure extrusion. Cells (ca. 20 g wet weight) were suspended in an equivalent volume of 0.05 M potassium phosphate buffer (pH7) containing 0.5 mM phenyl methyl sulphonyl fluoride and passed twice through a cooled French press (131,000 kPa). The disrupted suspensions were centrifuged at 17,300 x g for 20 min. In some instances, the cell-free suspensions were concentrated by centrifugation using a Centricon microconcentration system (3,500 x g for 2 h).

Analytical methods. Concentrations of cells, ethanol, D-glucose and D-xylose were determined as described previously (Alexander et al., 1987a). Alcohol dehydrogenase activity and protein concentration were determined as described by Vallee and Hoch (1955) and Bradford (1976) respectively. A unit (U) of ADH specific activity is defined as the utilization of one µmol ethanol per min per mg protein. The ADH isozymes (present in extracts of similar protein concentration) were separated between pH 5 and 8 using the Phast Gel isoelectric focusing system following the manufacturer's instructions (Pharmacia, Uppsala, Sweden). ADH isozymes were visualized by activity staining for 30 min in the dark (Ciriacy, 1975). The staining reaction was stopped by immersing the gel in 7% acetic acid. The gels were stored in 7% acetic acid. The carbon dioxide concentration in the fermentor off-gas was determined by infra-red gas analysis (Mine Safety

Appliances Lira 3000). Oxygen transfer rates were determined as described previously (Alexander et al., 1987).

RESULTS AND DISCUSSION

When *C. shehatae* was cultivated in continuous culture under carbon-limited conditions, similar ADH specific activity values were obtained irrespective of whether glucose or xylose were the limiting factor (Table 1). Higher ADH specific activity levels were observed under oxygen-limited conditions and when glucose was the carbon source. This suggests that conditions favouring ethanol production led to induction or derepression of ADH.

The reduction of oxygen supply to the continuous culture and switching to a fed-batch mode of operation resulted in the ethanol concentration in the reactor increasing from 0 to 20 $g.\ell^{-1}$ under oxygen-limited conditions within 11 h and subsequently increasing to 26 $g.\ell^{-1}$ under anoxic conditions (Fig. 1). The specific rate of ethanol production increased sharply under oxygen-limited conditions, attained a maximum after 6 h and subsequently declined. The specific rate of CO_2 production followed a similar pattern and both specific rates were considerably reduced under anoxic conditions. On the other hand, the specific activity of ADH increased during oxygen-limited and anoxic conditions and reached a maximum activity after 27 h.

Table 1 Relationship between growth conditions and alcohol dehydrogenase production by *Candida shehatae* in steady-state continuous culture at 30°C

Growth conditions					
Dilution rate (h^{-1})	Stirring speed ($rev.min^{-1}$)	Limitation	Carbon source ($g.\ell^{-1}$)	ADH specific activity ($U.mg^{-1}$)	Steady state ethanol concn. ($g.\ell^{-1}$)
0.09	(650)	Carbon (aerobic)	xylose, 20 $g.\ell^{-1}$	0.82	0
0.09	(800)	Carbon (aerobic)	glucose, 20 $g.\ell^{-2}$	0.83	0
0.05	(500)	Oxygen	xylose 93 $g.\ell^{-1}$	1.40	13
0.05	(500)	Oxygen	glucose 103 $g.\ell^{-1}$	3.00	20

In aerobically grown cells only one ADH isozyme could be detected whereas under oxygen-limited conditions, a second isozyme appeared after 1 h which coincided with increased total ADH activity. Under anoxic conditions,

three isozymes were observed. This pattern of ADH isozyme synthesis in *C. shehatae* differs from observations made in *S. cerevisiae* (Wills and Martin, 1984). *S. cerevisiae* has a constitutive ADH that is involved in the fermentative production of ethanol while a second ADH is only induced under aerobic conditions and is involved in the oxidation of ethanol. These two ADH's are cytoplasmic whereas a third ADH is located in the mitochondrion.

The kinetic properties of ADH differed in crude extracts prepared from cells grown aerobically and anoxically (Fig. 2). The ADH from aerobically grown cells attained a maximum specific activity at a much lower ethanol concentration (0.15 M) than anoxically grown cells (1.5 M). However, the ADH from aerobically grown cells was more susceptible to ethanol inhibition than the ADH from anoxically gram cells. These differences are reflected

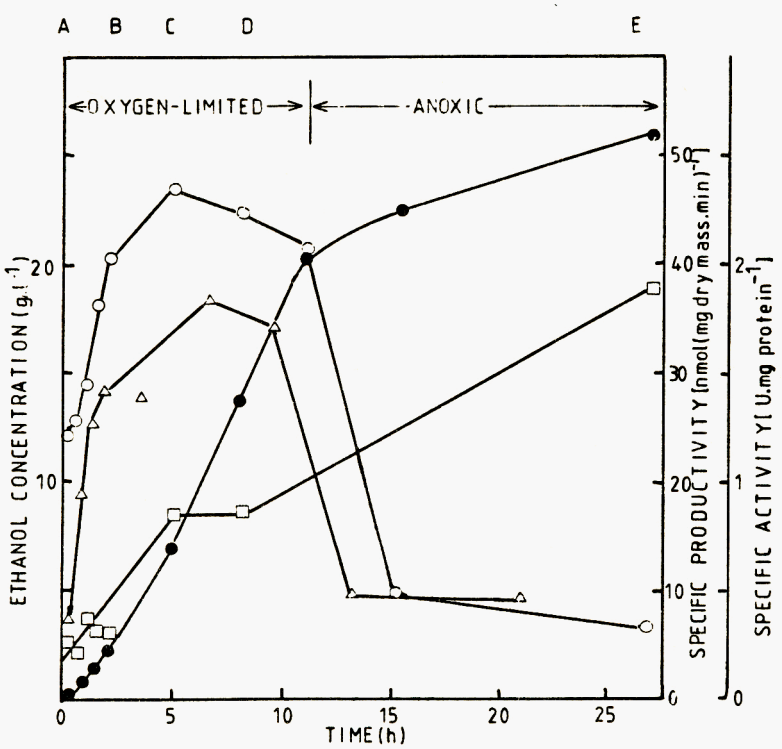

Figure 1. The response of an aerobic steady–state continuous culture (dilu–tion rate = 0.076 h^{-1}; oxygen transfer rate = 55 mmol. $\ell^{-1}h^{-1}$) growing on D–xylose (40 g. ℓ^{-1}) to a switch to fedbatch culture under oxygen–limited and anoxic conditions Under oxygenlimited conditions, D–xylose was added at a rate of 12.5 g.h^{-1} for 11 h and the culture was aerated by adding 0.4 ℓ pure oxygen.min^{-1} (oxygen transfer = 15 mmo1. $\ell^{-1}h^{-1}$) at a stirring speed of 300 rev.min^{-1} and at 20°C. Thereafter, the oxygen supply was stopped in order to create anoxic conditions but the culture was not deaerated. The effects on the specific rates of CO_2 production (○) and ethanol production (△), ethanol concentration (●) and alcohol dehydrogenase specific activity (□) are indicated. The alcohol dehydrogenase isozyme patterns are shown after 0 (A), 1 (B), 5 (C), 8 (D) and 27 h (E).

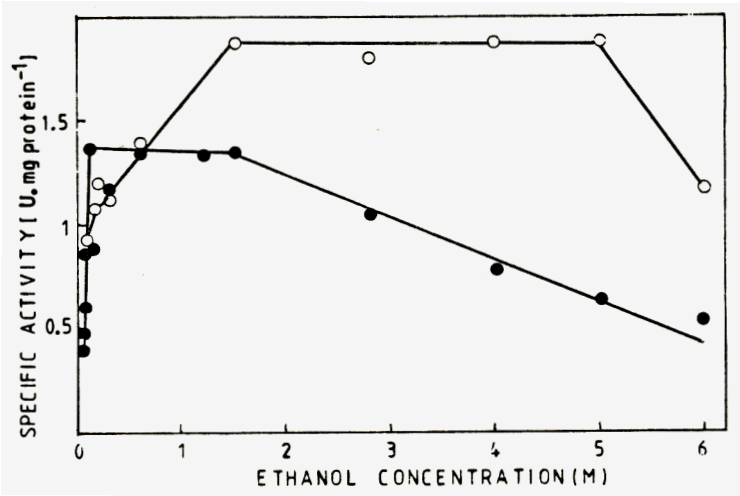

Figure 2. The effect of ethanol concentration on alcohol dehydrogenase specific activity from aerobic (●) and anoxic (○) *Candida shehatae* cultures. The aerobic cells were grown in D-xylose-limited (40 g. ℓ $^{-1}$) continuous culture while the anoxic cells were harvested from a fed-bat:) culture maintained for 16 h in the absence of oxygen and fed 12.5 g.h^{-1} D-xylose. Other growth conditions are as described in Fig. 1.

in the kinetic constants calculated from the reciprocal plots of the data. The affinity of ADH for ethanol was seven-fold greater in extracts in cells grown aerobically (K_m = 0.015 M; n = 6; r = 0.96) than anoxically (K_m = 0.10 M; n = 9; r = 0.95) whereas ethanol inhibited ADH in extracts in cells grown aerobically (K_i = 0.19 M, n = 4; r = 0.99) to a greater degree than in extracts from cells grown anoxically (K_i = 7.66 M, n = 5, r = 0.66). Furthermore the greater maximum specific activity observed in anoxically grown cells (V_{max} = 1.80 U mg^{-1}) than aerobically grown cells (V_{max} = 1.17 U mg^{-1}) confirms the data presented in Table 1 and Fig. 1. However, these kinetic constants were determined using crude extracts and require confirmation using purified isozymes. The results suggest that the ADH in aerobically grown cells may primarily be involved in the oxidation of ethanol whereas the additional ADH induced under oxygen-limited and anoxic conditions is involved primarily in ethanol production as found in *S. cerevisiae* (Wills and Martin, 1984).

As has been found in *S. cerevisiae* (Millar et al., 1982), alcohol dehydrogenase does not appear to be the rate-limiting step in the fermentation of D-xylose by *C. shehatae.* The maximum specific activity of ADH in anoxically grown cells {1.80 µmol(min.mg protein)$^{-1}$} is considerably greater than the maximum rate of ethanol production 136.6 nmol(min.mg dry biomass)$^{-1}$] observed. However, the *in vitro* enzyme assay is conducted under substrate saturating conditions and *in vivo* enzymes are often subject to allosteric control that would result in a lower specific activity. Furthermore, the *in vitro* ADH assay suggests that under fermentative conditions the enzyme is neither inhibited by ethanol nor responsible for the relatively low ethanol concentrations produced by this yeast. The

enzyme tolerated 5M ethanol *in vitro* before inhibition was observed whereas Du Preez et al. (1987) have shown that whole cells will only tolerate 1M ethanol before growth ceases. The tolerance of ADH to ethanol in aerobically–grown *C. shehatae* is similar to *S. cerevisiae* but the ADH of anoxically grown *C. shehatae* was more tolerant. Millar et al. (1982) reported that 3.5 M ethanol reduced ADH activity by 50% and 6 M ethanol almost completely inhibited ADH activity. Whether ADH isozymes differed in ethanol tolerance was not reported. The presence of higher intracellular ADH concentrations may relieve the NADH accumulation that occurs during the anoxic conversion of xylose to xylulose (Bruinenberg et al., 1984). Therefore, ADH may limit anoxic xylose metabolism even though it might not do so by limiting the rate of product formation.

ACKNOWLEDGEMENTS

Travel grants to BAP from the Foundation for Research Development, Council for Scientific and Industrial Research, Pretoria and the University of the Orange Free State are gratefully acknowledged. We thank J.C. du Preez for comments.

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