Journal of General Microbiology (1991), **137**, 2879–2883. *Printed in Great Britain*

Effects of alcohols on the respiration and fermentation of aerated suspensions of baker's yeast

HELLE N. CARLSEN,* HANS DEGN and DAVID LLOYD†

Institute of Biochemistry, Odense University, Campusvej 55, DK-5230 Odense M, Denmark

(*Received 10 July 1991; accepted 5 September 1991*)

The immediate effects of externally added alcohols on CO_2 production and O_2 consumption of suspensions of washed, aerated baker's yeast were studied by stopped-flow membrane inlet mass spectrometry. Glucose-supported fermentation was progressively inhibited by increasing ethanol concentration (0–20%, v/v). The inhibition by ethanol was quite different from that observed for acetaldehyde; thus it is unlikely that toxicity of the latter can account for the observed effects. For five different alkanols (methanol, ethanol, 1-propanol, 2-propanol and 1-butanol) increasing inhibition of anaerobic fermentation was correlated with increased partition coefficients into a hydrophobic milieu. This suggests that the action of ethanol is primarily located at a hydrophobic site, possibly at a membrane. Results for respiratory activities were not as definite as for those for anaerobic metabolism because some alkanols act as respiratory substrates as well as giving inhibitory effects.

Introduction

The accumulation of ethanol in cultures of yeast eventually leads to decreased rates of fermentation (production of ethanol and CO_2), decreased growth rates and loss of viability (Ingram & Buttke, 1984). Despite a great deal of research, it is still not clear exactly how ethanol inhibits various essential functions. Part of the difficulty in distinguishing mechanisms is due to the multiplicity of alcohol-sensitive sites.

Earlier claims that hexokinase and alcohol dehydrogenase are key sites for ethanol inhibition have been refuted by the detailed studies of Larue *et al.* (1984). However, competitive inhibition by ethanol of phosphoglycerate kinase, phosphoglycerate mutase and pyruvate decarboxylase, and non-competitive inhibition of the remaining nine enzymes clearly indicates a role for ethanol as an inhibitor of glycolysis (Millar *et al.*, 1982).

Other observations indicate that interaction of ethanol with membranes is also involved in the inhibition of glycolysis. There are several reports describing the inhibitory effect of ethanol on transport of sugars and amino acids (Thomas & Rose, 1979; Leao & Van Uden, 1982; Iglesias *et al.*, 1991). The inhibition pattern is non-competitive, ethanol decreasing the maximal uptake rate. The correlation found between the hydrophobicity and ability of alkanols to inhibit glucose utilization (Gray & Sova, 1956; Ingram & Buttke, 1984) and glucose uptake (Leao & Van Uden, 1982) seems to indicate a membrane effect. Yeast grown in the presence of increasing concentrations of ethanol has also been shown to increase the content of mono-unsaturated fatty acids in its membrane lipids in response (Beavan *et al.*, 1982). Non-specific effects due to reduced water activity and accumulation of toxic compounds such as acetaldehyde have also been proposed to be involved in inhibition by ethanol (Jones, 1989).

In this paper we report the early effects of added ethanol on CO_2 production by suspensions of aerated non-proliferating baker's yeast. We show that both aerobic and anaerobic CO_2 production from glucose is inhibited by ethanol and that increasing inhibitory effects of higher alkanols on CO_2 production correlate with increasing hydrophobicity. Our results favour the assumption of a membrane effect in inhibition of yeast metabolism by ethanol.

Methods

Organism. The organism used was a commercial strain of baker's yeast (*Saccharomyces cerevisiae*) (Danske Spritfabrikker, Grenaa, Denmark). A suspension (20%, w/v) in 10 mM-sodium citrate buffer, pH 4·4, was aerated vigorously (2 litres of air per litre of suspension per min) at room temperature (21 °C) for at least 18 h. The organisms were washed twice by centrifugation at 3000 *g* for 3 min, and finally

---

† Present address: Microbiology Group (PABIO), University of Wales College of Cardiff, PO Box 915, Cardiff CF1 3TL, UK.

0001-7035

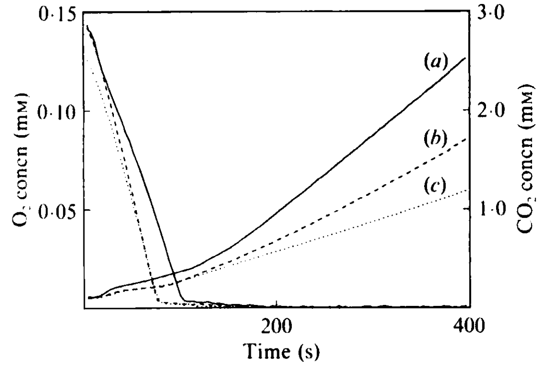

Fig. 1. Effects of ethanol on the rates of O_2 consumption and CO_2 production in a washed, aerated, non-proliferating suspension of baker's yeast after mixing with air-saturated glucose solution in a stopped flow cell. Time zero corresponds to cessation of the flow. The decreasing signals are O_2 consumption and the increasing signals are CO_2 production. Cell density was 5·5 mg dry wt ml^{-1}. The glucose concentration was 20 mM and the ethanol concentrations (v/v) were (*a*) 0%, (*b*) 5% and (*c*) 10%.

Table 1. *Effects of ethanol on O_2 consumption and aerobic and anaerobic CO_2 production in baker's yeast*

Mass spectrometric determinations typical of results obtained with three different yeast suspensions are shown. Values for the degree of inhibition (−) or stimulation (+) are given in parentheses.

Addition		Aerobic		Anaerobic
Ethanol (%, v/v)	Glucose (mM)	V_{O_2} [nmol O_2 min^{-1} (mg dry wt)$^{-1}$]	V_{CO_2} [nmol CO_2 min^{-1} (mg dry wt)$^{-1}$]	V_{CO_2} [nmol CO_2 min^{-1} (mg dry wt)$^{-1}$]
0	20	32	48	172
2·5	20	46 (+44%)	25 (−48%)	146 (−15%)
5·0	20	40 (+25%)	24 (−50%)	110 (−36%)
10	20	39 (+22%)	23 (−52%)	60 (−65%)
0	0	0·4	2·4	1·2
5	0	36	2·6	4·2
10	0	38	4·5	6·5
20	0	64	4·7	7·5
25	0	41	8·6	9·5
30	0	22	4·3	6·9

resuspended in the same buffer at a cell concentration of about 10 mg dry wt ml^{-1}.

Measurements of O_2 and CO_2. Details of the method used for simultaneous monitoring of O_2 and CO_2 by stopped-flow membrane inlet mass spectrometry have been published previously (Degn & Kristensen, 1986; Gaunt *et al.*, 1988). Aerated yeast suspension and 40 mM-glucose (both in 10 mM-sodium citrate buffer, pH 4·4) were equilibrated at 30 °C. Equal volumes of the two components were drawn into the reaction vessel of the mass spectrometer (VG Gas Analysis, UK). After arrest of the flow, effected by a magnetic valve, O_2 consumption and CO_2 evolution were monitored through a Teflon membrane (12 μm thick; Radiometer, Denmark) at *m*/*z* values 32 and 44 respectively. Calibration for O_2 was with air-saturated buffer. That for CO_2 employed standard gas mixtures or mixing 400 μM-$NaHCO_3$ with sodium citrate buffer (pH 4·4). Solubilities used for O_2 in air-saturated buffer and for CO_2 at 30 °C were 247 μM and 29·7 mM respectively in the absence of ethanol (Wilhelm *et al.*, 1977). For correction purposes the small consumption of gases by the measuring device was determined by monitoring the gases in citrate buffer in the absence of yeast.

Results

The early stages of the metabolism of glucose by washed, aerated, non-proliferating suspensions of baker's yeast are shown in Fig. 1 (curve *a*). In the absence of added ethanol, the rate of O_2 consumption accelerated gradually over the first 80 s. Aerobic CO_2 production also showed a gradual acceleration corresponding with that of O_2 utilization, but as O_2 became limiting, it underwent a rapid phase of increase to reach constant velocity after about 200 s. When the experiment was repeated in the presence of 5% (v/v) ethanol, the respiration rate of the organisms increased (Fig. 1, curve *b*), but aerobic CO_2 evolution was markedly inhibited. Exhaustion of O_2 was again accompanied by accelerated CO_2 production. The phase of constant anaerobic fermentation eventually attained (after about 200 s) also showed inhibition by comparison with the control rate in the absence of ethanol. When higher concentrations of ethanol were used (e.g. 10%; Fig. 1, curve *c*) effects on aerobic O_2 consumption were similar to those with 5% (v/v) ethanol. Aerobic CO_2 production was nearly the same, but anaerobic CO_2 evolution was further inhibited. In these experiments glucose and ethanol were presented simultaneously to the organisms. Pre-incubation of organisms with glucose gave some 'protection' against the effects of ethanol, so that somewhat lower figures for inhibition were obtained (not shown). However, the overall pattern of effects remained similar. Experiments done in the absence of glucose indicated that at low concentrations (e.g. 10%) ethanol was a respiratory substrate. At higher concentrations ethanol (> 20%) became inhibitory to O_2 consumption. Anaerobic CO_2 production progressively increased as the ethanol concentration was increased. These results are summarized in Table 1.

Acetaldehyde (12·5 mM) was a potent inhibitor of glucose-supported respiration, as shown by marked decreases in O_2 consumption. Aerobic and anaerobic CO_2 production was only marginally decreased (by a few per cent). At 125 mM-acetaldehyde, inhibition of both glucose respiration and fermentation was observed; O_2 consumption and CO_2 production of the starved yeast in the absence of glucose was stimulated by acetaldehyde (Table 2).

In order to determine whether the effects of ethanol can be modified by the long-term exposure of non-

Table 2. *Effects of acetaldehyde on O_2 consumption and aerobic and anaerobic CO_2 production in baker's yeast*

Mass spectrometric determinations typical of results obtained with three different yeast suspensions are shown. Values for the degree of inhibition (−) are given in parentheses.

Addition		Aerobic		Anaerobic
Acetaldehyde (mM)	Glucose (mM)	V_{O_2} [nmol O_2 min^{-1} (mg dry $wt)^{-1}$]	V_{CO_2} [nmol CO_2 min^{-1} (mg dry $wt)^{-1}$]	V_{CO_2} [nmol CO_2 min^{-1} (mg dry $wt)^{-1}$]
0	20	26	28	109
12·5	20	20 (−23%)	27 (−4%)	111 (−2%)
125	20	10 (−62%)	22 (−21%)	88 (−19%)
0	0	5·0	4·6	1·6
12·5	0	15	3·8	4·6
125	0	8·5	9·3	27

Table 3. *Effects of alkanols on glucose-supported respiration and CO_2 production by non-proliferating suspensions of baker's yeast*

Mass spectrometric determinations typical of results obtained with two different yeast suspensions are shown. The concentration of glucose was 20 mM in all experiments. Values are presented as percentages of the rates of respiration or CO_2 production when no alkanol was added [V_{O_2} = 28 nmol O_2 min^{-1} (mg dry $wt)^{-1}$; aerobic V_{CO_2} = 44 nmol CO_2 min^{-1} (mg dry $wt)^{-1}$; anaerobic V_{CO_2} = 136 nmol CO_2 min^{-1} (mg dry $wt)^{-1}$]. +, Stimulation; −, inhibition.

		Aerobic		Anaerobic
Alkanol added	Concn (M)	V_{O_2} (%)	V_{CO_2} (%)	V_{CO_2} (%)
Methanol	2·50	−6	−24	−20
Ethanol	0·86	+36	−91	−25
	1·72	+36	−86	−56
1-Propanol	0·67	+30	−67	−37
	1·33	+49	−28	−70
2-Propanol	0·66	−6	−32	−23
	1·31	−6	−32	−68
1-Butanol	0·55	+21	−28	−55
	1·09	−37	−51	−81
Isobutanol	0·27	−20	−21	−30
	0·54	−41	−32	−53

proliferating organisms, another series of experiments used yeast that had been starved in the usual way (18 h aeration), but was then aerated for 4 h in the presence of either 5% or 20% ethanol. Essentially similar results were obtained irrespective of pretreatment. Thus addition of 10% ethanol accelerated respiration and gave extensive inhibition of both aerobic and anaerobic CO_2 evolution; 20% ethanol gave some relief of the inhibition of aerobic CO_2 production but in other respects was more inhibitory (data not shown).

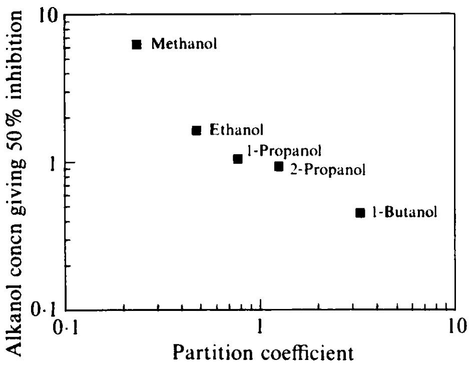

Fig. 2. Inhibition of anaerobic CO_2 production by various alkanols as a function of their partition coefficient. Anaerobic CO_2 production was measured as described in Fig. 1 in the presence of different concentrations of various alkanols, and the alkanol concentration that gave 50% inhibition of anaerobic CO_2 production was calculated. The logarithm of this concentration was plotted against the logarithm of the partition coefficient of the alkanol in the dimyristoyl lecithin/water system (Katz & Diamond, 1974).

Table 3 shows the effects of various alkanols on the metabolism of baker's yeast. Methanol was less inhibitory than ethanol towards anaerobic CO_2 production whereas higher alkanols were more inhibitory. When the logarithm of the alkanol concentration giving 50% inhibition of anaerobic CO_2 output was plotted against the logarithm of its partition coefficient between dimyristoyl lecithin and water (Katz & Diamond, 1974) a good correlation was obtained (Fig. 2). This relationship could not be established for respiratory functions where the effects of higher alkanols were more complex (Table 3).

Discussion

Whereas measurements of glucose uptake were used in previous studies of the effect of ethanol on yeast metabolism, we based the present study on measurements of CO_2 production. The onset of the inhibitory effects of ethanol on aerobic and anaerobic CO_2 production by intact non-proliferating cell suspensions of baker's yeast were rapid, occurring in less than 10 s. Utilization of ethanol as a mitochondrial respiratory substrate (Ohnishi *et al.*, 1966; Lloyd, 1974) masks possible inhibitory effects when measured by O_2 consumption and aerobic CO_2 production. As anaerobic CO_2 production is a result of glycolysis it is a direct measure of the fermentation rate.

The degree of inhibition of anaerobic CO_2 production was linear with ethanol concentration up to 10% ethanol and it was not qualitatively altered by previous exposure to ethanol for 4 h. The concentration of ethanol which reduced the fermentative activity by 50% was found, in our experiments, to be 8–10%. For baker's yeast Kalmokoff & Ingledew (1985) found 50% inhibition of fermentation at 13% ethanol. Growth was totally inhibited at 12% ethanol and the minimum level of ethanol at which losses in viability were detected was 18%. Their data indicate that fermentation ability is affected at a much lower ethanol concentration than viability. Decreased viability seems therefore not to be the cause of the observed inhibition of fermentation.

A number of mechanisms have been proposed to be involved in ethanol inhibition. These include alteration of membrane permeability properties, sugar transport systems, direct effects on enzymes, product inhibition or accumulation of toxic metabolites (Leao & Van Uden, 1982; Ingram & Buttke, 1984; Casey & Ingledew, 1986; Jones & Greenfield, 1987; Jones, 1989; Koukou *et al.*, 1990; D'Amore & Stewart, 1990). These various proposals are not mutually exclusive, and different inhibitory effects may assume greater or lesser significance in different yeasts and under different environmental situations.

That acetaldehyde (12·5 mM) gives a completely different pattern of effects from those shown by ethanol strongly indicates that the accumulation of this substrate cannot account for ethanol inhibition of baker's yeast, even though this has been claimed to be the case in some other strains of *S. cerevisiae* (Jones, 1989). Thus, in our experiments, acetaldehyde is a more powerful inhibitor of respiration than of anaerobic glycolysis, whereas ethanol has a much more pronounced effect on anaerobic CO_2 production.

The striking correlation between hydrophobicity within the alkanol series and inhibition of anaerobic glycolytic CO_2 production provides a strong indication that hydrophobic site(s) are the main target for the ethanol inhibition. At present it is not possible to decide whether interaction of ethanol with hydrophobic sites on, for example, the glycolytic enzymes or on a membrane is the cause of decreased fermentation. Ethanol inhibition of glucose uptake, as observed by Leao & Van Uden (1982), could account for inhibition of fermentation by ethanol if glucose transport is rate-limiting for glycolysis. Leao & Van Uden (1982) found that the ethanol concentration resulting in 50% inhibition of the glucose uptake rate was 1·1 M (6·3%, v/v). This value is in the same range as the ethanol concentration which, in our experiments resulted in 50% inhibition of glycolytic CO_2 production.

This work was supported by the Centre for Food and Process Biotechnology, The Technical University, Copenhagen, Denmark.

References

BEAVAN, M. J., CHARPENTIER, C. & ROSE, A. H. (1982). Production and tolerance of ethanol in relation to phospholipid fatty acyl composition in *Saccharomyces cerevisiae* NCYC 431. *Journal of General Microbiology* **128**, 1447–1455.

CASEY, G. P. & INGLEDEW, W. M. (1986). Ethanol tolerance in yeasts. *Critical Reviews in Microbiology* **13**, 219–280.

D'AMORE, T. & STEWART, G. G. (1990). A study of ethanol tolerance of yeast. *Critical Reviews in Biotechnology* **9**, 287–304.

DEGN, H. & KRISTENSEN, B. (1986). Stopped flow mass spectrometry: applications to the carbonic anhydrase reaction. *Journal of Biochemical and Biophysical Methods* **12**, 305–310.

GAUNT, D. M., DEGN, H. & LLOYD, D. (1988). The influence of oxygen and organic hydrogen acceptors on glycolytic carbon dioxide production in *Brettanomyces anomalous*. *Yeast* **4**, 249–255.

GRAY, W. D. & SOVA, C. (1956). Relation of molecule size and structure to alcohol inhibition of glucose utilization by yeast. *Journal of Bacteriology* **72**, 349–356.

IGLESIAS, R., FERREAS, J. M., ARIAS, F. J., MUÑOZ, R. & GIRBÉS, T. (1991). Effect of continued exposition to ethanol on activity of the ammonium and fructose transport systems in *Saccharomyces cerevisiae* var. *ellipsoideus*. *Biotechnology and Bioengineering* **37**, 389–391.

INGRAM, L. O. & BUTTKE, T. M. (1984). Effects of alcohols on microorganisms. *Advances in Microbial Physiology* **25**, 253–300.

JONES, R. P. (1989). Biological principles for the effects of ethanol. *Enzyme and Microbial Technology* **11**, 130–153.

JONES, R. P. & GREENFIELD, P. F. (1987). Specific and non-specific inhibitory effects of ethanol on yeast growth. *Enzyme and Microbial Technology* **9**, 334–338.

KALMOKOFF, M. L. & INGLEDEW, W. M. (1985). Evaluation of ethanol tolerance in selected *Saccharomyces* strains. *Journal of the American Society of Brewing Chemists* **43**, 189–196.

KATZ, Y. & DIAMOND, J. M. (1974). Thermodynamic constants for nonelectrolyte partition between dimyristoyl lecithin and water. *Journal of Membrane Biology* **17**, 101–120.

KOUKOU, A. I., TSOUKATOS, D. & DRAINAS, C. (1990). Effects of ethanol on the phospholipid and fatty acid content of *Schizosaccharomyces pombe* membranes. *Journal of General Microbiology* **136**, 1271–1277.

LARUE, F., LAFON-LAFOURCADE, S. & RIBEREAU-GAYON, P. (1984). Relationship between the inhibition of alcoholic fermentation by *Saccharomyces cerevisiae* and the activities of hexokinase and alcohol dehydrogenase. *Biotechnology Letters* **6**, 687–692.

LEAO, C. & VAN UDEN, N. (1982). Effect of ethanol and other alkanols on the glucose transport system of *Saccharomyces cerevisiae*. *Biotechnology and Bioengineering* **24**, 2601–2604.

LLOYD, D. (1974). *The Mitochondria of Microorganisms*, p. 101, London: Academic Press.

MILLAR, D. G., GRIFFITHS-SMITH, K., ALGAR, E. & SCOPES, R. K. (1982). Activity and stability of glycolytic enzymes in the presence of ethanol. *Biotechnology Letters* **4**, 601–606.

OHNISHI, T., KAWAGUCHI, K. & HAGIHARA, B. (1966). Preparation and some properties of yeast mitochondria. *Journal of Biological Chemistry* **241**, 1797–1806.

THOMAS, D. S. & ROSE, A. H. (1979). Inhibitory effect of ethanol on growth and solute accumulation by *Saccharomyces cerevisiae* as affected by plasma-membrane lipid composition. *Archives of Microbiology* **122**, 49–55.

WILHELM, E., BATTINO, R. & WILCOCK, R. J. (1977). Low-pressure solubility of gases in liquid water. *Chemical Reviews* **77**, 219–262.