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INHIBITION AND STIMULATION OF YEAST GROWTH BY ACETALDEHYDE

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SUMMARY

Acetaldehyde at above about 0.3 g/l inhibited yeast growth, suggesting that it may contribute to product inhibition in alcohol fermentations when present at high concentrations intracellularly. The toxic effects of acetaldehyde and ethanol were not mutually reinforcing, acetaldehyde appearing to alleviate slightly the effects of ethanol. In support of this, low concentrations of acetaldehyde greatly reduced the lag phase in ethanol-containing medium and increased the specific growth rate.

INTRODUCTION

It has been suggested that produced acetaldehyde may contribute significantly to the overall product inhibition effect in fermentations (Jones, 1989,1990). Although acetaldehyde has been shown to inhibit a wide variety of biochemical processes, relatively little has been done to characterise its inhibitory effects on ethanol-producing microorganisms *in vivo* (Jones 1989,1990). Recently, however, acetaldehyde was shown to be the principal cause of the inhibition of growth rate and cell yield in batch *Zymomonas mobilis* fermentations under conditions of high oxygen supply (Ishikawa *et al.*, 1990). Acetaldehyde has recently been shown to accumulate intracellularly in *Saccharomyces cerevisiae*, raising the possibility that it may also be a significant inhibitor in yeast alcohol fermentations (Stanley and Pamment, 1993).

There appear to be no published reports of the effects of acetaldehyde on the specific growth rate of yeast nor has the possibility of a synergistic inhibitory effect between acetaldehyde and ethanol (such as occurs between ethanol and other fermentation by-products) been examined. In this paper we report the inhibition constant for acetaldehyde inhibition of the specific growth rate of *Saccharomyces cerevisiae* in the presence and absence of ethanol. We also demonstrate that, in contrast to its inhibitory effects when present at relatively high concentrations, acetaldehyde at low concentrations actually increases the specific growth rate of yeast in the presence of added ethanol and greatly reduces the lag phase.

MATERIALS AND METHODS

Organisms, media and culture conditions *Saccharomyces cerevisiae* UNSW 706800 (University of NSW culture collection) was grown in a glucose/yeast extract/phosphate medium as described previously (Dasari *et al.*, 1983). *Saccharomyces cerevisiae* X2180–1A (University of California Yeast Genetic Stock Centre) was grown in a glucose/tryptone/yeast extract medium (Walker–Caprioglio and Parks, 1987) which was filter sterilised. The cultures were grown in 500 ml Erlenmeyer flasks containing 200 ml of medium under either aerobic or anaerobic conditions. For aerobic experiments, the flasks were stoppered with cotton wool plugs. For anaerobic experiments the flasks were fitted with rubber stoppers with ports for gas inlet and exit and sampling; a nitrogen atmosphere was maintained in the headspace. Inocula were grown under the same conditions as for the respective experimental cultures and were harvested in the late exponential phase. Inocula for the experiments in ethanol–containing medium were grown either in the presence of ethanol at the same concentration as used in the experiments (strain 706800) or without ethanol (strain X2180–1A). All flasks were agitated in a reciprocating shaker at 200 rpm and 30°C.

Acetaldehyde stock solutions Analytical grade acetaldehyde (BDH Ltd, Poole, U.K.) was redistilled when necessary according to Vogel (1962) and kept at 4°C. Stock solutions were prepared in distilled water at 4°C in precooled glassware. Acetaldehyde from the stock solutions was added volumetrically to the cultures immediately before inoculation.

Analyses Cell numbers were determined using a Coulter Counter as previously described (Dasari *et al.*, 1983). The maximum specific growth rate during the exponential phase μ_m was determined from a linear regression fit of the semilog plot of cell growth. Lag times (defined as the difference between the observed time t for an inoculum of size N_0 to reach a certain density N in the exponential phase and the "ideal time" that would have been taken if the culture had commenced growing immediately at the maximum specific growth rate) were determined according to the method of Lodge and Hinshelwood (1943). Ethanol concentrations were determined using gas chromatography (Dasari *et al.*, 1983). Acetaldehyde was analysed using an enzyme test kit (Boehringer, Mannheim, Germany).

RESULTS

In medium lacking added ethanol, added acetaldehyde lengthened the lag phase and reduced the exponential phase specific growth rate of both anaerobic and aerobic cultures of *Saccharomyces cerevisiae* 706800 (Figs 1, 2). The growth curves and measured acetaldehyde curves for aerobic conditions (not shown) were very similar to those for anaerobic conditions. There was no significant difference in the inhibitory effects of acetaldehyde under aerobic and anaerobic conditions (Fig. 2). The inhibition constant for growth (*i.e.* the concentration of acetaldehyde required to reduce the specific growth rate to half that of the control) was 0.5 g/l. Acetaldehyde had no effect on lag time or specific growth rate at concentrations below about 0.05 g/l. At low added acetaldehyde concentrations, acetaldehyde was produced during fermentation, while at initial added acetaldehyde concentrations between 0.35 and 0.68 g/l, portion of the added acetaldehyde was consumed by the cells (Fig. 1B). (Control experiments showed that evaporation contributed only slightly to the loss of acetaldehyde. This is confirmed by the acetaldehyde profile of the culture containing 0.9 g/l added acetaldehyde, which failed to grow).

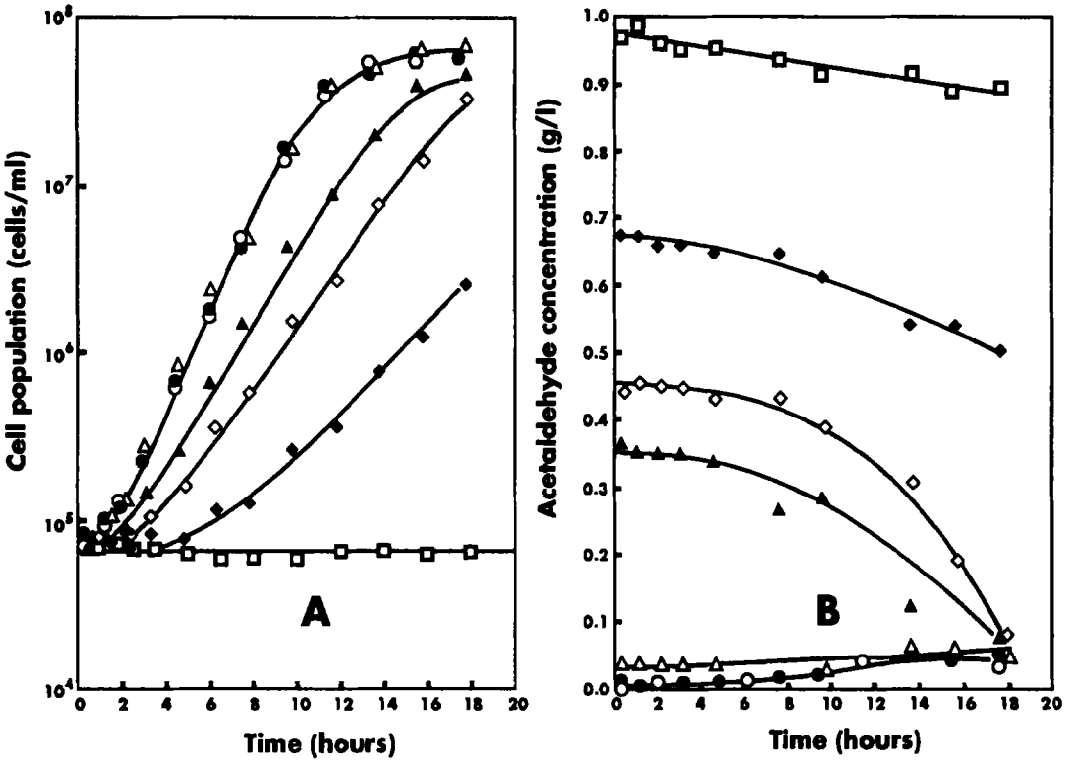

Fig.1 Time course of growth (A) and extracellular acetaldehyde concentration (B) in anaerobic cultures of *Saccharomyces cerevisiae* UNSW 706800 containing various initial concentrations of added acetaldehyde.

The effect of acetaldehyde on the lag time and specific growth rate was significantly different when the medium contained 4% w/v added ethanol. Under these conditions, added acetaldehyde at concentrations up to about 0.58 g/l markedly *reduced* the lag time of the cultures (Fig. 2B). The stimulatory influence of low concentrations of acetaldehyde on growth in ethanol–containing medium was also apparent from the specific growth rates of the cultures. Cultures containing low concentrations of added acetaldehyde (up to 0.08 g/l) had specific growth rates slightly higher than that of the control (Fig. 2A). Further, the concentration of acetaldehyde required to halve the specific growth rate (0.65 g/l) was higher than in the absence of ethanol (Fig. 2A). The ability of low concentrations of added acetaldehyde to increase the specific growth rate in ethanol–containing medium was confirmed in aerobic experiments using *S.cerevisiae* X2180–1A (Fig. 3).

DISCUSSION

The ability of added acetaldehyde to reduce markedly the lag phase of yeast in ethanol–containing medium under aerobic conditions was reported previously (Walker–Caprioglio and Parks, 1987). Our data extend this observation to anaerobic conditions and show that, at low concentrations of acetaldehyde, the specific growth rate is also significantly increased. The reasons for this effect are as yet unknown and are the subject of current research in our laboratory.

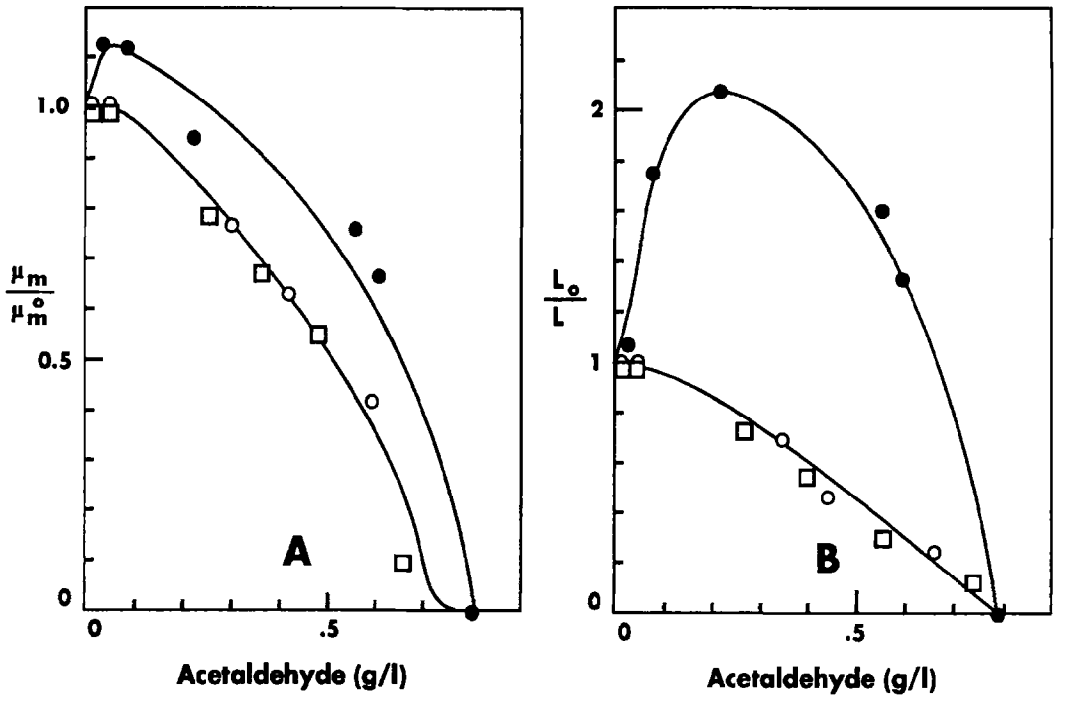

Fig.2 **A.** Effect of added acetaldehyde on the specific growth rate (μ_m) of *Saccharomyces cerevisiae* UNSW 706800 in the absence of added ethanol under aerobic (□) and anaerobic (○) conditions and in the presence of 4% w/v added ethanol under anaerobic conditions (•). Data are expressed relative to the specific growth rates (μ_m^o) of control cultures lacking added acetaldehyde. **B.** Ratio of the lag times of the control cultures (L_o) and acetaldehyde-containing cultures (L) in Fig. 2A. Acetaldehyde concentrations are the time-weighted average during the lag and exponential phases. The maximum concentration of ethanol produced during the exponential phase was less than 0.08% w/v. Control culture data: cultures without added ethanol, μ_m^o = 0.64 h^{-1}, L_o = 1.1 h (aerobic conditions) μ_m^o = 0.65 h^{-1}, L_o = 1.3 h (anaerobic conditions); culture with 4% w/v added ethanol, μ_m^o = 0.42 h^{-1} , L_o = 7.9 h.

The inhibition constant for inhibition of yeast specific growth rate by acetaldehyde has not been previously determined. However our estimate (0.5 g/l) accords with the report that the cell population in microaerobic continuous cultures of *Saccharomyces cerevisiae* began to fall when the concentration of acetaldehyde added to the reactor exceeded 0.4 g/l (Maiorella *et al.*, 1983).

Our finding that the inhibition constant for acetaldehyde is slightly increased in the presence of ethanol contrasts with the observations of other investigators who reported synergistic inhibitory effects between ethanol and various toxic byproducts including n-butanol and 3-methyl butanol (Okolo *et al.*, 1987) and certain higher fatty acids (Viegas *et al.*, 1985). (Synergistic inhibitory effects are here defined as those in which the inhibition observed in the presence of two or more inhibitors exceeds that predicted from

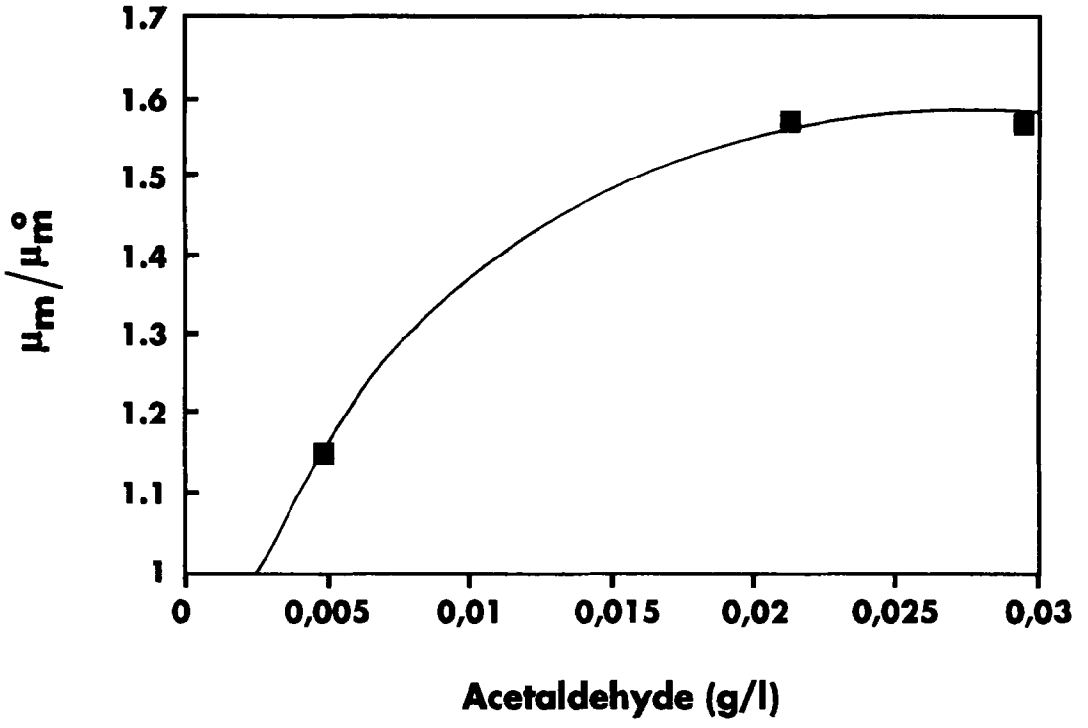

Fig. 3 Effect of small concentrations of added acetaldehyde on the specific growth rate of *Saccharomyces cerevisiae* X2180–1A in medium containing 3.2% w/v added ethanol under aerobic conditions (data from two separate experiments). Acetaldehyde concentrations are the time–weighted average during the lag and exponential phases. The control cultures (containing no added acetaldehyde) had an average specific growth rate (μ_m^o) of 0.22 ± 0.02 h^{-1} and a mean acetaldehyde concentration (due to acetaldehyde production) of 0.0025 ± 0.0003 g/l.

the sum of the inhibitory effects of each component acting alone). In contrast, the inhibitory effect of a given concentration of acetaldehyde was proportionately less in medium containing 4% w/v ethanol than in medium without ethanol (Fig. 2). While this suggests that acetaldehyde toxicity is reduced in the presence of ethanol, the alternative explanation – that acetaldehyde may reduce ethanol toxicity – is also consistent with the data. As the alleviating influence of acetaldehyde on ethanol inhibition is clearly demonstrated by the ability of low concentrations of acetaldehyde to reduce the lag phase and increase the specific growth rate in ethanol–containing medium, the latter explanation appears the more probable.

Whether produced acetaldehyde contributes to the overall inhibition in yeast fermentations as suggested by Jones remains to be determined. The acetaldehyde concentrations observed in fermentation broths are usually less than 0.1 g/l (Engan, 1981) *i.e.* well below the concentrations shown to cause significant inhibition in this work. However, recently we reported the occurrence of intracellular acetaldehyde concentrations as high as 0.33 g/l during yeast fermentations; this concentration was sustained during much of the

fermentation period (Stanley and Pamment, 1993). Assuming that it is intracellular acetaldehyde which is primarily responsible for acetaldehyde toxicity, a concentration of 0.33 g/l intracellular acetaldehyde would be predicted to contribute to the overall product inhibition at low ethanol concentrations, but to have a negligible effect once the ethanol concentration reached 40 g/l (Fig. 2). It is possible that inhibition due to acetaldehyde may account for the unexplained deactivation of cell replication observed at low ethanol concentrations in many batch fermentations (Jones, 1985). It might be argued that the intracellular acetaldehyde concentrations during our experiments with added acetaldehyde would have been higher than the extracellular concentration and that the tolerance of the cells to intracellular acetaldehyde may thus be higher than our data imply. This seems unlikely since, in the presence of added acetaldehyde concentrations above 0.3 g/l, the extracellular acetaldehyde concentration in actively growing cultures fell slightly during the experiments due to acetaldehyde uptake by the cells (Fig 1B). As acetaldehyde uptake against a concentration gradient is improbable, the intracellular acetaldehyde concentration in cultures containing significant amounts of added acetaldehyde would have been less than the extracellular concentration. Intracellular acetaldehyde concentrations have to date been measured only in high cell density fermentations (Stanley and Pamment, 1993); further proof of the role of acetaldehyde in product inhibition in yeast fermentations will require the development of methods for the determination of intracellular acetaldehyde at conventional cell population densities.

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