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ACTIVITY AND STABILITY OF GLYCOLYTIC ENZYMES IN THE PRESENCE OF ETHANOL

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SUMMARY

An investigation of the effects of ethanol on both the stabilities and activities of glycolytic enzymes of yeast and Zymomonas mobilis is presented. It is concluded that enzyme denaturation is unlikely to play a direct part in ethanol tolerance, but inhibition by ethanol may be responsible for slowing some of the glycolytic reactions.

INTRODUCTION

The biochemistry of ethanol production by yeasts has been studied for nearly a century, and is well understood in general terms. However, the effects of the end product, ethanol, on the enzymes involved has largely been ignored despite the fact that during fermentations to produce beverages or industrial alcohol, the concentration of ethanol inside the cell can become quite high, and may exceed the extracellular concentration substantially (Navarro & Durand, 1980; Nagodawithana & Steinkraus, 1976). Indeed the extent of ethanol production may be limited by inhibition of the enzymes that produce it, either as a direct enzymic inhibition, end-product inhibition (of alcohol dehydrogenase) or by denaturation. Nagodawithana et al. (1977) reported an effect of ethanol on hexokinase, and suggested that this was the most important inhibition by ethanol on glycolysis, hexokinase being the first enzyme in the sequence. However, the amount of hexokinase activity in yeast is several-fold greater than the maximum glycolytic flux attainable; control of glucose input into glycolysis is exerted at the site of its entry into the cell rather than at hexokinase. At high concentrations of ethanol it is to be expected that most enzymes will be inhibited; the present work was initiated to find which of the twelve Embden-Myerhoff glycolytic enzymes in yeast was most affected by ethanol in conditions similar to those existing in vivo.

Zymomonas mobilis ferments glucose to ethanol at a faster rate than yeasts (Rogers et al., 1979; Lee et al., 1979), and utilizes the Entner-Doudoroff pathway of glycolysis. Many of the enzymes are common to both pathways. A study of the activities of Zymomonas enzymes in the presence of ethanol has been carried out.

MATERIALS AND METHODS

Yeast extracts were made from either fresh baker's yeast or from dried nitrogen-packed cans of yeast obtained from the Australian Yeast Co. Suspensions were disrupted with the aid of a Vibrogen Cell Mill (Bühler, Tubingen). After centrifuging the homogenate at 30,000 g for 30 min, the extract was treated with phenylmethylsulphonyl fluoride

(0.5 mM) and Pepstatin A (10^{-7} M) to inhibit proteolytic activity.

Zymomonas mobilis (Strain ATTC 10988, ZM1) was grown in batch culture on a complete medium containing 1% yeast extract, salts and 15% w/v glucose. Cells were harvested at a cell density of 3-4 g dry weight per litre, and were extracted by osmotic shock/lysozyme treatment (Schwinghamer, 1980). The extract, containing 8 mg protein ml^{-1} was used for enzyme inhibition studies.

Individual yeast enzymes were purified by methods developed in this laboratory, which have been reported elsewhere (Welch & Scopes, 1981; Scopes et al., 1981; Welch, 1982). Most were in a state of 90% + homogeneity as determined by gel electrophoresis.

Enzyme assays were carried out at 20^{o} either in conditions which give optimum activity, or at a pH of 6.5, considered to be close to the intracellular pH of anaerobic yeasts (Navon et al., 1979). Except as noted, these were coupled spectrophotometric assays leading to oxidation or reduction of NAD(P). When assays were carried out in the presence of ethanol, coupling enzyme amounts were increased to ensure that their activity remained in sufficient excess of the activity of the enzyme being measured.

Denaturation studies involved incubating a sample of purified enzyme or cell extract with ethanol, in a buffer at pH 6.0. The concentration of protein in the incubation mixture was in the range 2-4 mg ml^{-1}. The samples were incubated for 30 min, then cooled in ice, five volumes of cold buffer added, and denatured protein removed by centrifugation. The amount of active enzyme remaining in the supernatant was measured by taking 1-10 µl aliquots and adding to 1 ml of the assay mixture.

The effect of alcohol on activity was studied by adding the alcohol to the assay mixture. In the short time of assay, no significant denaturation of the enzymes occurred, except in the case of yeast fructose 1,6-bis-P aldolase. The rates were compared to the rates in the absence of ethanol (except for alcohol dehydrogenase). Pyruvate decarboxylase could not be measured by the usual coupled assay in the presence of high levels of alcohol because the coupling enzyme, alcohol dehydrogenase, was product-inhibited. Consequently a stopped method was used for this enzyme, measuring pyruvate with NADH and lactate dehydrogenase, after stopping further reaction with EDTA.

RESULTS

1. Denaturation of yeast enzymes by ethanol

In a 30 min incubation at pH 6.0, 30^{o}, no yeast enzyme was denatured by 10% w/v ethanol. At 15% w/v ethanol, small losses of hexokinase, phosphofructokinase, enolase and pyruvate decarboxylase occured, and a larger proportion of glyceraldehyde phosphate dehydrogenase. At 20% ethanol, most of the aldolase, glyceraldehyde phosphate dehydrogenase, phosphoglycerate kinase and pyruvate decarboxylase was destroyed, but almost none of phosphoglucose isomerase, triose phosphate isomerase, phosphoglycerate mutase or alcohol dehydrogenase. The latter four mostly survived 30% ethanol also; a procedure for purifying alcohol dehydrogenase based on this ethanol denaturation has been reported (Scopes et al., 1981). Table 1 lists the % ethanol that caused 10%, 50% and 90% denaturation for each enzyme. On a few occasions when crude extract was used

rather than purified enzymes, very similar results ensued.

TABLE 1 Denaturation of yeast enzymes by ethanol. The % w/v ethanol required to cause 10%, 50% and 90% denaturation in 30 min under the condition described in Methods is listed for each enzyme.

Enzyme	10% loss	50% loss	90% loss
Hexokinase	16	19	25
Phosphoglucose isomerase	22	35	>40
Phosphofructokinase	14	19	22
Fructose 1,6-bis-P aldolase	15	18	20
Triose phosphate isomerase	25	>35	>40
Glyceraldehyde-P dehydrogenase	13	17	21
Phosphoglycerate kinase	16	19	21
Phosphoglycerate mutase	20	35	>40
Enolase	12	19	28
Pyruvate kinase	18	21	27
Pyruvate decarboxylase	14	17	19
Alcohol dehydrogenase	25	>35	>40

2. Inhibition of enzyme activity by alcohol

Two sorts of inhibition can be expected, competitive, in which the ethanol binds to the active site and prevents substrate binding, and general non-competitive effects due to various influences of ethanol on the enzyme's structure. Although we have not analysed each in detail, competitive effects would be expected to be gradual and express themselves in a hyperbolic decay of activity with increasing ethanol concentration. Non-competitive inhibition, if the result of a large number of alcohol molecules affecting the enzyme molecule, would be expected to increase sharply with ethanol concentration, following little effect at low concentration. On this basis, the results illustrated in Fig. 1 suggest that only phosphoglycerate kinase, phosphoglycerate mutase and pyruvate decarboxylase are inhibited competitively, the other enzymes being affected, to a greater or lesser degree, by various non-competitive influences of alcohol as its concentration rises. The least inhibited was fructose 1,6-bis-P aldolase, which had 100% activity up to the point at which denaturation occurred too quickly to allow an activity determination. Pyruvate kinase was also fully active up to 15% w/v ethanol. In contrast to previous reports (Navarro & Durand, 1980; Nagodawithana et al., 1977) hexokinase was not affected by 10% ethanol; at 20% ethanol a more detailed investigation of the inhibition indicated that the effect was non-competitive with glucose (Fig. 2a). The most significant inhibitions were of phosphoglycerate kinase, phosphoglycerate mutase, phosphofructokinase and pyruvate decarboxylase. Above 20% ethanol few enzymes would be active enough to allow glycolysis to continue.

The enzymes from Zymomonas mobilis that are common to the Embden-Myerhoff pathway showed essentially identical patterns of inhibition. Hexokinase was virtually unaffected by 15% ethanol (Fig. 2b). The enzymes unique to the Entner-Doudoroff pathway were only inhibited slightly in the 0-15% ethanol range.

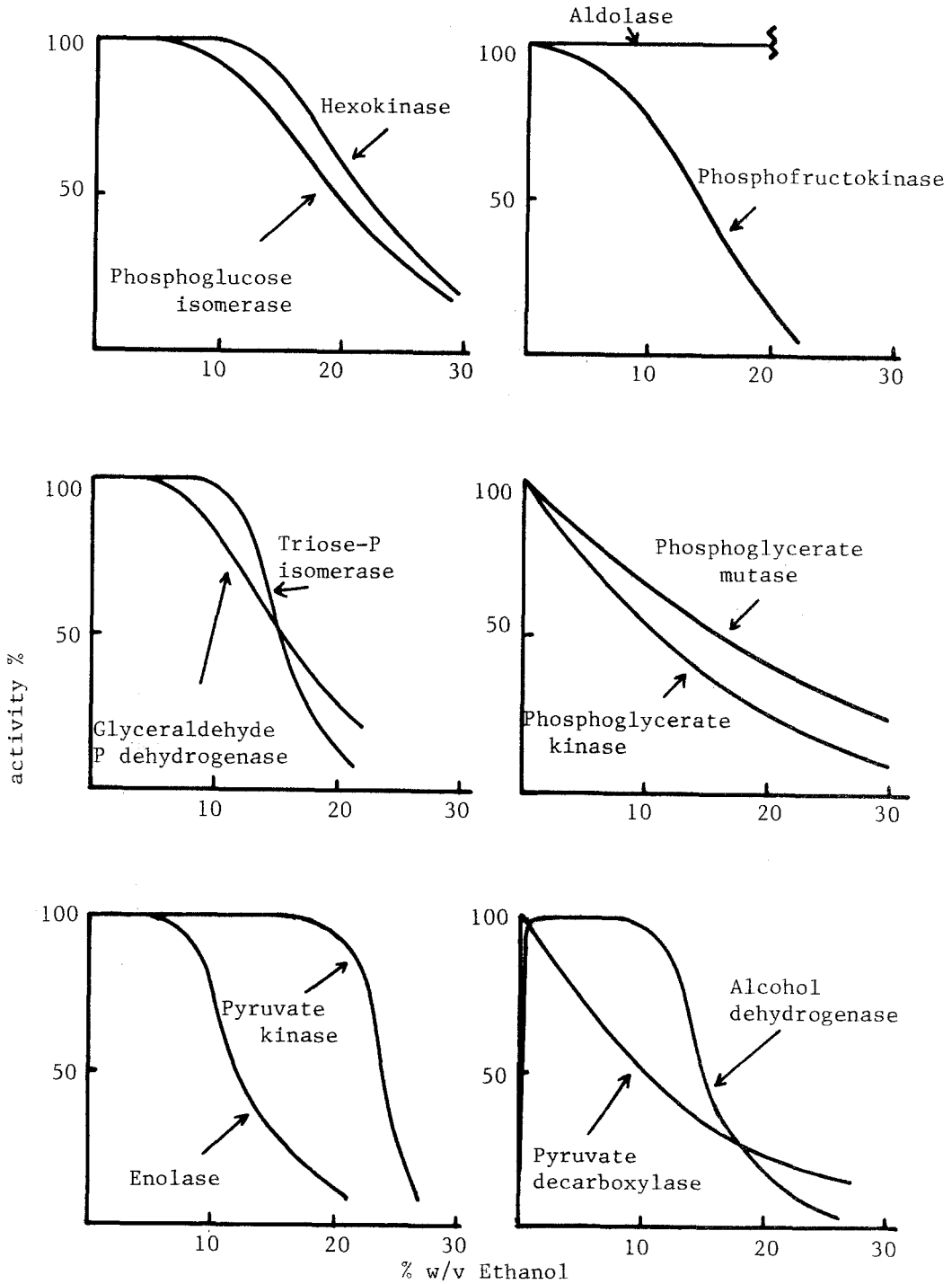

Fig. 1. Inhibition of the activities of the twelve yeast glycolytic enzymes by ethanol.

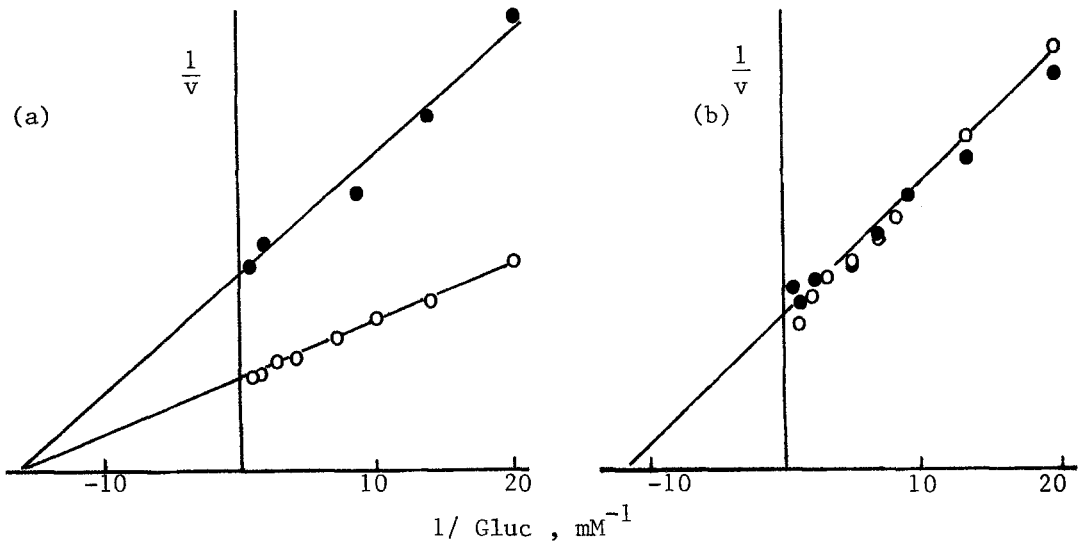

Fig. 2. Reciprocal plots for hexokinase activity. (a) Yeast enzymes, O-no ethanol; ●-20% w/v ethanol. (b) Zymomonas enzyme, O-no ethanol; ●-15% w/v ethanol.

DISCUSSION

The extent of ethanol production by yeasts or by bacteria is limited by causes which are not completely understood, but which include effects on membrane structure, direct product inhibition of alcohol dehydrogenase, and possibly inhibition and/or denaturation of other enzymes involved in ethanol production. The rate of ethanol production by yeast decreases steadily as the (exterior) ethanol concentration rises; even 1% w/v decreases the rate significantly (Moulin et al., 1980). The rate of ethanol production by Zymomonas mobilis is less affected by low concentrations, but drops sharply at concentrations of ethanol approaching those which cause fermentation to cease. Strain selection for ethanol stability can result in final concentrations of over 12% w/v with both yeast and Zymomonas (Lee et al., 1981).

The present investigation has indicated what degree of inhibition and denaturation of the ethanol-producing enzymes might be expected during fermentation. It seems improbable that denaturation plays any part in the loss of rates at least up to 12% w/v ethanol. At this point glyceraldehyde phosphate dehydrogenase, a key enzyme common to both pathways, becomes susceptible, and may represent an ultimate limitation on the ability of these organisms to produce high concentrations of ethanol. However, this argument becomes more complex if one assumes that the ethanol concentration inside the cell is substantially greater than outside. Although reported to reach a value of 30% w/v (Navarro & Durand), the present results suggest that this is improbable, since many enzymes would be denatured long before reaching such a value.

Activity inhibition is more significant, for although many enzymes seem to be present in much greater amounts than needed for the maximum glycolytic flux (especially in yeast), detailed consideration of the reactions catalysed, and the levels of substrates and products present, show that these apparently excessive amounts are mostly necessary to

sustain a high flux, often in thermodynamically unfavourable conditions. So a loss of, say 50% activity by alcohol inhibition could be partly responsible for slowing the overall fermentation rate. The results presented used assay methods in which substrate concentrations were relatively high; competitive-type inhibitions would be more significant at lower substrate concentrations occuring in the cell. However, most of the inhibition data suggested non-competitive inhibition, with little effect of ethanol on most enzymes below 5% w/v.

The most significant inhibitions as far as yeast glycolysis is concerned were of phosphofructokinase (because it is allosterically controlled to be rate-limiting), phosphoglycerate kinase (because of its importance in controlling ATP synthesis by reacting with minute concentrations of 1,3-bis-phosphoglycerate), and pyruvate decarboxylase (which is not present in amounts greatly in excess of the maximum flux rate). In Zymomonas the latter two are also considered to be significantly affected, for the same reasons.

Experiments with fermenting extracts have demonstrated that as alcohol concentration increased, fructose 1,6-bis-phosphate plus triose phosphates accumulated, despite the large amount of glyceraldehyde phosphate dehydrogenase and ample inorganic phosphate and NAD^+ present. Inhibition of phosphoglycerate kinase, slowing the removal of 1,3-bis phosphoglycerate, can be responsible for accumulation of these intermediates. Also, inhibition of pyruvate decarboxylase has been evident in fermenting extracts of both yeast and Zymomonas. Above about 5% w/v ethanol, pyruvate levels increased, reflecting inhibition of the decarboxylase. Pyruvate levels of over 10 mM at 10% ethanol have been found, which presumably is a response to overcome the competitive-type inhibition of pyruvate decarboxylase by ethanol evident in Fig. 1.

In conclusion, it is clear that enzyme inhibition could be responsible for declining rates of fermentation as ethanol concentration increases. Cessation of alcohol production could also be due to combined inhibition/denaturation of certain glycolytic enzymes, especially if the concentration of ethanol inside the cell is significantly higher than the 10-12% w/v in the medium when fermentation normally ceases.

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