Production of Extracellular Glutathione by *Candida tropicalis* Pk 233

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*Candida tropicalis* Pk 233 produced extracellular glutathione during growth in filamentous form caused by adding ethanol (≥ 2.5%, v/v). The intracellular concentration of glutathione also was greater in cultures with added ethanol. myo-Inositol added at a physiological concentration (5 µg ml⁻¹) prevented the ethanol-induced production of extracellular glutathione as well as the morphological change. In batch culture, production of extracellular glutathione was optimal at an ethanol concentration of 5%, and reached a maximum concentration of 42 mg ml⁻¹ after 96 h cultivation, before decreasing gradually.

INTRODUCTION

The usefulness of glutathione as a medicine for hepatic diseases has become of interest in recent years. There is also a continuous demand for the tripeptide as a potent and natural reductant in biological research. The industrial production of glutathione is currently either by chemical synthesis or by extraction from yeast cells. Production by chemical synthesis is inefficient, requiring the protection of chemically reactive groups during the process. To improve production from yeast, efforts have been made to select efficient strains and to establish optimal culture conditions. Recent studies have attempted to improve production of glutathione, using bacteria, by controlling the activities of enzymes concerned with its synthesis [γ-glutamylcysteine synthetase (EC 6.3.2.2) and glutathione synthetase (EC 6.3.2.3)] and with its degradation [γ-glutamyltranspeptidase (EC 2.3.2.2)]. γ-Glutamylcysteine synthetase from *Proteus mirabilis* (Kumagai et al., 1981a,b; Nakayama et al., 1981) and glutathione synthetase from *Escherichia coli* (Gushima et al., 1983b) have been purified and characterized extensively. The cloning of the genes for glutathione-synthesizing enzymes has been investigated to increase the productivity of glutathione in *E. coli* B (Murata & Kimura, 1982; Murata et al., 1983; Gushima et al., 1983a, b, c). In addition, immobilized cells of *E. coli* and *Saccharomyces cerevisiae* have been employed for the production of glutathione (Murata et al., 1979, 1980a, b, 1981a, b). It is of interest that the inhibition of γ-glutamyltranspeptidase with serine-borate or 6diazo-5-oxo-L-norleucine during cultivation of *P. mirabilis* causes the leakage of glutathione into the growth medium (Nakayama et al., 1984). Similarly, a methylglyoxal-resistant mutant of *E. coli* B excretes glutathione in the presence of methylglyoxal; this is ascribed to increases in the levels of the glutathione-synthesizing enzymes (Murata et al., 1980c).

This communication describes the production of extracellular glutathione by the yeast *Candida tropicalis* Pk 233, growing in filamentous form in the presence of ethanol (Tani et al., 1979). This phenomenon may provide a useful method for the industrial production of glutathione.
Table 1. Effects of ethanol and myo-inositol on glutathione production by C. tropicalis Pk 233

Cells were grown to the stationary phase in defined medium in the presence or absence of ethanol (2.5%, v/v) and/or myo-inositol (5 μg ml⁻¹). The cells grew in fully developed filamentous form with ethanol and maintained the yeast-like form in the control and myo-inositol-supplemented cultures. Intra- and extracellular glutathione was estimated as described in Methods. Each value is the mean of three determinations and maximum percentage variation is given in parentheses.

<table>
<thead>
<tr>
<th>Additions</th>
<th>Conc. of glutathione</th>
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<tbody>
<tr>
<td></td>
<td>Cells (mg g⁻¹)</td>
</tr>
<tr>
<td>None (control)</td>
<td>1.68 (47)</td>
</tr>
<tr>
<td>Ethanol</td>
<td>2.91 (13)</td>
</tr>
<tr>
<td>Ethanol + myo-inositol</td>
<td>1.46 (19)</td>
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</tbody>
</table>

METHODS

Organism and growth conditions. Candida tropicalis Pk 233 was grown in defined medium containing 2% (w/v) glucose as carbon source with or without addition of ethanol at indicated concentrations and/or 5 μg myo-inositol ml⁻¹ as described previously (Tani et al., 1979). Glucose and other components of the medium were sterilized separately at 120 °C for 5 min. Growth was followed by measuring OD₆00 and converting to mg dry wt from a standard curve. The relation between OD₆00 and mg dry wt was similar for yeast and filamentous forms.

Determination of glutathione. Culture supernatant was used for the assay of extracellular glutathione. Intracellular glutathione was extracted in boiling water (Owens & Belcher, 1965). Glutathione content was determined according to the method of Owens & Belcher (1965) by measuring the rate of glutathione reductase-catalysed reduction of 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB) in the presence of catalytic amounts of glutathione and an excess of NADPH.

Chemicals. Vitamin-free Casamino acids were purchased from Difco, and glutathione was a product of Kojin, Tokyo, Japan. Glutathione reductase was obtained from Sigma. All other chemicals used were of analytical reagent grade or of the highest purity available commercially.

RESULTS

Effects of ethanol and myo-inositol on glutathione production

Adding ethanol to the culture medium at a concentration of 2.5%, sufficient to cause a fully developed filamentous form of growth (Tani et al., 1979), brought about the production of extracellular glutathione (Table 1). The concomitant addition of myo-inositol reduced this effect of ethanol substantially, as well as preventing the morphological change. The intracellular concentration of glutathione nearly doubled in the presence of ethanol, but no increase was seen if myo-inositol was also added. The release of extracellular glutathione to the culture medium may have enhanced its biosynthesis within the cells.

Effect of ethanol concentration on the production of extracellular glutathione

Fig. 1 shows the time course for the production of extracellular glutathione with various concentrations of ethanol. Growth curves for corresponding cultures are given in the figure. In the control culture, where ethanol was not added, the production of extracellular glutathione was just detectable. In the presence of 2.5 or 3% ethanol, significant amounts of glutathione were excreted even during the early stages of growth. At higher concentrations of ethanol, the production of extracellular glutathione was low initially, but it increased rapidly during growth. The maximum concentration was attained in 5% ethanol culture after 96 h cultivation. Extracellular glutathione then decreased gradually. This was observed at all concentrations of ethanol used and was possibly due to the action of some peptidase(s) excreted into the medium during filamentous growth.
Extracellular production of glutathione

Fig. 1. Time course for the production of extracellular glutathione caused by ethanol. Cells were grown with the addition of ethanol at different concentrations, as described in Methods. Cultures with ethanol at 0\% (○), 2.5\% (●), 3\% (□), 4\% (■), 5\% (△) and 6\% (▲) were monitored for the content of glutathione in the supernatant (a), and for cell growth (b). Representative results are shown.

DISCUSSION

This communication demonstrates the production of extracellular glutathione associated with the ethanol-induced filamentous growth of C. tropicalis Pk 233. In addition to glutathione, C. tropicalis Pk 233 has been found to produce extracellular polysaccharides (Tani et al., 1979) and proteins (unpublished data) under the same cultural conditions. These observations offer a promising prospect for the production of useful substances by yeast.

The concomitant addition of myo-inositol and ethanol substantially reduced the production of extracellular glutathione and prevented the morphological change. Inositol is essential for normal growth and function of yeast cells (Becker & Lester, 1977; Hanson & Lester, 1980; Henry et al., 1977), serving as an important component of the membranes, mainly in the form of phosphatidylinositol (Becker & Lester, 1977). It is therefore probable that both the release of extracellular glutathione to the culture medium and the morphological change are consequences of the effects of ethanol on the structure and functions of cell membranes, especially the plasma membrane. At all the ethanol concentrations tested, cell yields increased continuously for 144 h and the maximum concentrations attained were maintained during further cultivation for at least 24 h (Fig. 1). Therefore, the accumulation of glutathione in the medium seems to be due to leakage or secretion, rather than cell lysis. myo-Inositol only partially improved the growth rate in the presence of added ethanol (data not shown) while the production of extracellular glutathione was markedly prevented by the addition of myo-inositol. This fact indicates that leakage of glutathione is not solely linked to a decrease in growth rate and, therefore, that myo-inositol counteracts an ethanol-induced change in the plasma membrane.

REFERENCES


