Journal of Industrial Microbiology, 10 (1992) 111–116

Published by Elsevier

SIM 00420

Effects of heat shock and ethanol stress on the viability of a *Saccharomyces uvarum (carlsbergensis)* brewing yeast strain during fermentation of high gravity wort

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(Received 24 September 1991; revision received 16 December 1991; accepted 31 December 1991)

Key words: Heat shock; Ethanol; *Saccharomyces*; Yeast; Fermentation; Viability; Wort

SUMMARY

The effects of heat shock and ethanol stress on the viability of a lager brewing yeast strain during fermentation of high gravity wort were studied. These stress effects resulted in reduced cell viability and inhibition of cell growth during fermentation. Cells were observed to be less tolerant to heat shock during the fermentation of 25 °P (degree Plato) wort than cells fermenting 16 °P wort. Degree Plato (°P) is the weight of extract (sugar) equivalent to the weight of sucrose in a 100 g solution at 20 °C. Relieving the stress effects of ethanol by washing the cells free of culture medium, improved their tolerance to heat shock. Cellular changes in yeast protein composition were observed after 24 h of fermentation at which time more than 2% (v/v) ethanol was present in the growth medium. The synthesis of these proteins was either induced by ethanol or was the result of the transition of cells from exponential phase to stationary phase of growth. No differences were observed in the protein composition of cells fermenting 16 °P wort compared to those fermenting 25 °P wort. Thus, the differences in the tolerance of these cells to heat shock may be due to the higher ethanol concentration produced in 25 °P wort which enhanced their sensitivity to heat shock.

INTRODUCTION

Ethanol and temperature exact a number of inhibitory effects on yeasts during fermentation. These include inhibition of cell growth, cell viability and fermentation performance [18,23,30,31]. A decrease in the rate of ethanol production has been correlated with loss of cell viability [22]. Cell growth and cell viability are inhibited at relatively low ethanol concentrations, whereas inhibition of fermentative capacity occurs at higher ethanol concentrations [1,3,24,37]. It has been suggested that these inhibitory effects result from ethanol- and temperature-induced alterations in cell membrane structure and permeability [12], inhibition of sugar and amino acid transport [37], inhibition of glycolytic enzymes [23] and glucose-induced protein fluxes [15], enhancement of thermal death [17], and petite mutations in yeasts [4]. It has also been demonstrated that the plasma membrane is a major target site of ethanol in yeasts [8,34,35].

Recently, there has been an increasing interest in high gravity brewing involving high substrate concentrations, in an attempt to improve ethanol yields. However, under the conditions of high gravity fermentation, yeasts are exposed to a variety of environmental stresses resulting from the high osmotic pressure and high ethanol concentration produced [26]. How these stress conditions affect yeast performance during high gravity fermentation is of prime concern. This paper examines the effects of heat shock and ethanol stress on the viability of a lager brewing yeast strain during fermentation. The effect of ethanol stress on the protein composition of this strain during fermentation was also examined.

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MATERIALS AND METHODS

Yeast strain

The yeast strain employed in this study was *Saccharomyces uvarum (carlsbergensis)* brewing lager strain 3021 which was obtained from the Labatt culture collection.

Growth and fermentation medium

The yeast cells were subcultured in PYN medium which consisted of: 3.5 g peptone; 3.0 g yeast extract; 2.0 g

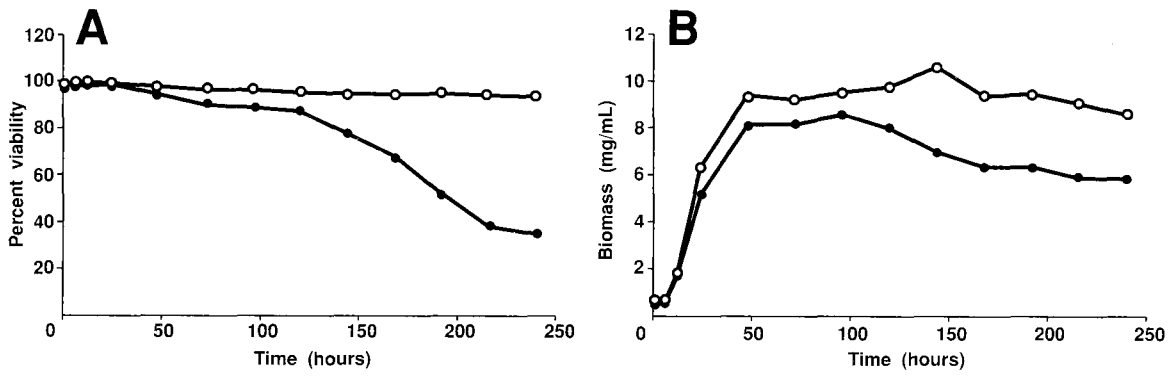

Fig. 1. Viability (A) and biomass (B) of lager strain 3021 during fermentation of 16°P (○) and 25°P (●) worts. Fermentations were conducted in shake flasks at 21 °C, inoculum 0.35% (w/v), and percent viability was determined by methylene blue stain method. The results are the average of three separate experiments.

KH_2PO_4; 1.0 g $(NH_4)_2SO_4$; 1.0 g $MgSO_4 \cdot 7H_20$; 20 g glucose; all dissolved in 1 l of distilled water and adjusted to pH 5.6. Fermentations were conducted in production 16° Plato wort and 25° Plato wort (prepared by addition of corn syrup to 16°P wort) at 21 °C with constant agitation at 150 rpm. Degree Plato (°P) is the weight of extract (sugar) equivalent to the weight of sucrose in a 100 g solution at 20 °C (e.g., 10% (w/w) sucrose = 10°P). The yeast inoculum employed was 3.5 g wet weight of cells/l.

Determination of viability

Cell viability was determined using 0.01% (w/v) methylene blue stain dissolved in 2% (w/v) sodium citrate solution. An equal volume of the stain was added to an aqueous suspension of the yeast to be counted. Approx. 500 cells were counted in the hemacytometer chamber for each determination. Cells that stained blue were considered non-viable. Percent viability was then calculated.

The colony forming units per ml of growth medium were determined by plating portions of samples diluted in sterile physiologic saline (0.89% (w/v) NaCl) on PYN agar plates containing 2% (w/v) glucose and incubating at 21 °C for 3 days.

Determination of biomass

Cell population densities during fermentation were determined by withdrawing 5 ml of cell suspension at various times, washing the portion twice with distilled water and weighing the pellet after drying in an aluminum dish at 100 °C for 4 h.

Determination of ethanol

Samples (5 ml) were taken at various times of fermentation and centrifuged at 4000 × *g* for 5 min at 4 °C. The supernatants were frozen for subsequent analysis. Ethanol was determined using a Carle A GC series 100 gas chromatograph operated at 150 °C and equipped with a 2.0 m long column packed with Chromosorb 102 column ($^{80}/_{100}$ mesh) and a Spectra-Physics SP4270 integrator.

Test for tolerance to heat shock and ethanol stress

Tolerance of yeast cells to heat shock and ethanol stress at various times during fermentation was tested by incubating samples (2 ml) in a 40 °C or 45 °C water bath for 5 min, or by adding ethanol to the sample up to 15% (v/v) concentration and incubating at 21 °C for 15 min. Cell viability was determined before and after heat shock and ethanol stress. Tolerance was measured as percentage of viable cells that survive heat shock and ethanol stress treatments.

Analysis of cell protein composition

Yeast cell proteins were extracted as described previously [9] and separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) [16]. Proteins were visualized by an ultra sensitive silver staining procedure [21].

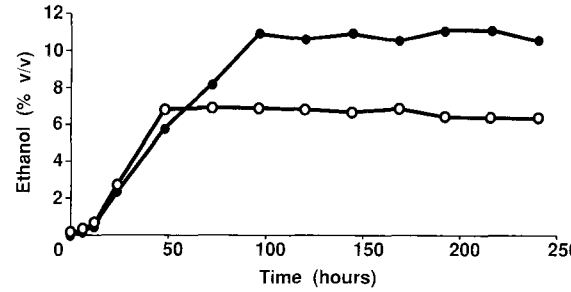

Fig. 2. Ethanol production of lager strain 3021 during fermentation of 16°P (○) and 25°P (●) worts. Fermentations were conducted in shake flasks at 21 °C, inoculum 0.35% (w/v). The results are the average of three separate experiments.

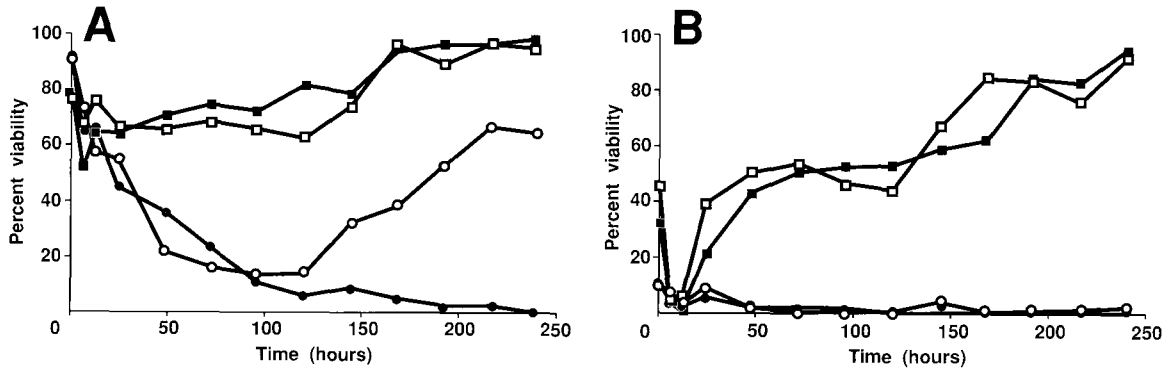

Fig. 3. Tolerance of lager strain 3021 to heat shock at 40 °C (A) and 45 °C (B) during fermentation of 16 °P and 25 °P worts. Fermentations were conducted in shake flasks at 21 °C, inoculum 0.35% (w/v). Cells obtained from various stages of fermentation, were heat shocked for 5 min. Viability of cells washed free of culture medium (16 °P (□), 25 °P (■)) prior to heat shock, was compared to that of unwashed cells (16 °P (○), 25 °P (●)). Percent viable cells that survived heat shock treatment was determined by colony count method. Each data point represents the average of three separate experiments.

RESULTS

Viability, biomass and ethanol production by lager brewing strain 3021

Cell viability, biomass and ethanol production by lager brewing strain 3021 during fermentation of 16 °P and 25 °P worts were determined. A decline in cell viability and cell growth was observed after 96 h of fermentation in the 25 °P wort. Cells fermenting 16 °P wort remained viable (greater than 90%) with little or no decline in cell growth after 240 h of fermentation (Fig. 1). The decrease in cell growth and cell viability in the 25 °P wort occurred when the concentration of ethanol approached 11% by volume (Fig. 2). Ethanol concentration in 16 °P wort reached a maximum of 7% volume after 48 h of fermentation. Ethanol at this concentration did not inhibit cell viability or cell growth. Similar results were obtained when cell viabilities were determined by either methylene blue staining or the colony count method.

Effects of heat shock and ethanol stress on cell viability

The effects of heat shock and ethanol stress on cell viability during fermentation were examined. Culture samples were taken at various times during fermentation and subjected to heat shock at 40 °C or 45 °C for 5 min, or to ethanol stress by adding 15% (v/v) exogenous ethanol, and incubating for 15 min at 21 °C. Cells fermenting 25 °P wort were observed to be less tolerant to heat shock at 40 °C or 45 °C than cells fermenting 16 °P wort (Fig. 3). However, when cells were washed free of culture medium with sterile water, their tolerance to this stress condition improved considerably. No differences were observed in the tolerance of cells fermenting either 16 °P or 25 °P worts to heat shock after washing cells free of culture medium (Fig. 3). Similar results were obtained when cells fermenting 16 °P and 25 °P worts were tested for their tolerance to 15% (v/v) ethanol (Fig. 4). Thus, the stress effects of high concentration of ethanol encountered by cells during fermentation of 25 °P wort enhanced their sensitivity to heat shock and resulted in reduced cell viability (Fig. 1). However, tolerance to heat shock and ethanol stress after washing cells free of culture medium or after exposure to non-inhibitory concentrations of ethanol produced in 16 °P wort increased with time of fermentation (Figs. 3 and 4). Thus, exposure of yeast cells to non-inhibitory concentrations of ethanol may enhance their tolerance to ethanol and heat shock. A lag period between the time ethanol concentration reached 7% (v/v) ethanol (Fig. 2) and protection of cells from heat shock (Fig. 3A) was observed.

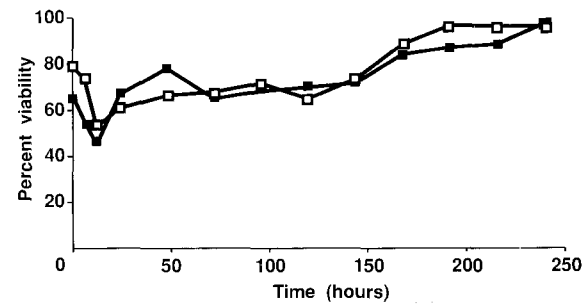

Fig. 4. Tolerance of lager strain 3021 to ethanol stress during fermentation of 16 °P and 25 °P worts. Fermentations were conducted in shake flasks at 21 °C, inoculum 0.35% (w/v). Cells obtained from various stages of fermentation were washed free of culture medium and then exposed to exogenously added ethanol (15% (v/v) for 15 min at 21 °C). Percent viable cells from 16 °P (□), 25 °P (■) wort, that survived 15% (v/v) ethanol shock was determined by colony count method. Each data point represents the average of three separate experiments.

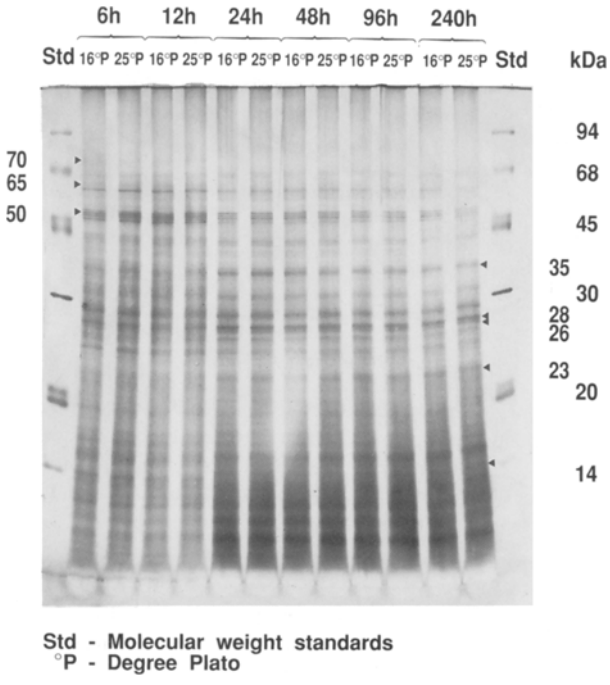

Fig. 5. Cell protein composition of lager strain 3021 at indicated hours of fermentation of 16°P and 25°P worts. SDS-solubilized proteins (10 μg per well) were fractionated on 15% polyacrylamide slab gels and visualized by silver stain.

This time may represent a period of adaptation to ethanol effects.

Analysis of cell protein composition during fermentation

The protein composition of cells during fermentation was analyzed by SDS-PAGE. Several low molecular mass proteins of approx. 35, 28, 26, 23 and 14 kDa appeared in increasing amounts after 24 h of fermentation (see arrows, Fig. 5). The concentration of ethanol in the culture medium at this time of fermentation was approx. 2% (v/v). Also, high molecular mass proteins of approx. 70, 65 and 50 kDa were present at 6 and 12 h of fermentation, but in lower amounts after 24 h of fermentation. These sets of proteins were probably induced in response to ethanol stress. Comparison of the protein composition of cells fermenting 16°P wort and those fermenting 25°P wort showed no differences (Fig. 5). Thus, the conditions of high gravity wort fermentation were not responsible for changes observed in the protein composition of these cells during fermentation.

DISCUSSION

Interest in high gravity brewing has increased considerably over the years because of the economic advantages of this type of brewing process. For example, high gravity brewing produces higher ethanol yields, increases plant efficiency, improves beer smoothness, and improves flavour and haze stability [5,6,26]. However, the high osmotic pressure encountered by yeast cells during fermentation of high substrate concentrations has been shown to inhibit cell growth and fermentation performance [10,14]. The results presented in this manuscript demonstrate that the elevated ethanol levels encountered by cells during fermentation of 25°P wort resulted in reduced cell viability and inhibition of cell growth. The viability of cells fermenting 16°P wort was approx. 90% after 240 h of fermentation, while the viability of cells fermenting 25°P wort declined to less than 40%. The ethanol concentration in 16°P wort reached a maximum of 7% (v/v) after 48 h of fermentation, and that of 25°P wort reached a maximum of 11% (v/v) after 96 h of fermentation. The decline in cell viability and growth coincide with the time the ethanol concentration in 25°P wort peaked at 11% (v/v). Brewing yeast strains are generally known to have relatively low tolerance to high ethanol concentrations. Cell growth is inhibited at concentrations above 10% (v/v), whereas fermentation capacity is inhibited in 20% (v/v) ethanol [13,30,32]. Although cell viability and growth are more sensitive to the toxic effects of ethanol than the fermentation activity of cells [32], a reduction in the rate of ethanol production has been correlated with loss of cell viability [22].

Yeast cells fermenting 25°P wort were found to be less tolerant to heat shock at 40°C or 45°C than cells fermenting 16°P wort. Relieving the stress effects of ethanol by washing the cells free of culture medium improved their tolerance to heat shock at these temperatures. Similar results were obtained when cells fermenting 16°P and 25°P worts were washed free of culture media and then tested for tolerance to 15% (v/v) ethanol. These results indicate that the ethanol level encountered by cells during fermentation of 25°P wort enhanced their sensitivity to heat shock and reduced cell viability. Ethanol has been shown to enhance the lethal effects of high temperatures (thermal death) and decrease the maximal temperature permitting growth [4,36]. Thus, cells in the presence of high concentrations of ethanol develop increased sensitivity to heat shock.

A relationship between tolerance to heat shock and the cell cycle of *Saccharomyces cerevisiae* has been reported [25,28]. Exponentially growing cells were shown to be more sensitive than stationary phase cells to a thermal shock. It is believed that thermotolerance is acquired as cells pass from exponential phase to stationary phase of growth. Thus, cells in stationary phase are more thermotolerant than actively growing cells. This is confirmed by our results which demonstrate that cells become more

tolerant to heat shock and ethanol stress at later stages of fermentation (Figs. 3 and 4). This may be due to the transition of cells to stationary growth phase or the exposure of cells to non-lethal concentrations of ethanol which induces tolerance of cells to heat and ethanol stress. It has been shown that exposure of yeast cells to low concentrations of ethanol induced synthesis of heat shock proteins (HSPs), which may serve to protect the cell from the lethal effect of high concentrations of ethanol and elevated temperatures [20,27,38]. A lag period between the time ethanol concentration reached 7% (v/v) and the subsequent protection of cells from heat shock, was observed. Although the basis for this is unclear, the time may represent a period of adaptation to ethanol effects.

In an attempt to determine whether changes in yeast cellular proteins occur during fermentation, cells obtained during various stages of fermentation were lysed and cellular proteins in the lysate analyzed by SDS-PAGE. Although no differences were observed in the protein profiles of yeasts fermenting 25°P wort compared to those fermenting 16°P wort, changes in yeast protein composition were observed during fermentation. Increase in the syntheses of several low molecular mass proteins of approx. 35, 28, 26, 23 and 14 kDa proteins were observed after 24 h of fermentation, at which time more than 2% (v/v) ethanol was produced. Also, high molecular mass proteins of approx. 70, 65 and 50 kDa were present at 6 and 12 h of fermentation, but in lower amounts after 24 h of fermentation. Some of these proteins (70, 35, 23, 26 and 14 kDa), have been shown to be heat and ethanol inducible [2,7,11,19,29,33]. Also, there are reports that the syntheses of these proteins are growth phase dependent [29,31,39]. The syntheses of proteins identified in the present study were either induced in response to ethanol stress during fermentation or were the results of the transition of cells from exponential phase to stationary phase of growth. The role of these proteins in yeast ethanol and thermotolerance is currently under investigation.

ACKNOWLEDGEMENTS

The authors would like to thank the management of John Labatt Limited for their support and permission to publish this manuscript. The authors would also like to thank their colleagues in the Brewing Research Department for helpful discussions during the preparation of this manuscript, and G. Celotto and I. Hancock for expert technical assistance. J. Odumeru is the recipient of an Industrial Research Fellowship from the Natural Science and Engineering Research Council of Canada for which gratitude is expressed.

REFERENCES

1 Benitez, T., L. Del Castillo, A. Aguilera, J. Conde and E. Cerdaolmedo. 1983. Selection of wine yeasts for growth and fermentation in the presence of ethanol and sucrose. Appl. Environ. Microbiol. 45: 1429–1436.

2 Bossier, P., I.T. Fitch, H. Boucherie and M.F. Tuite. 1989. Structure and expression of a yeast gene encoding the small heat shock protein Hsp 26. Gene 78: 323–330.

3 Brown, S.W., S.G. Oliver, D.E.F. Harrison and R.C. Righelato. 1981. Ethanol inhibition of yeast growth and fermentation: differences in the magnitude and complexity of the effect. Eur. J. Appl. Microbiol. Biotechnol. 19: 151–155.

4 Cabeca-Silva, C., A. Madeira-Lopes and N. van Uden. 1982. Temperature relations of ethanol-enhanced petite mutation in *Saccharomyces cerevisiae*: mitochondria as targets of thermal death. FEMS Microbiol. Lett. 15: 149–151.

5 Casey, G.P. and W.M. Ingledew. 1983. High gravity brewing: influence of pitching rate and wort gravity on early yeast viability. J. Am. Soc. Brew. Chem. 41: 148–152.

6 Casey, G.P., C.A. Magnus and W.M. Ingledew. 1983. High gravity brewing yeasts. Biotechnol. Lett. 5: 429–434.

7 Cavicchioli, R. and K. Watson. 1986. Loss of heat shock acquisition of thermotolerance in yeast is not correlated with loss of heat shock proteins. FEBS Lett. 207: 149–152.

8 D'Amore, T., C.J. Panchal, I. Russell and G.G. Stewart. 1990. A study of ethanol tolerance in yeast. CRC Crit. Rev. Biotechnol. 9: 287–304.

9 Dowhanick, T., J. Sobczak, I. Russell and G.G. Stewart, 1990. The rapid identification of protein fingerprinting of yeast and bacterial brewery contaminants. J. Am. Soc. Brew. Chem. 48: 75–79.

10 Hahn-Hagerdal, B., M. Larsson and B. Mattiason. 1982. Shift in metabolism toward ethanol production in *Saccharomyces cerevisiae* using alterations of the physical-chemical micro-environment. Biotechnol. Bioeng. Symp. 12: 199–202.

11 Hall, B.G. 1983. Yeast thermotolerance does not require protein synthesis. J. Bacteriol. 156: 1363–1365.

12 Ingram, L.O. 1986. Microbial tolerance to alcohols: role of the cell membrane. Trends Biotechnol. 4: 40–44.

13 Ismail, A.A. and A.M.M. Ali. 1971. Selections of high ethanol-yielding *Saccharomyces*. II. Genetics of ethanol tolerance. Folia Microbiol. 16: 350–354.

14 Jones, R.P., N. Pamment and P.F. Greenfield. 1981. Alcohol fermentation by yeasts: the effect of environmental and other variables. Process Biochem. 16: 42–49.

15 Juroszek, J.R., M. Feullat and C. Charpentier. 1987. Effect of ethanol on glucose-induced movements of protons across the plasma membrane of *Saccharomyces cerevisiae* NCYC 431. Can. J. Microbiol. 33: 93–97.

16 Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 277: 680–685.

17 Leao, C., and N. van Uden. 1982. Effects of ethanol and other alkanols on the kinetics and the activation parameters of thermal deaths in *Saccharomyces cerevisiae*. Biotechnol. Bioeng. 24: 1881–1884.

18 Luong, J.H.T. 1985. Kinetics of ethanol inhibition in alcohol fermentation. Biotechnol. Bioeng. 27: 280–285.
19 McAlister, L., S. Strausberg, A. Kulaga and D.B. Finkelstein. 1979. Altered patterns of protein synthesis induced by heat shock of yeast. Curr. Genet. 1: 67–74.
20 McAlister, L. and D.B. Finkelstein. 1980. Heat shock proteins and thermal resistance in yeast. Biochem. Biophys. Res. Commun. 93: 819–824.
21 Morrisey, J.H. 1981. Silver stain for proteins in polyacrylamide gels; a modified procedure with enhanced uniform sensitivity. Anal. Biochem. 117: 307–310.
22 Nagatani, M., M. Shoda and S. Aiba. 1968. Kinetics of product inhibition in alcohol fermentation. J. Ferment. Technol. 46: 241–248.
23 Nagodawithana, T.W., T. Whitt and A.J. Cutaia. 1977. Study of the feedback of ethanol on selected enzymes of the glycolytic pathway. J. Am. Soc. Brew. Chem. 35: 179–183.
24 Oliver, S.G., 1984. Economic possibilities of fuel alcohol. Chem. Ind. 12: 424–427.
25 Parry, J.M., P.J. Davies, and W.E. Evans. 1976. The effects of 'cell age' upon the lethal effects of physical and chemical mutagens in the yeast *Saccharomyces cerevisiae*. Mol. Gen. Genet. 146: 27–35.
26 Pfisterer, E. and G.G. Stewart. 1976. High gravity brewing. Brew. Dig. 51 (6): 34–42.
27 Plesset, J., C. Palm and C.S. McLaughlin. 1982. Induction of heat shock proteins and thermotolerance by ethanol in *Saccharomyces cerevisiae*. Biochem. Biophys. Res. Commun. 108: 1340–1345.
28 Plesset, J., R. Ludwig, B.S. Cox and C.S. McLaughlin. 1987. Effect of cell cycle position on thermotolerance in *Saccharomyces cerevisiae*. J. Bacteriol. 169: 779–784.
29 Praekelt, U.M. and P.A. Meacock. 1990. HSP 12, a new small heat shock gene of *Saccharomyces cerevisiae:* Analysis of structure, regulation and function. Mol. Gen. Genet. 223: 97–106.
30 Ranganathan, B. and J.V. Bhat. 1958. Ethanol tolerance of some yeasts. J. Indian Inst. Sci. 40: 105–110.
31 Rossi, J.M. and S. Lindquist. 1989. The intracellular location of yeast heat shock protein 26 varies with metabolism. J. Cell. Biol. 108: 425–439.
32 Stokes, S.L. 1970. Influence of temperature on the growth and metabolism of yeasts. In: The Yeasts (A.H. Rose and J.S. Harrison, eds.), pp. 119–134, Vol. 2. Academic Press, London.
33 Susek, R.E. and S.L. Lindquist. 1989. HSP 26 of *Saccharomyces cerevisiae* is related to the super family of small heat shock proteins but is without a demonstratable function. Mol. Cell. Biol. 9: 5265–5271.
34 Thomas, D.S., J.A. Hossack and A.H. Rose. 1978. Plasma-membrane lipid composition and ethanol tolerance. Arch. Microbiol. 117: 239–245.
35 Thomas, D.S. and A.H. Rose. 1979. Inhibitory effect of ethanol on growth and solute accumulation by *Saccharomyces cerevisiae* as affected by plasma-membrane lipid composition. Arch. Microbiol. 122: 49–55.
36 van Uden, N. 1984. Effects of ethanol on the temperature relations of viability and growth in yeast. CRC Crit. Rev. Biotechnol. 1: 263–272.
37 van Uden, N. 1985. Ethanol toxicity and ethanol tolerance in yeasts. Ann. Rep. Ferment. Processes. 8: 11–58.
38 Watson, K. and R. Cavicchioli. 1983. Acquisition of ethanol tolerance in yeast cells by heat shock. Biotech. Lett. 5: 683–688.
39 Watson, K. 1990. Microbial stress proteins. Adv. Microbial Physiol. 31: 183–223.