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Alterations in fatty acid composition and trehalose concentration of *Saccharomyces* brewing strains in response to heat and ethanol shock

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SUMMARY

The effects of heat and ethanol shock on fatty acid composition and intracellular trehalose concentration of lager and ale brewing yeasts were examined. Exposure of cells to heat shock at 37 °C or 10% (v/v) ethanol for 60 min resulted in a significant increase in the ratio of the total unsaturated to saturated fatty acyl residues and the intracellular trehalose concentration of cells. A similar increase in the amount of unsaturated fatty acids was observed in cells after 24 h of fermentation of 16 °P (degree Plato) or 25 °P wort, at which time more than 2% (v/v) ethanol was present in the growth medium. These results suggest that unsaturated fatty acids and high concentrations of intracellular trehalose may protect the cells from the inhibitory effects of heat and ethanol shock.

INTRODUCTION

Tolerance to high concentrations of ethanol and elevated temperature is one of the most desirable characteristics of a brewing yeast. Ethanol and temperature have been shown to exert several inhibitory effects on yeasts during fermentation. These include inhibition of cell growth, cell viability, accumulation of various nutrients, protein fluxes and fermentation performance [1,2,22, 24,28]. It has been suggested that the cell membrane is the primary target site of ethanol toxicity [12,27,28,30]. Ethanol is believed to interact with membranes by insertion into the hydrophobic interior, increasing the polarity of this region, weakening the hydrophobic barrier to the free exchange of polar molecules and affecting the positioning and type of membrane components [12]. Changes in the chain length and level of unsaturation of the fatty acids of phospholipids, and a decrease in the total lipid content of the cell membrane have all been shown to be induced by ethanol [8,13,27]. Thus, the structural alteration in the plasma membrane may be one of the mechanisms underlying ethanol toxicity.

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There are reports that yeast cells with high intracellular concentrations of the reserve carbohydrate trehalose are tolerant to adverse environmental conditions such as frost, heat and desiccation [6,9,10,29,32]. Trehalose is a non-reducing disaccharide consisting of two glucose moieties linked together by an α-1,1-glycosidic bond. This disaccharide has been shown to play a protective role in osmoregulation [29], in protecting cells during conditions of nutrient depletion and starvation [7,15], and in improving cell resistance to high and low temperatures [4,7, 9,14,25]. This protective role may be due to the stabilizing effect of trehalose on cell membranes. In the present study, the effects of heat and ethanol shock on the fatty acid composition and intracellular trehalose concentration of selected strains of brewing yeasts were examined. Changes in these cellular components during fermentation were correlated with the tolerance of yeasts to these stress conditions.

MATERIALS AND METHODS

Yeast strains and growth medium

The brewing yeast strains employed in this study were *Saccharomyces uvarum* (*carlsbergensis*) lager strain 3021 and *Saccharomyces cerevisiae* ale strain 3001. These strains were from the Labatt Culture Collection.

Growth and fermentation media

The yeast cells were subcultured in PYN medium which consisted of: peptone, 3.5 g; yeast extract, 3.0 g; KH_2PO_4, 2.0 g; $(NH_4)_2SO_4$, 1.0 g; $MgSO_4 \cdot 7H_2O$, 1.0 g; glucose, 20 g; all dissolved in 1 litre of distilled water and adjusted to pH 5.6. Fermentations were conducted in 100 ml production 16 °P wort and 25 °P wort at 21 °C with constant agitation at 150 rpm. The 25 °P wort was prepared by addition of high maltose corn syrup (Casco No. 1530, Casco Ltd., Etobicoke, Ontario) to 16 °P production wort. After 120 h of fermentation the yeasts were harvested by centrifugation at $4000 \times g$ for 10 min and washed twice with sterile physiologic saline (0.89% (w/v) NaCl), and freeze-dried for storage until required. 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 which is approx. 1.5×10^7 cells/ml.

Conditions of heat and ethanol shock

Cultures, grown to the exponential phase at 21 °C, were subjected to heat shock at 37 °C for 60 min or ethanol shock in 10% (v/v) ethanol at 21 °C for 60 min. Control cells were incubated at 21 °C without heat shock or ethanol addition. The yeast cells were harvested by centrifugation at $4000 \times g$ for 10 min immediately after the shocking period, washed twice with sterile physiologic saline and freeze dried for storage until required.

Fatty acid analysis

Fatty acids, before and after heat and ethanol shock, or during various stages of fermentation, were extracted and analysed as previously described [3], with some modifications. Approximately 200 mg of freeze-dried yeast powder was weighed into a vacuum hydrolysis tube (Pierce Chemical Co., Rockford, IL) and 5 ml of 1 M KOH in ethanol containing 10% (v/v) water was added along with 200 µl of tridecanoic acid solution (15 mg/ml) in a 2:1 (v/v) chloroform/methanol mixture, as internal standard. The tube was capped, placed in an ultrasonic bath (Canlab Baxter, Mississauga, Ontario) for 30 min and then vortexed for 30 s. The extraction and saponification were carried out at 100 °C for 30 min in a heating block (Pierce Chemical Co., Rockford, IL), with occasional stirring behind a Plexiglass safety shield. After cooling to room temperature, the tube was opened, 2 ml of 4 M HCl saturated with NaCl and 3 ml of petroleum-ether were added, and the mixture was vortexed for 30 s. The organic phase was transferred into a 15-ml tube and the extraction of the acidic aqueous phase was repeated with a fresh 3-ml portion of petroleum-ether. The organic extracts were combined, treated with 1.5 ml of iso-octane, dried over sodium sulphate (0.5 g) for 10 min, transferred to a clean 10-ml test tube and then concentrated under N_2 to about 1 ml. The sample was decanted into autosampler vials (2-ml capacity) and the fatty acid content was analysed by gas chromatography using a Varian model 3700 gas chromatograph equipped with a Chrompack FFAP-CB fused-silica capillary column (25 m × 0.32 mm × 0.3 µm film) and a flame ionization detector (FID). The sample injector was operated in the split mode at a ratio of 100 to 1. Carrier flow of hydrogen was maintained at 4 ml/min through the column. Injector and detector temperatures were set at 270 °C and 300 °C, respectively. The oven temperature during a chromatographic run, was controlled at 110 °C

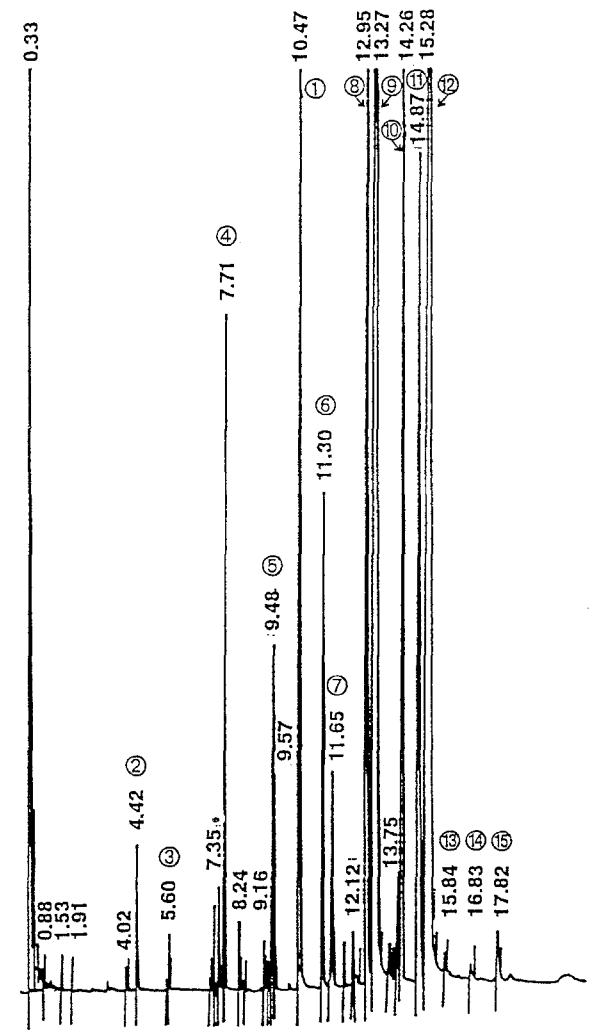

Fig. 1. Gas chromatogram of fatty acids of a *Saccharomyces uvarum* (*carlsbergensis*) lager brewing yeast strain 3021, cultured in PYN broth for 24 h. Peaks: 1 = C_{13} (internal standard), 2 = C_7, 3 = C_8 4 = C_{10}, 5 = C_{12}, 6 = C_{14}, 7 = $C_{14:1}$, 8 = C_{16}, 9 = $C_{16:1}$, 10 = C_{18}, 11 = $C_{18:1}$, 12 = $C_{18:2}$, 13 = $C_{18:3}$, 14 = C_{20}. Circled numbers are peaks and numerical print-outs are retention times in minutes.

for 2 min and then ramped to 220 °C at 10 °C/min. The temperature was held at 220 °C for 12 min, followed by a 5 min cool down time, giving a total cycle time of 30 min per run. A sample size of 2 μl was injected via an automatic sampler. The chromatograms were recorded by an SP4270 integrator (Spectra-Physics, San Jose, CA), which has been calibrated to report fatty acids in grams/100 grams of sample.

Trehalose assay

Trehalose, before and after heat shock at 37 °C or ethanol shock in 10% (v/v) ethanol at 21 °C for 60 min, was extracted from the yeast as described previously [6] and the concentration was determined by the anthrone method of Trevelyan and Harrison [26].

RESULTS

Effects of heat and ethanol shock on fatty acid composition of brewing yeasts

Fig. 1 shows the chromatogram of fatty acids of *Saccharomyces uvarum* (*carlsbergensis*) lager brewing strain 3021 after 24 h of growth in PYN media. Fatty acid groups ranging from C_6 to C_{20} were identified, which are similar to those repeated elsewhere [1,11,13]. Quantitatively, C_{16}, $C_{16:1}$, C_{18} and $C_{18:1}$ were the most predominant fatty acids identified in the yeast cells.

In order to determine the effects of heat and ethanol shock on the fatty acid composition of brewing yeasts, lager strain 3021 and ale strain 3001 were subjected to heat shock at 37 °C or ethanol shock with 10% (v/v) ethanol for 60 min, as described in Materials and Methods. The fatty acid content of the cells before and after exposure to these stress conditions was determined. Yeast cells exposed to heat or ethanol shock had a significant increase in the concentration of unsaturated fatty acids and a corresponding proportional decrease in the concentration of saturated fatty acids (Table 1). The increase in the concentration of unsaturated fatty acids was observed mainly in the mono-unsaturated fatty acids while the concentration of poly-unsaturated fatty acids declined to non-detectable levels after heat and ethanol shock. A similar increase in the ratio of unsaturated fatty acids to saturated fatty acids was observed in yeast cells after 120 h of fermentation of 16 °P or 25 °P worts (Table 2). Again, the increase in the unsaturated fatty acids was observed mainly in the percent mono-unsaturated fatty acids. Yeast cells fermenting 25 °P wort show slightly higher levels of unsaturated fatty acids in their cell membrane after 120 h of fermentation compared to those fermenting 16 °P wort. This difference is likely due to higher concentrations of ethanol produced in 25 °P wort.

In order to determine whether there is a relationship between the concentrations of ethanol in the fermentation medium and the amount of unsaturated fatty acids in the yeast cells, yeasts were harvested at various stages of fermentation and the fatty acid composition of each sample was determined. A dose-dependent increase in the con-

TABLE 1

Effect of heat shock and ethanol stress on the fatty acid composition of brewing yeast strains

Yeast and strain No.	Treatment for 60 min	Percentage of total fatty acids				
		Sat's	Mono-U	Poly-U	Total-U	mg/g*
Saccharomyces uvarum (*carlsbergensis*) 3021	control, 21 °C	31.7[a]	67.1	1.1	68.2	23.0
Saccharomyces uvarum (*carlsbergensis*) 3021	heat shock, 37 °C	16.4	83.6	0	83.6	41.0
Saccharomyces uvarum (*carlsbergensis*) 3021	10% (v/v) ethanol, 21 °C	16.0	84.0	0	84.0	38.0
Saccharomyces cerevisiae 3001	control, 21 °C	29.3	68.6	1.9	70.7	40.0
Saccharomyces cerevisiae 3001	heat shock, 37 °C	22.1	77.9	0	77.9	52.0
Saccharomyces cerevisiae 3001	10% (v/v) ethanol, 21 °C	20.7	79.3	0	79.3	47.0

Sat's = saturated fatty acids (C_6, C_8, C_{10}, C_{12}, C_{17} and C_{18}); Mono-U = mono-unsaturated fatty acids ($C_{10:1}$, $C_{16:1}$ and $C_{18:1}$); Poly-U = poly-unsaturated fatty acids ($C_{18:2}$ and $C_{18:3}$); Total-U = total unsaturated fatty acids; mg/g* = total fatty acids in milligrams per gram dry weight of yeast cells.

[a] Each figure represents the average of three determinations with a standard deviation of less than 5% of the average value.

TABLE 2

Fatty acid composition of *Saccharomyces uvarum* (*carlsbergensis*) 3021 before and after fermentation of 16°P and 25°P worts

Fatty acid type	0-h sample	16°P 120-h sample	25°P 120-h sample
Saturated	31.7[a]	15.2	12.0
Mono-unsaturated	67.1	84.1	87.7
Poly-unsaturated	1.1	0.4	0.7
Total-unsaturated	68.2	84.8	88.1
Total fatty acids (mg/g)	23.0	76.0	161.0

[a] Each figure, expressed as a percentage of total fatty acid mg/g of yeasts' dry weight, represents the average of three determinations with a standard deviation of less than 5% of the average value.

centration of unsaturated fatty acids followed by a corresponding decline in saturated fatty acids was observed in relation to an increase in the concentration of ethanol in the medium (Figs. 2 and 3). The ratio of unsaturated to saturated fatty acids in cells fermenting 16°P wort peaked after 72 h of fermentation, at which the concentration of ethanol in the medium reached a maximum of approx. 7% (v/v), while the ratio of these fatty acids in yeasts fermenting 25°P wort peaked after 96 h of fermentation, about the same time the ethanol concentration reached a maximum of approx. 11% (v/v). These data suggest that there is a direct correlation between the concentration of unsaturated fatty acids in yeast cells and the concentration of ethanol in the fermentation medium. Fatty acids most affected by the increase in ethanol concentration are shown in Fig. 4. The concentrations of palmitoleic ($C_{16:1}$) and oleic acids ($C_{18:1}$) increased, while that of palmitic acid (C_{16}) decreased during fermentation. The concentration of stearic acid (C_{18}) remained relatively constant.

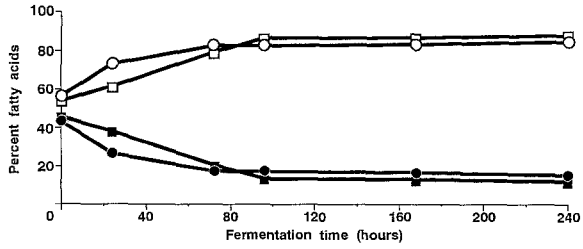

Fig. 2. Total fatty acid composition of *Saccharomyces uvarum* (*carlsbergensis*) lager strain 3021 during fermentation of 16 and 25°P worts. Total unsaturated fatty acids of cells fermenting 16°P (○) and 25°P (□) worts. Total saturated fatty acids of cells fermenting 16°P (●) and 25°P (■) worts.

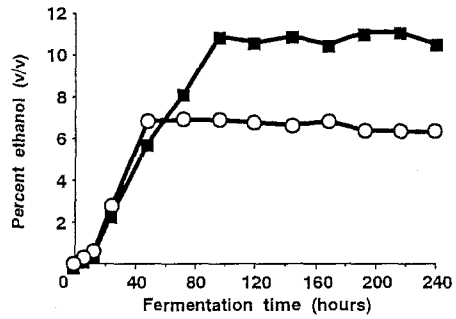

Fig. 3. Concentration of ethanol produced by *Saccharomyces uvarum* (*carlsbergensis*) lager strain 3021 during fermentation of 16°P (○) and 25°P (■) worts.

Effects of heat and ethanol shock on the intracellular trehalose concentration of brewing yeasts

The effects of heat and ethanol shock on the intracellular trehalose content of brewing yeasts, lager strain 3021 and ale strain 3001 are shown in Table 3. Exposure of yeast cells to heat shock at 37°C and ethanol shock for 60 min resulted in a significant increase in the trehalose composition of the cells. A similar increase in trehalose content, although to a lesser extent, was observed following exposure of cells to 10% (v/v) ethanol for 60 min. Exposure of cells to higher concentrations of ethanol (15% (v/v)), did not result in further increases in trehalose content (data not shown). These results indicate that heat or ethanol shock in addition to the induction of the synthesis of unsaturated fatty acids, also induces the accumulation of trehalose in yeast cells.

DISCUSSION

The mechanism of temperature and ethanol tolerance in yeasts is not well understood. A number of studies, however, have suggested that the plasma membrane is a major target site of ethanol toxicity in yeasts [12,18,27,28].

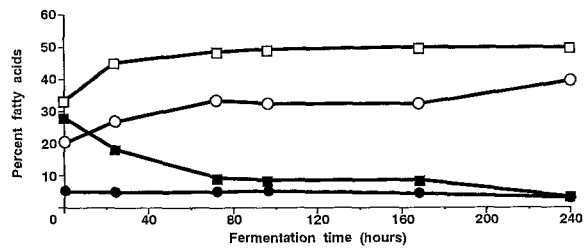

Fig. 4. Fatty acid composition of *Saccharomyces uvarum* (*carlsbergensis*) lager strain 3021 during fermentation of 25°P wort. Fatty acids: palmitoleic, $C_{16:1}$ (□); palmitic, C_{16} (■); oleic, $C_{18:1}$ (○); stearic, C_{18} (●).

TABLE 3

Effects of heat shock and ethanol stress on the trehalose concentration in brewing yeast strains

Yeast and strain No.	Treatment for 60 min	Trehalose (μg/mg dry wt.)
Saccharomyces uvarum (*carlsbergensis*) 3021	control, 21 °C	8.24[a]
Saccharomyces uvarum (*carlsbergensis*) 3021	heat shock, 37 °C	22.47
Saccharomyces uvarum (*carlsbergensis*) 3021	10% (v/v) ethanol, 21 °C	11.77
Saccharomyces cerevisiae 3001	control, 21 °C	6.53
Saccharomyces cerevisiae 3001	heat shock, 37 °C	13.66
Saccharomyces cerevisiae 3001	10% (v/v) ethanol, 21 °C	7.97

[a] Each figure, expressed in μg per mg dry weight of yeast, represents the average of three determinations with a standard deviation of less than 5% of the average value.

There are reports that ethanol tolerance in yeasts and bacteria can be influenced by the lipid composition of the plasma membrane [8,11,12,18,27,28]. Trehalose, a non-reducing disaccharide (α-D-glucopyranosyl-1,1-α-D-glucopyranoside), has also been shown to play a protective role in maintaining the yeast cytosolic structure under various stress conditions such as heat, desiccation and frost [7,9,14,29], in addition to its storage function [14,25,29]. This protective role appears to be the primary function of this disaccharide [32].

In the present study, the effects of heat and ethanol shock on fatty acid composition and trehalose concentration of two brewing yeasts were examined. Exposure of yeast cells to heat shock at 37 °C or 10% (v/v) ethanol at 21 °C for 60 min resulted in an increase in the ratio of unsaturated to saturated fatty acids in the cells and in the accumulation of intracellular trehalose. Heat shock induction of trehalose synthesis has been demonstrated previously [9]. An increase in the ratio of unsaturated to saturated fatty acids was also observed in cells after 24 h of fermentation at which time more than 2% (v/v) ethanol was present in the fermentation medium. The ratio of unsaturated to saturated fatty acids in the cell appears to increase in a dose-dependent fashion with an increase in the concentration of ethanol produced during fermentation.

A recent report [23] on the effect of ethanol on lipid structure and fatty acid composition of a strain of *S. cerevisiae* indicates that the exposure of yeast cells to various ethanol concentrations (5–25% (v/v)) induced an increase in the concentration of oleic acid ($C_{18:1}$) in all phospholipid structures, and palmitoleic acid ($C_{16:1}$) in sterol esters and triacylglycerols, with little effect on the amount of stearic acid (C_{18}). Increases in palmitic acid (C_{16}) were also observed after exposure of cells to ethanol concentrations above 15% (v/v), which resulted in the loss of cell viability and membrane reconstitution. In our study, ethanol induced increases in $C_{18:1}$ and $C_{16:1}$ fatty acids, while the concentration of C_{16} fatty acids declined. Ethanol had little effect on stearic acid (C_{18}), as noted previously [23]. The maximum ethanol concentration produced after 240 h of fermentation of 25 °P wort was 11% (v/v), hence the increase in C_{16} fatty acid reported at 15% (v/v) ethanol [23] was not observed.

Previous studies in our laboratory have demonstrated that yeast cells developed increased tolerance to heat and ethanol shock at later stages of the fermentation of 16 °P and 25 °P worts [19]. Increase in tolerance of these strains to these stress conditions correlated with increases in the concentration of unsaturated fatty acids in yeasts during fermentation. Ingram [11] demonstrated that increases in $C_{18:1}$ fatty-acyl residues, analogous to changes in fatty acid composition induced by ethanol, were beneficial for the growth and survival of *Escherichia coli* K-12 in the presence of ethanol. Mutant strains of *E. coli*, which were unable to synthesize oleic acid ($C_{18:1}$) fatty-acyl residues, were very sensitive to growth inhibition and cell death caused by ethanol [11]. Similarly, *S. cervisiae* grown under anaerobic conditions, which inhibit the synthesis of unsaturated fatty acids and sterol, developed increased sensitivity to the inhibitory effects of ethanol [31]. However, anaerobic yeast cultures supplemented with mono-unsaturated fatty acids displayed an increase in tolerance to ethanol and produced higher concentrations of ethanol than unsupplemented cultures [27,28,31]. These studies suggest that unsaturated fatty acids play an important role in yeast tolerance to ethanol.

Further studies, including those in our laboratory, have demonstrated that pre-exposure of yeast cells to heat shock at 37 °C or 10% (v/v) ethanol for 30–60 min resulted in the synthesis of a set of proteins referred to as heat shock proteins (hsps), and increased tolerance of cells to higher temperature and ethanol concentrations [17,19–21]. This increase in the yeasts' tolerance to these stress conditions correlated with an increase in the concentration of unsaturated fatty acids and trehalose in the yeast cell following heat and ethanol shock treatment. Thus, the increase in the synthesis of these cellular components, while part of an adaptive response of yeasts to these stress conditions, appears to play a protective role. The mechanisms by which these components protect the cell against heat and ethanol stress remain to be determined.

In summary, the data presented herein indicate that the

exposure of yeast cells to heat and ethanol shock results in: (a) an increase in the concentration of mono-unsaturated fatty acids in the yeast membrane with a corresponding decrease in the concentration of saturated fatty acids, and (b) an increase in the accumulation of trehalose in the cell. The concentration of mono-unsaturated fatty acids in yeast cells increased in a dose-dependent fashion with an increased concentration of ethanol in the fermentation medium. The stress conditions described in this report have been shown previously to induce the synthesis of hsps and thermotolerance in yeasts [16,17,20,21]. Thus, an increase in these cellular components appears to play a protective role against heat and ethanol shock.

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