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The Effects of Osmotic Pressure and Ethanol on Yeast Viability and Morphology

Patricia L. Pratt[1], James H. Bryce[1] and Graham G. Stewart[1,2]

ABSTRACT

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The selection of a brewing yeast strain with the required fermentation and recycling characteristics is critical. The yeast strain will influence the rate and extent of fermentation, the flavour characteristics and the overall quality and stability of the finished beer, and consequently, the economic viability of the brewery. Since high gravity worts can have a deleterious effect on yeast fermentation performance, it is imperative that the strain selected be suitable for this environment, which includes a capacity to withstand high osmotic pressures and elevated ethanol levels. Under controlled *in vitro* osmotic and ethanol induced stresses, there was a decline in mean cell volume in both lager and ale yeast strains. Whilst significant reductions in viability were observed in the lager strains, the ale strains studied were not affected. Cell surface investigations revealed shrinkage of the yeast cells and crenation of the outside envelope under both stresses, although exposure to ethanol had a more marked effect on the yeast cell surface than sorbitol-induced elevated osmotic pressure.

Key words: Cell viability, cell volume, ethanol, high gravity wort, osmotic pressure.

INTRODUCTION

In brewing, yeast is recycled and the fact that it is cropped and repitched into subsequent fermentations is one of the differentiating features between brewing and the production of many other alcoholic beverages including Scotch whisky[37]. Consequently, the quality of yeast cropped will not only affect the overall performance of most fermentations but also the quality and stability of the resulting beer. It is important therefore that the factors which influence yeast performance in the brewing process, and particularly in high gravity brewing, are considered, to ensure efficient fermentation and the production of a beer of a consistently high quality and stability. Breweries, the world-over, are continually seeking ways to reduce capital expenditure, labour, utilities, effluent and other operational costs and at the same time ensuring that the quality of their beers remains consistently high. As a result, many are employing the process of high gravity brewing due to its many advantages[39]. Similarly, some breweries have not implemented this process due to its disadvantages[40]. Of significance, are the deleterious effects of this process on yeast fermentation performance[41,43]. Fermentations have been reported to be sluggish due to elevated ethanol levels and high osmotic pressure, resulting in yeast viability and vitality reductions[6].

Osmotic pressure is the force that develops between two solutes of differing concentration separated by a semi-permeable membrane[20]. When yeast is exposed to wort it is subjected to an osmotic pressure. Very high osmotic pressures such as those encountered in high gravity worts, may distort yeast metabolism or decrease yeast viability. The extent of the osmotic pressure will depend on the concentration of solutes surrounding the cell[20,23]. It has been shown that increases in wort osmotic pressure produced a concomitant decrease in yeast viability, growth and fermentation performance[10]. Yeast responds to the effects of solute concentration in the growth medium in many ways. Yeast cells alter their cell volume in response to osmotic challenges, decreasing volume in response to hypertonic stress and increasing volume in the presence of hypotonic stresses[28]. Rose[34] proposed that this decrease in cell volume was caused by the equilibration of the intracellular solute concentration with that of the environment by osmotic water loss. The walls and plasma membranes of *Saccharomyces cerevisiae* cells are relatively elastic and weakly buffered against water loss and thus, one would expect differences in cytoplasmic volume under external osmotic pressures. Furthermore, Marechal & Gervais[27] observed cell volume variation in *S. cerevisiae* during the first 24 h when exposed to various water potentials and attributed their observations to the exit of water flow from the cell. They concluded that hyperosmotic shock in *S. cerevisiae* resulted in the loss of cell turgor pressure and subsequently a rapid decrease in cytoplasmic water content and cell volume.

The study of ethanol tolerance in yeast is quite difficult due to the many inhibitory effects on this organism[11,13,14]. Additionally, there is no universally accepted method to define ethanol tolerance. Ethanol inhibits cell growth, cell viability and fermentation rate, therefore, the method one uses must depend on the parameter that is being assessed. Louiero and Ferreira[25] have reported that exogenous ethanol added to *S. cerevisiae* is less toxic than endogenous ethanol produced during fermentation, as it does not truly reflect the tolerance of yeast under fermentation conditions. Withstanding this, it is still the most widely employed method for determining ethanol tolerance as it in-

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volves the suppression of cell growth in the presence of exogenous ethanol[14]. Ethanol tolerance is strain dependent and *S. cerevisiae* is believed to be intolerant to ethanol[3,42]. In general, ethanol inhibits yeast growth above concentrations of 10% (v/v), while fermentation capacity is inhibited at 20% (v/v) ethanol[7]. The degree of inhibition is related to environmental factors and is strain dependent. Although the effects of high gravity brewing have been extensively studied[3,5,9,19,32,40] and numerous morphological studies have been able to successfully link changes in morphology to some physiological feature of the yeast cell[1,15,21,29,30,35], little research has been conducted on the effect of this process on yeast cell volume and the applicability of this tool in the selection of yeast strains that will successfully ferment high gravity worts in a production environment. In this study, we examined the effects of osmotic and ethanol stresses on the cell volume and viability of lager and ale yeast strains.

MATERIALS AND METHODS

Yeast strains

The yeast strains employed in this study were brewing strains of *S. cerevisiae* lager strains NCYC 1324, and 1342; industrial strain (B), and ale strains LCC 70 and 3, and industrial strain (G). The strains were obtained from the National Collection of Yeast Cultures (NCYC), Labatt Culture Collection (LCC) and from the ICBD Culture Collection. The yeast cultures were stored in 1 mL ampoules at –70°C.

Yeast strain identification and verification

The strains were verified as ale or lager by growth on peptone-yeast extract glucose nutrient agar plates for 48 h at 25°C and 37°C. Lager strains grew at 25°C and not at 37°C, whereas, ale strains grew at both temperatures[38].

Production of wort (natural medium) for yeast biomass production

All malt 12°Plato brewer's wort was produced in the ICBD 2 hL pilot brewery, employing the procedures described by Stewart *et al.*[40]. The boiled worts were frozen and held at –20°C until required for use.

The production of yeast biomass

Yeast biomass was obtained following the method described by Younis and Stewart[46].

Determination of yeast viability during fermentations

Viability of the diluted samples of yeast was determined using the citrate methylene violet technique described by Smart *et al.*[36] employing the EBC Analytica Microbiologica Method[16]. The assays were conducted in triplicate.

Examination of yeast cell surface changes using environmental scanning electron microscopy (ESEM)

The ESEM method employed for the examination of yeast cell surfaces was a modification of the method reported by Barker and Smart[1]. Ten mL of sample was transferred to 15 mL centrifuge tubes and mixed. An appropriate aliquot, based on the time of incubation was transferred to a 1.5 mL Eppendorf tube and diluted with deionised water. Approximately 15 µl of diluted sample was applied onto a 3.5 mm × 9 mm aluminum stub (Oxford Instruments, U.K.) and placed under the Phillips XL 30 (Lab 6) Environmental Scanning Electron Microscope (FEI UK Ltd.) for examination. In order to achieve accuracy in the assessment of cell surface changes, a minimum sample size of 100 objects (single and budded) was used for each assessment. All assays were conducted in duplicate.

The determination of yeast cell volume during fermentation using image analysis

The analytical method employed to determine yeast cell volume was a modification of the method reported by Cahill *et al.*[4]. Microscopic images of diluted yeast samples were recorded using a JVC KY-F55B colour video camera (Victor Company of Japan Ltd., Yokohama, Japan) attached to a Zeiss Optiphot microscope (Zeiss, West Germany) at 400× magnification. The images were stored and processed with KS image analysis software (Imaging Associates Ltd., Thames, U.K.) using a Pioneer (300 MHz) PC. In order to achieve accuracy in the measurement of mean cell volume, a minimum sample size of 300 objects (single or budded cells) was used for all measurements[29]. A total of 20 microscopic fields were recorded for each sample.

Application of osmotic stress to yeast cultures

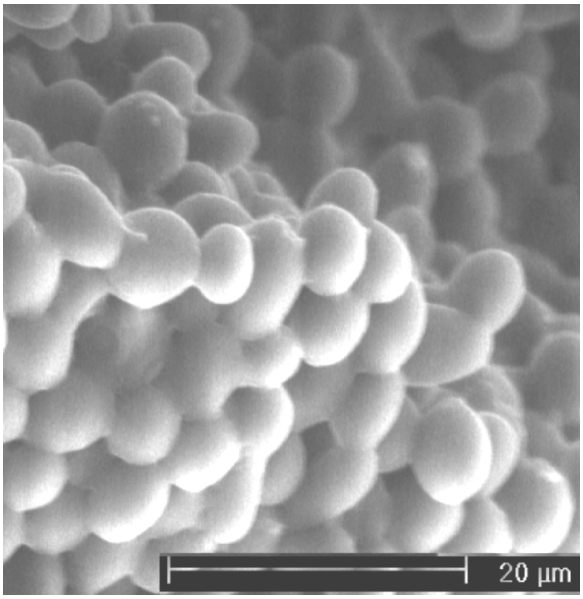
The method employed for the application of osmotic stress on yeast was a modification of the method reported by Cahill *et al.*[4]. One hundred mL of citrate phosphate buffer solution was transferred to 250 mL Erlenmeyer flasks. Twenty % (w/v) sorbitol was added to each flask and yeast was pitched at a rate of 0.35 g/100 mL (wet weight). The flasks were incubated at 21°C for 96 h at 150 rpm. Samples were retrieved daily for cell viability and mean cell volume determinations. The experiments were conducted in triplicate and each assay was conducted in triplicate.

The effect of exogenous ethanol on yeast

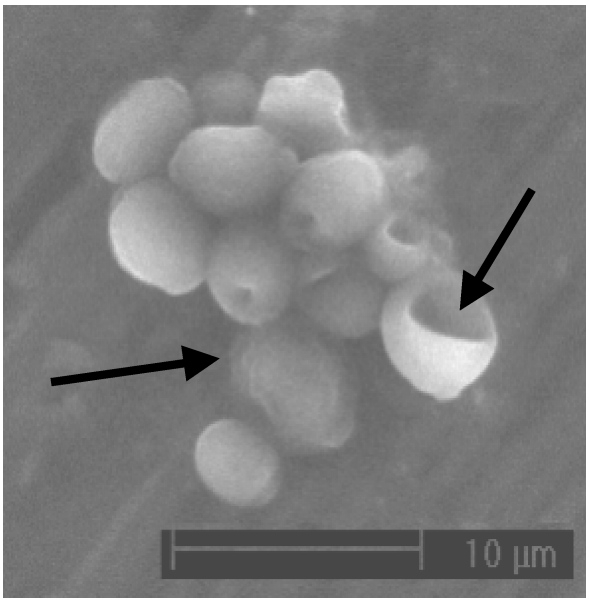
The method employed for the application of ethanol stress on yeast was a modification of the method reported by Cahill *et al.*[4]. One hundred mL of citrate phosphate buffer solution was transferred to 250 mL Erlenmeyer flasks. Ten % (v/v) ethanol was added to each flask and yeast was pitched at a rate of 0.35 g/100 mL (wet weight). The flasks were incubated at 21°C for 96 h at 150 rpm. Samples were retrieved daily for cell viability and mean cell volume determinations. The experiments were conducted in triplicate and each assay was conducted in triplicate.

Analysis of variance (ANOVA) statistical methods

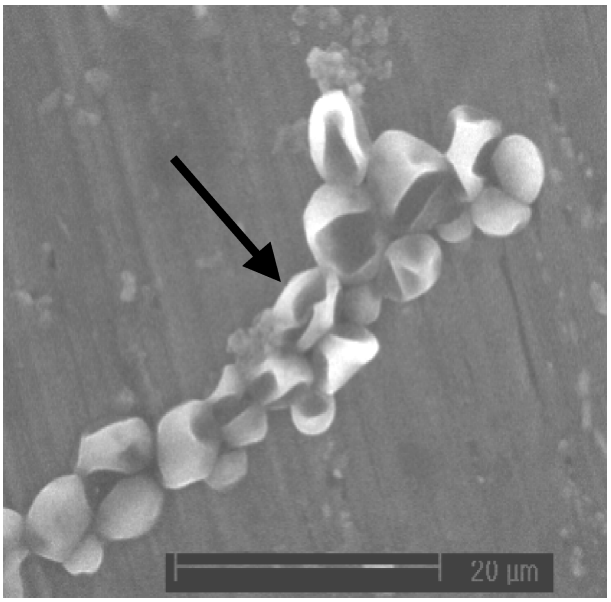
The data from each experiment was statistically analysed using the Analysis of Variance Method (ANOVA) described by Fowler and Cohen[17]. In all cases, the homogeneity of variance was determined to ensure that the sample variances were similar to each other before ANOVA was



A. Control cells



B. Exposure to 20% (w/v) sorbitol for 15 minutes



C. Exposure to 10% (v/v) ethanol for 15 minutes

Fig. 1. Morphological changes of lager yeast cells exposed to 20% (w/v) sorbitol and 10% (v/v) ethanol solutions.

conducted. The results were expressed using the Levene's Test, which determines equal variances of any continuous distribution.

RESULTS

Effect of osmotic pressure and ethanol stress on cellular morphology of lager yeast cells in 20% (w/v) sorbitol and 10% (v/v) ethanol

Variations of yeast morphology as a function of solute concentrations in their growth media are important in understanding the impact of morphology on fermentation performance. Therefore, studies of yeast cells in 20% (w/v) sorbitol and 10% (v/v) ethanol solutions were conducted. Morphological changes of lager yeast cells, exposed to 20% (w/v) sorbitol and 10% (v/v) ethanol solutions for 15 min (Figure 1, micrographs A through C) were examined, using Environmental Scanning Electron Microscopy (ESEM).

Figure 1, micrograph A shows yeast cells of control samples taken prior to application of osmotic and ethanol stresses. Oval shaped yeast cells with apparent smooth cell surfaces are visible. After 15 min exposure to 20% (w/v) sorbitol solution, shrinkage of yeast cells, crenation of the outside envelope and presence of invaginations were observed (Figure 1, micrograph B). Moreover, shrinkage of yeast cells and invaginations were also detected on sur-

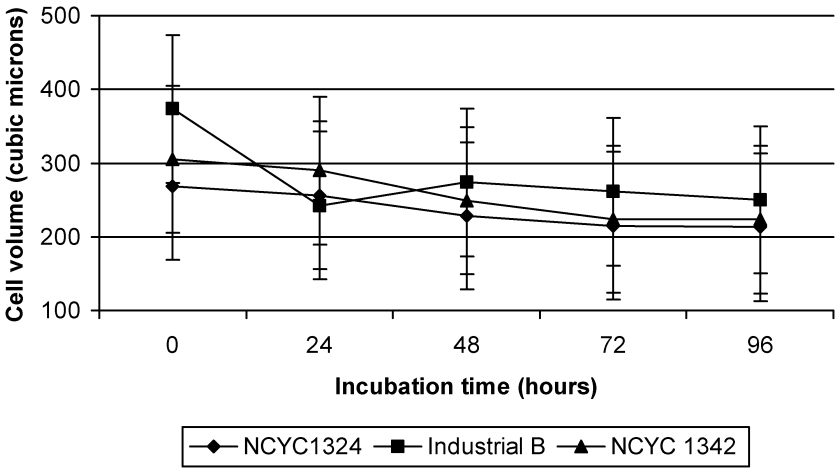


Fig. 2. Effect of 20% (w/v) sorbitol on mean cell volume of lager yeast strains.

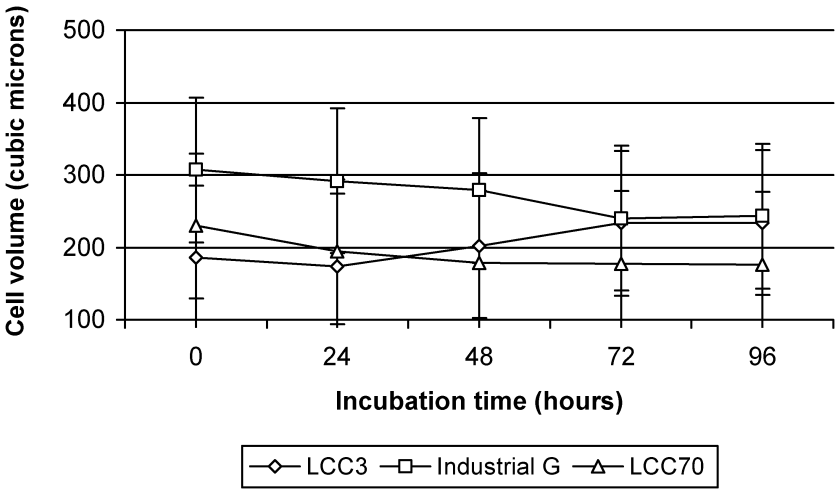


Fig. 3. Effect of 20% (w/v) sorbitol on mean cell volume of ale yeast strains.

faces of yeast cells when subjected to 10% (v/v) ethanol solution for 15 min (Figure 1, micrograph C).

The changes in morphology of the yeast cells in response to osmotic pressure are consistent with the findings of Morris *et al.*[28]. These researchers also reported shrinkage of yeast cells and differences in the organization of the cell envelope upon examining the freeze fracture images of shrunken cells. Walker and Birch[44] reported aberrations in cell envelope topology when yeast cells of a wine strain was exposed to 10% (v/v) ethanol. These findings imply that exposure to ethanol has a more discernible effect on yeast cells than sorbitol and that there is good correlation between morphology and the environment.

Effect of osmotic stress on mean cell volume of lager and ale yeast strains

The findings presented above have shown that exposure to osmotic stress resulted in significant changes to the shape and features of the yeast cells (Figure 1, micrographs A and B). As a result, it was proposed that shrinkage of yeast cells under exposure to osmotic stress must in some way affect the cell volume of yeast cells. As a consequence, the effect of osmotic stress on cell volume was investigated.

Three lager yeast strains were exposed to 20% (w/v) sorbitol solution. Figures 2 and 3 show the effect of osmotic stress on mean cell volume of lager strains and ale yeast strains, respectively. The mean cell volume decreased at 24 h, in yeast cells of lager strain Industrial B in response to osmotic stress ($p \leq 0.05$). Following this period, mean cell volume increased at 48 h, suggesting that yeast cells of Industrial B had recovered from the osmotic shift ($p \leq 0.05$). However, after this period, mean cell volume decreased significantly, indicating that osmotic equilibrium was difficult to maintain ($p \leq 0.05$). In contrast, mean cell volume of yeast strains NCYC 1324 and NCYC 1342 remained stable ($p \geq 0.05$) until declining at 48 h post pitching ($p \leq 0.05$). This indicated that yeast cells were capable of maintaining osmotic balance across the cells for longer periods, compared to yeast cells of Industrial B. Further decreases in mean cell volume were seen at 72 h in yeast cells of NCYC 1324 and NCYC 1342 ($p \geq 0.05$). When yeast cells were incubated in the buffer in the absence of sorbitol or ethanol there were no changes in mean cell volume during the incubation period (data not shown).

Osmotic stress on ale yeast strains resulted in a reduction in cell volume at 24 h, in all three strains ($p \leq 0.05$).

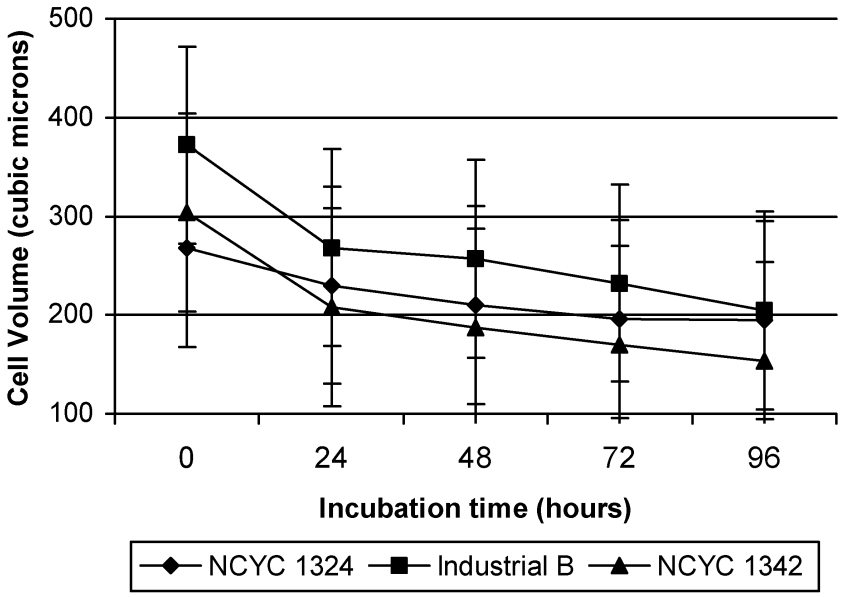


Fig. 4. Effect of 10% (v/v) ethanol on mean cell volume of lager yeast strains.

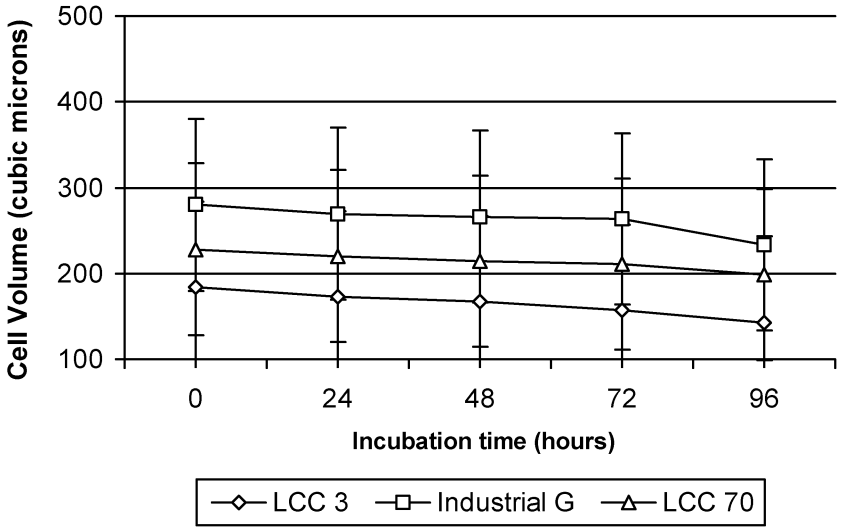


Fig. 5. Effect of 10% (v/v) ethanol on mean cell volume of ale yeast strains.

Additionally, significant decreases in mean cell volume were observed at 48 h, for yeast strains Industrial G and LCC 70, respectively. However, LCC 3 showed an increase in mean cell volume at the same time ($p \leq 0.05$). At 72 h, there was a further reduction in mean cell volume in yeast cells of Industrial G whilst mean cell volume of LCC 70 remained constant ($p \geq 0.05$). Conversely, another increase in mean cell volume was observed in yeast cells of LCC 3 ($p \leq 0.05$). This was surprising, as one would have anticipated a decline in mean cell volume by this period. These findings suggested that the exposure to sorbitol-induced osmotic stress had a more marked effect on ale yeast strains LCC 70 and Industrial G whilst increases in the water concentration gradient of LCC 3 resulted in cell swelling and increased turgor pressure. There were no further changes in mean cell volume after this time in all ale yeast strains studied ($p \geq 0.05$).

These data demonstrated that effects of osmotic stress on mean cell volume of yeast cells of lager and ale strains were inter-strain dependent. The effect of osmotic stress on mean cell volume was in agreement with the findings of Morris *et al.*[28] with the exception of strain LCC 70. They provided evidence that increases in wort osmotic pressure produced shrinkage in the yeast cells. Additionally, Cahill *et al.*[4] also observed decreases in cell volume when yeast cells were exposed to varying concentrations of osmotic stress. The findings indicate that osmotic stress has a more marked effect on the mean cell volume of the lager strains than ale strains studied.

Effect of ethanol stress on mean cell volume of lager and ale yeast strains

The experiments described above have shown that exposure to ethanol stress resulted in significant changes to

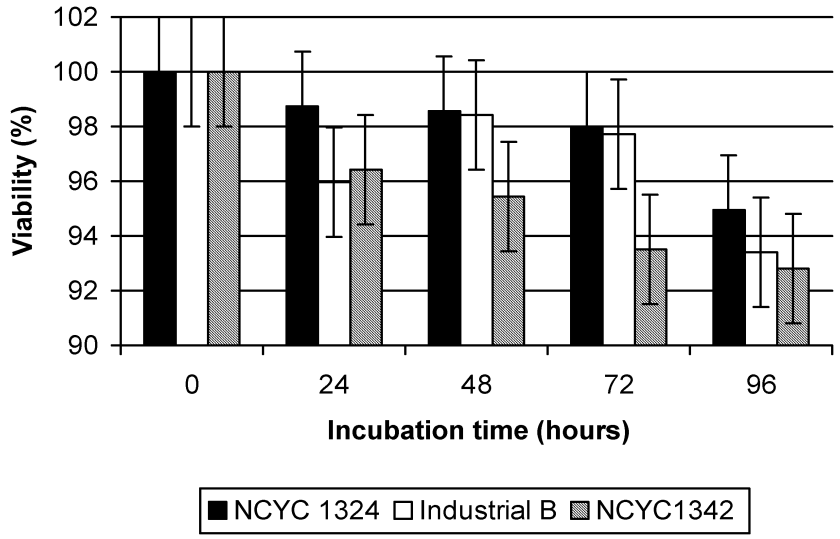


Fig. 6. Effect of 20% (w/v) sorbitol on viability of lager yeast strains.

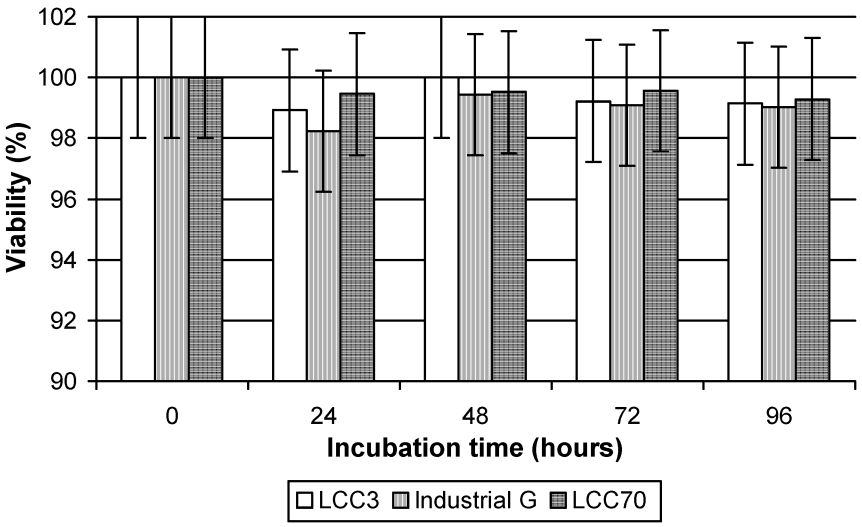


Fig. 7. Effect of 20% (w/v) sorbitol on viability of ale yeast strains.

the shape and features of the yeast cells (Figure 1, micrographs A and C). As a result, it was proposed that shrinkage of yeast cells during exposure to ethanol stress caused a reduction in cell volume and thus the effect of ethanol stress on mean cell volume was investigated.

Yeast strains were exposed to 10% (v/v) ethanol solution. Figures 4 and 5 show the effect of ethanol stress on mean cell volume of lager strains and ale yeast strains, respectively. The effect of exogenous ethanol resulted in continuous decreases in mean cell volume throughout the incubation period in cells of yeast strains Industrial B and NCYC 1342 ($p \leq 0.05$), whilst declines in mean cell volume for NCYC 1324 ceased after 72 h ($p \leq 0.05$). These findings indicated that unlike NCYC 1342 and Industrial B, NCYC 1324 was affected less by ethanol than sorbitol. Ale yeast strains showed different mean cell volume patterns under ethanol exposure compared to their lager counterparts. In all cases, ale yeast cells' response to ethanol stress were not evident until 96 h of exposure ($p \leq 0.05$) whereas, lager strains showed immediate responses to ethanol stress by reductions in mean cell volume at 24 h ($p \leq 0.05$).

The data demonstrated that effects of ethanol stress on cell volume of cultures of lager and ale strains were inter-strain dependent. The effect of ethanol stress on mean cell volume was in agreement with the findings of Cahill *et al.*[4]. They provided evidence that increases in ethanol concentration resulted in a corresponding decrease in mean cell volume. The findings indicate that ethanol exposure has a more marked effect on mean cell volume of lager than ale strains.

Effect of osmotic stress on viability of lager and ale yeast strains

The studies described above have shown that exposure to osmotic stress resulted in significant changes to the shape and features of yeast cells (Figure 1, micrographs A and B). As a result, it was proposed that crenation or wrinkling, visible on the cell surface of yeast cells may have played a significant role in the survival of the yeast cells

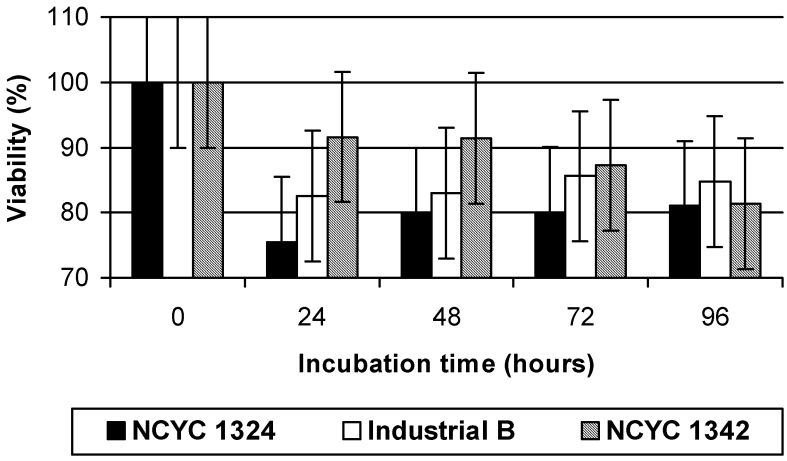


Fig. 8. Effect of 10% (v/v) ethanol on viability of lager yeast strains.

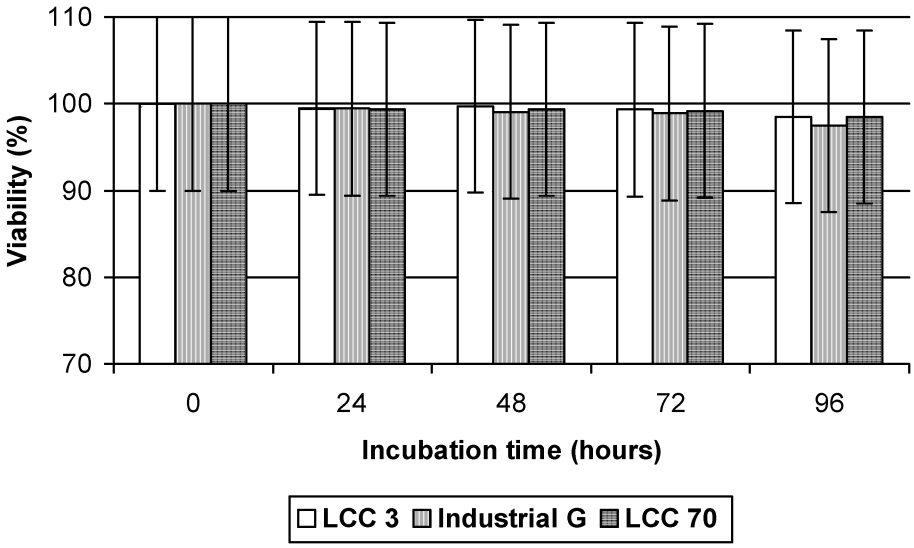


Fig. 9. Effect of 10% (v/v) ethanol on viability of ale yeast strains.

during osmotic stress. As a consequence, the effect of osmotic stress on yeast viability was investigated.

Yeast cultures were exposed to 20% (w/v) sorbitol solution. Figures 6 and 7 show the effect of osmotic stress on viability of lager and ale strains, respectively. Marked reductions in viabilities were apparent in all lager strains studied by the end of the incubation period ($p \leq 0.05$). Conversely, viability of ale yeast strains remained relatively constant throughout the incubation period ($p \geq 0.05$). The effects of osmotic stress on survival of cells of lager and ale strains were inter-strain dependent. The effect of osmotic stress on yeast viability was in agreement with the findings of Beney *et al.*[2]. They provided evidence, which showed that increases in osmotic pressure resulted in corresponding decreases in yeast viability. These findings indicate that ale strains are more tolerant to osmotic stress than lager strains.

Effect of exogenous ethanol on viability of lager and ale yeast strains

Previous studies have shown that exposure to osmotic stress resulted in significant changes to the shape and features of the yeast cells (Figure 1, micrographs A and C). As a result, it was proposed that the collapse of the entire cell envelope could be a contributory factor in the survival of the yeast cells during exposure to ethanol stress. As a consequence, the effect of ethanol stress on yeast viability was investigated.

The yeast strains were exposed to 10% (v/v) ethanol solution. Figures 8 and 9 show the effect of ethanol stress on viability of lager and ale yeast strains, respectively. Marked declines in viability were seen at 24 h in NCYC 1324, Industrial B and NCYC 1342 ($p \leq 0.05$). Yeast cells of NCYC 1324 and Industrial B did not recover from the effects of ethanol, as viabilities remained constant ($p \geq 0.05$). However, viability of NCYC 1342 remained consistent ($p \geq 0.05$) until declining at 96 h ($p \leq 0.05$). In contrast to the lager strains, there were negligible decreases in viability with all the ale strains studied ($p \geq 0.05$).

The effects of ethanol stress on survival of yeast cells of lager and ale strains were inter-strain dependent. The effects of ethanol stress on yeast viability were in agreement with the findings of Jiménez and Benítez[24]. They showed that increases in ethanol in the growth medium

Table I. General linear model for effects of 20% (w/v) sorbitol and 10% (v/v) ethanol solutions on mean cell volume of lager and ale yeast strains.

Factors	Responses	Probability (p value)
Time	Cell volume	Time vs cell volume: ($p \leq 0.05$) significant for all yeast strains.
Strain	Cell volume	Strain vs cell volume: ($p \leq 0.05$) significant for all yeast strains.
Time × strain	Cell volume	Interaction of time × strain on cell volume: ($p \leq 0.05$) significant for lager strains. However, interaction of time × strain on cell volume: was not significant for ale strains under ethanol stress ($p \geq 0.05$).

Table II. General linear model for effects of 20% (w/v) sorbitol and 10% (v/v) ethanol solutions on viability of lager and ale yeast strains.

Factors	Responses	Probability (p value)
Time	Viability	Time vs viability: ($p \leq 0.05$) significant for all yeast strains.
Strain	Viability	Strain vs viability: ($p \leq 0.05$) significant for all yeast strains.
Time × strain	Viability	Interaction of time × strain on viability: ($p \leq 0.05$) significant for lager strains under ethanol stress. However, interaction of time × strain on viability: was not significant for lager and ale strains ($p \geq 05$).

resulted in a corresponding decreases in yeast viability. It is important to emphasize that the ability of the ale yeast strains to outperform the lager strains and maintain higher viabilities under both osmotic and ethanol stresses may be linked to the 20°C incubation temperature. Ale fermentations are normally conducted between 18°C–20°C, whilst lager temperatures are at a much lower range of 10°C–15°C. These findings indicate that ale strains are more tolerant to ethanol stress compared to lager strains at higher incubation temperatures.

Statistical summary of the effects of osmotic and ethanol stress on mean cell volume and viability of lager and ale strains

The Levene's Test was significant for 95% confidence level for effects of both osmotic and ethanol stress on cell volume and for effects of osmotic and ethanol stress on cell viability of lager and ale yeast strains. ANOVA, using the general linear model is tabulated in Tables I and II.

DISCUSSION

During wort fermentation, yeast cells are exposed to variable environmental conditions[4,6,12,23,31]. These conditions are exacerbated by high wort gravities. As a consequence, factors in the environment must be considered in order to understand the performance of yeast cells. There are many features, which contribute to successful high gravity fermentations[39]. However, this study focused primarily on osmotic and ethanol stresses as they are interrelated.

During the early stages of wort fermentation, yeast cells must adapt to osmotic stress induced by high concentrations of sugars and later to the elevated levels of ethanol present at the end of fermentation. In order to perform efficiently, the yeast cell is expected to maintain its metabolic activity by not only sensing these stresses but also rapidly responding and adapting in order to ensure continued viability and vitality. Le Chatelier's Principle states[8], "If an external stress is applied to a system at equilibrium, the system will adjust itself in such a way to partially offset the stress". The relevance of this principle in yeast cells exposed to 20% (w/v) sorbitol and 10% (v/v) ethanol solutions will be discussed.

Data originating from the ESEM *in situ* studies were consistent with those of Morris *et al.*[28] (Figure 1, micrographs A through C). Although, their studies differed with respect to the *S. cerevisiae* strain employed, the concentrations and types of hypertonic solutions used and the duration of the experiments. It should be noted that during exposure to hypertonic solutions of NaCl and glycerol, shrinkage of yeast cells occurred. However, in contrast to the results being reported here, were the findings of Morris *et al.*[28] comparing the surface of shrunken cells fixed in 3 osM–NaCl to those of cells fixed in isotonic medium. There were no marked differences in morphology, such as crenation of the outside envelope on cells exposed to hypertonic solutions compared to those fixed in isotonic solutions. However, when they examined freeze fractures of shrunken cells, they reported major differences in the organization of the cell envelope. Although invaginations were not visible, structures emanating from these invaginations were observed, proving that there was a good correlation between the presence of invaginations in the cell envelope and resistance to hypertonic stress. Walker and Birch[44], conducted a similar study on the effect of 10% (v/v) ethanol exposure on a wine yeast strain and observed aberrations in cell envelope topology. It is worthy to note from the findings reported here, that osmosensing occurred at the cell surfaces.

In order to understand the morphological abnormalities which occurred under both osmotic and ethanol stresses, it was important to determine the physiological impact of these stresses on cell volume and viability. As a consequence, the three lager and three ale strains were incubated in 20% (w/v) sorbitol and 10% (v/v) ethanol solutions as previously described. Decreases in mean cell volume and viabilities of yeast cells of lager and ale strains were observed, upon exposure to osmotic and ethanol stresses (Figures 2 through 9). Hyperosmotic shock, or osmotic upshift, is the phenomenon, which occurs when yeast cells are placed in a medium and there is rapid water outflow from the cell resulting in cell shrinkage[22]. Conversely, hypo-osmotic shock, or osmotic downshift, increases the water concentration gradient of the cell and leads to rapid influx of water, cell swelling and increased turgor pressure[22]. Although reductions in mean cell volume and viability decreases were strain and stress dependent, the findings were consistent with those of Morris *et al.*[28] and Cahill *et al.*[4]. Morris *et al.*[28] compared cell viability after osmotic stress with the reduction in mean cell volume and surface area in hypertonic conditions. They reported cell shrinkage and loss in viability when hypertonic solutions of NaCl, glycerol and methanol were used to exert osmotic pressure on *S. cerevisiae* strains. In contrast to the studies being reported here, they studied reduction in mean cell

volume within the first five minutes of exposure, using various osmolarities (0 osM–6 osM) of NaCl. Although, there were differences in the method employed, the results were similar. Shrinkage of the cells (reduction in mean cell volume) when exposed to hypertonic solutions of NaCl at 21°C occurred within the first minute and remained stable with no increase or reswelling for up to 5 minutes. Cell viability after exposure to NaCl or glycerol decreased with increasing osmolarity. Their findings were consistent with our results, as decreases in cell viability with longer exposure times were also observed.

After initial exposure to osmotic shock, mean cell volume of lager strain Industrial B (Figure 2) recovered and an increase in mean cell volume was observed. This phenomenon of reswelling was reported by Morris and colleagues[28] and was observed upon resuspension of the cells in an isotonic medium. Additionally, ale strain LCC 3, (Figure 3) continually increased its mean cell volume under osmotic stress conditions. These findings not only suggested that the response of yeast cells to osmotic shock was strain dependent but leaves unanswered questions. Firstly, do some yeast strains tolerate, or perhaps even prefer, much lower water activities than others? Secondly, after the initial shock, was there a second shock, which caused continuous reductions in water activity (a_w) at 24 h and 48 h resulting in further reductions in cell volume observed in the other two ale yeast strains? The answer to these questions can be found in the work of Marañon *et al.*[26]. They proposed that the ability of yeast cells to withstand osmotic stress could be dependent on the physiological state of the yeast cells. Yeast cells in the exponential phase had weak turgor pressure compared to turgor pressure of stationary phase yeast cells. The yeast cells of all strains employed in this study were propagated under similar growth conditions and pitched when in stationary growth phase. However, the recovery of ale yeast strain LCC 3 following the osmotic shift, and its increased turgor pressure and the ability of all ale yeast strains to maintain higher viabilities than the lager strain strains under both stresses, suggests that the ale yeast cells are capable of withstanding a much higher gradient of osmotic pressure and ethanol stress than the lager yeast strains studied.

When yeast cells were first inoculated into, either 20% (w/v) sorbitol or 10% (v/v) ethanol solutions, the passive exit of water resulted in a reduction in mean cell volume and a rapid decrease in cell viability. The loss of viability in all strains after hypertonic stress was directly related to a reduction in mean cell volume in the shrunken state. Moreover, the enhancement of the survival of these strains indicated that there was a relationship between mean cell volume and cell viability. Significant decreases in viability were observed in lager strains under both stresses. In contrast, the impact of both stresses was minimal on ale yeast strains. Lager yeast wort fermentation temperature ranges from 10°C to 15°C while ale yeast fermentations are conducted between 18°C and 22°C[20]. These findings indicated that there could be a relationship between temperature and cell viability under hyperosmotic stresses[2] as this study was performed at 21°C. Additionally, the ability of the ale strains to maintain high viabilities may be in agreement with Gervais *et al.*[18] and Marañon *et al.*[26]. Both groups postulated that the ability of a yeast strain to support a higher gradient of osmotic pressure could be due to turgor pressure and thickness of the cell membrane. Cells, which normally have high turgor, have membranes with higher tensile strength. Thus, stationary phase cells, which have been reported to be more stress tolerant than actively growing exponential phase cells, must have a more rigid cell membrane in order to maintain the same volume than exponential cells[45]. The effect of ethanol stress on mean cell volume of lager and ale strains was consistent with the studies of Cahill *et al.*[4]. They reported a decline in mean cell volume of both lager and ale strains when exposed to various concentrations of ethanol. The marked losses in viability of lager strains and the significant changes of morphology of the yeast cells under 10% (v/v) ethanol exposure compared to 20% (w/v) sorbitol exposure confirmed that the effect of exogenous ethanol on the yeast cells was more marked than that of sorbitol.

CONCLUSIONS

Optimal growth conditions are associated with low stress tolerance and sub-optimal with high stress tolerance. Consequently, the morphological changes observed in the yeast cells, from smooth to wrinkly with crenulations, together with extreme changes to cell envelope topology during exposure to sorbitol and ethanol induced stresses, implied that the yeast cells were experiencing adverse environmental conditions. The more marked effect of ethanol on cellular morphology compared to sorbitol confirmed that yeast cells experienced severe alterations in cell envelope structure. The findings demonstrated that morphological responses to stress by yeast cells occurred at the cell surface, whilst implying, that the ability to withstand such stresses is strongly influenced by plasma membrane permeability.

The survival patterns of lager and ale yeast strains in aqueous solutions of sorbitol and ethanol showed that ale strains exhibited higher viabilities and therefore, appeared to be more tolerant to applied osmotic and ethanol stresses. The low cell volumes of the ale strains, particularly Industrial G and LCC 70 under both stresses, could be one of the characteristics that enabled these strains to remain more viable. In contrast, increases in cell volume by LCC 3 under osmotic stress indicated that this yeast strain maintained a much higher turgor pressure under this stress than the other ale yeast strains studied. However, the lower viabilities observed with the lager strains during both stress conditions, implied that the necessary adaptive responses to counteract the effects of osmotic and ethanol stresses may not have been activated in a timely fashion to ensure continued growth and metabolic activity.

The survival patterns of lager and ale yeast strains in aqueous solutions of sorbitol and ethanol were examined as a means to identify suitable strains that might be employed in high gravity fermentations. The performance of LCC 3 under osmotic stress suggests that this strain maintains a higher turgor pressure than the other strains. However, it also implied that this strain exhibited a greater sensitivity to high osmolarity by the continuous uptake of water into the cell resulting in a much higher final mean cell volume than original mean cell volume. It is believed that as a result of this behavior pattern this strain may not

be suitable for high gravity fermentations compared to the other yeast strains studied as they exhibited more desirable characteristics. Future work will be required to investigate the validity of such predictions.

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