The Yeast Vacuole – A Scanning Electron Microscopy Study During High Gravity Wort Fermentations

Patricia L. Pratt[1,2], James H. Bryce[2] and Graham G. Stewart[2,3]

ABSTRACT

J. Inst. Brew. 113(1), 55–60, 2007

The yeast vacuole has been shown to exhibit morphological responses to environmental conditions when exposed to worts of different gravity during fermentation. Marked effects of high gravity wort (20° Plato) on yeast morphology compared to more conventional wort gravity (12° Plato) were observed. High gravity worts caused vacuolar enlargement compared to conventional gravity wort. These results suggested that yeast cells experienced severe alterations with the vacuolar tonoplast when exposed to high osmotic pressure and elevated levels of ethanol.

Key words: High gravity wort, ethanol, morphology, osmotic pressure, vacuole, yeast stress.

INTRODUCTION

Yeast cells must be able to cope with constantly changing environmental conditions during fermentation such as temperature, pH, osmolarity, oxygen, ethanol and nutrients to ensure continued growth and metabolic activity. The cells are expected to sense these environmental changes by evoking the appropriate responses to ensure continued survival. There have been a variety of cellular responses by yeast to different stress situations[12]. These responses include the immediate production of compounds such as glycerol[10,12], significant changes in cellular morphology[11,12], and the activation of signalling pathways[4] all of which indicate that the yeast cells are experiencing adverse conditions.

The vacuole designates an optically empty space within the cytoplasm. This space is encircled by the vacuolar membrane called the tonoplast. The membrane contains specific membrane-bound proteins and several permeases[14]. It is more elastic than the plasma membrane and has a different composition of phospholipids, unsaturated fatty acids and sterols[6]. The tonoplast plays an important role in the metabolic processes associated with the vacuole[6]. The tonoplast usually remains intact when yeast protoplasts lyse under hypoosmotic conditions. However, tonoplasts will disintegrate under prolonged nutrient starvation and other conditions which result in the autolysis of the cell by the release of vacuolar enzymes.

The vacuole is an inherited organelle in yeast. Vacuolar segregation begins with elongation of the vacuole toward and/or into the emerging bud as a tubule or line of vesicles (Fig. 1, monograph A). The large vacuole in unbudded cells shrinks and fragments into small vacuoles as bud initiation occurs and cell density increases. Small vacuoles move into the bud, followed by fusion and expansion of the vacuole as cell density decreases[6,13]. Vacuoles may exist in cells as a single large compartment or as several smaller compartments. Vacuolar volume changes with cell growth phase and growth conditions and are visible when growing in a rich medium[21]. Stationary phase cells contain only one or two large vacuoles[19], whilst cells growing under stressful conditions have very large vacuoles[7,12].

The internalization of the lipophilic dye FM4-64 by endocytosis, observed by fluorescence microscopy, was used to study the effect of ethanol stress and heat shock in *Saccharomyces cerevisiae*[7]. This dye showed that the rate and pattern of endocytosis of the yeast cells may be an indicator to determine the physiological state of the cell. In the presence of both ethanol and heat shock conditions, vacuolar morphology was altered from segregated structures to a single large organelle. Nass and Rao[9] concluded that both ethanol and heat shock induced similar responses in yeast.

Nass and Rao[9] provided further evidence to substantiate these postulates and demonstrated that yeast endosomal/prevacuolar Na^+/H^+ exchanger Nhx1 contributed to osmotolerance following sudden exposure to hyperosmotic media. The vacuoles from yeast strain K601, labelled with the styryl dye FM 4-64, upon exposure to a hypertonic medium, shrank within 6 min, with cell surfaces changing from smooth to crenellated.

Vacuolar morphology changes during growth phase and growth conditions such as elevated osmotic pressure, nutrient limitation and high levels of ethanol all of which are characteristics of high gravity worts. To understand the role of this organelle during brewery wort fermentations, we examined the effects of high gravity worts on yeast vacuolar morphology when exposed to both conventional gravity (12° Plato) and high gravity (20° Plato) worts. The objective of this study was to elucidate the

[1] Molson Coors Brewing Company, Golden, CO 80401.
[2] International Centre for Brewing and Distilling, School of Life Sciences, Heriot-Watt University, Riccarton, Edinburgh, EH14 4AS, Scotland.
[3] Corresponding author. E-mail: g.g.stewart@hw.ac.uk

Publication no. G-2007-0416-467

influence of high gravity wort on overall yeast morphology including the vacuole.

MATERIALS AND METHODS

Yeast strains

The yeast strains employed in this study were brewing strains of *S. cerevisiae.* The lager strains were NCYC 1056, 1126 and 1342 and Industrial Strain B. The ale stains were NCYC 1006, LCC 70 and Industrial Strain G. The NCYC yeast strains were obtained from the National Collection of Yeast Cultures (NCYC), the LCC strains from the Labatt Culture Collection (LCC) and the Industrial strains 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 identified (ale or lager type) by attempting to grow them 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[16].

Preparation of synthetic medium

Chemicals used in the preparation of peptone-yeast extract nutrient synthetic medium, or peptone-yeast extract

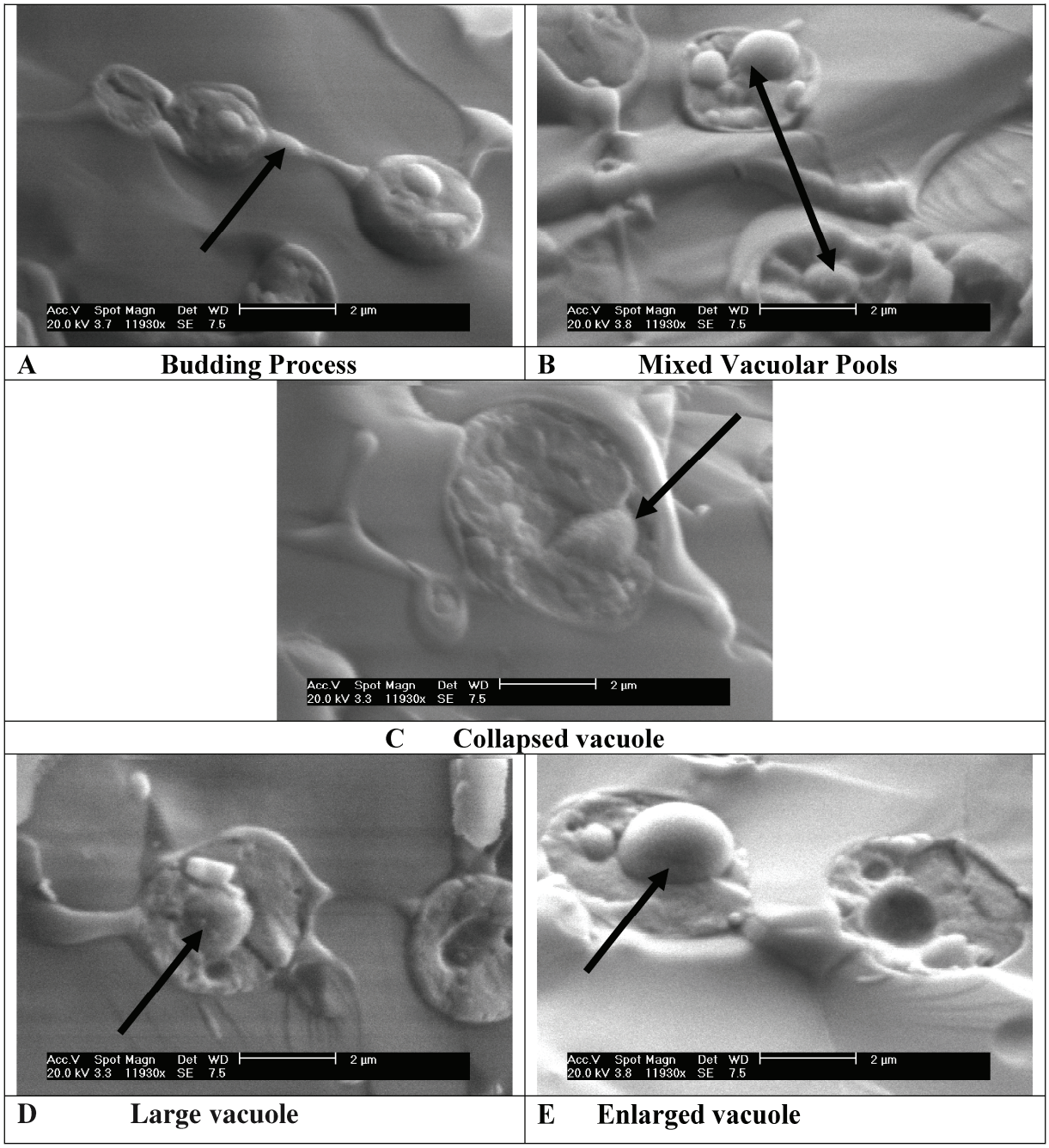

Fig. 1. Typical changes associated with vacuolar diameter of lager yeast cells during fermentation in high gravity (20° Plato) all malt wort.

nutrient agar (Table I) were obtained from BDH/Merck (Poole, Dorset, UK) or those chemicals indicated by asterisk (*) were obtained from Oxoid Ltd (Basingstoke, UK). The chemicals were dissolved in one litre of distilled water. The medium was sterilised by autoclaving at 121°C (15 psi) for 15 min.

All malt 12° Plato and 20° Plato brewer's wort was produced in the ICBD, 2 hL pilot brewery facility, employing the procedures described by Stewart et al.[17] The malt specifications are listed in Table II.

The barley was provided by Hugh Bairds and Sons (Witham, England) and was milled using a 2 row mill (Fraser M5 Bruiser Agricultural Mill (Fraser Agricultural Ltd., Inverurie, Scotland). The grist was kept overnight at room temperature prior to mashing. The milled malt was mashed at a grist to liquor ratio of 1 to 3.5 for 12° Plato wort and 1 to 2.5 for 20° Plato wort. The mashing temperature was maintained at 65°C for 1 h. The temperature was raised to 74°C and held constant for 1 min. Water at 80°C–100°C was pumped to the Briggs (Burton-upon-Trent, U.K.) 200 L lauter tun to approximately one cm above the false bottom. The converted mash was transferred from the mash-mixing vessel to the lauter tun, where it settled on the false bottom or filter plate, until the grist bed was formed.

The wort from the grist bed was recirculated for 20 min; once a satisfactory clarity was achieved, circulation ceased and the first wort (main mash) was pumped to the kettle. As the spent grains still contained fermentable extract, the grist bed was sparged with water at 77°C–80°C. The rakes were used to increase filtration. Once the last wort or runnings had reached a gravity between 2.5 °Plato and 1.5 °Plato and the desired volume in the combined Briggs Kettle/Whirlpool was achieved, this indicated that wort separation was completed and sparging was stopped. Hops were added in the form of pre-isomerised Target pellets 11% alpha (The Wigan Hop Company Ltd. England) to obtain a final beer bitterness level of 20 bitterness units (BU). The wort was boiled for 1 h at an evaporation rate of 10% per hour. At the end of the boiling time, the concentrated wort was allowed to stand for 30 min. The wort was transferred through an APV Paraflow Heat Exchanger System (Derby, England) and collected at 4°C–8°C in a Briggs 2 hL (Burton-upon-Trent, U.K.) fermenter. It was cooled to a temperature depending on the beer type. Temperatures ranged from 12°C–15°C for lagers and 18°C–20°C for ales. The boiled worts were transferred to two litre bottles, frozen and held at –20°C until required for use.

Production of yeast biomass

Yeast biomass was determined following the method of described by Younis and Stewart[20].

Fermentation procedure with tall-tubes

Static wort fermentations were conducted in 2 L custom-built tall-tubes (1585 mm × 50 mm, sample port at 55 mm, Scotia Glass Technology, Stirling, U.K.). The tubes were steam sterilized for 30 min prior to the addition of wort. Fermentations were conducted using 1.5 L of sterile wort. Yeast was pitched as described previousl`[20] Oxygenation was based on 1 mg dissolved oxygen/L per degree Plato of extract as reported by Stewart[15]. Normal gravity (12 °Plato) wort was oxygenated to 12 ppm whilst high gravity (20 °Plato) wort to 20 ppm. The addition of oxygen was carried out using 100% oxygen flowing through a 0.45 µm sterile air filter. The air filter was connected to sterile oxygen permeable hose, which was attached to a stainless steel scinter. The oxygen levels were measured using a model 3650 Micro O_2 Logger (Orbisphere U.K. Ltd., Chesterfield, U.K.). Fermentations were held at constant controlled temperatures of 13°C and 20°C for a period of 192 h and 96 h for lager and ale yeast strains, respectively.

Determination of the diameter of yeast vacuoles during fermentation using SEM

The analytical method employed for the examination and measurement of yeast vacuoles was a modification of the SEM technique reported by Barker and Smart[1]. Approximately 15 µL of diluted yeast sample was transferred into a standard rivet (Oxford Instruments, U.K.) and another rivet placed on top and rapidly frozen in liquid nitrogen slush. The sample was transferred to the cryostage of CT 1500 cryostation (Oxford Instruments, U.K.) and fractured under vacuum. The fractured yeast cell was gold coated 3 times for 20 sec and returned to the Phillips XL 30 SEM microscope for examination. The vacuoles were measured using the measuring software supplied. In order to achieve accuracy for measurement of the vacuoles, a minimum sample size of 100 objects (single and budded) was used for each measurement. The assay was conducted in duplicate.

Determination of the diameter of yeast vacuoles during fermentation using a Zeiss Axiphot microscope

Ten mL of fermentation sample was retrieved daily and transferred to a 15 mL centrifuge tube. The sample was properly mixed. An aliquot based on the time of fermentation (usually between 100 µL to 250 µL) was pipetted and dispensed into a 1.5 mL Eppendorf tube. The sample was diluted with deionised water (usually between 750 µL to 900 µL) based on sample volume. The diluted yeast mixture was transferred to a Neubauer Improved Haemo-

Table I. Composition of peptone yeast extract nutrient agar.

Chemicals	Concentration (g/L)	Grade
Bacteriological peptone*	3.50	GPR
Yeast extract	3.0	GPR
Potassium phosphate (KH_2PO_4)	2.0	GPR
Ammonium sulphate ($(NH_4)2SO_4$)	1.0	GPR
Magnesium sulphate ($MgSO_4 \cdot 7H_2O$)	1.0	GPR
Glucose	100	GPR
Agar*	20	Technical

Table II. Malt specifications.

Parameters	Specifications
Malt variety	Maris Otter (British Pale Ale)
Extract	298L°kg
Colour	5.0 EBC
Moisture	4%
Total nitrogen	1.51% db

cytometer grid and the yeast cells were examined. Microscopic images of the 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 using the Zeiss measuring software supplied. In order to achieve accuracy in the assessment of yeast vacuoles, a sample size of 10 pictures was taken, each representing 100 to 400 cells (single or budded cells). The assay was conducted in duplicate.

Analysis of variance (ANOVA) statistical methods

The data from each experiment was statistically examined using the Analysis of Variance Method (ANOVA) described by Fowler and Cohen[3].

In all cases, the homogeneity of variance was determined to ensure that the sample variances were similar to each other before ANOVA was conducted. The results were expressed using the Levene's test, which determined equal variances of any continuous distribution.

RESULTS

Changes associated with yeast vacuolar morphology during fermentation in high gravity all malt wort using scanning electron microscopy

The vacuole has been postulated to function during periods of osmotic and ethanol stresses to ensure continued metabolic activity and viability of yeast cells[7,9]. Since both of these stresses are characteristics of high gravity worts, morphological investigations into the role of the vacuole during high gravity fermentations were performed. Yeast cells of strain NCYC 1056, retrieved from tall-tube high gravity fermentations, were fractured using Scanning Electron Microscopy (SEM) to monitor the morphological changes of the vacuoles during fermentation. Changes associated with vacuolar morphology are depicted in Fig. 1.

During the budding process, vacuolar fragments, were distributed between mother and daughter cells and subsequently fused to form single vacuoles (Fig. 1A). Fig. 1B depicts yeast cells exhibiting mixed vacuolar pools, indicating that yeast cells were growing under optimal conditions. Contrariwise, Fig. 1C shows a single vacuole, which has shrunk, probably, as a result of elevated osmotic pressure brought about by the high concentrations of wort sugars. As ethanol concentrations increased, during stationary growth phase, the vacuoles became less fragmented and large, single vacuoles were visible (Fig. 1D). By the end of stationary growth phase, as nutrients became limiting and conditions less favourable, vacuolar morphology was altered, resulting in a swollen, single vacuole, taking up almost the entire width of the cell (Fig. 1E).

Effect of wort gravity on vacuolar morphology of lager and ale yeast strains in all malt wort

The diameter of yeast vacuoles of the three lager yeast strains; Industrial B, NCYC 1342 and NCYC 1126 and the three ale yeast strains; LCC 70, Industrial G and NCYC 1006 were measured at specified times, during static fermentations in 12 °Plato and 20 °Plato all malt worts. Figs. 2–7 show the effect of wort gravity on vacuolar diameter of the lager and ale yeast strains.

At 0.5 h post pitching, there was likely a passive flow of water from yeast cells to the outside medium, causing reductions in vacuolar volumes of yeast cells fermenting 12° Plato wort[2]. Decreases in vacuolar volumes at 0 h, were observed in all six yeast strains studied ($p \leq 0.05$). Vacuolar diameter remained relatively constant during lag phase, as yeast cells adapted to the new environment ($p \geq 0.05$). As budding commenced, at 6 h into fermentation, small fragmented vesicles were distributed between

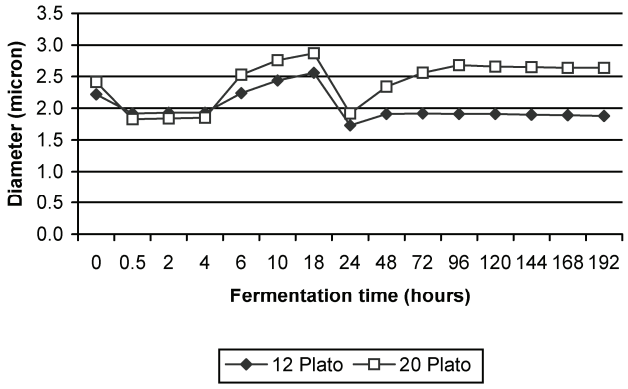

Fig. 3. Effect of wort gravity on vacuolar diameter of lager yeast strain NCYC 1342 in all malt worts.

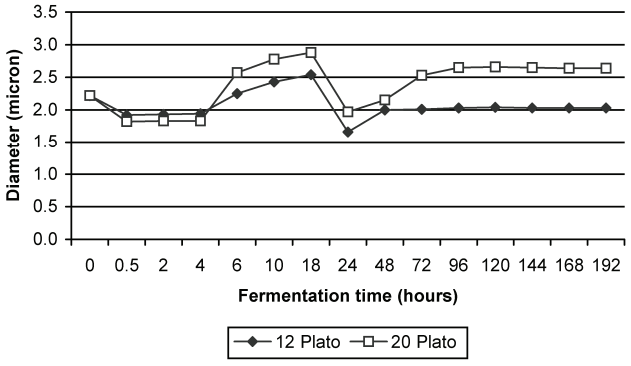

Fig. 2. Effect of wort gravity on vacuolar diameter of lager yeast industrial strain B in all malt worts.

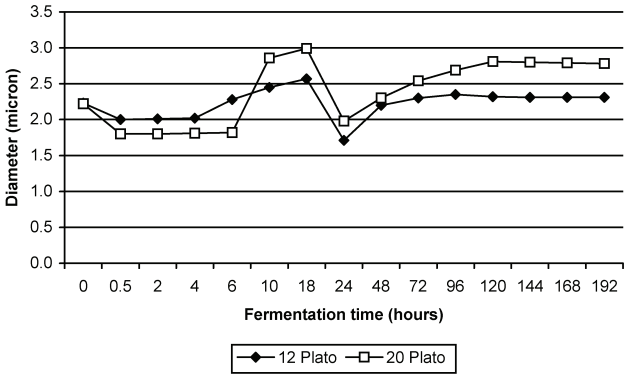

Fig. 4. Effect of wort gravity on vacuolar diameter of lager yeast strain NCYC 1126 in all malt worts.

mother and daughter cells, subsequently fusing to form large vacuoles. Consequently, vacuolar volumes increased and continued until 18 h into fermentation ($p \leq 0.05$). During active growth phase, yeast cells were more sensitive to stresses and thus sudden changes in osmolarity at 24 h, resulted in rapid reductions in vacuolar volumes in all six yeast strains ($p \leq 0.05$). There were increases in vacuolar volumes, following the osmotic shift, at 48 h, as the recovered cells entered stationary growth phase ($p \leq 0.05$). There were no subsequent changes in vacuolar dynamics after this time in lager yeast strains Industrial B and NCYC 1342 and in the three ale yeast strains ($p \geq 0.05$).

Conversely, there was a continuous increase in vacuolar size in yeast cells of strain NCYC 1126 fermenting normal gravity worts until the end of fermentation ($p \leq 0.05$). A similar trend in vacuolar dynamics was observed in all yeast cells fermenting high gravity worts. However, for lager strain NCYC 1126 and ale strain NCYC 1006, the lag phases were extended beyond 6 h post pitching. Consequently, budding was not initiated until 10 h, indicating that the ability of these yeast cells to adapt to high solute concentrations, to ensure continued metabolic activity and cell growth, was hindered by wort gravity. In the presence of elevated ethanol levels, there were continuous increases in vacuolar volumes between 48 h and 96 h, in all six yeast strains ($p \leq 0.05$). There were no subsequent changes in vacuolar volumes after this period ($p \geq 0.05$). These findings indicate that wort gravity has a significant negative effect on vacuolar volume of yeast cells of both lager and ale yeast strains during fermentation.

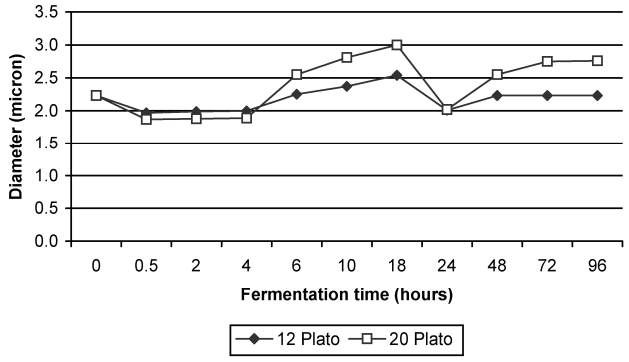

Fig. 5. Effect of wort gravity on vacuolar diameter of ale yeast strain LCC 70 in all malt worts.

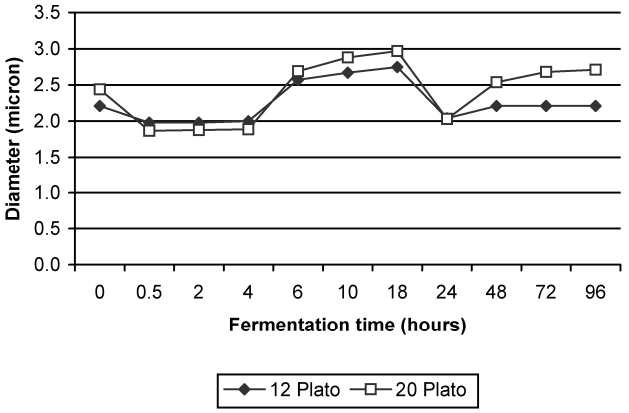

Fig. 6. Effect of wort gravity on vacuolar diameter of ale yeast industrial strain G in all malt worts.

Statistical summary for the effect of wort gravity on vacuolar morphology of lager and ale yeast strains in all malt wort

Levene's test, which determines equal variances of any continuous distribution, was significant for 95% confidence level for the effect of wort gravity on vacuolar morphology of lager and ale yeast strains. ANOVA, using the general linear model, is tabulated in Table III.

DISCUSSION

The importance of the yeast vacuole has been widely discussed[2,5,6,12,13]. The effect of wort gravity on changes associated with vacuolar morphology have been studied in this laboratory using a number of techniques, all of which have demonstrated that the vacuole had adjusted to altered osmolarity (Fig. 1 A–E and Figs. 2–7).

The morphological picture displayed by the vacuoles in the present study, was in agreement with evidence presented by Matile[6], Meaden et al.[7] and Nass and Rao[9]. In the study conducted by Meaden et al.[7], the effect of ethanol stress on the accumulation in endocytic intermediates of the styryl dye FM 4-64 was assessed. They reported that vacuolar morphology was also less affected, with a more swollen and less fragmented pattern than that found in cells perfused in the absence of ethanol. Exposure to heat shock at 42°C led to the vacuoles in each cell combining to form a single large structure[7]. Whilst Nass and Rao[9] reported that in the absence of hypertonic stress or re-suspension in isotonic medium, the yeast cells became

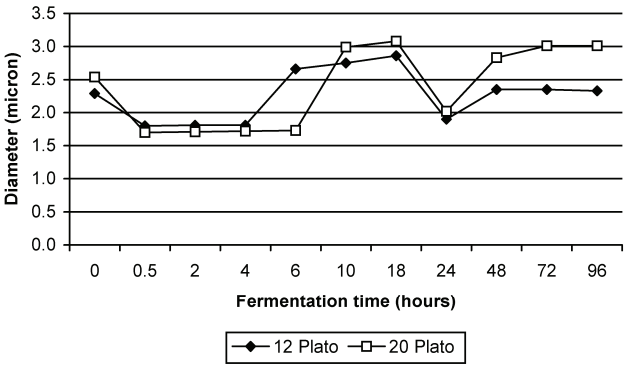

Fig. 7. Effect of wort gravity on vacuolar diameter of ale yeast strain NCYC 1006 in all malt worts.

Table III. General linear model for the effect of wort gravity on vacuolar morphology of lager and ale yeast strains in all malt worts.

Factors	Responses	P
Time	Diameter	Time vs. diameter: ($p \leq 0.05$) significant for all yeast strains.
Gravity	Diameter	Gravity vs. diameter: ($p \leq 0.05$) significant for all yeast strains.
Time × gravity	Diameter	Interaction of time × gravity on diameter: ($p \leq 0.05$) significant for all yeast strains.

swollen and the vacuoles were apparent. All the studies confirmed that upon hyperosmotic shock, the vacuole shrinks or fragments and during hypoosmotic shock, smaller vacuolar fragments fuse to form a larger structure. These findings indicated an obvious role of yeast vacuoles as one involved in osmoregulation. However, it should be noted, that there is also evidence arguing against a primary involvement of the vacuole as an osmoregulator[2]. During fermentation, the vacuolar volume varied suggesting that the same amount of vacuolar membrane (tonoplast) accommodated different vacuolar volumes (Figs. 3–7). The ability of the yeast vacuole to maintain its shape is perhaps due to a slightly higher turgor pressure inside the vacuole, than the surrounding cytosol. Consequently, shrinkage of the yeast vacuole and tonoplast, after an osmotic shock and subsequent recovery supports the theory that the tonoplast is water permeable. However, it strongly disputes the hypothesis of Coury et al.[2] that yeast cells do not control their internal osmolarity through facilitated rapid water or solute transport across vacuolar membranes. Morris et al.[8] studied the effect of exposure to hypertonic stress on vacuolar size of living cells and observed shrinkage of the vacuoles upon exposure within 6 min, with the surface changing from smooth to crenellated. These observations are consistent with our findings which have been reported previously[11].

It is believed that the vacuole is another possible target site that is affected by ethanol toxicity in addition to the plasma membrane. Secretion and endocytosis are major mechanisms of membrane flow to and from the plasma membrane in eukaryotic cells. Eukaryotic cells must balance secretory and endocytic traffic in order to maintain the appropriate protein and lipid content in the plasma membrane[18]. The second step of the endocytic pathway is the transport of ions and small molecules into the vacuole[16]. The maintenance of turgor pressure in the vacuole may also be important for the proper trafficking of proteins essential for normal cellular function. If this hypothesis is correct, then the continuous intake of water into the vacuoles, observed for example, in NCYC 1006 yeast cells fermenting high gravity worts (Fig. 7), indicated abnormal functioning of the vacuolar membrane.

Changes associated with vacuolar morphology, confirmed some possibilities for osmosensing of the yeast cells, during the fermentation of high gravity worts (>16 °Plato) at the vacuolar membrane, as evident by the studies presented in this paper.

ACKNOWLEDGEMENTS

Gratitude is extended to Graham McKernan and Margaret Stobie for technical assistance and advice, Dr. James Buckman for technical aid with the Scanning Electron Microscopy, and to Heineken International for financial support.

REFERENCES

1. Barker, M. G. and Smart, K. A., Morphological changes associated with the cellular aging of a brewing yeast strain. *J. Am. Soc. Brew. Chem.,* 1996, **54**, 121–126.
2. Coury, L. A., Hiller, M., Mathai, J. C., Jones, E. W., Zeidel, M. L. and Brodsky, J. L., Water transport across yeast vacuolar and plasma membrane-targeted secretory vesicles occurs by passive diffusion. *J. Bacteriol.,* 1999, **181**, 4437–4440.
3. Fowler, J. and Cohen, L., Practical Statistics for Field Biology. John Wiley & Sons Ltd: Chichester, England, U.K., 1992, pp. 179–196.
4. Francois, J. and Parrou, J. L., Reserve carbohydrates metabolism in the yeast *Saccharomyces cerevisiae. FEMS Microbiol. Rev.,* 2001, **25**, 125–145.
5. Kida, K., Gent, D. and Slaughter, J. C., Effect of vacuoles on viability of *Saccharomyces cerevisiae. J. Ferment. Bioeng.,* 1993, **76**, 284–288.
6. Matile P., Biochemistry and function of vacuoles. *Ann. Rev. Plant Physiol.,* 1978, **29**, 193–213.
7. Meaden, P., Arneborg, N., Guldfeldt, L. U, Siegumfeldt, H and Jakobsen, M., Endocytosis and vacuolar morphology in *Saccharomyces cerevisiae* are altered in response to ethanol stress or heat shock. *Yeast,* 1999, **15**, 1211–1222.
8. Morris, G. J., Winters, L., Coulson, G. E. and Clarke, K. J., Effect of osmotic stress on the ultrastructure and viability of the yeast *Saccharomyces cerevisiae. J. Gen. Microbiol.,* 1986, **129**, 2023–2034.
9. Nass, R. and Rao, R., The yeast endosomal Na^+/H^+ exchanger, Nhx1, confers osmotolerance following acute hypertonic shock. *Microbiol.,* 1999, **145**, 3221–3228.
10. Petrovska, B., Winkelhausen, E. and Kuzmanova, S., Glycerol production by yeasts under osmotic and sulfite stress. *Can. J. Microbiol.,* 1999, **45**, 695–699.
11. Pratt P. L., Bryce, J. H. and Stewart, G. G., The effects of osmotic pressure and ethanol on yeast viability and morphology. *J. Inst. Brew.,* 2003, **109**, 218–228.
12. Pratt-Marshall, P. L., Bryce, J. H. and Stewart, G. G., High gravity brewing – effects on yeast vacuolar morphology. Proceedings of the 27th Convention of the Institute and Guild of Brewing, IGB Asia Pacific Section: Adelaide, Australia, 2002, Contribution 21.
13. Pringle, J. R., Broach, J. R. and Jones, E. W., The Molecular and Cellular Biology of the Yeast Saccharomyces. Cell Cycle and Cell Biology. Vol. 3. Cold Spring Harbour Laboratory Press: Cold Spring Harbour, U.S.A., 1987, pp. 364–367, 443.
14. Schwencke, J., Vacuoles, internal membranous systems and vesicles. In: Yeasts Organelles, 2nd ed., A. H. Rose and J. S. Harrison, Eds., Academic Press Inc: London, U.K., 1991, pp. 347–432.
15. Stewart, G. G., High gravity brewing. *Brewers' Guardian,* 1999, **128**, 31–37.
16. Stewart, G. G. and Russell, I., An Introduction to Brewing Science and Technology Series III, Brewers' Yeast. Institute of Brewing: London, U.K., 1998.
17. Stewart, G. G., Bryce, J., Cooper, D., Monagas, M. and Younis, O., High gravity brewing: Its influence on beer and yeast quality. Proceedings of the 7th Scientific and Technical Convention of the Institute of Brewing, Africa Section: Nairobi, Kenya, 1999, pp. 100–110.
18. Vida, T. A. and Emr, S. D., A new vital stain for visualizing vacuolar membrane dynamics and endocytosis in yeast. *The Journal of Cell Biology,* 1995, **128**, 779–792.
19. Walker, G. M., Yeast Physiology and Biotechnology, Wiley: Chichester, U.K., 1998.
20. Younis, O. S. and Stewart, G. G., Sugar uptake and subsequent ester and higher alcohol production by *Saccharomyces cerevisae. J. Inst. Brew.,* 1998, **104**, 283–287.
21. Zalewski, K. and Buchholz, R., Morphological analysis of yeast cells using an automated image processing system. *J. Biotechnol.,* 1996, **48**, 43–49.

(Manuscript accepted for publication March 2007)