The Impact of Extracellular Osmolality on *Saccharomyces* Yeast Populations during Brewing Fermentations

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Left running head: Zhuang, S., Smart, K. A., and Powell, C. D.

Right running head: Osmolality and brewing fermentations

Abstract

Osmotic stress represents one of the major environmental challenges experienced by yeast during industrial fermentations. This stress is particularly associated with high gravity processes which utilise concentrated substrates to yield products with elevated concentrations of ethanol. The aims of this work were to quantitatively measure factors affecting extracellular osmotic pressure (osmolality) during brewing fermentations, and to determine their effects on yeast at the physiological and molecular level. Osmolality was observed to increase during fermentation due predominantly to ethanol production, indicating a strong relationship between these environmental parameters. High osmolality was shown to have a negative impact on yeast physiology, viability and vitality and although genome integrity was unaffected, cell membrane fluidity became altered. This data not only demonstrates the occurrence of an increase in osmotic pressure during fermentation, but provides an explanation for the decrease in yeast quality typically observed under high gravity conditions. The results presented here are directly relevant to all brewery fermentations worldwide and have applications within associated industries where microorganisms are used for ethanol production, including food products, alcoholic beverages and biofuels.

Keywords

Genome integrity, high gravity, membrane fluidity, osmotic stress, yeast physiology

Introduction

Osmotic pressure can be defined as the force exerted by the flow of water through a semi-permeable membrane separating two solutions with different concentrations of solute, and is a major environmental stress factor experienced by yeast cells during industrial batch fermentations (38,52,64). Within the brewing industry, as well as other related sectors, there is a growing trend to employ high gravity (HG) and very high gravity (VHG) substrates (worts) as a means of energy-saving, process optimisation (53,70) and capacity optimisation. Such worts are concomitant with elevated osmotic pressure (osmolality), potentially leading to a greater influence on yeast physiology and fermentation characteristics (6,13,71,82).

Current understanding within the brewing industry is that osmotic pressure is encountered when a yeast culture is first inoculated into brewers wort (a malt-based liquid extract) at the beginning of fermentation, after which

42 osmotic pressure reduces as fermentable sugars are assimilated (15). The immediate consequence of exposing
43 yeast cells to high osmotic pressure is a rapid increase in osmotic potential within the cell, resulting from the
44 outflow of water, as well as a decrease in cell volume and turgor (33,37,39). The yeast response is to trigger
45 extensive gene regulation leading to the production of a range of protective compounds including glycerol
46 (17,37,55,80) and trehalose (18,34,35). Glycerol is known to act as a compatible solute to counterbalance
47 external pressure and is produced via activation of the High Osmolarity Glycerol (HOG) pathway (9,17,83),
48 whilst trehalose has been shown to stabilise proteins, internal membrane structures and the plasma membrane
49 (8,35). This is significant since during osmotic adaptation the plasma membrane functions as a chemi-osmotic
50 barrier, providing a major interface between the organism and its external environment (67). Consequently,
51 yeast membrane fluidity has been proposed to be an essential parameter for survival under osmotic stress, as
52 well as other extreme environments (24,74). Under normal conditions, the phospholipid bilayers of biological
53 membranes are believed to be structured in a liquid-crystalline state, whereas during stress conditions
54 phospholipid head-groups are forced together, leading to a phase transition from liquid-crystalline to gel-phase,
55 characterised by decreased membrane fluidity (4,66). It has been suggested that phase transition within the cell
56 membrane could be a key step in the induction of cell death as a result of changes in osmotic pressure
57 (4,24,66,67). In addition to effects on the cell membrane, it has been shown that DNA damage can be induced
58 by osmotic stress in laboratory yeast strains (11,12,23,38,57). Similarly, Miermont *et al.* (38) demonstrated that
59 DNA damage may be related to cell volume reduction caused by severe osmotic stress, and analysis of
60 chromosomal DNA from yeast cells under hyperosmotic shock has indicated that such conditions can directly
61 result in DNA breakage, leading to the production of fragments of several hundred kilobases in size (57).

62 Previous studies of osmotic stress in industrial polyploid brewing yeast strains have typically involved the
63 examination of fermentations conducted using worts of different sugar concentration, and the subsequent effects
64 on product characteristics and yeast health (10,43,46,51,64,81,82). However, in these studies the net effects of
65 osmotic stress alone may have been masked by the combined effects of ethanol toxicity, oxygen availability and
66 nutritional requirements. Furthermore, given that the presence of wort sugars is not the only factor involved in
67 determining osmotic potential (20), a precise characterisation of the external osmotic pressures occurring during
68 brewing fermentations has not previously been achieved. In addition, there have been few reports on the
69 relationship between external osmotic pressure and the changing environmental conditions occurring during
70 VHG brewing, or the effects of this on brewing yeast cell integrity, damage to DNA and membrane structures.
71 In this study we aimed to determine the precise changes in osmotic pressure encountered by lager

(*Saccharomyces pastorianus*) and ale (*S. cerevisiae*) yeast populations during brewery fermentations, and to investigate the impacts of these stress factors on cells at the physiological and molecular level.

EXPERIMENTAL

Yeast strains and growth media

Lager yeast (*S. pastorianus*) W34/70 was obtained from Hefebank Weihenstephan (Germany) and ale yeast (*S. cerevisiae*) NCYC1332 was collected from the National Collection of Yeast Culture (UK). Yeast strains were maintained on YPD agar slopes containing 1 % (w/v) yeast extract, 2 % (w/v) neutralized bacteriological peptone, 2 % (w/v) D-glucose and 1.2 % (w/v) agar at 4 ºC. All chemicals were purchased from Fisher Scientific, UK and media was sterilised by autoclaving at 121 ºC and 15 psi for 15 min immediately after preparation.

Wort preparation

Standard gravity (13 ºP) and high gravity (25 ºP) all-malt worts were obtained from Molson Coors Brewing Company (UK). The 13 ºP wort was used without modification, while the 25 ºP wort was diluted with sterile reverse-osmosis (RO) water to produce 18 ºP (HG) and 24 ºP (VHG) worts. All worts were supplemented with 0.2 mg/L Zn^{2+} by the addition of $ZnSO_4 \cdot 7H_2O$ (Fisher Scientific, UK) and oxygenated prior to use. For the latter, in order to mimic typical oxygen concentrations employed within industrial fermentations, 18 ºP and 24 ºP worts were oxygenated, whilst 13 ºP worts were aerated for 3 hours (to achieve saturation) prior to pitching.

Fermentations

Fermentations were carried out in triplicate in glass hypo-vials according to the method previously described (50,54). Well-mixed yeast slurry was pitched into 100 mL wort to create five different experimental conditions (Table I). All fermentations (lager and ale) were performed at isothermal 15 ºC with constant stirring (350 rpm). It is acknowledged that this temperature is not reflective of industrial ale-type fermentations, however it was applied consistently across yeast strains to standardise environmental conditions. Samples were taken at regular intervals for up to 120 hours after pitching and cooled immediately on ice. Wort was separated from yeast cells by centrifugation at 4 ºC and stored at -80 ºC prior to analysis.

Measurement of osmolality

Throughout this study osmotic pressure is determined and expressed in the form of osmolality, commonly used in practical osmometry, and defined as the number of milliosmoles of osmotically active particles per kilogram of solvent. Osmolality was determined using a micro-osmometer (Model 3300, Advanced Instrument, USA) and

applied to analysis of worts and component analyses, including 24 ºP wort (in dilutions of 100, 75, 50 and 25 % [v/v]), ethanol (10, 20, 30, 40, 50, 60, 70, 80 and 90 g/L) and glycerol (1, 2, 3, 4 and 5 g/L). These values were selected to cover the typical range of individual concentrations observed throughout standard, HG and VHG fermentations.

Osmotic challenge using sorbitol

In order to mimic the range of extracellular osmolality observed during fermentations, sorbitol solutions (10, 20, 30, 40 and 50 %, w/v) were prepared, alongside sterile RO water as a baseline control. Solutions were sterilised by autoclaving at 121 ºC and 15 psi for 15 min prior to use. Populations of yeast cells harvested at both exponential and stationary phase were washed twice in sterile RO water and re-suspended in 100 mL of either sterile RO water or sorbitol solutions (10-50% as above) to achieve a concentration of 5.0×10^8 cells/mL, and incubated at 15 ºC using an orbital shaker at 120 rpm for 48 hours prior to analysis.

Yeast viability determination

Yeast viability was determined using brightfield methylene blue staining according to the method of Pierce (47). Methylene blue (Sigma, UK) was dissolved in 2 % (w/v) sodium citrate to a final concentration of 0.01 % (w/v). A washed cell suspension (0.5 mL, 1.0×10^7 cells/mL) was mixed with an equal amount of methylene blue solution and gently agitated. The solution was incubated for 5 min and cells were examined using a microscope at a magnification of 400× (Nikon, Japan). Dark blue cells were considered to be dead cells and those which remained unstained were counted as viable. A minimum of 200 cells were enumerated for each sample and viability was expressed as a percentage of the total population, representing the mean of triplicate samples.

Yeast vitality determination

Yeast vitality was determined by proton efflux using acidification power, according to Siddique and Smart (63). The assay contains two components, a base-line water acidification power (WAP) and a sugar-related glucose acidification power (GAP). For determination of WAP, a pre-calibrated pH probe (Mettler Toledo, UK) was placed into a sterile universal bottle, containing 19 mL sterile RO water and a magnetic flea, on a magnetic stirrer. Following equilibration of the bottle at room temperature, 1 mL of the cell suspension (5.0×10^8 cells/mL) was added followed by the immediate measurement of an initial pH, defined as WAP0. At the end of 10 min, 5 mL of sterile RO water was added and pH measurement continued until the end of 20 min (WAP20). A final WAP value was obtained: WAP=WAP0-WAP20. Determination of GAP was similar to the method for

WAP; however, at the end of 10 min, 5 mL of sterile 20.2 % (w/v) glucose solution was added instead of 5 mL sterile RO water. GAP was calculated as follows: GAP=GAP0-GAP20. Consequently, net glucose induced proton efflux (GIPE) was obtained by subtracting WAP from GAP: GIPE=GAP-WAP, where GIPE provides a relative indication of cellular vitality.

Analysis of intracellular trehalose and glycerol

Intracellular trehalose was quantified using the method of Parrou and Francois (45). Aliquots of 1.0×10^9 total cells were suspended in Na_2CO_3 (0.25 mL, 0.25 M) and incubated in a 95 ºC water bath for 2 hours followed by the addition of sodium acetate (0.6 mL, 0.2 M) and acetic acid (0.15 mL, 1 M). Subsequently 0.5 mL of the mixture was transferred to an Eppendorf tube and incubated at 37 ºC for 10 hours in the presence of 3 mU of freshly prepared trehalase (Sigma, UK). After incubation, samples were centrifuged at 13,000 rpm for 2 min and the liberated glucose in suspension was determined using a glucose assay kit (Megazyme, Ireland) at an optical density of 510 nm. The concentration of trehalose in samples was expressed as µg glucose derived from 10^8 cells.

Intracellular glycerol was quantified according to the method of Hounsa, Brandt, Thevelein, Hohmann and Prior (18). Aliquots of 1.0×10^9 total cells were suspended in 1 mL boiling Tris-HCl (pH 7.0) for 10 min. The supernatant was harvested by centrifugation for 10 min and glycerol concentration was determined using a glycerol assay kit (Megazyme, Ireland). Results were expressed as µg glycerol per 10^8 cells.

Measurement of membrane fluidity

Yeast membrane fluidity was determined by fluorescent staining using laurdan (6-lauroyl-2-dimethylamino naphthalene), based on the methods of Learmonth and Gratton (27), and Walker *et al* (75). 5 mM of laurdan stock solution was prepared by the addition of laurdan (Molecular Probes, Invitrogen, USA) into absolute ethanol. Cell suspension (OD_{600} = 0.1) was mixed with the laurdan solution to achieve a final concentration of 5 µM and incubated in the dark for 1 hour. Fluorescence was measured at an excitation wavelength (350 nm) and two emission wavelengths (440 nm and 490 nm) using a Varioskan Flash micro-plate reader (Thermo Fisher Scientific, UK). Consequently, Generalized Polarization (GP) was obtained to provide an index of membrane fluidity:

$$GP = \frac{I_{440} - I_{490}}{I_{440} + I_{490}}$$

Where I_{440} and I_{490} indicate relative fluorescence intensities at wavelengths of 440 nm and 490 nm, respectively.

Analysis of yeast DNA integrity via inter-delta sequences

Yeast genomic DNA was extracted according to the method described by Powell and Diacetis (48) and stored at 4 ºC prior to PCR amplification. The primers (delta12: 5′-TCAACAATGGAATCCCAAC-3′ and delta21: 5′-CATCTTAACACCGTATATGA-3′) (28) were obtained from Eurofins MWG, UK. Each reaction contained 1 µM primer delta12, 1 µM primer delta21, 1× Phusion Master Mix (New England Biolabs, UK) and 100 ng of DNA in a total volume of 25 µL. The amplification reaction was conducted using a TC-512 thermal cycler (Techne, UK) using the following conditions: 98 ºC for 30 sec, followed by 35 cycles of 98 ºC for 10 sec, 48 ºC for 30 sec and 72 ºC for 3 min, and a final extension at 72°C for 10 min. PCR products were resolved by electrophoresis on a 1.5% agarose gel.

Statistical analysis

The mean and standard deviation of each data set were calculated using Excel (Microsoft, USA). Statistical analyses were performed using SPSS version 20.0 for windows (Chicago, USA). Data was subjected to one-way analysis of variance (ANOVA) with either least significant difference (LSD) or Student's t-test used to determine the significant differences between samples. The null hypothesis was that there was no significant difference between data sets, and differences were considered significant at $P < 0.05$.

RESULTS AND DISCUSSION

Extracellular osmolality during fermentation and potential contributors

Consideration of the range and extent of osmotic pressure that brewing yeast cells encounter in fermentations is critical since this can not only affect the movement of solutes and water across the cell membrane, but may also cause cell damage (11,67). Damage to yeast cells is extremely undesirable in brewing fermentations since lysis can lead directly to haze production (56) and poor head retention (5), while poor population health is known to result in a number of process related abnormalities, including atypical yeast flocculation, poor sugar utilisation and inappropriate flavour production (31,71).

A series of lab-scale fermentations were conducted using 13 ºP, 18 ºP and 24 ºP worts. Fermentation progression was determined by monitoring carbon dioxide evolution (data not shown), and utilisation of wort sugars over time. Analysis indicated that fermentations were complete within an acceptable period of time, and uptake of wort sugars was as expected (Fig. 1). To determine the potential impact of osmotic stress on yeast health, the

osmolality of wort was determined by analysis of samples obtained during fermentation. It was observed that increasing wort density resulted in an elevated starting osmolality, as expected. However, once sugars began to be utilized, the extracellular osmolality was observed to increase during fermentation, irrespective of original wort gravity or the yeast strain applied (Fig. 2). In 13 ºP, 18 ºP and 24 ºP fermentations, extracellular osmolality increased from approximately 700 to 1500 mOsm/kg, from 800 to 1800 mOsm/kg and from 1100 to 2500 mOsm/kg, respectively. This observation was perhaps surprising given that sugar concentration, believed to be a major contribution to environmental osmotic pressure, is reduced during fermentation. To investigate this further, the contribution of potential compounds to osmolality was investigated in more details, specifically focusing on the major components of fermenting wort, i.e. the wort itself, ethanol and glycerol (Table II). It was observed that each of these components exerts an osmotic pressure (Fig. 3): wort osmolality became reduced in response to the dilution of an original 24 ºP wort, whereas the osmolality of ethanol and glycerol were observed to increase at higher concentrations. The extent to which this occurred was dependent on the type of solute, as indicated by variation in the scale of the y-axis in each instance. Given the concentration range of each component observed during fermentations, it can be concluded that ethanol is the major contributor to extracellular osmolality during fermentation, whereas wort sugars and glycerol have a comparatively minor effect. This finding is in accordance with Jones and Greenfield (20), who reported a non-specific inhibitory effect of ethanol on yeast growth, resulting from reduced water activity (i.e. increased osmolality). The same authors also provide evidence to suggest that the inhibitory effects of ethanol on biomass yield were significantly greater than the relative concentration of substrate sugars, again providing partial support to the results presented here. This data therefore indicates that osmotic pressure derived from ethanol may play an important role in brewing fermentations, and that this effect may be exacerbated by the use of HG and VHG worts. Furthermore, it is suggested that since internal (cellular) ethanol forms an equilibrium with external ethanol during fermentation, due to passive diffusion at the cell membrane, this does affect our overall understanding of osmotic stress during fermentation. Yeast cells are subject to a significant ‘internal’ as well as general osmotic shock, which cannot be countered simply by the movement of water. Ethanol-derived osmotic stress will therefore have a direct impact on cellular organelles, an observation which provides a likely explanation for the production of compounds known to protect internal membrane structures such as trehalose (18,34,35).

The effect of extracellular osmolality on yeast cells

Sorbitol was selected as an osmotic agent since it does not dissociate into ions in liquids, and has no nutritional role or toxic effect on yeast cells (16). Furthermore this sugar has been used previously for the analysis of osmotic stress in a variety of yeast strains (13,16,38,40,52,79). As shown in Fig. 4, sorbitol at various concentrations (10, 20, 30, 40, 50 %, w/v) provided a range of osmolality levels ranging from approximately 560 to 2800 mOsm/kg, alongside a baseline control comprising sterile RO water at 0 mOsm/kg. These concentrations were selected as they represent the range of external osmolality levels (approximately 650-2500 mOsm/kg) observed during fermentations (Fig. 2).

Yeast viability

In order to assess the impact of extracellular osmolality on cell survival, yeast viability was determined using three different staining techniques: the brightfield stain methylene blue, and the fluorophores MgANS (36) and Oxonol (30). This approach was taken to eliminate the effect of the mode of action of individual stains on determining the relationship between osmolality and cell health. However, although variations in viability were observed when different methods of assessment were used, the application of different staining protocols yielded comparable results, hence only data from methylene blue staining is displayed here. Cell viability was observed to decrease with increasing external osmolality for each yeast strain, irrespective of growth phase (Fig. 5). Although viability loss was strain-dependent, the general trend was consistent with the findings of Panchal and Stewart (43), who observed a decline in cell viability with increasing sorbitol-induced osmotic challenge. The data presented here therefore indicates that increased external osmolality could be one of the primary causes of viability loss occurring during fermentation. It is likely that the reduction in viability can be directly apportioned to the external osmotic pressure and, as such, acts as a retrospective indicator of the impact of osmotic stress on cell physiology. Perhaps unsurprisingly, the data also demonstrates that stationary phase cells of ale and lager yeast are more tolerant to external osmotic pressure than their exponential counterparts. For example, stationary phase cells from lager yeast populations (W34/70) exhibited a 10-20 % reduction in viability following exposure to 30 % (w/v) sorbitol, whereas a 30-40 % reduction in viability was observed for the corresponding exponential phase populations. Similarly for the ale strain NCYC1332, a 20-30 % and 40-50 % viability loss was observed for stationary and exponential phase populations, respectively, in the presence of 30 % (w/v) sorbitol. The difference in resistance is likely to be due to intrinsic physiological changes and altered expression levels of general stress response genes that are concomitant with the onset of stationary phase (42,76-78). These modifications provide cells with the ability to survive for extended periods of time during stressful

conditions (3,78), and are likely to aid cells against stress occurring during the latter stages of fermentation when cell activity slows.

Yeast vitality

The acidification power test, based on proton efflux in response to sugar utilisation, was used as a measure of yeast activity related to the fermentation capabilities of yeast cultures (14,29,62,65). In the current study this assay was performed to obtain net glucose induced proton efflux (GIPE), as a measure of population vitality, where a higher GIPE value indicates greater yeast vitality.

In general, GIPE values were observed to decrease with increasing osmolality, regardless of yeast strain and growth phase applied (Fig. 6). This suggests that the capacity of yeast to utilize exogenous glucose may become reduced with elevated extracellular osmolality and is consistent with previous findings showing that the rate of proton efflux is inhibited during the latter stages of HG and VHG brewing fermentations (81). It should be noted that although these authors (81) provided evidence that higher concentrations of ethanol had a significant inhibitory effect on proton efflux, they did not link this to osmotic stress *per se*. This has major implications for yeast fermentation performance and suggests that cells may exhibit a reduction in the ability to pump hydrogen ions out of the cell, potentially impacting on internal pH homeostasis and the uptake of wort sugars by proton mediated active transport. Such effects are likely to be exacerbated during HG and VHG brewing and may contribute to the elongated production times frequently associated with such fermentations (64,82).

Intracellular trehalose and glycerol

Trehalose and glycerol are regarded as important stress protectants in brewing yeast (10,35,46,82), however the precise relationship between their production and osmolality within the range associated with industrial brewery fermentations has yet to be reported. To address this, concentrations of these compounds were determined in yeast cells pre-exposed to a variety of osmolality environments. A marked decrease in trehalose was observed in cells following exposure to 0 % (w/v) sorbitol, irrespective of growth phase and yeast strain applied (Fig. 7AB), indicating potential utilisation of trehalose as a carbohydrate source due to nutrient limitation. However, trehalose was observed to accumulate when cells were subjected to 10-50 % (w/v) sorbitol (corresponding to 563 to 2813 mOsm/kg), although the final concentration was similar regardless of the range of sorbitol concentration applied. This reinforces the view that trehalose plays an important role in protecting cells from damage even at very low levels of external osmotic pressure (34), likely to be a factor of non-specific stress

responses pathways (34,35). Moreover, accumulation of trehalose was more marked in stationary phase cells than in exponential phase cultures, consistent with the enhanced osmo-tolerance associated with stationary phase cells, as indicated during yeast viability analysis. It should be noted that despite accumulation of this sugar, decreased cell viability with increasing osmolality was still observed. Indeed, it has been previously reported that the hyper accumulation of trehalose in yeast does not necessarily improve survival rates when compared to wild-type strains (18). Although trehalose has been regarded as a membrane stabilizer and stress protectant under stressful fermentations (34,35), it remains unclear to what extent this molecule acts to preserve yeast cells in industrial fermentations, or the minimum and maximum effective concentrations required to protect individual cellular components.

There was a dramatic decrease in the concentration of glycerol following exposure to 0 % (w/v) sorbitol, regardless of growth phase (Fig. 7CD). This is likely to be explained by the opening of Fps1p driven glycerol channels (32) due to hypo-osmotic shock, required to export glycerol to prevent cell lysis (32,73). However, irrespective of this, glycerol production became elevated as the concentration of sorbitol was increased from 10 to 50 % (w/v), indicating a relationship between the levels of external osmolality encountered and the concentration of internal glycerol produced. In addition, the lager strain W34/70 produced higher amounts of glycerol than the ale strain NCYC1332 (within the range of sorbitol concentrations utilized), irrespective of growth phase applied, which provides some explanation for the different osmo-sensitivities observed between the two strains. Given that glycerol is primarily produced as a result of the activation of the HOG pathway, this result could be related to the expression levels of genes involved in the cellular signalling route, including *GPD1* and *GPP1*, involved in catalysing the conversion of dihydroxyacetonephosphate (DHAP) via glycerol-3-phosphate (G3P) to glycerol (22).

Membrane fluidity

The fluidity of the plasma membrane can be affected by various types of stress, leading to cellular damage and cell death (4,27,74). However, no analysis of the effect of external osmolality on this cell parameter in brewing yeast strains has been performed previously. To achieve this, membrane fluidity was determined using laurdan generalized polarization (GP) to represent membrane fluidity by indicating different emission spectra in the liquid-crystalline (490 nm) and gel phases (440 nm) of the membrane, where a low GP value indicates high membrane fluidity and *vice versa*.

As shown in Fig. 8, the lipid bilayer of both yeast cells was composed of a mixture of liquid-crystalline and gel phases, since the GP value was intermediate between the theoretical range from -1 to +1 (27,44). Although fluidity was not observed to vary when cells were exposed to low osmolality conditions (0-10% sorbitol), the predominant trend was that membrane fluidity gradually decreased with increasing osmotic pressure. Although cells of W34/70 and NCYC1332 were affected in slightly different ways, the membrane fluidity within populations was gradually reduced to a similar degree under high osmolality conditions (40 % and 50 % [w/v] sorbitol). This data confirmed the existence of a link between the membrane state and the extracellular osmolality, indicating that yeast cells are able to implement a change in membrane fluidity during growth in response to external osmotic pressure. The reduced membrane fluidity resulting from the modification of membrane structure and composition may act to compensate for changes in osmolality and to govern cell resistance to stress both directly and via cell signalling pathways (4). Indeed, it was observed that stationary phase W34/70 cells displayed lower membrane fluidity than NCYC1332 in the presence of 10 and 20 % (w/v) sorbitol. Simultaneously, stationary phase cells of W34/70 displayed a higher viability and vitality, as well as higher glycerol accumulation than their exponential phase counterparts. Interestingly, it has been proposed that a heat-induced signal for pathway activation is generated in response to a weakness in the cell wall (21), perhaps a consequence of decreased membrane fluidity. Moreover, the degree of lipid unsaturation and the presence of ergosterol in the membrane of *S. cerevisiae*, have been related to stress tolerance (72). Other studies have also demonstrated a relationship between the composition of membrane lipid and expression of stress-induced proteins (7,58,59). Nevertheless, it should be noted that membrane fluidity can be modified transiently or permanently by environmental stress factors (24-26,61). In the case of extreme stress conditions, regulation may not compensate for the changes in the physical membrane characteristics (4,24), and may result in cellular damage or death in industrial fermentations as shown here.

DNA inter-delta regions

Although genome instability, as a consequence of environmental stresses, has been suggested to provide stress adaptation in yeast strains, it inevitably has a negative effect on beer quality (68). Inter-delta sequences are DNA repeats that flank the Ty1 yeast retrotransposon and are known to be subject to frequent positional change within the genome (41). Consequently these elements are good candidate targets for analysis of genetic variation and have been used previously to investigate potential genetic changes during serial repitching (48), as well as the analysis of the effect of dehydration and rehydration on active dried yeast genetic integrity (19). In order to further investigate potential damage caused by changes in extracellular osmolality, analysis of inter-delta

sequences was performed in the two brewing yeast strains. Analysis of DNA from cells subjected to extracellular osmotic pressure revealed that there were no detectable genetic changes within these regions, suggesting that cells were genetically stable over the range of osmolality tested (0 to 2813 mOsm/kg) (Fig. 9). Although this result is important, it was perhaps not entirely surprising, given that previous results have shown evidence to suggest that DNA is not impacted by stress factors associated with dehydration and rehydration of cells (19). It should also be recognised that there are significant differences between industrial yeast strains in terms of their propensity to form genomic mutants through successive generations (1,2,48,49,60,69) and that other strains may be more susceptible to osmotic stress related DNA damage. Finally, it must be recognized that due to the nature of PCR based techniques, which require analysis of cell populations rather than individuals, DNA damage occurring at an extremely low rate may not be detected. However, this data does indicate that genomic DNA damage should not be directly associated with the range of osmolality encountered within standard, HG and VHG brewery fermentations.

CONCLUSIONS

The move towards higher gravity fermentations within the brewing industry has resulted in a change in the demands placed on industrial yeast strains. In particular, stress factors associated with fermentation are exacerbated, which can directly impact on yeast health and subsequently fermentation performance. Consequently, there is significant interest within the industry in understanding the response of yeast to stress factors associated with high gravity brewing, and specifically those associated with osmotic pressure.

Measurement of wort osmolality indicated that external osmotic pressure increased throughout brewing fermentations, with the largest contributor appearing to be derived from ethanol. This observation highlights the osmotic potential of ethanol during fermentation and has implications with regard to protracted osmotic stress for both brewery and other yeast-based fermentation systems, including wines, distilled spirits and biofuels. The effects of extracellular osmolality on the physiological manifestations of the yeast stress response were investigated by analysis using media containing various concentrations of sorbitol. Cell viability and vitality (proton efflux) were both negatively affected, while cells responded to osmotic shock by production of both trehalose and glycerol. Although glycerol production appeared to be directly correlated to the level of external osmolality, trehalose was observed to be produced to maximum concentration once low levels of stress were encountered, potentially indicating an important but less specific stress response. Although analysis of DNA inter-delta regions indicated that brewing yeast cells were genetically stable under the effects of increased

osmolality, it was demonstrated that the cell membrane was affected. Membrane fluidity was observed to decrease with increasing osmolality, indicating potential changes to the membrane structure which may be either a function of damage, or a manifestation of the cellular response to this specific stress. Irrespective, such impacts on cellular physiology have implications for the brewing process since performance is largely dictated by membrane health and cell vigour in general. Importantly, the response of yeast cells to osmotic stress may also have a direct impact both on performance and final product characteristics, particularly since many metabolic pathways are repressed in the presence of trehalose, and since the production of carbon-based metabolites typically occurs at the expense of ethanol production. It is suggested that mitigation of osmotic stress by ensuring yeast health prior to and during fermentation may become increasingly important within the industry both to ensure that brand specifications are met, and to ensure fermentation efficiency.

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Figure Captions

Fig. 1. Concentrations of glucose (A, B), maltose (C, D) and maltotriose (E, F) during fermentations using lager strain W34/70 (A, C, E) and ale strain NCYC 1332 (B, D, F). Data points represent the mean of triplicate samples, with error bars indicating the standard deviation.

Fig. 2. Wort osmolality during fermentations using lager strain W34/70 (A) and ale strain NCYC1332 (B) as a function of time. Data points represent the mean of triplicate samples, with error bars indicating the standard deviation.

Fig. 3. Osmolality of individual fermentation components including 24 ºP brewer wort (A), ethanol (B) and glycerol (C). Data points represent the mean of triplicate samples, with error bars showing the standard deviation. Mean values and linear equations (y and R^2 values) are shown.

Fig. 4. Osmolality induced by 0-50 % (w/v) sorbitol solutions. Data points represent the mean of triplicate samples, with error bars showing the standard deviation. Mean values and linear equations (y and R^2 values) are shown.

Fig. 5. Viability of exponentially-growing (A) and stationary phase yeast cells (B) following exposure to a range of osmolality levels induced by sorbitol. Yeast viability was determined using methylene blue staining. Error bars represent standard deviation from the mean values of triplicate samples.

Fig. 6. Vitality analysis by determination of glucose induced proton efflux (GIPE) for W34/70 and NCYC1332 yeast cells prior to (control) and post exposure to a range of osmolality levels induced by sorbitol. Exponential (A) and stationary phase (B) cells were examined using the acidification power test. Values represent the mean of triplicate samples and the standard deviation is indicated by error bars.

Fig. 7. Concentrations of trehalose (A, B) and glycerol (C, D) in W34/70 and NCYC1332 yeast cells prior to (control) and post exposure to a range of osmolality levels induced by sorbitol over 48 hours. Exponential (A, C) and stationary phase (B, D) cells were examined. Values represent the mean of triplicate samples and the standard deviation is indicated by error bars.

Fig. 8. Membrane fluidity of yeast strains W34/70 and NCYC1332 prior to (control) and post exposure to a range of osmolality levels induced by sorbitol over 48 hours, as indicated by Generalized Polarization (GP). Exponential (A) and stationary phase (B) cells were examined and data represents the mean of triplicate samples and the standard deviation is indicated by error bars.

Fig. 9. Analysis of DNA inter-delta regions derived from exponential phase lager yeast W34/70 (A) and NCYC1332 (B). In each instance, lane 1: 100bp ladder; lane 2-3: 0 % sorbitol; lane 4-5: 10 % sorbitol; lane 6-7:

584 30 % sorbitol; lane 8-9: 50 % sorbitol; lane 10-11: fresh cells (control); lane 12: blank (pure water); lane 13:

585 1Kb marker. Note that stationary phase cells yielded identical results (data not shown).

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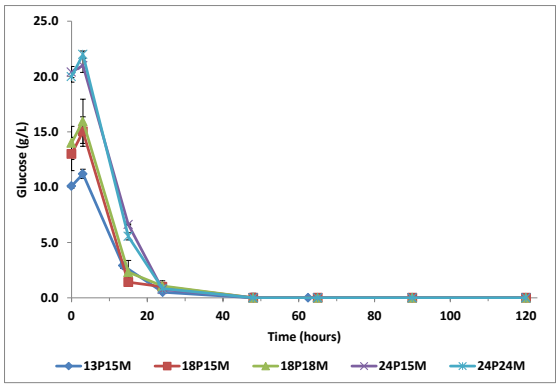
(A)



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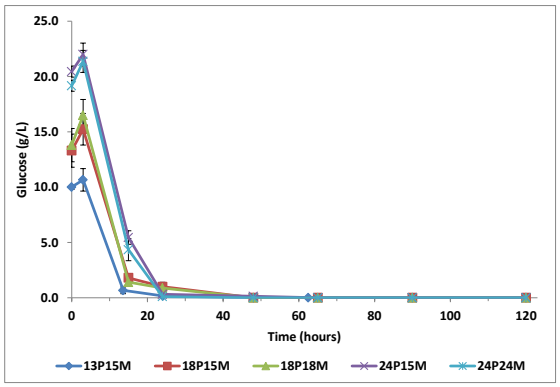

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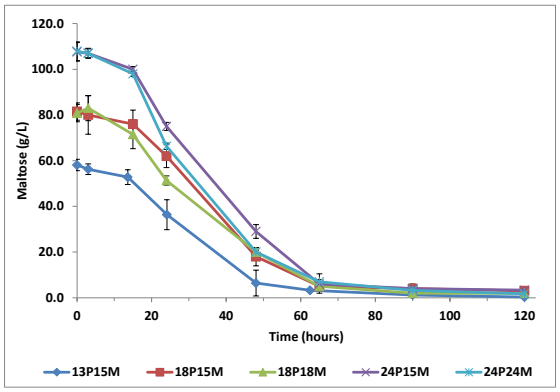
(C)



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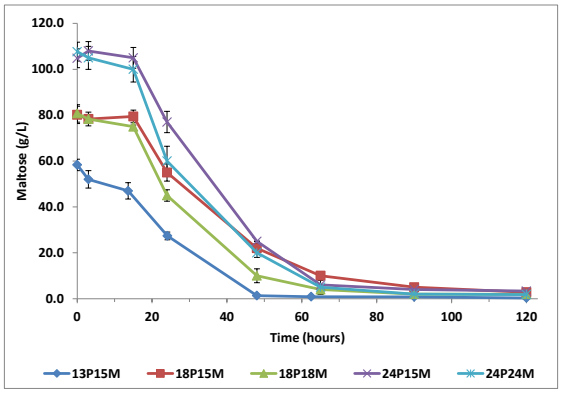

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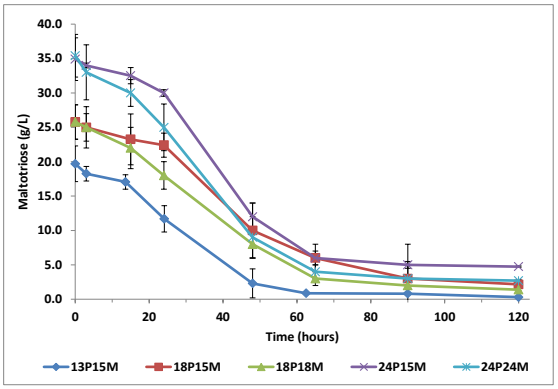
(E)



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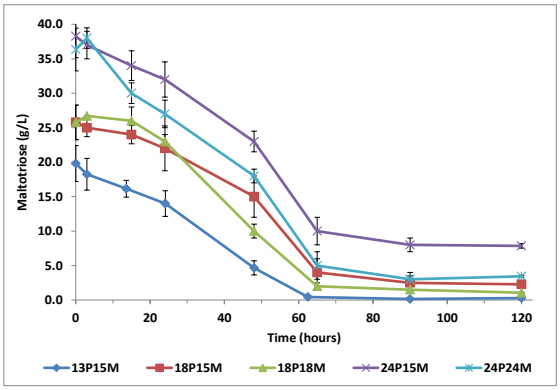

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594 **Fig. 1**

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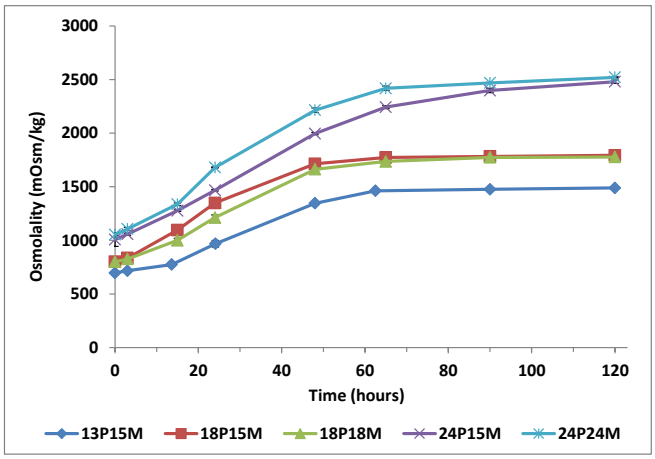
(A)



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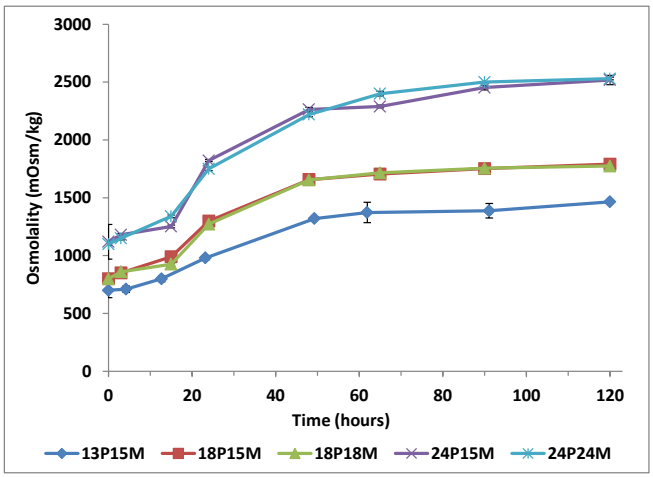
(B)



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601 **Fig. 2**

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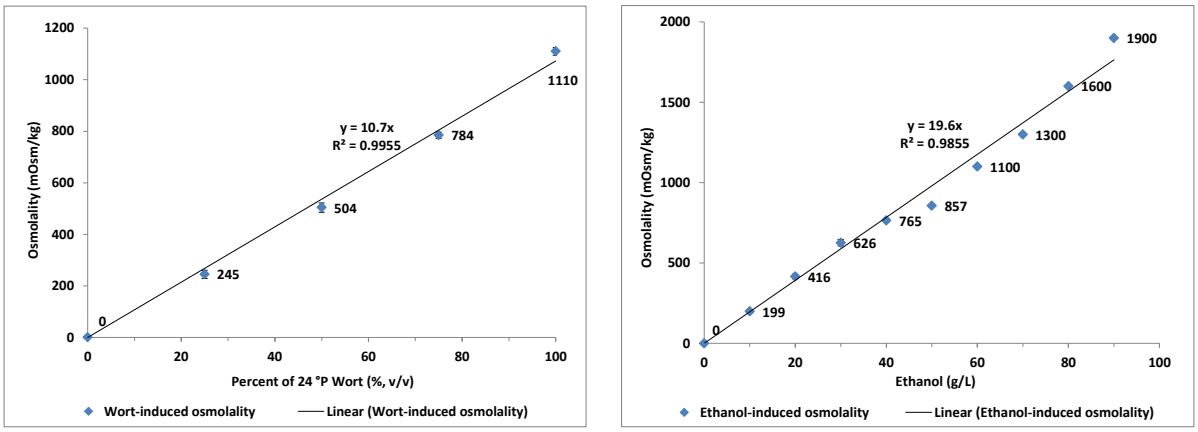
603 **(A)** **(B)**



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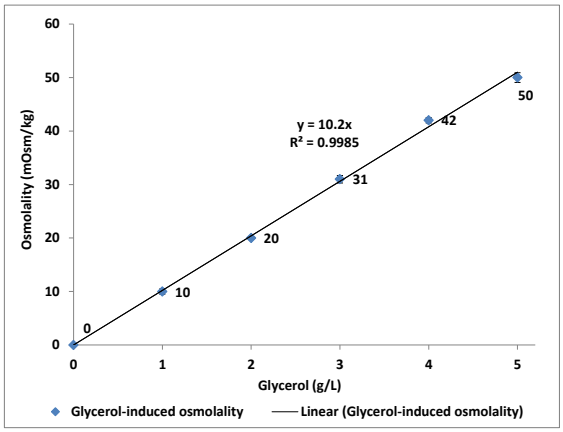
606 **(C)**



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609 **Fig. 3**

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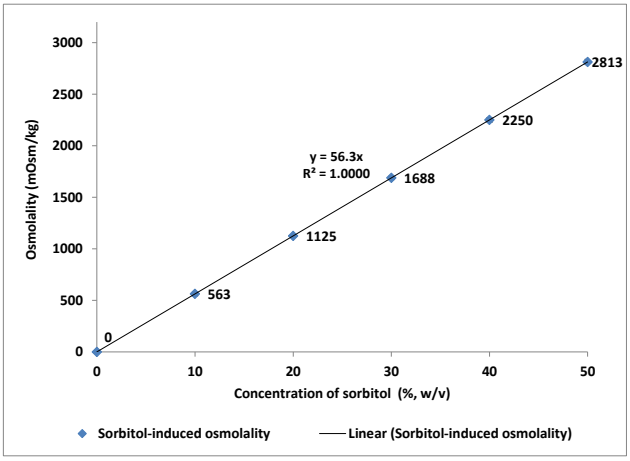

612

613 **Fig. 4**

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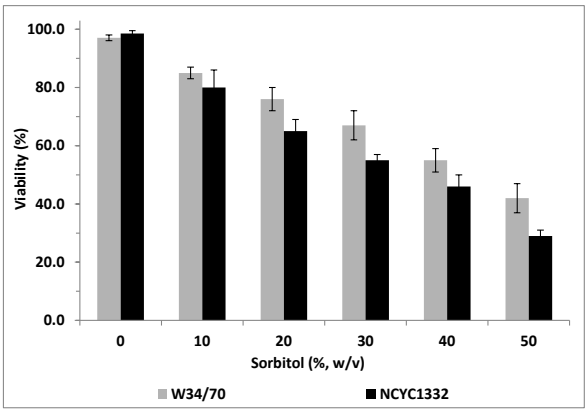
615 **(A)**



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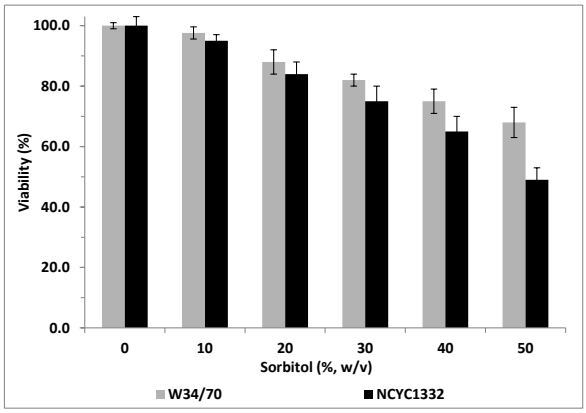
618 **(B)**



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621 **Fig. 5**
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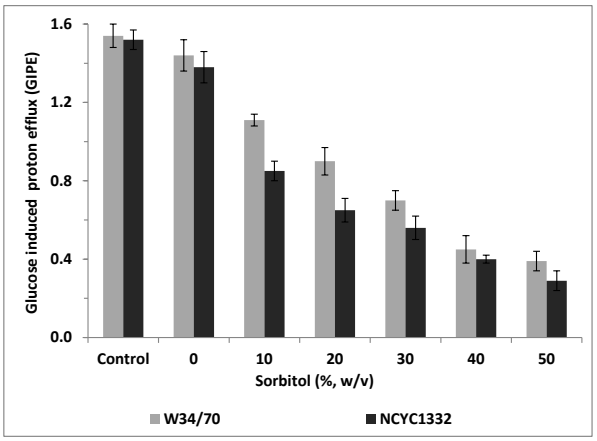
623 **(A)**



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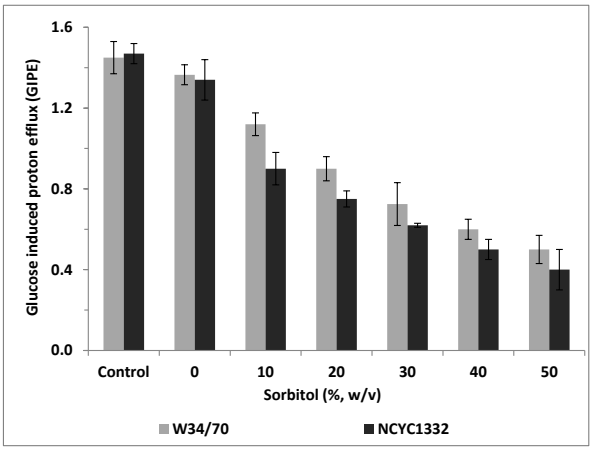
626 **(B)**



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628

629 **Fig. 6**

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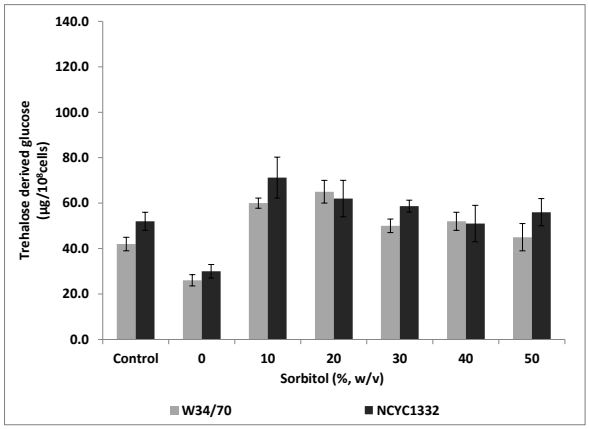
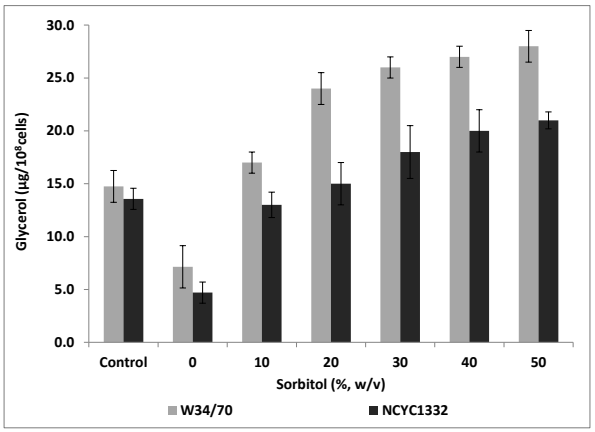
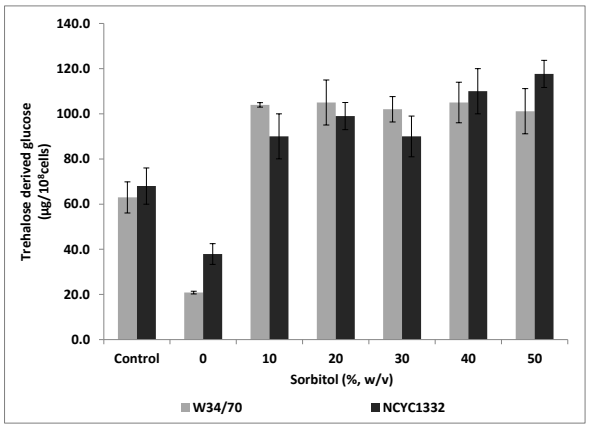
(A)


Trehalose derived glucose (µg/10⁶cells)
140.0
120.0
100.0
80.0
60.0
40.0
20.0
0.0
Control
0
10
20
30
40
50
Sorbitol (%, w/v)
W34/70
NCYC1332

(C)


Glycerol (µg/10⁶cells)
30.0
25.0
20.0
15.0
10.0
5.0
0.0
Control
0
10
20
30
40
50
Sorbitol (%, w/v)
W34/70
NCYC1332

(B)


Trehalose derived glucose (µg/10⁶cells)
140.0
120.0
100.0
80.0
60.0
40.0
20.0
0.0
Control
0
10
20
30
40
50
Sorbitol (%, w/v)
W34/70
NCYC1332

(D)

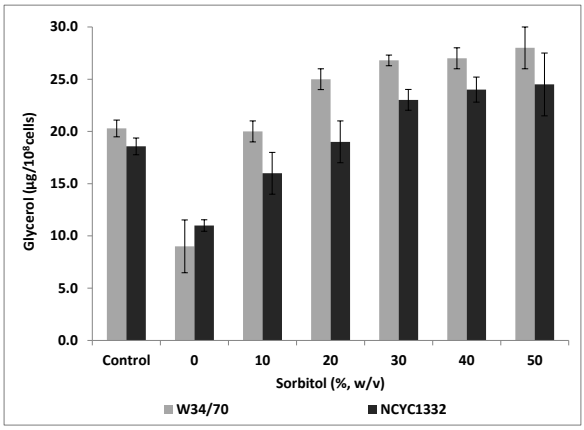
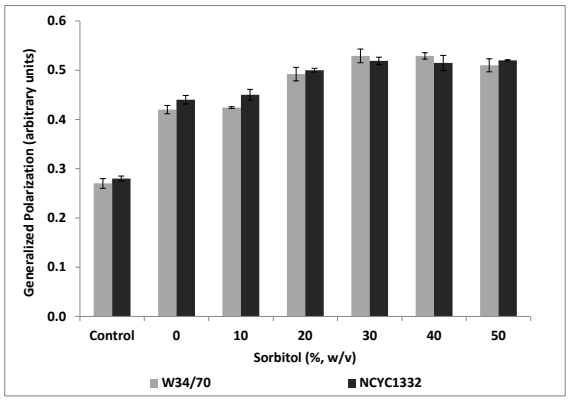

Glycerol (µg/10⁶cells)
30.0
25.0
20.0
15.0
10.0
5.0
0.0
Control
0
10
20
30
40
50
Sorbitol (%, w/v)
W34/70
NCYC1332

Fig. 7

(A)


Generalized Polarization (arbitrary units)
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Control
0
10
20
30
40
50
Sorbitol (%, w/v)
W34/70
NCYC1332

(B)

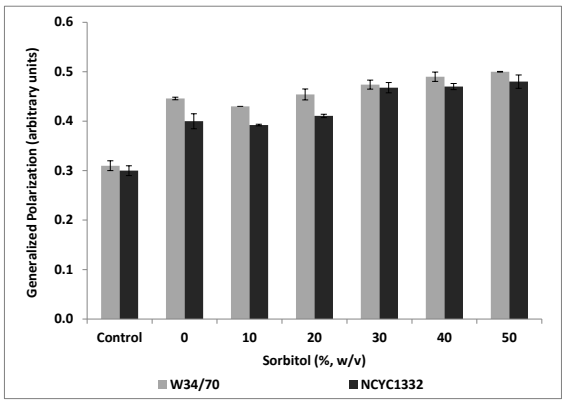
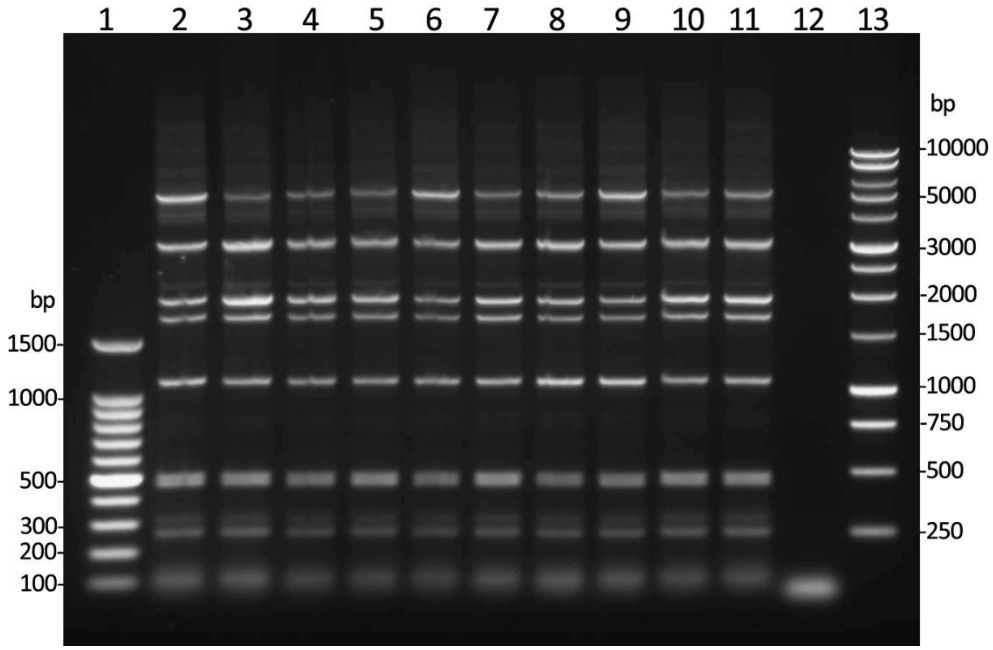

Generalized Polarization (arbitrary units)
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Control
0
10
20
30
40
50
Sorbitol (%, w/v)
W34/70
NCYC1332

Fig. 8

(A)


1 2 3 4 5 6 7 8 9 10 11 12 13
bp
1500
1000
500
300
200
100
bp
10000
5000
3000
2000
1500
1000
750
500
250

(B)

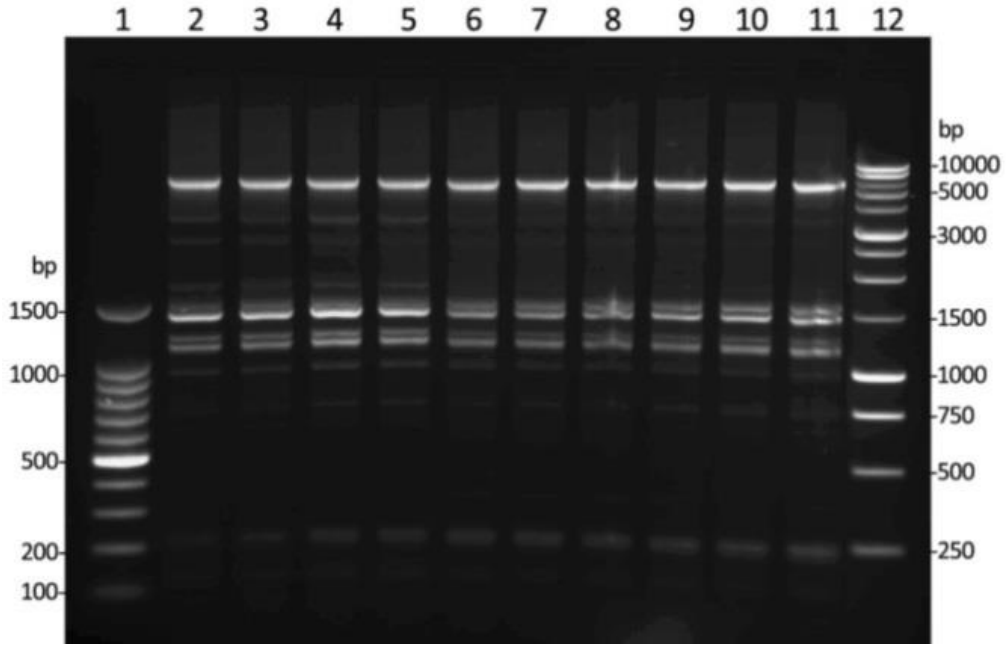

1 2 3 4 5 6 7 8 9 10 11 12
bp
1500
1000
500
200
100
bp
10000
5000
3000
1500
1000
750
500
250

Fig. 9

672 **TABLE I**

673 **Experimental conditions applied**

674

Test conditions	*Wort gravity (°P)*	*Pitching rate (Viable cells per mL)*
13P15M	13	1.5×10^7
18P15M	18	1.5×10^7
18P18M	18	1.8×10^7
24P15M	24	1.5×10^7
24P24M	24	2.4×10^7

675

676

677 **TABLE II**

678 **Final fermentation characteristics. Data represents the mean of triplicate independent samples ±**
679 **standard deviation**

Parameters	*Fermentation conditions*				
	13P15M	***18P15M***	***18P18M***	***24P15M***	***24P24M***
			W34/70		
Final wort gravity (°P)	1.8 ± 0.3	2.5 ± 0.1	2.5 ± 0.1	4.2 ± 0.1	3.6 ± 0.2
Final ethanol yield (g/L)	44.0 ± 0.9	58.8 ± 0.5	60.0 ± 0.8	80.8 ± 0.5	84.5 ± 0.7
Final glycerol yield (g/L)	3.0 ± 0.2	3.6 ± 0.1	3.3 ± 0.1	3.9 ± 0.1	4.5 ± 0.2
			NCYC1332		
Final wort gravity (°P)	1.7 ± 0.3	2.4 ± 0.2	2.5 ± 0.1	4.7 ± 0.3	3.5 ± 0.1
Final ethanol yield (g/L)	43.0 ± 1.0	54.9 ± 0.8	57.3 ± 1.1	72.4 ± 0.8	79.5 ± 0.7
Final glycerol yield(g/L)	3.2 ± 0.1	4.3 ± 0.2	3.9 ± 0.2	4.6 ± 0.2	5.1 ± 0.2

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