REVIEW ARTICLE

Yeast responses to stresses associated with industrial brewery handling

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Received 5 February 2007; revised 4 May 2007; accepted 9 May 2007.
First published online August 2007.

DOI:10.1111/j.1574-6976.2007.00076.x

Editor: Martin Kupiec

Keywords
fermentation; yeast brewing; stress.

Abstract

During brewery handling, production strains of yeast must respond to fluctuations in dissolved oxygen concentration, pH, osmolarity, ethanol concentration, nutrient supply and temperature. Fermentation performance of brewing yeast strains is dependent on their ability to adapt to these changes, particularly during batch brewery fermentation which involves the recycling (repitching) of a single yeast culture (slurry) over a number of fermentations (generations). Modern practices, such as the use of high-gravity worts and preparation of dried yeast for use as an inoculum, have increased the magnitude of the stresses to which the cell is subjected. The ability of yeast to respond effectively to these conditions is essential not only for beer production but also for maintaining the fermentation fitness of yeast for use in subsequent fermentations. During brewery handling, cells inhabit a complex environment and our understanding of stress responses under such conditions is limited. The advent of techniques capable of determining genomic and proteomic changes within the cell is likely vastly to improve our knowledge of yeast stress responses during industrial brewery handling.

Introduction

Effective brewery fermentation requires conditions appropriate for ensuring the production of high-quality beer and for maintaining yeast vitality. However, optimal conditions for the former can be suboptimal for the latter and, consequently, brewery fermentation imposes a variety of stresses on the yeast cell. During industrial fermentation brewing strains of yeast (typically members of the *Saccharomyces cerevisiae s.s.* complex) are exposed to fluctuations in oxygen concentration, osmotic potential, pH, ethanol concentration, nutrient availability and temperature (Briggs *et al.*, 2004). As a nonmotile, unicellular organism, yeast relies on physiological mechanisms to cope with these environmental changes. The aim of this paper is to review our current knowledge of the stresses experienced by brewing yeast during industrial batch fermentation and related processes, and their molecular and physiological responses to these stresses. This review will focus on stresses which may be encountered during industrial-scale production of pilsener-type lager beers with lager (bottom fermenting) yeast, although many of the stresses detailed will also be relevant to smaller scale fermentations and fermentations involving ale (top-fermenting) yeast.

Environmental changes experienced by yeast during industrial brewery handling

Modern industrial brewing practices are essentially derivatives of those used in traditional brewing. In both cases, wort, an aqueous extract of malted barley containing extracts of hops, is fermented with a specific *Saccharomyces* brewing yeast strain to produce an alcoholic beverage (beer).

The quantities of yeast required for industrial-scale fermentation (typically $> 10\,000$ L of yeast slurry) necessitate the propagation of yeast in specially designed vessels prior to fermentation or, less commonly, the use of dried yeast as a direct inoculum. Yeast is propagated in wort under aerobic conditions, achieved through the addition of sterile air or oxygen (Boulton *et al.*, 2000). Following propagation, the fresh yeast slurry is transferred (pitched) into aerated wort in a cylindroconical fermentation vessel. After a brief lag phase the yeast grows exponentially, rapidly depleting the available oxygen and creating an anaerobic environment.

The fermentable wort sugars and assimilable nutrients are also rapidly utilized resulting in carbon and nutrient limitation, typically prompting the cell to enter a quiescent state. These carbon- and nutrient-limited conditions coincide with an increase in ethanol concentration (Casey *et al.*, 1984). On completion of fermentation, yeast which has sedimented in the cone at the bottom of the cylindroconical vessel is removed (cropped) from the base of the cone and a portion of this yeast is stored (under beer) at low temperature (3–4 °C) until required for use in subsequent fermentations (Briggs *et al.*, 2004). Before repitching, yeast is often washed with food-grade acids (pH 2.2) in order to remove any bacterial contaminants (Boulton & Quain, 2001). The acid-washed cells are then repitched into a fermentation vessel and are subjected to the same procedures as before. Serial repitching, whereby yeast cropped at the end of the fermentation is reused in subsequent fermentations, is a process unique to brewery fermentations and can only be conducted a finite number of times before the yeast quality deteriorates and fermentation performance is compromised.

In the modern brewery, techniques have been developed to improve the efficiency of wort fermentation leading to a reduction in fermentation time and/or an increase in yield. The latter requirement has been addressed by the use of high-gravity worts, produced via the addition of sugar adjuncts that result in higher ethanol concentrations towards the end of fermentations, (Casey & Ingledew, 1983; Casey *et al.*, 1984; Stewart *et al.*, 1988; Stewart, 2001). Traditional and more recent innovative developments in practice are usually adopted for process efficiency; however, the fermentation of wort by yeast and the handling of yeast between fermentations results in exposure to numerous stresses (Fig. 1).

The general stress response

Two major stress response pathways in *Saccharomyces cerevisiae* are the heat shock response (HSR), which is activated in a complex manner by sublethal heat stress (Chatterjee *et al.*, 2000) mediated by the so-called heat shock transcription factor (HSF) (Bienz & Pelham, 1986; Morimoto *et al.*, 1996). Alternatively, the general (or global) stress response (GSR) is activated by a number of environmental stresses including oxidative, pH, heat and osmotic stresses as well as nitrogen starvation (Ruis & Schuller, 1995; Martinez-Pastor *et al.*, 1996; Schmitt & McEntee, 1996). The GSR is believed to be an evolutionary adaptation that allows yeast to respond to adverse environmental conditions in a nonspecific manner, in order that cellular fecundity is retained whilst specific responses are activated (Ruis & Schuller, 1995; Martinez-Pastor *et al.*, 1996). The GSR is typified by the up-regulation of *c.* 200 genes and their corresponding proteins, which are involved in a diverse array of cellular functions (Gasch *et al.*, 2000; Causton *et al.*, 2001). The expression of these genes has been demonstrated to occur in a process dependent upon the pentameric *cis*-acting sequence CCCCT within the promoter region of the induced genes. This so-called stress responsive element (STRE) was first identified in reference to the stress-induced expression of the *CTT1* gene encoding cytosolic catalase T (Marchler *et al.*, 1993) and subsequently in control of expression of the *DDR2* gene, which encodes a putative chaperone protein (Kobayashi & McEntee, 1993). It has subsequently been demonstrated that the activation of the STRE element of inducible genes is dependent upon two zinc finger transcriptional activators (Msn2p and Msn4p) (Martinez-Pastor *et al.*, 1996; Schmitt & McEntee, 1996; Treger *et al.*, 1998),

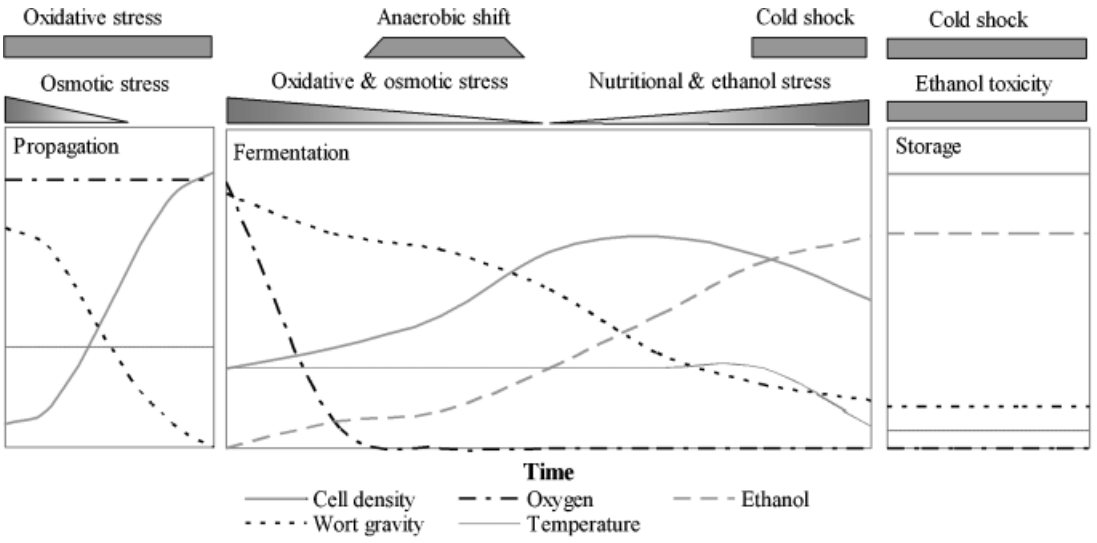

Fig. 1. Schematic representation of the temporal and sequential nature of potential stresses encountered by yeast during brewery propagation, fermentation and storage.

which are active during a wide array of stresses (Ruis & Schuller, 1995; Hohmann, 2002), including those associated with yeast handling in the industrial brewery and during diauxic shift (Boy-Marcotte *et al.*, 1998). This mode of activation is one reason why exposure to one type of stress often confers resistance to another, unrelated form of stress (Lindquist, 1986). The GSR is a transient phenomenon (Gasch *et al.*, 2000) and Msn2p is rapidly degraded following the stress response (Bose *et al.*, 2005).

Oxidative stress

While molecular oxygen (O_2) is necessary for the release of energy during aerobic respiration, derivative forms of O_2, termed reactive oxygen species (ROS) are produced endogenously by cells under aerobic conditions. These ROS include the superoxide radical ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($^{\bullet}OH$), which can damage cell components, contribute to cellular ageing and ultimately lead to cell death (Beckman & Ames, 1998). Specific effects include lipid peroxidation (Girotti, 1998), protein inactivation (Cabiscol *et al.*, 2000) and nucleic acid damage (Salmon *et al.*, 2004; Ribeiro *et al.*, 2006), including damage to mtDNA, which can lead to the generation of respiratory deficient 'petites' (O'Rourke *et al.*, 2002a, b; Doudican *et al.*, 2005; Gibson *et al.*, 2006). Oxygen's apparently contradictory roles within the cell, i.e. being essential for aerobic respiration and other metabolic processes, whilst being inherently toxic, have been referred to as the 'oxygen paradox' (Davies, 1995).

Oxygen and oxidative stress in the brewing process

Despite its potential toxicity, oxygen plays an essential role in the brewing process. A supply of oxygen is necessary during brewery propagation and early fermentation to generate yeast biomass and ensure that yeast is in optimum physiological condition for effective fermentation (Hammond, 2000; Hulse, 2003). Oxygen is required for lipid synthesis, which is necessary to maintain plasma membrane integrity and function, and consequently for cell replication (Hammond, 2000; Briggs *et al.*, 2004). Oxygen is required for the biosynthesis of both sterols and unsaturated fatty acids (Lorenz & Parks, 1991). However, overexposure of yeast to oxygen in the fermentation vessel can result in excessive yeast growth at the expense of ethanol production (Briggs *et al.*, 2004). Optimum oxygen levels are therefore necessary for successful beer production.

After fermentation is complete the yeast cells that have sedimented out of the wort are reused in subsequent fermentations. Serial repitching, a procedure unique to brewery fermentation, may have important implications for yeast physiological state and fermentation performance (Powell *et al.*, 2003). The free radical theory of ageing proposes that ROS produced under aerobic conditions are the primary cause of cellular ageing (Halliwell & Gutteridge, 1999). Oxidative stress may therefore have a role in the progressive deterioration of cells as they are serially repitched, particularly as the techniques employed to collect sedimented yeast from the fermentation vessel may potentially select yeast with a high proportion of aged cells (Powell *et al.*, 2000). The replicative lifespan of brewing yeast (determined by the number of times a cell is capable of producing daughter cells) is strain-dependent (Maskell *et al.*, 2001) and related to the antioxidant potential of the cell (Barker *et al.*, 1999; Van Zandycke *et al.*, 2002). It is likely that the number of pitches that a particular brewing yeast culture can tolerate is determined by its replicative lifespan, which may be determined by the strain's antioxidant potential and sensitivity to oxidative stress (Gibson *et al.*, 2006).

Cellular antioxidant defences

The importance of oxidative stress to the cell is illustrated by the number and diversity of antioxidant molecules that are synthesized by *S. cerevisiae* (Dawes, 2004). These antioxidant defences include the nonenzymatic molecules D-erythroascorbic acid, flavohaemoglobin, glutathione, metallothioneins, polyamines, ubiquinol, trehalose and ergosterol, and enzymatic defences such as catalase, cytochrome *c* peroxidase, superoxide dismutase, glutaredoxin, glutathione peroxidase, glutathione reductase, thioredoxin, thioredoxin peroxidase and thioredoxin reductase (Higgins *et al.*, 2003; Kwon *et al.*, 2003; Dawes, 2004; Saffi *et al.*, 2006). Antioxidants synthesized by brewing strains have been measured in both small-scale and industrial fermentations and results indicate that brewing yeast strains respond rapidly to oxidative stress. Clarkson *et al.* (1991) demonstrated that cellular Cu/Zn superoxide dismutase, Mn superoxide dismutase and catalase activities of brewing yeast strains changed rapidly with the addition or removal of O_2 from a semidefined wort medium during small-scale fermentation, indicating that enzymatic antioxidant activity is regulated by exposure to O_2. During industrial-scale propagation, catalase activity increases as propagation proceeds (Martin *et al.*, 2003). Whether this result is due directly to oxidative stress is debatable as catalase is an STRE-regulated protein and its synthesis may increase in response to a number of stress factors. Interestingly, Swan *et al.* (2003) observed that cellular levels of thioredoxin, a small, ubiquitous antioxidant protein, remained unchanged throughout an industrial fermentation, although extracellular concentrations increased as fermentation proceeded. It was also observed in laboratory-scale fermentations that the increase in extracellular thioredoxin concentration was greater with

higher wort dissolved oxygen concentrations (Swan *et al.*, 2003). Cellular glutathione concentrations vary during the brewing process (Gibson *et al.*, 2006) falling in the early stages of both propagation and fermentation before increasing steadily as each of these stages progresses. These results indicate that glutathione content of cells may be reduced during exponential growth, followed by an accumulation as cells respond to nutrient depletion and enter stationary phase (Gibson *et al.*, 2006). Whether glutathione is directly affected by O_2 during brewery fermentation is unknown and requires further investigation.

It has also been observed that the relative gene expression of several antioxidant-encoding genes is high at the beginning of pilot-scale fermentations. Expression of antioxidant-encoding genes may be up to 53-fold greater in the first hour of fermentation relative to their activities after 23 h, a response attributed to oxidative stress (Higgins *et al.*, 2003), but which may also be related to the transition from stationary to exponential phase growth. A second study revealed a strong up-regulation of oxidative stress response genes in the later stages of a small-scale fermentation, in this case attributed to the effect of residual ROS within cells or these genes responding to other stresses during fermentation (James *et al.*, 2003). As well as their role in the oxidative stress response, antioxidants have been implicated in the protection of cells exposed to reducing environments (Rand & Grant, 2006) and ethanol stress (Costa *et al.*, 1993, 1997; Pereira *et al.*, 2001), both of which may be encountered by cells in the later stages of an industrial brewery fermentation. Furthermore, a number of antioxidant encoding genes, including *CTT1*, contain the STRE promoter sequence and are involved in the GSR (Costa & Moradas-Ferreira, 2001). Another possibility is that the change in antioxidant activity is related to the nutritional status of the wort, with transcription of antioxidant-encoding genes being up-regulated when glucose and other fermentable carbon sources are exhausted (Costa & Moradas-Ferreira, 2001).

It should also be noted that the responsiveness of brewing yeast strains to changes in oxygen concentration is likely to be strain-dependent. Of the two strains used in a study of antioxidant enzyme activity changes in response to O_2 concentration, it was found that while catalase activity of one strain was reduced immediately after O_2 removal, a change in activity in a second strain was not affected until 30 h later (Clarkson *et al.*, 1991). Likewise, the response to exogenous ROS is strain-dependent, with sensitivities of individual brewing strains being associated with cellular catalase activity and glutathione concentration (Gibson *et al.*, 2006). Sensitivity to oxidative stress has also been associated with growth phase and medium composition, both of which influence antioxidant levels and hence the resistance of individual strains (Gibson *et al.*, 2006).

Trehalose and oxidative stress

Trehalose is an important stress protectant conferring stability to the plasma membrane (Mansure *et al.*, 1994) and enzymes (Sola-Penna & Meyer-Fernandes, 1998) as well as ensuring the proper folding (Singer & Lindquist, 1998) and repair (Simola *et al.*, 2000) of proteins; and additionally acting as a carbon source during starvation (Fales, 1951; Eaton, 1960; Chester, 1963; Panek, 1963). The level of trehalose is thought to be related to stress tolerance and adaptation (Hottiger *et al.*, 1987a, b; Van Laere, 1989; D'Amore *et al.*, 1991; Majara *et al.*, 1996a, b; Conlin & Nelson, 2007). Trehalose is accumulated in response to heat shock (Hottiger *et al.*, 1987a, b; Iwahashi *et al.*, 1995; Majara *et al.*, 1996b), exposure to toxic chemicals (Attfield, 1987), ethanol stress (Eleutherio *et al.*, 1993; Mansure *et al.*, 1994; Majara *et al.*, 1996b) and osmotic stress (MacKenzie *et al.*, 1988; Majara *et al.*, 1996a).

Trehalose has also been implicated in the protection of yeast cells and cellular components against ROS. Increased cellular accumulation of trehalose has been associated with increased resistance to oxidative stress in the presence of a hydroxyl radical-generating system (H_2O_2/FeCl) (Benaroudj *et al.*, 2001) and the superoxide-generating drug menadione (Herdeiro *et al.*, 2006), though not to the organic peroxide *tert*-butyl hydroperoxide (Herdeiro *et al.*, 2006). This increased resistance may be due to protection against protein carbonylation (Benaroudj *et al.*, 2001) and/or lipid peroxidation (Herdeiro *et al.*, 2006). The genes involved in trehalose synthesis (*TPS1*, *TPS2*, *TSL1*, *TPS3*) and degradation (*NTH1*, *NTH2*, *ATH1*) (Zähringer *et al.*, 2000; for a review see François & Parrou, 2001) are regulated by STRE elements (Winderickx *et al.*, 1996) and are up-regulated in response to various stresses, including oxidative stress (Parrou *et al.*, 1997; Pedreño *et al.*, 2002). Strains lacking the transcriptional activators Msn2p and Msn4p are unable to accumulate trehalose in response to stress (Parrou *et al.*, 1997). An increase in the transcription of genes encoding enzymes involved in trehalose biodegradation, as well as synthesis, on exposure to stress, indicates that rapid trehalose turnover rather than accumulation may be the factor governing cellular protection against oxidative stress in yeast (Parrou *et al.*, 1997; Pedreño *et al.*, 2002). Trehalose accumulation in response to stress is a transient phenomenon (Parrou *et al.*, 1997). The rapid degradation of trehalose may be essential for the resumption of normal cellular activity as trehalose is known to inhibit the activity of essential enzymes, including glutathione reductase, which is involved in maintaining cellular homeostasis and reducing oxidative damage within the cell (Sebollela *et al.*, 2004). This hypothesis is supported by evidence that a mutant strain (Δ*nth1*) of *S. cerevisiae* unable to synthesize the cytoplasmic neutral trehalase enzyme was found to be sensitive to H_2O_2

exposure, despite the greater accumulation of trehalose in this strain (Pedreño *et al.*, 2002). In addition, Pereira *et al.* (2001) found that the Δ*tps1* mutant of *S. cerevisiae*, which has little appreciable trehalose-synthesizing ability, was still capable of acquiring tolerance to H_2O_2 stress, presumably due to an accumulation of antioxidants and/or heat shock proteins.

Mitigation of oxidative stress

While oxygen cannot be eliminated from the brewing process, it may be possible to minimize its use without any detrimental effect on fermentation performance. Yeast propagation typically involves continuous aeration or oxygenation of yeast but it has been suggested that a relatively short aeration period may be sufficient. Maemura *et al.* (1998) found that the performance of yeast during small-scale wort fermentation was unimpaired when yeast had been propagated with only limited aeration (1 h at the beginning of propagation) compared with yeast exposed to a continuous supply of air. Little difference was seen in terms of cell density, carbohydrate reserves or unsaturated fatty acid (UFA) level (Maemura *et al.*, 1998).

The presence of O_2 in wort at the beginning of fermentation allows yeast cells to synthesize lipids, thereby revitalizing the sterol-deficient cell population and ensuring that fermentation can proceed efficiently. An alternative approach involves oxygenation of the stored yeast prior to pitching, thereby reducing the O_2 concentration necessary in the fermentation wort (Boulton *et al.*, 2000; Depraetere *et al.*, 2003). In this case, UFA synthesis occurs prior to pitching and the pitched yeast, being sterol-replete, has a reduced requirement for wort oxygenation. Trials have found that preoxygenated yeast in unoxygenated wort performs as well as normal yeast in oxygenated wort in terms of fermentation profile, ester synthesis and alcohol production; the only apparent difference in this investigation was a reduced yeast growth in the unoxygenated wort (Boulton *et al.*, 2000). While yeasts are still exposed to O_2, exposure is more readily controlled in the storage vessels than in larger, industrial-scale fermentation vessels and the use of excess O_2 can be avoided (Boulton *et al.*, 2000). The potential reduction in fermentation rate caused by the reduced cell density may be overcome by adjusting the pitching rate (Boulton *et al.*, 2000). The reduction in cell growth in that investigation may have been due to excessive O_2 consumption, which can result in depletion of the reserve carbohydrate trehalose. Optimum aeration of yeast prior to pitching has been shown to increase cell growth in unoxygenated wort (Fujiwara & Tamai, 2003).

It has also been suggested that the cellular requirement for O_2 can be reduced by supplementation of stored yeast or wort with sterol (David & Kirsop, 1972), lipids derived from spent grains (Taylor *et al.*, 1979) or UFA (Moonjai *et al.*, 2003). Moonjai *et al.* (2003) have, for example, proposed the use of linoleic acid supplements as an alternative to wort oxygenation and demonstrated that preconditioning yeast in this fashion removed the requirement for wort oxygenation. Viability and fermentation performance of supplemented cells in nonaerated wort were similar to those of unsupplemented cells in aerated wort (Moonjai *et al.*, 2003). Consequently, such supplementations may have potential in industrial fermentations by obviating the requirement for O_2, thereby mitigating the effect of oxidative stress to yeast cells. It should, however, be noted that the β-oxidation of fatty acids within peroxisomes of yeast cells can generate ROS such as H_2O_2. Koerkamp *et al.* (2002) detected an oxidative stress response in yeast cells in chemostat cultures when the carbon source within the growth medium was switched from glucose to the fatty acid oleate.

Osmotic stress

Osmotic stress can be defined as any situation where there is an imbalance of intracellular and extracellular osmolarities, sufficient to cause a deleterious change in physiology (Csonka & Hanson, 1991). In natural environments, yeasts are continuously subjected to changes in external osmolarity that can be extremely detrimental to cellular functioning (Hounsa *et al.*, 1998; Beney *et al.*, 2000; Tamás & Hohmann, 2003). Indeed, osmotic stress may occur when there is a low external osmotic potential, for example in deionized water, and this is characterized by an influx of water into the cell resulting in hypo-osmotic stress (Csonka & Hanson, 1991; Dihazi *et al.*, 2001). Conversely, osmotic stress may also result from exposure to environments comprising high solute concentrations leading to hyperosmotic stress characterized by the loss of cellular water and subsequently turgor (Blomberg & Adler, 1992; Wood, 1999; Klipp *et al.*, 2005).

Osmotic stress and brewing

During the brewing process, there are two principal sources of osmotic stress. The first involves the practice of acid washing that is employed by some brewers to remove bacterial contaminants (Hammond *et al.*, 2001) and/or to fluidize yeast slurries so that dispersal is more efficient following inoculation into the fermentation vessel. Acid washing regimes involve the submersion of yeast slurries in food-grade acids to achieve a pH within the range 2.2–2.4, which may impart osmotic stress due to the abundance of dissociated H^+ ions. The second, and by far the most important, source of osmotic stress involves the process step in which the yeast is inoculated into wort, a complex and highly concentrated medium containing high concentrations of sugars (Briggs *et al.*, 2004). The use of high-gravity

(or higher solute) worts (Panchal & Stewart, 1980) has been suggested to increase external osmotic pressure resulting in a deterioration of viability, growth and fermentation performance (D'Amore, 1992). In support of this Cahill *et al.* (2000) have demonstrated that an increase in gravity of the propagation medium results in decreased viabilities in subsequent high-gravity [17.5°P (17.5 g of extract per 100 mL liquid)] fermentations. More recently, Dumont *et al.* (2003) suggested that hyperosmotic stress may reduce the loss of yeast viability incurred in response to ultrarapid cooling rates during freezing as a consequence of the reduction in intracellular water. The impact of hyperosmotic stress on resistance to brewing yeast tolerance to cold shock has not been previously investigated but it is suggested that the negative impacts of the former would outweigh the positive effects of the latter during temperature downshift and maintenance.

Osmotolerance and osmoadaption

Osmoregulation in yeast is dependent on the capacity to sense external stimuli and the resultant changes in physiology, biochemistry and other cellular functions to meet the modified needs of the cell in light of that environmental change. There are two forms of 'response' which any cell may apply following exposure to osmotic challenge: osmotolerance and osmoadaption.

Osmotolerance may be defined as the innate physiological resistance to osmotic stress in response to both acute and chronic environmental hyperosmotic pressure and represents the mechanism by which cells maintain viability in the presence of deleterious solute concentrations. The intrinsic osmotolerance of brewing yeast strains has received little attention despite the recognition of this form of stress within the process. Recent results from our laboratory suggest that brewing yeast osmotolerance is strain-dependent (P. White and K.A. Smart, unpublished data); furthermore, stationary phase populations of ale and lager yeast were observed to be consistently more osmotolerant than their exponential phase counterparts when exposed to osmotic stress (P. White & K.A. Smart, in preparation) supporting previous reports concerning the reduced sensitivity of stationary-phase populations of laboratory haploid strains to this and other forms of physiological stress (Werner-Washburne *et al.*, 1993; Hounsa *et al.*, 1998; Gasch & Werner-Washburne, 2002).

Enhanced tolerance to hyperosmotic stress is derived from an innate ability to withstand the deleterious effects of hyperosmotic pressure as a consequence of intrinsic factors such as 'superior' membrane structure (Sharma *et al.*, 1996), vacuolar functioning (Latterich & Watson, 1993; Nass & Rao, 1999) and residual trehalose levels (Singer & Lindquist, 1998). Indeed, osmotolerance is promoted by the abundance of osmoprotectant macromolecules that stabilize cellular membranes, enzymes, other proteins and possibly nucleic acids, with little effect on the intracellular water potential (Hernández-Saavedra *et al.*, 1995).

In contrast, osmoadaption represents a highly refined sensing and response system that may also be activated, and for *S. cerevisiae* this takes the form of an acute or chronic response. Nass & Rao (1999) define the chronic response (or acquired osmotolerance) as a signal transduction-mediated pathway that alters the levels of specific proteins. Conversely, the acute response is invoked when nonosmotically stressed cells are suddenly exposed to high external osmolarity and survival is determined by intrinsic physiological characteristics (Nass & Rao, 1999).

Osmoadaption represents the processes by which cells adjust their normal physiology in order to survive (if not to proliferate) in conditions of adverse water potential (Poolman & Glaasker, 1998). In many instances this will involve the accumulation of one or more types of molecule, termed 'osmoticum' (pl = 'osmotica'), within the cell in order to increase intracellular osmotic potential, and thus prevent cellular water loss (Yancey *et al.*, 1982; Wegmann, 1986; Blomberg & Adler, 1992; Hernández-saavedra *et al.*, 1995). Osmotica can be further categorized according to their specific effects upon cellular physiology; one subclass of osmotica, the compatible solutes, have very little effect on normal cellular functioning when accumulated at high levels (Poolman & Glaasker, 1998) and their role in stabilizing the cell during hyperosmotic stress is crucial to the cell.

Compatible solutes and the circumvention of osmotic stress

The major benefits of accumulating compatible solutes appear to be twofold: first, these molecules are able to effect an increase in intracellular osmotic potential without the need for the production of stabilizing proteins within the cell (Yancey *et al.*, 1982) and, secondly, they can be readily assimilated following the removal of the osmotic stress (Kempf & Bremer, 1998).

In *S. cerevisiae* the polyhydric alcohol glycerol is the compatable solute accumulated during osmotic stress (Brown, 1978; Brown *et al.*, 1986, Blomberg & Adler, 1989, 1992, Albertyn *et al.*, 1994; Hohmann, 1997); indeed, mutants deficient in enzymes of the glycerol biosynthetic pathway are unable to survive hyperosmotic conditions (Albertyn *et al.*, 1994; Eriksson *et al.*, 1995; Liden *et al.*, 1996; Ansell *et al.*, 1997; Hounsa *et al.*, 1998). Furthermore, glycerol has an important secondary role to play in anaerobic stress as the requirement of NAD^+ in its production enables glycerol to serve as the final product in a 'redox dump' pathway (Ansell *et al.*, 1997) and this is relevant to brewing yeast fermentations.

As might be expected, high levels of intracellular glycerol appear to confer the capacity to withstand high extracellular osmolarity; glycerol has also been demonstrated to be quickly accumulated following exposure to unfavourably high osmotic potential (Albertyn *et al.*, 1994; Norbeck & Blomberg, 1996). An increase in osmotic stress resistance can therefore be correlated with accumulation of intracellular glycerol in haploid yeast strains (Albertyn *et al.*, 1994; Eriksson *et al.*, 1995; Liden *et al.*, 1996; Ansell *et al.*, 1997; Hounsa *et al.*, 1998) as well as ale and lager strains (P. White & K.A. Smart, unpublished data).

Cells subjected to high external osmolarity are able to sense this external stimulus effectively and respond by activation of the high-osmolarity glycerol (HOG) [mitogen-activated protein (MAP) kinase] pathway (for reviews see Hohmann, 2002; O'Rourke *et al.*, 2002a, b; Saito & Tatebayashi, 2004). The effects of the initiation of the HOG pathway are all designed to promote survival of the organism during a period of physiological stress. The HOG pathway is required for the activation of the Msn2p and Msn4p, suggesting that Hog1p may have a role in regulating the GSR via STREs (Schüller *et al.*, 1994).

The induction of the HOG pathway ultimately results in stimulating the hyperproduction and hyperaccumulation of glycerol as a compatible solute in order to balance the external and internal osmolarities. The HOG pathway also appears to regulate the activity of the glyceroaquaporin Fps1p (Luyten *et al.*, 1994; Tamás *et al.*, 1999; Karlgren *et al.*, 2005), which reduces the net efflux of glycerol and thus aids intracellular accumulation.

Osmoadaptation and yeast organelles

It has been previously shown that despite the rigid cell wall, yeast cells show a change in cell size concurrent with external osmolarity changes (Hohmann, 1997). An increase in external osmolarity results in a rapid loss of intracellular water and thus cell shrinkage (Morris *et al.*, 1986; Blomberg & Adler, 1992; Hohmann, 1997). Restoration of favourable (isotonic) solute conditions to hyperosmotically stressed cells does not however result in cells regaining their former volume (Hohmann, 1997). Conversely, when cells are exposed to low external solute concentrations, the net influx causes a relative increase in cell size. Despite recognition that the cell wall is essential for osmotolerance due to its rigidity, its role with respect to sensing and osmoadaption has only recently received significant attention. For example, this organelle is intimately involved in the HOG 1 MAP kinase pathway (Alonso-Monge *et al.*, 2001; Klis *et al.*, 2002; Levin, 2005) and Wsc1p, which is involved in the reorganization of the actin cytoskeleton during osmotic stress, resides in the cell wall (Gualtieri *et al.*, 2004). Furthermore remodelling of the cell wall in response to changes in cellular turgor has been suggested to represent one potential form of osmoadaptation (García-Rodríguez *et al.*, 2005), though this has not been investigated in the brewing context. The main reason for this is that multiple effectors can modify yeast cell size including progression through the cell cycle, yeast cell age (Barker & Smart, 1996) and even process parameters. For instance, freshly propagated yeast are generally reduced in size when compared with yeast in the fermentation vessel (C.A. Boulton, pers. commun.).

Aquaporins are transmembrane proteins which facilitate the movement of water across the plasma membrane (Agre *et al.*, 1998), and represent the largest group in the major intrinsic protein (MIP) family of integral transmembrane proteins (Laizé *et al.*, 2000), which also include the plasma membrane intrinsic proteins (PIPs) and the tonoplast intrinsic proteins (TIPs) in yeast. Aquaporins mediate rapid fluxes in water (and solute) balance within cells and vacuoles (Borginia *et al.*, 1999; Laizé *et al.*, 2000) and consequently have important roles in water, ionic, solute and hence osmotic balance. There is a high degree of genetic variability and polymorphism within the aquaporin genes *AQY1* and *AQY2* in *S. cerevisiae* (Bonhivers *et al.*, 1998; Laizé *et al.*, 2000). In production brewing yeast, the aquaporin *AQY2* appears to be missing or nonfunctional leaving only *AQY1* to mediate water fluxes (P. White & K.A. Smart, unpublished data). In contrast Tanghe *et al.* (2002) have correlated *AQY1* expression to the ability of industrial bakers' yeast to withstand freezing, by facilitating rapid efflux of water, a preventative measure against ice crystal formation (Tanghe *et al.*, 2002).

The presence or absence of these aquaporin channels in the *S. cerevisiae* plasma membrane has been demonstrated by Carbrey *et al.* (2001) to influence cell surface properties including flocculence and hydrophobicity. This observation is interesting and, of course, directly relevant to brewing, since flocculation is required for rapid yeast sedimentation towards the end of the fermentation, permitting beer clarification and the accumulation of yeast 'crop' in the base of the vessel for subsequent collection. It is tempting to suggest that aquaporins may play a role in cell-wall remodelling in response to solute availability, which might explain the modifications in cell surface hydrophobicity, charge and flocculation observed in brewing yeast during starvation (Smart *et al.*, 1995; Rhymes & Smart, 1996) and the impact this has on osmotolerance and cell-wall remodelling during osmotic stress remains to be investigated.

In addition to the outer cell envelope, the vacuole has been suggested to play a role in osmoregulation (Chiang, 1995; Klionsky, 1998). Brewing yeast vacuolar morphology may be modified in response to shifts in the osmotic potential of the environment (Bone *et al.*, 1998; Nass & Rao, 1999; Bonangelino *et al.*, 2002), though more recent evidence suggests that these changes, viewed in the first hours of fermentation, may also be attributed to vacuole

morphological changes associated with progression through the cell cycle (P. White & K.A. Smart, unpublished data). Indeed, this organelle is prone to flux between fragmented and nonfragmented forms during the cell cycle and under stress conditions (Schwencke, 1977, 1991; Guthrie & Wickner, 1988; Spormann *et al.*, 1992; Wonisch *et al.*, 2001). The dynamic nature of the vacuole is, in part, due to its association with other cellular organelles. Indeed, the vacuole, endoplasmic reticulum, Golgi complex, secretory and endocytic vesicles are all interrelated, as they are components of interconnecting branched systems (Schwencke, 1991).

pH downshift

Wort acidification during fermentation

Wort pH during a typical lager fermentation is reduced from 5.5 to *c.* 4.1 (Coote & Kirsop, 1976; Rowe *et al.*, 1994). This decrease in pH occurs as a result of carbonic acid production from CO_2, secretion of organic acids and the consumption of buffering compounds (basic amino acids and primary phosphates) in the wort (Coote & Kirsop, 1976). Coote & Kirsop (1976) found a similar buffering capacity in wort and beer and calculated that the removal of buffering materials and release of organic acids are insufficient to account for the magnitude of the pH drop typically observed during fermentation. They concluded that the secretion of H^+ by yeast accounts for the discrepancy.

pH can have a sizeable impact on the production of flavour components by yeast. A shift in wort pH during brewery fermentation from pH 5.75 to 5.46 can result in a reduction in dimethyl sulfide production (up to 50%) (Anness & Bamforth, 1982). Furthermore, the rate of conversion of α-acetolactate to the detrimental butterscotch flavour diacetyl was increased by a factor of four when wort pH was lowered from pH 5.5 to 4.0 (Haukeli & Lie, 1978).

The final pH is dependent upon the buffering capacity of the wort, initial wort pH and the extent of yeast growth (Narziss *et al.*, 1983; Heggart *et al.*, 1999). Although it has been suggested that brewing yeast readily tolerate the 1.5–2.0 U downshift in pH, which occurs during fermentation (Boulton & Quain, 2001), recent evidence suggests that brewing lager yeast strains demonstrate some sensitivity to changes in this parameter, manifesting in modified specific growth rates and reduced replicative lifespans (Maskell, 2003).

pH downshifts of a magnitude observed during a typical lager fermentation are known to cause a sizeable change in gene expression. Haploid strains of *S. cerevisiae* experiencing a shift from pH 5.5 to 3.5 in minimal media demonstrate an increase (1.4-fold or greater) in the expression of 36 genes, including four that are cell wall-related (*CWP1*, *HOR7*, *SPI1*, *YGP1*) (Kapteyn *et al.*, 2001). Other groups of genes that were induced are involved in carbohydrate metabolism (*GCY1*, *GPD1*, *GPP2/HOR2*, *GRE3*, *HXK1*, *HXT6*, *SOL4*, *TPS1*, *TSL1*), redox metabolism (*ALD2*, *ALD3*, *MCR1*, *YER053C*, *YNL134C*), control of gene expression (*CUP1*), nuclear pore transport (*GSP2*), protection against various stress conditions (*CTT1*, *DDR2*, *GRE2*, *HSP12*, *HSP42*, *YDL204W*, *YDR453C*, *YML128C*) or encode proteins of unknown function (*YDR070C*, *YDR533C*, *YGL037C*, *YGR043C*, *YHR056C*, *YHR087W*, *YIL169C*) (Kapteyn *et al.*, 2001). Despite this knowledge, the impact of pH downshift during fermentation on brewing yeast stress responses is not known. This omission is perhaps a consequence of the extreme pH downshift that occurs during acid washing, which, in contrast, has been the subject of some debate in the industry.

Acid washing

During brewery handling, yeast may be exposed to exceptionally low pH during acid washing, which is often required to eliminate the occurrence of contaminating bacteria. When the yeast biomass is exposed to low pH during acid washing, several physiological effects are apparent. Some breweries have noted that the performance of the yeast was stimulated in the fermentation immediately after the acid wash (Brown, 1916; Jackson, 1988; Russell & Stewart, 1995), others suggest that it may be diminished (Bruch *et al.*, 1964; Roessler, 1968). However, more recent studies by Simpson & Hammond (1989) and Cunningham & Stewart (1998) suggest that there are no significant differences in the fermentation profiles between washed and unwashed cells when the guidelines on acid washing duration and conditions were followed (Simpson & Hammond, 1989; Cunningham & Stewart, 1998). Despite this, there is little doubt that the low pH conditions associated with acid washing affect cellular physiology: proteins involved in plasma membrane integrity may be susceptible to denaturation, particularly in the presence of ethanol (Jones, 1988; Simpson & Hammond, 1989); a reduction in viability (Casey & Ingledew, 1983; Cunningham & Stewart, 1998) or fermentation performance (Fernandez *et al.*, 1993; Cunningham & Stewart, 1998) may occur and newly formed daughter cells tend to exhibit poor survival rates (D.L. Maskell & K.A. Smart, unpublished data).

The addition of diethylstilbestrol during acid washing dramatically reduces yeast viability (Uchida *et al.*, 1988). Diethylstilbestrol is an inhibitor of plasma membrane H^+-ATPase and it has therefore been suggested that yeast death was most probably due to intracellular acidification as a consequence of the inability of the cell to release protons to maintain its intracellular pH. Evidence to support the hypothesis that plasma membrane H^+ATPase is essential for survival during acid washing comes from the studies showing that plasma membrane H^+ATPase is stimulated by low pH (Eraso & Gancedo, 1987; Carmello *et al.*, 1996). Carmello *et al.* (1996) observed that *S. cerevisiae* cells grown

in media with a low initial pH of 2.5 exhibited relatively low H^+ATPase activity compared with cells grown at an optimal initial pH. This may indicate that stimulation of H^+ATPase activity at low pH may only occur with short-term exposure. There have been no reports on the effect of phosphoric acid on the plasma membrane H^+ATPase, but it is probable that acid washing is a practice that can be highly damaging to plasma membrane functionality if not applied with due care.

Anaerobic shift

Within a fermentation vessel, initial oxygen content is around 8–12 $\mu g\,mL^{-1}$, or higher depending on wort gravity, but is rapidly depleted within the first few hours after pitching, leading to an anaerobic environment (Boulton & Quain, 2001) and the accumulation of CO_2. On completion of wort fermentation, yeast is transferred to a storage vessel and held under anaerobic conditions. As a consequence, brewing yeast is predominantly exposed to anaerobic environments in the brewery, but with intermittent periods of exposure to high O_2 concentrations. In addition, exogenous CO_2 can be used as a controlling agent in the production of alcoholic beverages by the brewing industry (Hoggan, 1980). The fermentation rate, the rate and extent of yeast growth, and the final concentration of fusel oils are all decreased by increasing CO_2 pressure, whilst the final pH is decreased (Kunkee & Ough, 1966; Jones & Greenfield, 1982; Arcay-Ledezma & Slaughter, 1984). As a consequence, the final concentration of many flavour compounds and esters may be decreased by increasing the CO_2 pressure during fermentation (Drost, 1977). Although its impact has not been extensively elucidated, anaerobiosis influences several cellular functions in yeast including modifications in cell division (Norton & Krauss, 1972), cell volume (Lumsden *et al.*, 1987), gene expression (Abramova *et al.*, 2001a; James *et al.*, 2003), metabolism (Stewart *et al.*, 1983; Hammond, 1993; Zheng *et al.*, 1994; Lewis & Young, 1995), amino acid uptake (Slaughter *et al.*, 1987) and the cell wall (Abramova *et al.*, 2001a; James *et al.*, 2003).

Stress associated with the anaerobic shift

Increases in the partial pressure of CO_2 (pCO_2) result in decreases in the cell yield and growth rate of *Saccharomyces* yeast in beer fermentation (Nakatani *et al.*, 1984; Knatchbull & Slaughter, 1987), although little change in fermentation activity due to pCO_2 change was observed. Norton & Krauss (1972) showed that cell growth stopped at 280 kPa of CO_2 pressure metabolically produced during ethanol fermentation, but a culture pressurized at 280 kPa using N_2 gas did not reduce the cell growth rate (Norton & Krauss, 1972). Inhibition of budding and cell division by *S. cerevisiae* is therefore a result of increased CO_2 concentration rather than pressure. Although cell division ceases, doubling of the DNA content of the cells still occurs, indicating that the inhibition of cell division is not due to a lack of DNA replication (Norton & Krauss, 1972). Despite the increase in DNA content of cells, the amount of RNA and protein per cell decreases (Lumsden *et al.*, 1987). Lumsden *et al.* (1987) also found that after 1 h at elevated CO_2 pressures, the mean cell volume of *S. cerevisiae* increased. This indicated that the influence of CO_2 upon cell characteristics may be associated with a change in cell size (Lumsden *et al.*, 1987). Under a CO_2 pressure of 198 kPa, fermentation of malt extract medium by *S. cerevisiae* results in a changed pattern of absorption of amino acids in the first 4 h, with a general excretion of amino acids thereafter (Slaughter *et al.*, 1987).

Transcriptome remodelling in response to anaerobiosis

While the shift from an aerobic to anaerobic environment may not pose an immediate threat to the yeast cell compared with other forms of stress, the loss of oxygen will necessitate a significant change in cellular metabolism. In an investigation of the transcriptomic response of *S. cerevisiae* to anaerobic shift, Lai *et al.* (2005) found that 938 genes showed a significant change in expression after O_2 depletion, with a transient change in expression occurring shortly after O_2 depletion and a chronic change in expression occurring after 45 min, presumably as a metabolic adaptation to anaerobiosis. These changes were observed when the cells had been growing on the poorly fermentable carbohydrate galactose. The loss of O_2 therefore resulted in a change from mixed respiro-fermentative to purely fermentative metabolism. Interestingly, the response to anaerobiosis was similar in many ways to the GSR. A down-regulation of rRNA processing genes, DNA transcription and repair genes occurred along with an up-regulation of Msn2/4-regulated genes involved in the synthesis of reserve carbohydrates and energy metabolism. It is likely that the observed changes allowed the cell to transiently suspend growth while modifying its metabolism to cope with the lack of O_2 and the inability to respire. This adaptation to anaerobic conditions was much delayed in an *MSN*2/4 mutant strain. The same investigation carried out using cells grown with glucose revealed a less dramatic remodelling of the transcriptome, with only 352 genes displaying a significant change in expression. This is most likely due to the fact that cellular metabolism is fermentative in the presence of glucose, regardless of the presence/absence of O_2, and a major retooling of metabolism is consequently not required when O_2 is lost from the system (Lai *et al.*, 2005). This latter case is more relevant to brewery fermentations where the anaerobic shift occurs while the fermentable carbohydrates glucose and fructose are present in the wort (Briggs *et al.*, 2004) and

a shift from respiro-fermentative to strictly fermentative metabolism is not expected.

ter Linde *et al.* (1999) and James *et al.* (2003) studied the transcription profiles of yeast during anaerobic incubation in chemostat culture and tall tube fermentations, respectively. During anaerobiosis, a number of genes, previously shown to respond to hypoxic conditions, were induced. These include *ERG11*, *NCP1*, *AAC3*, *COX5*, *HEM13*, *OLE1* and the *PAU* gene family (Rachidi *et al.*, 2000) which encodes the seripauperin proteins. Interestingly, 13 ORFs of unknown function, demonstrating homology to the *PAU* genes, are also induced under these fermentation conditions. Members of the anaerobiosis-inducible mannoprotein *DAN*/*TIR* family are also highly induced under these conditions. Additionally, transcript levels for the hypoxic gene *AAC3*, which is transcribed optimally under anoxic or microaerophilic conditions, were also up-regulated.

Under anaerobic conditions, cells cannot synthesize UFAs and sterols (Lorenz & Parks, 1991) and an exogenous source of UFA and sterol is essential for long-term anaerobic growth in *S. cerevisiae* (Andreasen & Stier, 1953, 1954). Lai *et al.* (2005) showed that transcriptomic remodelling during anaerobiosis is fairly specific for sterol and sphingolipid pathways, with very few genes specific for phospholipid or fatty acid synthesis (apart from *OLE1* and *AAC1*) showing changes in expression. Genes in the early portion of the sterol synthesis pathway exhibit complex expression patterns, with some showing transient (*ERG10*) or chronic up-regulation (*IDI1* and *HMG2*) and others showing transient (*ERG8* and *MVD1*) or chronic down-regulation (*ERG13*, *ERG20* and *HMG1*). Nearly all the genes in the later portion of the pathway were chronically up-regulated, as were genes involved in transport (*PDR11* and *AUS1*) and their primary regulator (*UPC2*).

Anaerobiosis and the yeast cell wall

The yeast cell wall represents a dynamic organelle which modifies composition and functionality in response to external stimuli (Klis, 1994; Cabib *et al.*, 1997; Heleen *et al.*, 1998; Abramova *et al.*, 2001a; Rhymes & Smart, 2001; Smart, 2003; Boorsma *et al.*, 2004; Lesage & Bussey, 2006). Under aerobic growth conditions, Cwp1p and Cwp2p, encoded by the genes *CWP1* and *CWP2*, respectively, are the most abundant cell wall mannoproteins and are involved in cell wall biosynthesis during cell replication (van der Vaart *et al.*, 1995). During environmental stress the composition of the cell changes resulting in the increase in cell wall mannoproteins other than Cwp1p and Cwp2p (Abramova *et al.*, 2001a). The most extensive response is the induction of several homologous mannoproteins (Dan1p, Tip1p, Tir1p and Tir2p) during anaerobic growth. The genes encoding these proteins are referred to as the *DAN*/*TIR* genes. This group of genes also includes genes for five other mannoproteins, designated Dan2p, Dan3p, Dan4p, Tir3p and Tir4p. The Tir1p, Tir3p and Tir4p proteins are required for anaerobic growth, with knockout yeast becoming arrested at G_1 in anaerobic conditions (Abramova *et al.*, 2001a).

During N_2-induced anaerobic adaptation, the *CWP1* and *CWP2* genes are down-regulated and the *DAN*/*TIR* genes are up-regulated allowing an extensive remodelling of the yeast cell wall (Abramova *et al.*, 2001a). The *DAN* genes, *DAN1* and *DAN4*, are expressed within 1 h of a N_2-induced anaerobic shift, and *DAN2* and *DAN3* are expressed after *c.* 3 h (Abramova *et al.*, 2001b). No functions associated with anaerobiosis have been assigned to the products of *DAN2*, *DAN3* or *DAN4*, despite their responsiveness to this condition. The remodelling of the cell wall is important in the brewing context as it has been demonstrated that the flocculation potential of brewing yeast strains is altered by growth in aerobic and anaerobic conditions (Lawrence & Smart, in press). The modified expression of these genes may alter cell wall composition and structure, favouring lectin–receptor interactions (Smart, 2003).

Ethanol toxicity

The primary purpose of brewery fermentation is the synthesis of alcohol from fermentable sugars and the production of particular flavour-active compounds by *Saccharomyces* yeast. However, an important product of fermentation is a suitably viable and vital population of yeast that can be used in subsequent fermentations (Stewart, 2001). Production of the desired end-product should therefore not be at the expense of yeast quality. As fermentation progresses, the ethanol concentration of wort increases and cells are exposed to increasingly toxic levels of ethanol (Briggs *et al.*, 2004). Under normal fermentation conditions final ethanol concentrations fall in the range of 3–6%, though under high-gravity fermentation this concentration may be $>$ 10% (Briggs *et al.*, 2004).

Occurrence and cellular targets

The effects of ethanol toxicity on yeast physiology are diverse, though cellular membranes appear to be the main sites of ethanol damage. Specific effects include growth inhibition, reduced cell size (Canetta *et al.*, 2006), reduced viability, reduced respiration and glucose uptake (Pascual *et al.*, 1988), reduced fermentation (Fernandes *et al.*, 1997), enzyme inactivation, lipid modification, loss of proton motive force across the plasma membrane (Petrov & Okorokov, 1990; Mizoguchi & Hara, 1997) and increased membrane permeability (Marza *et al.*, 2002), lowering of cytoplasmic pH and the induction of respiratory-deficient mutants (Jiménez *et al.*, 1988; Ibeas & Jiménez, 1997; Chi & Arneborg, 1999).

High-gravity brewing has been adopted as standard practice in many modern breweries due to improved productivity and efficiency and reduced labour, energy and capital costs (Casey *et al.*, 1984). High-gravity brewing involves a higher initial sugar concentration in wort, with values of 18°P being typical. Such worts will produce a final product containing *c.* 7.5% ethanol (v/v), which can then be diluted to the required volume before packaging. Beers produced through high-gravity fermentation have also been found to have a greater flavour stability and consistency than those produced using normal gravity brewing (McCaig *et al.*, 1992). This improved productivity may however be at the expense of yeast physiological state and hence performance in subsequent fermentations. Ethanol concentrations in beer may feasibly be as high as 16% (v/v) at the end of high-gravity fermentation (Casey *et al.*, 1984), far greater than the 3–6% (v/v) achieved during standard 12°P wort fermentations (Briggs *et al.*, 2004). Yeast cells are therefore exposed to potentially toxic levels of ethanol during high-gravity brewing, which has implications for fermentation performance. High concentrations of endogenously produced ethanol during fermentation are associated with reduced cell viability, growth rate and fermentation rate (Nagodawithana & Steinkraus, 1976; Kalmokoff & Ingledew, 1985; Dombek & Ingram, 1986; Cahill *et al.*, 2000; Stewart, 2001). It should, however, be noted that loss of cell viability may be related to other factors such as fermentation temperature and, in particular, nutrient status of the wort (discussed below).

Ethanol toxicity and cellular membranes

Numerous studies have identified plasma membrane composition as being central to the ethanol tolerance of yeast strains, with yeast responding to increased ethanol concentration, in a dose-dependent manner, by increasing the unsaturation index, and hence fluidity, of their membranes (Beaven *et al.*, 1982; Šajbidor & Grego, 1992; Lloyd *et al.*, 1993; Odumeru *et al.*, 1993; Alexandre *et al.*, 1994). The predominant mono-UFAs in the *S. cerevisiae* plasma membrane are the palmitoleic (16 : 1) and oleic (18 : 1) acids, formed by the desaturation of the saturated palmitic (16 : 0) and stearic (18 : 0) acids, respectively, a reaction catalysed by a membrane desaturase encoded by the gene *OLE1* (Stukey *et al.*, 1989, 1990). This gene is known to be up-regulated during fermentation (James *et al.*, 2003). Growth of *S. cerevisiae* in rich medium (with 10% ethanol) led to an increase in unsaturation index to 0.74, compared with a value of 0.57 for cells grown in YPD medium, mainly due to an increase in the percentage of the mono-UFAs palmitoleic acid (16 : 1) and oleic acid (18 : 1) and a corresponding reduction in the percentage of the saturated palmitic (16 : 0) and stearic acids (18 : 0) (Alexandre *et al.*, 1994). More recent evidence, from studies involving modification of lipid composition through genetic manipulation and UFA supplementation, suggests that oleic acid (18 : 1) is the main determinant of ethanol tolerance in *S. cerevisiae*, rather than unsaturation index *per se* (You *et al.*, 2003).

Growth in the presence of ethanol also resulted in an overall reduction in sterol levels due to reduced levels of squalene, zymosterol, fecosterol and ergost-5,7-Dien 3βol, though increases in ergosterol and lanosterol were observed (Alexandre *et al.*, 1994). When exposed to increased ethanol concentrations, these preadapted cells showed no change in membrane fluidity, as measured by fluorescence anisotropy, compared with control cells (Alexandre *et al.*, 1994). *Saccharomyces cerevisiae* cells also exhibit an increase in plasma membrane H^+ATPase activity in response to ethanol exposure (Rosa & Sá-Correia, 1991; Alexandre *et al.*, 1993; Rosa & Sá-Correia, 1996; Monteiro & Sá-Correia, 1998). This may represent a further adaptation process, whereby increased H^+ATPase activity counteracts the increased influx of protons across the plasma membrane of ethanol-exposed cells (Leão & Van Uden, 1984). Further evidence for the role of H^+ATPase in ethanol tolerance was shown by Fujita *et al.* (2006), who found that homozygous diploid mutant strains of *S. cerevisiae* lacking genes involved in vacuolar H^+-ATPase function were sensitive to ethanol, 1-propanol and 1-pentanol (Fujita *et al.*, 2006).

Increases in mono-UFAs and corresponding decreases in saturated fatty acids have also been observed in ale and lager yeast strains exposed to ethanol, either directly through supplementation or during fermentation (Odumeru *et al.*, 1993). The membrane composition of brewing yeast is influenced by wort composition, and supplementation of high-gravity wort with ergosterol and oleic acid (in the form of Tween 80) has been shown to significantly improve fermentation rate (Casey & Ingledew, 1985) and ethanol productivity (Dragone *et al.*, 2003). However, it is still unclear as to whether this improved fermentation performance is due to an increase in ethanol tolerance because of changes to lipid membrane composition, or simply due to improvement of the nutritional status of the growth medium, as suggested by Casey & Ingledew (1985).

Magnesium ions have a role in maintaining membrane integrity, and reduce the proton, anion and nucleotide permeability of membranes exposed to ethanol (Salgueiro *et al.*, 1988; Petrov & Okorokov, 1990; Hu *et al.*, 2003). Increasing the bioavailability of Mg prior to or during ethanol shock reduces the synthesis of the heat shock proteins (Birch & Walker, 2000) and increases the viability (Walker, 1998; Birch & Walker, 2000; Hu *et al.*, 2003) and growth (Ciesarova *et al.*, 1996) of cells. In addition, supplementation of fermentation media with magnesium has been shown to increase fermentation rate and ethanol

productivity (Dombek & Ingram, 1986; Stewart *et al.*, 1988; D'Amore *et al.*, 1990; D'Amore, 1992; Ciesarova *et al.*, 1996; Rees & Stewart, 1997; Walker & Maynard, 1997). Impaired fermentation rates in the absence of sufficient concentrations of Mg may also be related to its role in regulating the activity of glycolytic enzymes such as pyruvate kinase (Morris *et al.*, 1984).

Increased temperatures and ethanol exposure have a similar effect on yeast cells, causing an increase in membrane permeability, reduced proton motive force, reduced intracellular pH and inhibition of glycolysis, leading to reduced fermentation rates (Piper, 1995). Likewise, yeast cells respond to both forms of stress in an identical manner. The synthesis of heat shock proteins is increased, lipids become less unsaturated and sterol levels are reduced (Piper, 1995). Heat and ethanol stress consequently have a synergistic effect and the toxic effects of ethanol are influenced by temperature. Higher temperatures during high-gravity fermentations typically result in reduced viability, reduced cellular glycogen levels, reduced alcohol dehydrogenase activity and an increase in the incidence of petite mutation (Nagodawithana & Steinkraus, 1976; Casey *et al.*, 1984; Stewart, 2001). Cell viability is particularly susceptible to ethanol toxicity at elevated temperatures. Rapid fermentations of 25 °C Brix honey solutions resulted in a logarithmic loss of yeast cell viability at 30 °C; the same fermentations at 15 °C caused no loss in viability (Nagodawithana & Steinkraus, 1976). A similar result has been obtained with high-gravity (28°P) wort (Casey *et al.*, 1984; Casey & Ingledew, 1985). While ethanol productivity of brewing yeast during high-gravity fermentations can be improved by raising temperature, e.g. from 10 to 15 °C (Dragone *et al.*, 2003), at higher temperatures wort attenuation, fermentation rates and alcohol productivity are reduced (Casey *et al.*, 1984; Almeida *et al.*, 2001; Stewart, 2001). It is likely that the reduced fermentation performance under these conditions is due to the synergistic effect of ethanol and temperature on yeast physiology. The fermentation performance of yeast is also reduced in standard brewery wort supplemented with high initial ethanol concentrations (White, 1978). In addition, there is evidence that temperature and ethanol concentration can affect the stability of yeast during storage. Cropped yeast is typically stored at 4 °C, usually in beer (Boulton & Quain, 2001). Increased yeast storage temperatures exacerbate the toxic effects of ethanol, with protease release increased and acidification power and oxygen uptake rates being reduced (Lentini *et al.*, 2003).

Trehalose and ethanol tolerance

Trehalose is involved in reducing membrane permeability (Mansure *et al.*, 1994) and increasing ethanol tolerance (Mansure *et al.*, 1994; Sharma, 1997), as well as preventing endocytosis inhibition in yeast cells exposed to toxic concentrations of ethanol (Lucero *et al.*, 2000). Exposure of cells to ethanol stress (16%) induces the synthesis of trehalose (Sharma, 1997) and increased accumulation of trehalose has also been observed in ale and lager brewing yeast strains exposed to 10% ethanol (as well as heat shock) (Odumeru *et al.*, 1993). Majara *et al.* (1996a, b) reported an increase in trehalose synthesis and accumulation with increasing wort gravity. This was, however, interpreted as a response to the higher osmotic stress in these worts. While Alexandre *et al.* (2001) have shown that genes involved in trehalose synthesis in yeast are up-regulated in response to 30 min of ethanol stress (7%), it was found that trehalose synthase-encoding genes are down-regulated as wort fermentation proceeds (James *et al.*, 2003), though the reduction in wort gravity may also have elicited the down-regulation of these genes. The trehalose response of brewing yeast cells to ethanol during fermentation is poorly understood, though an increase in transcription of trehalose synthase genes and cellular trehalose has been observed in yeast during storage when exposed to relatively high ethanol concentrations (10%) and storage temperatures (10 °C) (Lentini *et al.*, 2003).

Antioxidants and ethanol tolerance

Transcription analysis demonstrated an up-regulation of several antioxidant-encoding genes in the later stages of wort fermentation (James *et al.*, 2003). This investigation showed an up-regulation of nine such genes after either 3 or 8 days of fermentation, despite anaerobic conditions. An increase in expression of antioxidant proteins, including Sod1p and Ctt1p, has also been observed with *S. cerevisiae* wine yeast under semianaerobic conditions (Trabalzini *et al.*, 2003). In both of these studies the increase in expression was attributed to the presence of other stresses (James *et al.*, 2003) or the direct exposure to ethanol (Trabalzini *et al.*, 2003). Exposure to 7% ethanol for 30 min has also been shown to result in an almost 12-fold induction in the expression of the *CTT1* gene (Alexandre *et al.*, 2001). Ethanol tolerance of *S. cerevisiae* cells has been associated with manganese superoxide dismutase (MnSOD) activity and mutant cells unable to synthesize this protein are hypersensitive to ethanol stress (Costa *et al.*, 1993, 1997; Pereira *et al.*, 2001). Glutathione levels and copper/zinc superoxide dismutase (Cu/ZnSOD) activity had little or no influence on ethanol toxicity (Costa *et al.*, 1993). The importance of the MnSOD enzyme may be related to its location within mitochondria, the principal producers of ROS during respiration (Halliwell & Gutteridge, 1999). While the exact cause of increased ROS production by yeast cells during ethanol exposure is not known, there are a number of possibilities including interaction of

acetaldehyde with proteins and lipids, direct mitochondrial damage, increased bioavailability of metals (facilitating reduction of O_2 and ROS) and impairment of the function of certain antioxidants such as glutathione (Wu & Cederbaum, 2003).

Ethanol toxicity and mitochondrial function

The cell population within a yeast slurry can contain a high frequency of respiratory deficient 'petite' cells, with values of 0.5–5% being typical (Silhankova *et al.*, 1970). Rho^- petites, while being physiologically diverse, are typically associated with reduced fermentation rate and unfavourable flavour changes (Silhankova *et al.*, 1970; Morrison & Suggett, 1983; Debourg *et al.*, 1991; Ernandes *et al.*, 1993)

Ethanol is one of the most potent inducers of the petite phenotype in yeast (Jiménez *et al.*, 1988; Ibeas & Jiménez, 1997; Chi & Arneborg, 1999; Castrejon *et al.*, 2002) and it is therefore likely that the generation of petites during brewery handling is strongly influenced by repeated exposure to high ethanol concentrations during fermentation. Despite the association of ethanol toxicity with petite frequency, the actual mechanism of damage is unclear. Ethanol is not a powerful mutagen of yeast mtDNA, as determined by pulsed field gel electrophoresis (Ristow *et al.*, 1995). It has been suggested that induction of petites may be related to the effects of ethanol on the mitochondrial membrane rather than on the mtDNA itself (Ibeas & Jiménez, 1997). It has been shown that lipid composition influences both ethanol sensitivity and susceptibility to petite mutation (Chi & Arneborg, 1999). An ethanol-tolerant yeast strain with a low level of ethanol-induced mitochondrial petite mutation was found to have a relatively high ergosterol/phospholipid ratio, high phosphatidylcholine content and high long-chain fatty acid component compared with an ethanol-sensitive strain (Chi & Arneborg, 1999), though how these membrane characteristics affect mitochondrial integrity in the presence of ethanol has yet to be elucidated. It may be that long-chain fatty acids have a role in counteracting the fluidizing affect of ethanol on membranes. The important role of the mitochondrial membrane in maintaining mtDNA integrity has been demonstrated by the fact that strains with reduced ergosterol-synthesizing ability are more susceptible to petite mutation (Jiménez *et al.*, 1988). It has also been suggested that acetaldehyde, the first product of ethanol metabolism, may have a direct mutagenic effect on mtDNA (Ristow *et al.*, 1995).

The propensity of cells to succumb to ethanol-induced petite mutation is a strain-specific phenomenon. Wine yeast strains have been shown to be more tolerant than laboratory strains. These strain differences appear to be related to the tolerance of the mitochondria themselves, as evidenced by the fact that laboratory yeast/wine yeast hybrids containing mitochondria derived from the ethanol-tolerant wine yeast were found to have a greater resistance to petite mutation (Jiménez *et al.*, 1988).

Nutritional stress

Fermentable carbohydrate limitation

Wort is a complex growth medium, consisting mainly of carbohydrate (*c.* 90% of wort solids) and nitrogen-containing substances (*c.* 5% of wort solids) as well as phosphates, inorganic ions, lipids, organic acids, polyphenols and nucleic acid derivatives (Briggs *et al.*, 2004). While wort composition is highly variable, there are features common to the majority of worts. The principle carbohydrates are the trisaccharide maltotriose (*c.* 15%), the disaccharides maltose (45–65%) and sucrose (*c.* 5%) and the monosaccharides glucose and fructose (*c.* 10%). The nonfermentable carbohydrate fraction is composed mainly of dextrins (20–30% of carbohydrate fraction) along with several saccharides including arabinose, xylose, ribose, isomaltose, panose and isopanose (Boulton & Quain, 2001). Carbohydrate utilization proceeds sequentially, with those most readily assimilated taken up first. The uptake of certain sugars may be inhibited by the presence of others (Boulton & Quain, 2001). Sucrose is typically taken up first by the yeast cell followed by fructose and glucose. Maltotriose and maltose are the last of the fermentable carbohydrates to be utilized by the yeast cells, and residual levels of these two carbohydrates may remain in the beer at the end of the fermentation (Patel & Ingledew, 1973). This is particularly the case with maltotriose, which may not be assimilated by cells until all of the wort maltose has been depleted.

Assimilable nitrogen limitation

Principal nitrogen-containing compounds in wort include proteins, polypeptides, small peptides and an array of amino acids including glutamic acid and glutamine, leucine, serine, alanine, aspartic acid, and asparagine, proline and valine (Yokoi *et al.*, 1988) as well as nucleic acids and their breakdown products (Boulton & Quain, 2001).

In the brewing industry wort nitrogen is generally classified as assimilable or nonassimilable. Assimilable nitrogen in wort consists of a diverse collection of nitrogen-containing compounds including ammonium salts, amino acids and peptides. Brewing yeasts have limited extracellular proteolytic capability (Jones, 1991) and polypeptides and proteins present in wort will invariably be present in the beer after fermentation is complete (Briggs *et al.*, 2004). The nitrogen composition of wort is dependent on a number of factors including barley variety, malting conditions, mashing conditions and the inclusion of adjuncts (O'Connor-Cox &

Ingledew, 1989). As is the case with wort fermentable carbohydrates, assimilable nitrogen sources are utilized sequentially. Irrespective of brewing conditions, amino acids are taken up in a strict order and have been classified into four categories (Pierce, 1987). Group A amino acids are utilized early and rapidly during fermentation, group B amino acids are taken up more slowly and group C amino acids are utilized only when group A amino acids have been depleted. Ammonium uptake is similar to that of the class C amino acids, i.e. it is only taken up when class A amino acids have been removed from the wort. Proline, whilst being an abundant wort amino acid, requires aerobic conditions for its assimilation (Wang & Brandriss, 1987) and is typically not utilized by brewing yeast during fermentation. This amino acid is therefore included separately in group D (Pierce, 1987). Regulation of amino acid uptake is complex and involves several different transport systems, most of which are specific for individual amino acids (Horak, 1986). When amino acid nitrogen becomes limited, yeast cells respond by synthesizing the general amino acid permease (GAP) which is involved in the active and nonselective uptake of amino acids (Prasad & Rose, 1986). The yeast cell therefore responds to nitrogen limitation by initiating a nitrogen-scavenging strategy: switching from selective to nonselective uptake of amino acids. Under nitrogen-limited conditions it may also be possible that proline utilization will become derepressed (Schwencke & Magana-Schwencke, 1969), though its assimilation rate is likely to be limited under the anaerobic conditions coincident with nitrogen limitation during fermentation. It has also been reported that amino acid limitation stimulates the uptake of peptides from wort (Island *et al.*, 1987). While amino acid transport by brewing yeast has been well characterized, relatively little is known about the utilization of peptides from wort. This is due to the complexity of the assimilable peptide fraction of wort (hundreds and thousands of dipeptides and tripeptides, respectively, are known to be present in wort). It is likely that the peptide fraction of wort is a significant source of assimilable nitrogen and it has been shown that up to 40% of the peptides present in the wort at the beginning of fermentation are absent at the end of fermentation (Clapperton, 1971). Peptide utilization is a complex process and is controlled by a number of factors, including peptide size (only peptides with less than five amino acid residues can be taken up), the composition and concentration of other nitrogen sources (Ingledew & Patterson, 1999; Patterson & Ingledew, 1999) and proteolytic activity of the cells (Stewart *et al.*, 2005). The homodipeptides $(Ala)_2$, $(Asp)_2$ and $(Leu)_2$ are capable of supporting growth of brewing yeast when present as the sole sources of nitrogen, albeit to different degrees; growth on $(Leu)_2$-containing media, for example, is more rapid than with media containing $(Ala)_2$ (Patterson & Ingledew, 1999). Growth rates are, however, relatively low in the presence of dipeptides compared with media containing ammonium sulphate or amino acids as the sole N sources. Growth is markedly improved when peptides are introduced in combination or in the presence of other amino acids, indicating that yeast prefer a combination of nitrogen sources, presumably as this will help in anabolic processes required for normal cell growth (Patterson & Ingledew, 1999). While growth rates and peptide utilization are stimulated in the presence of amino acids, the presence of ammomium nitrogen has been shown to inhibit the uptake of homodipeptides and heterodipeptides (Ingledew & Patterson, 1999; Patterson & Ingledew, 1999). Likewise, internal peptidase activity is inhibited by the presence of excess ammonium, while excess amino acid in the form of leucine stimulates peptidase activity (Ingledew & Patterson, 1999). The evidence therefore suggests that while peptides and amino acids can be taken up by the cells simultaneously, this is unlikely to occur during the early stages of wort fermentation due to the repressive effects of the ammonium ion, which persists in wort until group A amino acids are depleted.

It has been suggested that brewing yeast under certain conditions, e.g. when exposed to stresses associated with high-gravity brewing, can increase the availability of peptides by releasing proteases into wort. A study involving small-scale 15°P wort fermentations with both ale and lager yeast strains showed an increase in extracellular proteinase activity when ammonium and amino acid nitrogen was depleted. This proteolytic activity resulted in an increase in wort peptides, presumably due to larger peptides being broken down into smaller, more readily assimilable peptides (Stewart *et al.*, 2005).

Nitrogen limitation is particularly salient in the case of high-gravity fermentations where the use of adjuncts has the effect of increasing the concentration of fermentable carbohydrate but in doing so dilutes the noncarbohydrate fraction of the wort. Poor yeast viability, and stuck or sluggish fermentations have been attributed to low nitrogen availability in high-gravity worts (Casey *et al.*, 1984), a situation which may be remedied by the addition of nitrogen-rich supplements to the wort (Chen *et al.*, 2006).

Stationary phase as a response to nutrient depletion

Brewing yeast cultures respond to the depletion of fermentable sugar or assimilable nitrogen from wort by entering the stationary phase in which proliferation ceases and individual cells enter a 'quiescent' state (Briggs *et al.*, 2004). Cells in this state have essentially exited the cell cycle and entered the G_0 or quiescence cycle and remain in this state until the depleted nutrient or nutrients are replenished. Quiescence is a common response to nutrient depletion in yeast cells and

the cells of other organisms. In rich medium and under anaerobic conditions, the depletion of a particular nutrient leads to characteristic cell phase changes. Cells provided with all the nutritional elements necessary for growth proliferate rapidly (exponential phase); as fermentable sugars become depleted the cells experience diauxic shift, which typically involves a reduction in growth as cell metabolism is modified to utilize nonfermentable carbon sources such as ethanol. The postdiauxic phase involves the growth of cells at a reduced rate until entering the stationary phase proper, when the lack of usable carbon prevents further cell proliferation. Brewing yeast cells respond in a similar way to depletion of fermentable sugars from wort except that growth of cells in the postdiauxic phase will be limited or absent due to the anaerobic conditions coincident with nutrient depletion, which will prevent the utilization of nonfermentable carbon sources such as ethanol. Entry into stationary phase is not merely a default mechanism of cells, but is highly regulated at the genomic and proteomic levels; i.e. in yeast, as in other organisms, mechanisms have evolved to allow the cell to cope with extended periods of nutrient limitation and depletion (for reviews see Werner-Washburne *et al.*, 1993; Gasch & Werner-Washburne, 2002; Gray *et al.*, 2004).

Entry into G_0 is necessary for the maintenance of viability over extended periods in the absence of all the necessary elements for growth, and cells with an inability to exit the normal cell cycle in response to nutrient depletion rapidly lose viability (Werner-Washburne *et al.*, 1993). Quiescence is an adaptive mechanism which has evolved to protect cells experiencing nutrient depletion and is characterized by cessation of cell proliferation, thickening of cells walls, reduced cell porosity, and increased resistance to the effects of lytic enzymes such as zymolyase due to changes in wall mannoprotein structure, intracellular accumulation of proteases, accumulation of polyphosphates within vacuoles, an increase in 'storage' carbohydrates and a general increase in stress resistance (Gray *et al.*, 2004). These changes render quiescent cells less sensitive to environmental changes, a feature which is important in the absence of essential nutrients when cells may be less equipped to respond to specific stresses.

Of particular relevance in the brewery are the changes in intracellular glycogen and trehalose that accompany growth phase shifts. Levels of both intracellular carbohydrates have been used to assess vitality and predict fermentation performance of yeast batches (Quain & Tubb, 1982; Sall *et al.*, 1988; O'Connor-Cox *et al.*, 1996; Majara *et al.*, 1996a, b; Boulton, 2000).

Glycogen, a polymer of α-D-glucose, is the principal storage carbohydrate in yeast and therefore has a critical role in maintaining cellular metabolic function during periods of nutrient starvation (Boulton & Quain, 2001). Glycogen levels are determined by growth rate and nutrient or carbon levels of the growth medium. Glycogen accumulation has been observed to occur in response to nitrogen or carbon limitation under both aerobic (Becker *et al.*, 1979; Lillie & Pringle, 1980; Parrou *et al.*, 1999) and anaerobic conditions (Thomsson *et al.*, 2005).

In the brewery, yeast glycogen levels are rapidly depleted in the first hours of wort fermentation (Boulton, 2000; Boulton & Quain, 2001). It is likely that this mobilization of glycogen provides carbon and energy for UFA and sterol synthesis while oxygen is present in the wort. It has been observed that low glycogen levels in pitched yeast cells result in an inadequate sterol synthesis, which, in turn, results in poor fermentation performance (Quain & Tubb, 1982). Yeast cells respond to nutrient limitation later in the fermentation cycle by increasing synthesis of glycogen, and glycogen content of cells can be as high as 40% of the yeast dry mass in the later, anaerobic phase of fermentation (Quain *et al.*, 1981). Glycogen synthesis and degradation is under the control of several genes (François & Parrou, 2001), most of which exhibit an increase in transcription in response to nutrient limitation during industrial fermentation (James *et al.*, 2003; Peng *et al.*, 2003). Accumulation of glycogen persists until the yeast batch has entered the stationary phase of growth, after which point glycogen levels slowly decrease, presumably to provide a source of carbon and energy for maintenance of cellular metabolism until nutritional conditions improve. Prolonged periods of nutrient starvation in the fermentation vessel or in storage vessels after cropping, particularly at higher temperatures ($> 4\,^{\circ}C$) or in the presence of oxygen, result in glycogen mobilization and potentially compromised performance in subsequent fermentations (Boulton & Quain, 2001).

Unlike glycogen, the dissaccharide trehalose only accumulates within cells when fermentable sugar is depleted from the wort. The different accumulation and breakdown pattern of both carbohydrates suggests that they perform different functions within the cell. It would also seem redundant to have two systems for the storage of carbohydrate. A number of studies have shown intracellular trehalose to accumulate in response to nutrient depletion; these have included studies in defined or rich media (Panek, 1975; Lillie & Pringle, 1980; Mansure *et al.*, 1997; Parrou *et al.*, 1999) and in wort fermentations (Majara *et al.*, 1996b; O'Connor-Cox *et al.*, 1996). Reduced fermentation performance in high-gravity worts has been attributed to a deficiency in assimilable nitrogen (Casey *et al.*, 1984) and this may contribute to the relatively high levels of trehalose accumulation observed in such worts (Majara *et al.*, 1996a; Stewart *et al.*, 1999; Boulton, 2000; Reinman & Londesborough, 2000; Van Eunen *et al.*, 2005). Increases in transcription of the genes involved in trehalose synthesis (and degradation) have been observed towards the end of an

industrial high-gravity fermentation (Peng *et al.*, 2003) and very high-gravity fermentations (Devantier *et al.*, 2005). As nitrogen starvation is an inducer of the GSR in yeast (Marchler *et al.*, 1993), it is possible that accumulation and degradation of trehalose is an STRE-mediated response to nitrogen limitation. Trehalose may also have an additional role in the regulation of glycolysis. There is evidence that trehalose-6-phosphate inhibits hexokinase activity and restricts the influx of sugar to glycolysis and has an important role in the initiation of fermentation (Hohmann *et al.*, 1996; Ernandes *et al.*, 1998). The significance of this regulatory mechanism during brewery fermentation is, as yet, unknown.

Starvation and flocculation

Flocculation, the coagulation of dispersed cells into discrete flocs, is necessary for the sedimentation of yeast and its separation from beer towards the end of the fermentation cycle. The timely onset of flocculation is critical for efficient and effective commercial production of beer.

Despite the potential stress experienced by yeast when nutrients are limited or depleted, there is ample evidence to suggest that this form of stress is essential for optimal flocculation and hence fermentation performance of brewing yeast. Flocculation is determined by a number of environmental factors including the presence of calcium ions (Bidard *et al.*, 1995), absence of mannose, and in certain strains glucose, sucrose and maltose as well (Stratford & Assinder, 1991), carbon and nitrogen starvation (Smit *et al.*, 1992; Stratford, 1992a; Soares *et al.*, 1994; Soares & Mota, 1996; Barton *et al.*, 1997), oxygen content or the presence of compounds produced under aerobic conditions such as ergosterol (Miki *et al.*, 1982; Straver *et al.*, 1993), changes in pH (Stratford, 1992a; Soares *et al.*, 1994; Jin & Speers, 2000; Jin *et al.*, 2001), acid washing (Cunningham & Stewart, 1998), temperature changes (Stratford, 1992a; González *et al.*, 1996; Jin & Speers, 2000; Jin *et al.*, 2001) and changes in ethanol concentration (Kamada & Murata, 1984; Dengis *et al.*, 1995; D'Hautcourt & Smart, 1999; Jin & Speers, 2000; Jin *et al.*, 2001) as well as the presence of premature yeast flocculation-inducing factor (PYF) originating from malt husks during brewing (Herrera & Axcell, 1989; Axcell *et al.*, 2000; van Nierop *et al.*, 2004; Jibiki *et al.*, 2006).

The majority of brewing yeast strains express the NewFlo phenotype (Stratford & Assinder, 1991). This phenotype is characterized by a loss of flocculation in the presence of fermentable carbon sources, including those found in wort, i.e. glucose, fructose, maltose and sucrose (Stratford & Assinder, 1991; Soares *et al.*, 2004). The flocculation of such strains is cyclical, with flocculation lost on entering the exponential growth phase in rich medium and regained towards the end of this phase (Soares & Mota, 1996). The flocculation of single dispersed cells is believed to occur through a mechanism involving the binding of cell surface lectins (flocculins) to carbohydrate residues on the walls of surrounding cells (Day *et al.*, 1975; Miki *et al.*, 1982; Kihn *et al.*, 1988; Masy *et al.*, 1992; Stratford, 1992a; Farias & de Nadra, 2003; Touhami *et al.*, 2003). The inhibition of flocculation by fermentable sugars is believed to occur as a result of competition between these sugars and the cell's sugar residues for lectin binding sites (Stratford & Assinder, 1991).

Flocculation occurs towards the end of the fermentation cycle when fermentable sugar concentration in wort is minimal and cells are in the stationary phase of growth. It is likely that flocculation is induced by the lack of fermentable sugars as these are known to inhibit flocculation. Indeed, it has been shown that high-gravity worts, which contain higher concentrations of fermentable carbohydrate towards the end of the fermentation cycle, are often associated with poor yeast flocculation (Suihko *et al.*, 1993). In defined media the onset of flocculation has been seen to coincide with glucose or maltose depletion (Sampermans *et al.*, 2005). The same effect was not observed in cultures where the nonfermentable carbon source glycerol was present as the sole source of carbon (Sampermans *et al.*, 2005). Likewise, the addition of fermentable sugars to flocculant yeast cultures is known to induce loss of flocculation.

It has been observed that the addition of the fermentable sugars glucose, fructose, maltose or sucrose to flocculant, carbon-starved yeast cultures leads to a rapid loss of flocculation (Soares & Duarte, 2002; Soares *et al.*, 2004). The same authors noted that the addition of poorly metabolizable galactose and nonmetabolizable lactose resulted in limited and no loss in flocculation, respectively, indicating that loss of flocculation is an energy-dependent process (Soares *et al.*, 2004). Cycloheximide treatment also demonstrated that the glucose-induced loss of flocculation required *de novo* protein synthesis (Soares *et al.*, 2004), again demonstrating the requirement of energy for flocculation loss. While it is clear that the change from a flocculant to nonflocculant state and the maintenance of the latter state are energy-dependent, it should also be noted that the reverse is also true, i.e. flocculation does not occur in the absence of residual carbohydrate (Sampermans *et al.*, 2005). The onset of flocculation in the brewery is most likely triggered by the loss of fermentable carbohydrates from wort. However, other, concomitant changes in wort are also likely to influence flocculation. Nitrogen starvation, for example, also results in increased flocculation ability (Smit *et al.*, 1992), even in the presence of glucose (Sampermans *et al.*, 2005).

Flocculation onset occurs in wort with minimal fermentable carbohydrate but a relatively high concentration of

ethanol, particularly after high-, and very high-gravity fermentation. Unlike the fermentable sugars, which have an inhibitory effect on flocculation, there is evidence to suggest that ethanol has a positive effect on the flocculation phenotype (Jin & Speers, 2000; Soares & Vroman, 2003; Sampermans *et al.*, 2005; Claro *et al.*, 2007). Flocculation can occur in media containing ethanol (but not glycerol) as the sole carbon source (Sampermans *et al.*, 2005). Also, while flocculation ability is lost rapidly when cells are transferred to media containing fermentable sugars, the same is not true for cells transferred to media containing ethanol (4%) as the sole carbon source (Soares & Vroman, 2003). In addition, increasing concentrations of ethanol can increase the degree of flocculation (Jin & Speers, 2000). The positive effect of ethanol on flocculation is, however, concentration-dependent and 10% ethanol has been shown to inhibit the extent of flocculation significantly (Claro *et al.*, 2007).

Oxygen content of pitching wort has also been implicated in the induction of flocculation in wort (Straver *et al.*, 1993). It was found that cells pitched into air-depleted wort flocculated relatively early but to a limited extent. The addition of ergosterol or Tween 80 to the same wort restored the normal flocculation behaviour of the cells. It was concluded that lack of oxygen prohibited the synthesis of ergosterol and UFAs, thereby limiting cell growth and resulting in the early entry to stationary phase and early onset of flocculation (Straver *et al.*, 1993).

Cold shock

Low temperature environments in the brewery

In *S. cerevisiae* cold shock occurs with a downshift to temperatures of 20 °C or below (Kondo & Inouye, 1991; Kondo *et al.*, 1992; Kowalski *et al.*, 1995; Abramova *et al.*, 2001b). Lager fermentations are carried out at temperatures between 6 and 15 °C, whilst ale fermentations are conducted at higher temperatures, between 18 and 25 °C. Once the fermentation is complete breweries must maintain the yeast in the same physiological state it was at the time of cropping (O'Connor-Cox, 1998) to prevent contamination and minimize physiological changes (Boulton & Quain, 2001). The temperatures during storage in the bottom of the cylindroconical fermentation vessel are more extreme, ranging from 2 to 11 °C. Upon cropping, the yeast may be passed through a chiller to chill it uniformly to 4 °C before it is pumped into the yeast storage tank (or yeast collection vessel) where it is held as agitated slurry at 3–4 °C. Furthermore, acid washing is conducted at 4 °C. Yeast handling, therefore, consists of a series of low-temperature environments which may lead to the yeast slurry experiencing cold shock, a phenomenon though well documented in other organisms (Phadtare *et al.*, 1999; Rodriguez-Vargas *et al.*, 2002), has not been extensively studied in yeast.

Physiological consequences of low temperature

The principal fatty acyl chains in the plasma membrane of *S. cerevisiae* are oleic acid (18 : 1) and palmitoleic acid (16 : 1), with trace amounts of palmitic acid and stearic acid also present. Membrane fluidity is largely determined by the packing of these molecules. Lowering of the temperature leads to a more ordered membrane structure and hence a reduction in fluidity (Shinitsky, 1984). The membrane is essentially modified from a liquid crystalline form to a gel state (Thieringer *et al.*, 1998). This transformation alters various functions of membrane-bound proteins, such as the import and export of metabolites and proteins across the plasma membrane. The mechanism by which microorganisms tolerate changes in membrane fluidity has been well characterized and is termed 'homeoviscous adaptation' (Sinenski, 1974). Depending on the microorganism, this process involves increasing proportions of UFAs and/or *cis* double bonds into lipids, chain shortening and methyl branching (Shaw & Ingraham, 1967; Sinenski, 1974; McElhaney, 1982; Shinitsky, 1984; Russell, 1989). Another physiological effect of lowering the temperature is the reduction in the hydrophobic interactions between the carbon skeleton of the polypeptide and the side chains of amino acids, exposing nonpolar regions to water and risking protein denaturation (Gounot & Russell, 1999).

Impact of low temperature on genome-wide expression

In response to an abrupt drop in temperature, a change in gene expression has been postulated to allow for adaptation to the low-temperature environment (Sahara *et al.*, 2002; Schade *et al.*, 2004). This response is gene- and time-specific and involves the differential regulation of certain genes (Zhang *et al.*, 2001; Sahara *et al.*, 2002; Zhang *et al.*, 2003; Schade *et al.*, 2004), presumably as a result of the altered physiological state of the cell caused by reduced membrane transport, accumulation of misfolded proteins and reduced enzyme activity (Sahara *et al.*, 2002; Schade *et al.*, 2004). The early phase of the cold shock response involves adjustments to membrane fluidity and prevents destabilization of RNA secondary structures to allow efficient protein translation. The late phase involves the up-regulation of genes involved in the GSR, including those encoding heat shock proteins and metabolic enzymes engaged in glycogen and trehalose metabolism (Sahara *et al.*, 2002; Schade *et al.*, 2004). Sahara *et al.* (2002) demonstrated that the genes involved in the GSR are up-regulated following 4 h of low-temperature exposure, whilst Schade *et al.* (2004) showed that they are still being up-regulated following 12 and/or 60 h of low-

temperature exposure. The gene expression profile during growth at low temperatures (Homma *et al.*, 2003) has been reported but this is to be differentiated from cold shock gene expression profiles as the former does not involve a sudden downshift in temperature.

Induction of cold shock genes

Since 1991 when the first *S. cerevisiae* cold shock gene was cloned (*TIP1*) (Kondo & Inouye, 1991) several other genes involved in yeast cold shock have also been identified. These include the *NRS1* gene (Lee *et al.*, 1991; Kondo & Inouye, 1992; Kondo *et al.*, 1992), the *LOT* genes (*LOT1*, *LOT2* and *LOT3*) (Zhang *et al.*, 2001) and the *TIR* genes, *TIR1* (Kowalski *et al.*, 1995; Abramova *et al.*, 2001a), *TIR2* (Kowalski *et al.*, 1995; Abramova *et al.*, 2001a) and *TIR4* (Abramova *et al.*, 2001a).

Translation efficiency is greatly reduced at low temperatures due to the formation of secondary structures in RNA molecules and the inactivation of ribosomes (Jones & Inouye, 1996). To combat this, an early response to cold involves an increased expression of the majority of the genes involved in rRNA synthesis and processing (Sahara *et al.*, 2002; Schade *et al.*, 2004). For instance, Nsr1p (nuclear localisation sequence recognition) was identified as a cold-induced protein (Kondo *et al.*, 1992; Schade *et al.*, 2004). Nsr1p is required for normal pre-rRNA processing and ribosome biogenesis and cell growth upon a temperature drop (Lee *et al.*, 1991, 1992; Yan & Melese, 1993). In addition, genes encoding ribosomal proteins also show an increase in expression (Sahara *et al.*, 2002; Schade *et al.*, 2004), indicating that ribosomes may be synthesized at this time to compensate for the possible inefficient translation following exposure to cold (Sahara *et al.*, 2002). Indeed, it appears that restoring translation is vital to maintain and execute biochemical and physiological processes at low temperatures (Sahara *et al.*, 2002).

Mga2p represents the only putative eukaryotic cold sensor reported to date (Nakagawa *et al.*, 2002). Mga2p is a multicopy suppressor of Gam1p (Snf2p). Snf2p is required for transcription of *STA1*, which encodes an extracellular glucoamylase in *S. cerevisiae* (Yoshimoto & Yamashita, 1991). Mga2p has been demonstrated to activate *OLE1* transcription in response to low temperature and hypoxia signals (Nakagawa *et al.*, 2002). *OLE1* encodes for Δ9 fatty acid (FA) desaturase, an intrinsic membrane enzyme that converts saturated FA acyl-CoA substrates to mono-UFA species via an oxygen-dependent mechanism (Chellappa *et al.*, 2001). Mono-UFA species can comprise 75–80% of the fatty acyl groups in membrane lipids (Chellappa *et al.*, 2001) and since the ratio of saturated FAs and UFAs is important for maintaining optimum levels of membrane fluidity and curvature, it is suggested that Mga2p regulates this ratio in low-temperature environments (Nakagawa *et al.*, 2002). Indeed, it is postulated that Mga2p is essential for a number of cellular processes, such as membrane-associated enzyme activity and transport processes, during cold temperature exposure via the regulation of *OLE1* expression (Zhang *et al.*, 1999). Furthermore, both Mga2p and Ole1p are induced within 2 h following a downshift in temperature from 30 to 10 °C (Schade *et al.*, 2004). Interestingly, a large increase in *OLE1* expression has been demonstrated during fermentation (Higgins *et al.*, 2003; James *et al.*, 2003), particularly during the initial stages (Higgins *et al.*, 2003). Brewery fermentation is considered to be a low temperature environment (Leclaire *et al.*, 2003) where cold induced Mga2p activation of *OLE1* expression is likely to occur.

Tip1p (temperature shock-inducible protein) is an extracellular esterase (Horsted *et al.*, 1998) that is covalently bound to the cell wall glucan (van der Vaart *et al.*, 1995). *TIP1* gene expression is induced by cold shock as well as heat shock (Kondo & Inouye, 1991) and anaerobic conditions (Donzeau *et al.*, 1996). The expression of the *TIP1* gene is cell cycle-regulated and occurs in G_1 phase (Caro *et al.*, 1998). This protein is of significance in the brewery since esters are produced by yeast during fermentation and impart aroma and flavour to the beer (Suomalainen & Lehtonen, 1979). Indeed, the expression of *TIP1* was found to increase during fermentation (Higgins *et al.*, 2003; James *et al.*, 2003).

The Tir protein family includes the cold shock-inducible Tir1p, Tir2p and Tir4p, all of which exhibit a high level of homology (Kowalski *et al.*, 1995). The cold shock and anaerobic induction of the *TIR* genes requires the activator factor Mox4p (mannoprotein regulation by oxygen) whilst under these conditions *TIP1* gene expression does not; therefore, if a common signal pathway exists it must diverge at the level of the transcriptional regulators (Abramova *et al.*, 2001a, b). Interestingly, the *TIP1*, *TIR1* and *TIR2* genes were observed to be up-regulated during growth at 4 °C relative to 25 °C (Homma *et al.*, 2003). Thus, Tip1p, Tir1p and Tir2p may be necessary for growth at low temperatures as well as adapting to rapid downshift in temperature (Homma *et al.*, 2003).

Cell wall composition is modified in response to nutrient availability and environmental conditions such as pH, temperature and availability of oxygen (Smits *et al.*, 1999; Klis *et al.*, 2002). A remodelling of the cell wall appears to take place when cells are subjected to cold shock, Cwp1p is down-regulated and expression of the *TIP1*, *TIR1*, *TIR2* and *TIR4* genes is induced (Abramova *et al.*, 2001a). This indicates that cell wall mannoproteins are important for cold stress adaptation, and thus an understanding of the stress-conditional nature of *TIR* gene expression would be valuable. Evidence suggests that cell wall turnover is

modified, resulting in compositional (Abramova *et al.*, 2001a) and cell surface physical property changes (Rhymes & Smart, 2001).

Of the candidate genes involved in the specific response to cold, the *TIR* gene family are arguably the most interesting to the brewer, as they are putative mannoproteins, which may influence flocculation performance. Flocculation of lager yeast has been shown to be increased at lower temperature (González *et al.*, 1996). Here it was demonstrated that a settling temperature of 5 °C considerably increased the volume of sedimented yeast. The temperature of growth has also been reported to affect the final flocculation capacity (Van Iersel *et al.*, 1998).

Trehalose and glycerol regulation at low temperature

Trehalose accumulation occurs at temperatures of 10 °C and below, and coincides with induction of the trehalose-synthesizing enzymes, Tps1p and Tps2p (Kandror *et al.*, 2004). This is believed to be an adaptive response which increases tolerance to low temperatures as well as freezing. Accumulation of trehalose protects yeast against loss of viability at low temperatures, an effect also found in *Escherichia coli* (Kandror *et al.*, 2002). Interestingly, this response is slow, with adaptation requiring at least a day. However it is argued that rapid and large decreases in temperatures rarely occur *in situ* and an adaptive mechanism requiring a number of days is of significance for protection (Kandror *et al.*, 2004). Since trehalose and Hsp104 have a synergistic effect in protecting the yeast against heat denaturation it is suggested that they may also function together at low temperatures to protect cell proteins and membranes (Elliot *et al.*, 1996).

Recent evidence suggests a role for the HOG pathway in detecting cold and regulating the expression of cold shock genes (Panadero *et al.*, 2006). Cells of *S. cerevisiae* exposed to a downshift in temperature (from 30 to 12/4 °C) exhibit an increase in activation and a transient increase in MAPK Hog1p phosphorylation. The increase in expression of cold shock-associated genes such as *OLE1*, *TIP1* and *NSR1* does not occur in strains lacking the *HOG1* gene. Also, cells lacking the *HOG1* gene were found to be incapable of accumulating glycerol at low temperatures and these cells exhibited a reduced viability when exposed to freezing conditions compared with wild-type cells (Panadero *et al.*, 2006). Hyperosmolarity is known to reduce the fluidity of the yeast plasma membrane (Laroche *et al.*, 2001) and increased rigidity of the plasma membrane may be a key factor in stimulating the Hog1p response to cold. Panadero *et al.* (2006) found that membrane rigidification caused by exposure to Me_2SO at 30 °C also resulted in Hog1p phosphorylation.

Cold shock and the antioxidant response

Elevated temperatures can stimulate the production of ROS within yeast mitochondria (Davidson & Schiestl, 2001a, b) and antioxidant defences are known to be essential in protecting cells against these heat-induced ROS (Weiser *et al.*, 1991; Lee & Park, 1998; Sugiyama *et al.*, 2000a, b; Pereira *et al.*, 2001; Moraitis & Curran, 2004). However, relatively little is known about the relationship between cold and the antioxidant response. Zhang *et al.* (2003) have found that a reduction in temperature from 30 to 10 °C elicits an antioxidant response in yeast. This response involves the up-regulation of the antioxidant-encoding genes *SOD1*, *CTT1* and *GSH1* as well as an increase in cellular superoxide dismutase and catalase activities. Cold-shocked cells were also found to have an increased resistance to hydrogen peroxide (Zhang *et al.*, 2003). The exact role of antioxidants in protecting cells against cold shock is not known but may be related to an increased level of cellular hydrogen peroxide at lower temperature (Zhang *et al.*, 2003). Free radicals are also generated during freezing and thawing of cells, and cells with limited antioxidant capability are particularly sensitive to this form of stress (Park *et al.*, 1998). Deletion mutant strains of *S. cerevisiae* lacking the *SOD1* gene and double mutants lacking the *SOD1* and *SOD2* genes were found to be hypersensitive to freeze/thaw stress (Park *et al.*, 1998). The acetyltransferase-encoding gene *MPR1* has also been implicated in the protection of yeast cells to freeze/thaw stress by the elimination of ROS (Du & Takagi, 2005).

Stress by desiccation

Although not widely considered to be a stress associated with beer production, the increasing employment of Active Dried Yeast (ADY) within the brewery supply chain has resulted in growing interest in yeast stress associated with the production of dried yeast for brewing (Debourg & Van Nedervelde, 1999; Fels *et al.*, 1999; Finn & Stewart, 2002). ADY is typically produced by aerobic propagation followed by a series of preliminary water removal phases based on filtration and centrifugation (Bayrock & Ingledew, 1998a). Remaining extra- and intracellular water is then removed by drying and the final product is maintained at 4 °C, generally in the form of small beads or short noodle-like structures (Quain, 2006). Although there are several means of drying a yeast culture, the preferred method is by employing a fluidized bed (Grabowski *et al.*, 1997; Bayrock & Ingledew, 1998a, b), whereby warm dehumidified air is passed through the yeast culture until a final moisture content of between 4% and 8% is achieved. While a yeast culture can survive the removal of more than 94% water and still revert to its original functional state (Bayrock & Ingledew, 1998b), water is essential for the efficient functioning of individual cells

within the population. Even the removal of a small proportion of intracellular water can cause cellular damage (Bayrock & Ingledew, 1998b) and result in a corresponding cellular response, aimed at minimizing such adverse effects (Singh *et al.*, 2005). Given that yeast cells in their dehydrated form are not amenable to many types of analysis, the majority of studies investigating stress and desiccation typically refer to the rehydrated form of yeast as exhibiting a dehydrated phenotype. Consequently, analysis of stress associated with the production of dried yeast tends to include the response to both desiccation and rehydration.

Cell damage caused by desiccation

As a consequence of drying, yeast cells are susceptible to damage, which primarily occurs due to water loss and the consequential changes which affect cell size and shape. Such effects include cell wall crenellation (Finn & Stewart, 2002), cytoplasmic crowding (Billi & Potts, 2002), DNA supercoiling (Shirkey *et al.*, 2003), membrane disruption (Beker *et al.*, 1984; Attfield *et al.*, 2000), phase transitions (Van Stevenick & Ledeboer, 1974; Leslie *et al.*, 1995), and ultimately cell death. In addition, and as a direct consequence of the drying and rehydration process, cells can experience stress from heat (Bayrock & Ingledew, 1998b) and free radicals (Pereira *et al.*, 2003; Hansen *et al.*, 2006; Franca *et al.*, 2007), as well as hyper- and hypo-osmotic shock. Several of these stress factors are discussed elsewhere in this review and will not be covered in detail here, although it should be noted that desiccation can be seen to encompass a complex mixture of a number of stresses.

Although the presence of combined stress can affect cells in a number of ways, it is widely believed that the most detrimental consequence of the drying and rehydration process is the movement of water into and out of the cell, respectively. This hypothesis is supported by evidence that cell death occurs at a greater rate once a moisture content of $< 15\%$ is reached during the drying process (Bayrock & Ingeldew, 1998b). This point is believed to correspond to the time at which external moisture has been removed from around cells, leaving only intracellular water (Bayrock & Ingledew, 1998a). During fluidized bed drying, water is likely to move rapidly across the membrane with only limited cellular regulation. While it is possible that aquaporins mediate the transport of water during the early stages of dried yeast production, they are unlikely to have an important role when the rate at which internal water is removed increases during the latter stages of drying (Bonhivers *et al.*, 1998; Meyrial *et al.*, 2001). Such a vigorous flow of water is believed to be a source of physical damage to the cell membrane as well as causing disruption to the phospholipid bilayer (Attfield *et al.*, 2000). Similarly, other intracellular membrane structures and proteins within the cell are also likely to be affected (Prestrelski *et al.*, 1993; Allison *et al.*, 1999). Disruption of the phospholipid component of the cell membrane is of particular significance as, if the damage is such that nucleotides, ions and other solutes are able to diffuse freely into and out of cells, the ability to maintain membrane potential is likely to be severely compromised with a corresponding loss of viability (Beker *et al.*, 1984; Rapoport *et al.*, 1995; Attfield *et al.*, 2000). As mentioned previously, damage during drying can be augmented by the presence of additional stresses. Drying temperature in particular has been implicated in playing a prominent role in viability determination of dried yeast (Beker & Rapoport, 1987; Poirier *et al.*, 1999; Beney *et al.*, 2001; Oshita *et al.*, 2002). Bayrock & Ingledew (1998a) demonstrate that, due to evaporative cooling at the cell surface, the effect of heat only becomes apparent once intracellular water begins to be removed, which serves to illustrate the interactive relationship between different stress factors during drying. Characterization of the effects of stress associated with desiccation of yeast cells is further complicated by the fact that before dried yeast can regain functionality, it must be rehydrated. The cellular response to the sudden influx of water is as complex as that which occurs during the drying process itself (Shaw *et al.*, 2003; Rossignol *et al.*, 2006), and the manner of rehydration can significantly affect the rate of survival (Crowe *et al.*, 1992; Poirier *et al.*, 1999; Schebor *et al.*, 2000; Mille *et al.*, 2005). In a similar fashion to dehydration, cells are subjected to a rapid movement of water, which can again lead to transcriptional changes (Singh *et al.*, 2005; Rossignol *et al.*, 2006), membrane damage (Attfield *et al.*, 2000) and cell death as described above.

Yeast response to desiccation and rehydration

Depending on the precise regime employed, the production of dried yeast can occur within a time period of between 10 min and several hours. Given that the rate of water removal is significantly faster than would be encountered in a natural environment, yeast cells may have a limited period of time in which to respond actively to the environmental change. However, the transcriptional response of yeast strains dried using different regimes has been shown to be surprisingly similar, suggesting that the reaction of yeast to desiccation and rehydration may be a rapid and coordinated event, irrespective of the method of drying (Singh *et al.*, 2005). It is widely believed that yeast cells possess intrinsic response mechanisms to withstand desiccation, some of which are believed to be elicited as part of a GSR. In response to desiccation, yeast cells cease cell division, activate a number of stationary-phase essential genes and show characteristics typical of G_0 phase cells (Singh *et al.*, 2005). In addition, a number of genes associated with fatty

acid catabolism, gluconeogenesis and the glyoxylate cycle are also activated during desiccation and the subsequent rehydration of yeast (Singh *et al.*, 2005). Transcription of a number of cell-wall-related genes are also altered, in particular *PMT1-5* (Protein O-glyosylation), *CWP1* (wall mannoprotein) and genes involved in β-glucan synthesis, such as *FKS1*, *EXG1* and *SCW10*, all of which are down-regulated. Although the reasons for these changes are not known, it is possible that they may result in altered cell wall rigidity (Smits *et al.*, 2001), allowing for greater flexibility and allowing cells to distribute wall stabilizing factors more effectively. Interestingly, another set of cell wall genes are up-regulated during rehydration of yeast, suggesting that a similar reorganization must occur during recovery from desiccation (Singh *et al.*, 2005). These genes include *URA7*, which aids in synthesis of membrane phospholipids, *SPS100*, involved in the organization of cell wall during spore formation, and *HOR7*, which codes for a small depolarizing plasma membrane-bound protein. Singh *et al.* (2005) also identify a candidate 'desiccation gene' (YOR309C) which is down-regulated during dehydration and up-regulated during rehydration, although its current function is unknown. The same authors also suggest that potential changes in the cytoskeleton may occur during rehydration, due to elevated expression of *TUB3* and *SRO9*, which encode α-tubulin, responsible for mitotic segregation and nuclear migration, and a protein involved in the organization of actin filaments, respectively.

Yeast anhydrobiosis defence mechanisms

Although yeast cells possess their own means to withstand desiccation, many manufacturers of dried yeast include a series of preconditioning steps aimed at optimizing the condition of yeast prior to drying. Such measures include subjecting yeast cultures to nutrient deprivation, mild heat (Hottiger *et al.*, 1987b) or osmotic stress (Eleutherio *et al.*, 1997) to increase the concentration of compatible solutes or other protective compounds (Crowe *et al.*, 2001; Elbein *et al.*, 2003). Trehalose, which can be incorporated up to around 15–25% of the dry weight of cells, is thought to play a particularly important role in the protection of cells against desiccation damage (Gadd *et al.*, 1987; Eleutherio *et al.*, 1993; Crowe *et al.*, 2001), due to water replacement (Crowe *et al.*, 1984, 1998) and by the formation of glass structures (Crowe *et al.*, 1998; Schebor *et al.*, 2000). Trehalose may also play a role during rehydration as it has been shown to prevent phase transition events in the phospholipid bilayer, thereby protecting membranes from damage (Leslie *et al.*, 1995; Poirier *et al.*, 1999).

Despite the supposed importance of trehalose to desiccation resistance, there have been no direct reports to suggest that the transcriptional levels of genes associated with the production and mobilization of trehalose are up-regulated during drying. As production of trehalose is elicited by the GSR, and STRE promoters have been identified in upstream regions of genes associated with trehalose production, this is rather surprising, particularly as trehalose levels are known to increase during stationary phase (Gadd *et al.*, 1987) and genes associated with the GSR have been shown to be highly expressed in dried yeast cultures (Rossignol *et al.*, 2006). However, while there is a wealth of data to support the role of trehalose in desiccation resistance, the possibility that trehalose is not required in some strains (Ratnakumar & Tunnacliffe, 2006) or that it is not the only protectant (reviewed by Crowe *et al.*, 2001) is acknowledged.

It has been demonstrated that stress proteins also function to confer desiccation tolerance to mammalian cells (Ma *et al.*, 2005), and analysis of gene transcription has revealed several candidate proteins in yeast. In particular, *SIP18*, which has been suggested to be part of an osmotic stress response locus regulated by the *HOG2* signalling cascade (Miralles & Serrano, 1995), is greatly up-regulated during drying (Singh *et al.*, 2005; Rossignol *et al.*, 2006).

The abundance of several heat shock protein gene transcripts was also increased in response to drying (Singh *et al.*, 2005; Rossignol *et al.*, 2006), including *SSA3*, which encodes a chaperone protein within the HSP70 family (Werner-Washburne *et al.*, 1989), and *HSP12*, which acts to protect liposomal membrane integrity against desiccation (Sales *et al.*, 2000). Consequently, it is likely that these proteins may also play a role in protecting the cell, either independently or in conjunction with other protective agents.

Rehydration of yeast and fermentation performance

When dried yeast is rehydrated and subsequently inoculated into wort, the culture must revert to its fully functional state and adapt to its new environment before it can perform efficiently. In order to achieve this, cells must repair any damage sustained as well as removing the (now defunct) products of the stress response. Of particular significance is the presence of trehalose, which must be removed either by cellular utilization or by transporting the molecule outside the cell, in order to ensure efficient recovery from stress (Wera *et al.*, 1999) and allow fermentation to proceed as normal. Trehalose, despite protecting cells during adverse conditions, is known to inhibit some cellular defence mechanisms, such as the production of glutathione reductase (Sebollela *et al.*, 2004) as well as protein refolding (Singer & Lindquist, 1998) and a number of enzymes required for efficient metabolism, such as glucose 6-phosphate dehydrogenase (Sola-Penna *et al.*, 1997) and cytosolic pyrophosphatase (Lopes *et al.*, 1999).

Analysis of the genomic response to rehydration has indicated that rapid transcriptional changes occur according to the nutritional composition of the environment in which the yeast are placed (Rossignol *et al.*, 2006). It is also likely that genes involved in protein and nucleic acid synthesis are rapidly up-regulated and stress-related genes are down-regulated (Rossignol *et al.*, 2006). Despite changes in expression, cells may also retain some of the physiological effects of desiccation. As a consequence of the drying process yeast cells typically adopt a more crenellated, wrinkled appearance (Finn & Stewart, 2002). Although it is unknown whether this occurs as a result of water removal from the vacuole (resulting in a less turgid interior), or damage to the wall, this may be of significance to fermentation performance. Flocculation capacity is intrinsically related to the cell wall, being influenced by hydrophobic proteins (for initial attraction) and the presence of flocculins (Miki *et al.*, 1982; Stratford & Assinder, 1991; Stratford, 1992b) and it has been demonstrated that the flocculation characteristics of a yeast culture can become altered during drying (Finn & Stewart, 2002). It is possible that damage occurring at the cell wall may disrupt flocculins, resulting in a reduced capacity for cells to adhere to one another and consequently influencing the fermentation characteristics of some yeast strains.

It can be seen that stress by 'desiccation' is difficult to define, due to the combined effects of multiple stresses during drying and uncertainty as to whether more serious cell damage occurs during dehydration or rehydration of a yeast culture. While it is possible to characterize broadly the cellular response to desiccation, it is important to note that using current techniques, a proportion of cells within the population are typically unable to survive the drying process. The precise nature of these cells is unknown, but is likely to comprise physiologically weak individuals, or cells which are in a closer proximity to the drying airflow, for example those being on the outer surface of clumps of cells. Irrespective of this, it should be emphasized that desiccation stress is unique in that it only applies to a dried brewing yeast culture when the yeast is first employed. The effects of drying on a working culture are not permanent and if a slurry is used for serial repitching, subsequent generations do not exhibit biomarkers of a desiccated phenotype, but display qualities which are typical of a normal pitching yeast (Debourg & Van Nedervelde, 1999).

Conclusions

The brewing yeast cell inhabits a highly complex environment and is exposed to temporal changes in oxygen availability, solute concentration, pH, ethanol concentration, carbon and nutrient availability, and temperature (Briggs *et al.*, 2004). The complexity of fermentation and the practice of serial repitching mean that the stresses that yeast cells are exposed to are more akin to those experienced in natural environments than in the controlled environments experienced during laboratory experimentation. A further complication is that the exact composition of wort is not known (Boulton & Quain, 2001). Caution must therefore be exercised in predicting the responses of yeast during brewery handling based on conclusions drawn from laboratory experiments or small-scale wort fermentations.

The complex array of stresses that the brewing yeast cell is exposed to during brewery handling is matched by the array of defence mechanisms that are possessed by the cell. The stress response mechanisms of *Saccharomyces* species may be either general or specific. The GSR is initiated in response to a number of conditions associated with brewery handling, and the up-regulation of STRE-controlled genes and synthesis of associated molecules will be indicative of stress and may be of use in predicting fermentation performance. The accumulation of trehalose in response to the up-regulation of several STRE-controlled genes, for example, has been observed in yeast exposed to hyperosmotic conditions immediately after pitching into high-gravity wort (Majara *et al.*, 1996a), as well as nutrient limitation (Majara *et al.*, 1996b) and ethanol toxicity (Odumeru *et al.*, 1993; Lentini *et al.*, 2003). Of potentially more use as a diagnostic tool is the assessment of genes or gene products associated with specific stress responses. The measurement of specific stress responses to indicate the presence of specific stresses during brewery handling is, however, complicated by the ancillary roles of these defence mechanisms. An example is the up-regulation of antioxidant-encoding genes and synthesis of antioxidants in response to osmotic stress (Garay-Arroyo *et al.*, 2003), cold shock (Park *et al.*, 1998; Zhang *et al.*, 2003) and ethanol toxicity (Alexandre *et al.*, 2001; Trabalzini *et al.*, 2003) as well as dehydration and rehydration (Pereira *et al.*, 2003; Hansen *et al.*, 2006; Franca *et al.*, 2007).

A perennial difficulty with research involving industrial-scale fermentation is the inability to conduct experiments at the industrial scale and much of our knowledge concerning the responses of yeast to environmental changes during fermentation are inferred from small-scale fermentations, which may be of limited relevance to industrial-scale fermentations. In addition, much of our knowledge of brewing yeast stress responses at the molecular or physiological level is inferred from laboratory experiments involving the aerobic growth of haploid laboratory yeast strains on defined or rich media such as YPD. In recent years the use of techniques for measuring the genomic and proteomic changes of the yeast cell during full-scale fermentation has become more common and the results of such investigations are likely to increase substantially our knowledge of the responses of production yeast strains to the complex

environment in which they reside and allow for the modification of the brewing process to attenuate those stresses which may have a detrimental effect on fermentation performance.

The focus of brewing yeast research should be the mitigation of those stresses that may influence fermentation performance of the brewing yeast rather than the elimination of cellular stress *per se*. Indeed, cellular stress may be an integral part of the brewing process as shown in the case of fermentable carbohydrate limitation and the onset of flocculation.

The now widespread use of high-gravity wort in brewery fermentation has resulted in an increase in the magnitude of stress experienced by the brewing yeast cell. Such intensification of the brewing process has many benefits in terms of reduced capital, energy and labour costs and is likely to continue in the future, thereby posing new challenges for the brewing yeast cell and brewing scientist alike.

Acknowledgements

Katherine Smart is the SAB-Miller Professor in Brewing Science and gratefully acknowledges the support provided by SAB-Miller. Katherine Smart also gratefully acknowledges the support of the Royal Society, BBSRC and EPSRC for the award of Royal Society Industrial Fellow. We gratefully acknowledge the support of the J & J Morison Trust, The Institute of Brewing and Distilling Grant Fund and the University of Nottingham. Dr Chris Powell is grateful to Lallemand Inc for permission to publish.

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