Stress tolerance: The key to effective strains of industrial baker's yeast

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Application of yeasts in traditional biotechnologies such as baking, brewing, distiller's fermentations, and wine making, involves them in exposure to numerous environmental stresses. These can be encountered in concert and sequentially. Yeast exhibit a complex array of stress responses when under conditions that are less than physiologically ideal. These responses involve aspects of cell sensing, signal transduction, transcriptional and posttranslational control, protein-targeting to organelles, accumulation of protectants, and activity of repair functions. The efficiency of these processes in a given yeast strain determines its robustness, and to a large extent, whether it is able to perform to necessary commercial standards in industrial processes. This article reviews aspects of stress and stress response in the context of baker's yeast manufacturing and applications, and discusses the potential for improving the general robustness of industrial baker's yeast strains, in relation to physiological and genetic manipulations.

Keywords: food biotechnology, *Saccharomyces cerevisiae*, fermentation

The worldwide baker's yeast industry, valued at over $2 billion, produces in excess of 2 million tons of yeast (based on 30% dry weight of yeast) per year[1,2]. It is currently expanding at about 4% per year as a result of increasing population, industrialization, and dietary changes, with major growth in bread consumption occurring throughout Asia. About 80% of the yeast sold globally is in the form of cream yeast (an aqueous suspension at approximately 20% dry weight of yeast) or compressed yeast (blocks of approximately 30%)[3,4]. The remainder is sold as viable dried yeast (<5% water)[1–4]. Consumption of dried yeast in particular will increase over the coming years due to greater convenience of its storage and transport, and enhanced shelf life relative to wet yeast. There is also the potential for greatly increased use of yeast in specialty baking processes such as frozen doughs, which offer convenience, automation, and economy of scale to bakers.

Baker's yeast (mostly strains of *Saccharomyces cerevisiae*) is required to exhibit efficient respiratory metabolism during its production in order to yield biomass economically from raw substrates[3,4]. It must also be able to leaven bread efficiently by producing considerable quantities of carbon dioxide, mostly via ethanolic fermentation of various sugars in doughs, plus desirable flavor and aroma compounds from by-products of secondary metabolism[1,2,4]. Optimal physiological conditions for yeast can be described in general terms as being incubation with shaking in complete nutrient medium that provides a plentiful supply of easily fermented sugar (glucose, fructose, or mannose) at about pH 5 and approximately 25°C. Under these conditions yeast ferments and grows most rapidly, while showing little sign of stress response. When grown outside of these optimal conditions, yeast strains exhibit a complex stress response. In industrial practices, yeast never encounters the physiologically optimum environment and instead is exposed to a variety of stresses. Nevertheless strains are expected to yield biomass economically and to leaven doughs in a variety of baking processes. Thus, their ability to withstand stressful environments and still function to a commercially acceptable standard is crucial to their usefulness in the baking industry.

This review focuses on stresses encountered by yeast strains as they are used in the baking industry, and considers stress tolerance mechanisms in terms of their importance to the efficient performance of baker's yeasts. The potential for baker's yeast strain improvement through enhancement of stress responsiveness is also discussed.

Stresses encountered by baker's yeast during production and use

Production of yeast biomass. Baker's yeast is produced by aerobic fed-batch methods using molasses as the sugar source, although other cost-effective sugar syrups can be used. The fed-batch process[3–5] involves the addition of incremental amounts of a sugar source and supplementation with other key nutrients (including nitrogen and phosphate sources, and some minerals and vitamins). The process is ultimately limited by the aeration capacity of the fermenter system. Batches of molasses are highly variable depending on factors such as type (sugar cane or beet), geographical origin, climactic conditions, sugar milling and refining processes, and storage conditions[4–6]. In addition to sugars (mostly sucrose, glucose, fructose, and in the case of beet molasses, raffinose), they contain metabolizable and nonmetabolizable inhibitors such as volatile and nonvolatile organic acids, aliphatic and aromatic esters, aldehydes, ketones, furans, furfurals, phenolics, and SO_2, that can accumulate during the fed-batch process and result in significant exposure of yeast to their damaging effects[1,4–6]. Added to this is accumulation of secondary metabolites by yeasts (e.g., alcohols and organic acids), and the fact that manufacturers grow baker's yeast at supraoptimal temperatures and often add salts[7]. The result is that yeasts are exposed to an increasing plethora of stresses, simultaneously and sequentially during their growth. These include, for example, oxidation (derived from oxidizing compounds in molasses, but more significantly through the aerobic metabolism of yeast), hyperosmotic stress, ionic stress (e.g., Na^+), raised temperatures, organic acids, alcohols, and nutrient limitation and starvation (as yeast are forced into stationary phase at the end of the biomass production process).

Following growth, yeast is separated from spent medium, washed, and processed to form cream yeast, compressed yeast blocks, or dried yeast. Harvesting and processing wet yeast products can take several hours and resultant yeast is stored chilled. Production of compressed yeast involves removal of some extracel-

lular water by vacuum filtration or filter pressing. Cream and compressed yeast products are transported and stored chilled, and are used relatively rapidly (usually within 10 to 28 days, respectively)[4,5]. Dried yeast manufacture is more complex, involving dewatering of the yeast (removal of extracellular and intracellular water by application of salts during filtration) and subsequent warm air-drying. The drying process relies on evaporative cooling to minimize heating of yeast during dehydration. Food-grade additives, such as emulsifying agents and antioxidants, are used to enhance the drying process and to minimize damage to yeast[4,5]. The final product is vacuum-packed in a low-oxygen atmosphere and can be stored at ambient temperatures for prolonged periods (months to years)[5]. Clearly then, downstream processing results in further stressing of yeast including prolonged retention under starvation conditions albeit at low temperatures (during processing, transport, and storage prior to use by bakers), hypo- and then hyperosmotic stress (washing, storage as cream, and then dewatering, and drying, respectively), high-temperature stress (during late stages of drying as evaporative cooling diminishes while bound water is removed), and oxidative stress and desiccation (during drying).

Baking. Application of yeast in baking is extremely varied depending upon regional traditions. Examples of dough types include: (1) plain doughs, in which no sugar is added to flour, and yeast fermentation rely mostly on maltose derived from action of amylases on starch, and also on internal storage carbohydrates and intrinsic sugars available in flour (e.g., glucose and fructose); (2) sweet doughs, in which sucrose is added over a concentration range of 1% to 40% per weight of flour; (3) rye breads and wheat sour doughs, in which yeast is required to ferment doughs in acidic (pH<4) conditions generated traditionally through cofermentation with lactic acid bacteria[4,8,9]. Dough-making methods also vary greatly[8,9]. There are rapid dough-leavening processes involving relatively short fermentation times of 1.5 to 2.5 hours, and longer processes of 4 to 8 hours. Some dough-making methods rely on mechanical mixing to generate the desired dough structure for entrapment of carbon dioxide, whereas others employ food-grade oxidizing and reducing agents to assist in this process[8,9]. Certain doughs contain antifungal preservatives such as calcium propionate or acetic acid. In the case of dried yeast, bakers either add yeast directly to flour with other dry ingredients prior to adding water, or rehydrate yeast prior to mixing dough. In the latter case, rehydration can be across a range of temperatures depending on geographical location and level of technology and control being applied. It may also be necessary to rehydrate dried yeast in doughs containing different concentrations of added sucrose (2 to 40 g per 100 g flour). Dried yeast must therefore be able to rehydrate with maximum vitality across a diverse range of conditions. Another extreme condition for yeast (usually cream or compressed yeast) is the manufacture of doughs that are frozen[8,9]. These may be plain or variously sweet, and are prepared and blast frozen as soon as possible (preferably <30 minutes) after mixing to give a dough core temperature of –18°C within 2 to 4 hours, prior to storage (lasting weeks or months) at –18°C and slow (overnight) thawing.

From these examples, it is apparent that yeast encounter various stresses while being used in the production of leavened baked products. These stresses include high osmotic pressure, which increases with sugar content of doughs (the water activity of 25% added sugar doughs is close to the limit for growth of *S. cerevisiae*[10]); hyperosmotic stress during slow freezing and thawing of doughs, rehydration stress (particularly at cooler temperatures, which do not provide for rapid reconstitution of liquid-crystalline membrane status and structure); salt (particularly NaCl) stress; low pH (especially in sour doughs); and organic acid stress. Nevertheless, bakers require yeast to leaven doughs without significant lag in onset of fermentative activity. Moreover, yeast must produce the correct amount of leavening at the right time regardless of the type and level of bread-making technology being employed. Given the range of sequential and simultaneous stresses encountered by yeast it is remarkable that strains are able to provide the necessary performance characteristics. Arguably, this is due mostly to the ability of strains of *S. cerevisiae* both to withstand and to adapt to the various stresses encountered.

Consequences of stress and mechanisms of stress tolerance in yeast

Knowledge of stress and stress responses is crucial to our understanding how single-cell and multicellular organisms adapt to changing environmental and physiological conditions. It has implications in biotechnology, agrobiology, and medicine[11,12]. Yeast, and *S. cerevisiae* in particular, is the best genetically and biochemically characterized eukaryote; is easy to manipulate genetically through construction of defined mutants, reporter genes, etc., and physiologically through manipulation of growth and other environmental conditions; and has a relatively rapid generation time. Thus, it represents a very important organism for studies of stress and stress response, both from the perspective of its prominence in biotechnology and its role as a model eukaryote[13].

Yeasts have evolved to be extremely responsive and adaptive to environmental changes, including harsh industrial conditions

Table 1. Physiological consequences of key stresses encountered by baker's yeast during industrial applications.

Stress type	Major occurrence	Damaging consequences
Supraoptimal temperatures	During biomass production, drying of yeast, early stages of baking	Leakiness of membranes, loss of internal solutes, ionic imbalance, internal acidification, generation of free radicals, loss of mitochondrial function, damage to proteins and enzyme activities
Oxidation	During biomass production (e.g., respiration), during drying of yeast	Formation of free radicals; damage to proteins, lipids, and nucleic acids; damage to mitochondria; membrane leakage
Hyperosmolarity	During biomass production, downstream processing, drying, inoculation into doughs (particularly sugar doughs), freezing/ thawing of yeast and doughs	Reduction of cell volume and loss of turgor, growth inhibition, disturbance of metabolite concentrations, reduction of fermentative activity
Desiccation/rehydration	During dewatering of yeast in downstream processing, production of dried yeast and reconstitution in doughs (or prior rehydration), freezing and thawing of yeast and doughs	Similar to hyperosmotic pressure but with extreme concentration of internal solutes, disruption of macromolecular structure including membranes, loss of internal solutes upon rehydration, breakdown of vacuolar structure and release of degradative enzymes
Freezing/thawing	In storage of yeast blocks in some bakeries but especially in frozen dough technology	Low internal pH, imbalance of metabolites, dehydration, ionic toxicity, damage to essential membrane processes

described above. In fact, it is apparent that many of the so-called stress response processes involve, or overlap with, normal physiological transitions, e.g., stress factors are naturally induced when yeast transfers from growth on glucose to respiratory carbon sources such as ethanol or acetate. Thus, processes that have been labeled "stress response" through experimentation alternatively can be looked upon as naturally evolved genetic and physiological adaptations to changing environmental conditions that are likely to prevail in normal ecological niches (as well as industrial processes)[14–17].

Stress-associated damage. There is some degree of commonality in the nature of damage or inhibition resulting from the various stresses encountered by baker's yeast (Table 1), and this means that common mechanisms of protection and repair[18] can provide for tolerance of a broad range of environmental insults. Sublethal stresses can predispose to protection against subsequent lethal exposures to the same, or other types of stresses. The acquisition of broad stress tolerance following exposure to a single stress type is termed "cross protection"[18] and is extremely important for physiological conditioning of yeast during biomass production (see below).

Many stresses (e.g., heat, alcohol, organic acids, desiccation, and freezing) can lead to decline in intracellular pH[19]. A consequence of this is that cells must use ATP to expel protons via plasma membrane H^+-ATPase activity[19–22]. Subsequent survival of cells may therefore be determined by the balance between maintenance of intracellular ATP levels for repair and growth processes and expenditure of ATP for pH homeostasis[19,21]. Control of internal pH is clearly very important; in yeast cells growing normally (unstressed) the plasma membrane H^+-ATPase uses about 15% of ATP produced, but this can rise to about 50% in some conditions[21]. Internal acidification can lead to degradation of storage carbohydrates[23], and potentially increased glycolysis and greater provision of ATP[21]. It also stimulates the Ras-adenylate cyclase pathway leading to increased activity of protein kinase A[21]. It has been reported that thermotolerance can be induced in yeast by protein kinase A-dependent processes, probably in a cAMP-independent manner[24]. Because high protein kinase A activity can lead to down-regulation of expression of stress-response genes (Fig. 1), yeast must achieve a subtle balance between the need for energy-supplying pathways and expression of genes encoding stress protection/damage repair functions.

Table 2. Some key factors and their roles in protection or repair of stressed *S. cerevisiae*.

Factor	Function
Hsp104	Disaggregation of denatured proteins
Hsp70 representatives	Disassembly and refolding of denatured proteins, prevention of misfolding of proteins
Ubiquitin	Tagging of damaged and potentially toxic proteins for degradation
Catalase A (peroxisomes)	Detoxification of reactive oxygen species (ROS)
Catalase T (cytosol)	Detoxification of ROS
Cytochrome C peroxidase	Detoxification of ROS
Cu/Zn SOD (cytoplasm)	Detoxification of ROS
MnSOD (mitochondria)	Detoxification of ROS
Tsap	Protection against oxidation involving thiol groups
Atxp	Heavy metal (and associated ROS) detoxification
Metallothioneins	Heavy metal (and associated ROS) detoxification
H^+-ATPase	Efflux of protons and regulation of internal pH
Other membrane ATPases	Efflux of ions (e.g., Na^+, Li^+)
Glutathione	Detoxification of ROS
Trehalose	Protection of proteins and lipids—compatible solute
Glycerol	Osmotic equilibration and retention of turgor pressure in hyperosmotic conditions—compatible solute

Heat and oxidative stresses give rise to macromolecular damage (Table 1). Reactive oxygen species appear to play a somewhat common role in stress-induced injury derived from exposure to low water activity, freezing, organic acids, heat, dehydration, or alcohol[25–30]. Certainly, yeasts generate numerous antioxidant defenses (Table 2) when exposed to different types of stresses[30]. Moreover, baker's yeast is highly tolerant of oxidative stress. Compressed baker's yeast suspended in phosphate buffer and challenged with 1 M hydrogen peroxide for 1 hour, or 100 mM menadione for 1 hour, exhibited >90% survival (such oxidative treatments are highly lethal to laboratory strains) (unpublished observation). This high-level resistance to oxidative stresses may provide an insight into the general environmental robustness of industrial yeast strains relative to standard laboratory strains.

Exposure of *S. cerevisiae* to hyperosmotic conditions results in rapid water efflux, reduction in cell volume and associated cell shrinkage, loss of turgor pressure, and increase in internal solute concentrations[31–34] . The initial shrinkage of yeast cells is extremely rapid (minutes) and is the result of high water permeability of biological membranes. Internal solutes concentrate because of their relatively low permeability coefficients[32]. There is considerable overlap, but not identity, between the consequences of hypersosmotic stress and those of desiccation and freeze-thaw stresses. Thus, during drying of yeast or application in frozen doughs, yeasts are exposed to water loss, increased concentrations of internal solutes, lowering of intracellular pH, ionic toxicity, impairment of glycolysis, macromolecular damage, aggregation of cytoskeletal elements, and organelle disruption[35–40]. Membranes are particularly sensitive to these stresses, and yeast can become very leaky for intracellular components upon rehydration or thawing[36,41].

Stress tolerance mechanisms. Response of cells to stress entails processes of growth control, cell sensing, signal transduction, transcription, and posttranslational control[14–17,42,43]. When cells are growing exponentially by fermentation under optimum conditions the Ras-adenylate cyclase pathway is fully operative leading to cAMP-dependent activation of protein kinase A (Fig. 1). This leads to transcription and posttranslational events that support growth and proliferation, but also to down-regulation of stress-tolerance factors. Although the Ras-adenylate cyclase pathway is crucial, other important signal pathways also influence the physiological status of yeast cells during growth, the most prominent example being the glucose-repression pathway involving Hxk2 function, and the Cat1/Snf1 protein kinase[14].

When yeast enters suboptimal growth conditions, or is starved or otherwise stressed, a complexity of responses can occur (Fig. 1). These include a lowering of cAMP-dependent protein kinase A activity as well as activation of other cAMP-dependent and -independent processes. Specific and general stress responses can be activated depending upon the stress type. Cell sensing and mechanisms of signal transduction leading to the expression of specific and general stress tolerances are being unravelled but we are far from fully understanding the molecular bases of these processes. This area is complicated by the fact that there often are indistinct boundaries between stress types and responses, which probably reflects the need for cells to repair specific primary damage and to adjust to various other metabolic disturbances when exposed to a particular stressing agent. Thus, an individual stress such as heat can cause cellular damage either directly or indirectly through various modes (e.g., direct damage to membranes or thermal inhibition of enzyme activity, plus secondary oxidative damage through formation or accumulation of reactive oxygen species) and this requires a diversified response from yeast[30] (Fig. 1).

In *S. cerevisiae* there are at least three positive transcriptional elements activated by stresses. These are heat-shock elements (HSEs), stress-response elements (STREs), and AP-1 responsive elements (AREs). They appear to have overlapping but separable functions. For example, some genes containing HSE promoter sequences are expressed under moderate stress and their products have functions

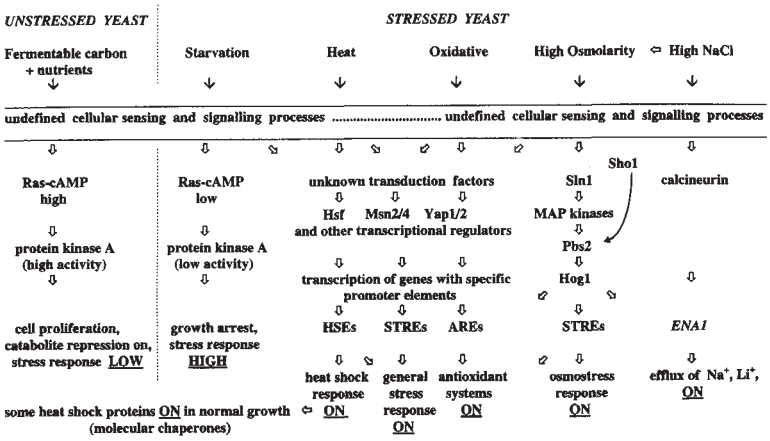

Figure 1. A simplified summary of stress response processes in *S. cerevisiae*.

in normal cell growth and development as well as in stress recovery; those with STREs are crucial to survival of cells that have been exposed to severe (potentially lethal) stress; and the ARE-controlled genes appear to function during oxidative stress[42,43]. Specific factors that are involved in the activation/expression of HSE-, STRE- or ARE-controlled genes are known. These include heat-shock transcription factor (Hsf), STRE-transcriptional factors (designated Msn2/4), and transcriptional activators (Yap1/2) that bind to AREs[44–48]. Yap1 appears to interact somewhat with STREs. Moreover, yeast strains with deleted Msn2/4 function are hypersensitive to carbon starvation and other stress types such as heat, and oxidative and osmotic stresses. These observations indicate, again, the overlap between apparently distinct stress-response processes.

Stress responses lead to the accumulation of a number of proteins and other molecules involved in cellular survival and restoration of function (Table 2). Heat-shock genes are prevalent among those that are expressed under stressful conditions[49–51]. However, to date only Hsp104 has been shown to be essential for thermotolerance[52]. Moreover, several of the heat-shock proteins function in normal cell growth and development, acting as molecular chaperones[50]. The more general STRE-dependent response involves diverse genes such as *UBI4* (ubiquitin); *DDR2* (DNA damage repair); *HSP104, 26,* and *12; CTT1* (catalase T); *TPS1* and *TPS2* (trehalose-6-phosphate synthase and trehalose phosphate phosphatase); *CYC7* (iso-2-cytochrome C); and many others[14,18,33,42]. The diversity of these gene functions indicates the generality of the STRE-dependent response. The oxidative response includes expression of genes coding for enzymes involved directly in detoxifying reactive oxygen species, or the synthesis of antioxidants such as glutathione[53], but also impacts on the heat shock and general stress response (Fig. 1).

Response of yeast to changing osmotic pressures is one of the better understood stress responses[33,54]. Hypoosmotic stress response is governed in part by the protein kinase C pathway and includes modulation of synthesis of cell-wall-assembly enzymes[33]. The hyperosmotic response of yeast includes expression of genes involved in glycerol synthesis, efflux of cations, and more general stress response (Fig. 1). There is a specific Na^+ efflux response that is modulated by calcineurin, which is a Ca^{2+}/calmodulin-dependent phosphoprotein phosphatase consisting of catalytic subunits (encoded by *CNA1* and *2*) and a regulatory subunit (*CNB1*)[34,54,55]. This leads to expression of *ENA1*, which encodes a Na^+, Li^+ -ATPase (Fig. 1). Hyperosmolarity exerted by salt and other osmolytes can also induce a more general osmotic response that involves an osmosensing transmembrane protein termed Sln1; a second transmembrane protein, Sho1; and specific MAP kinases[33,34]. This cascade results in activation of the high-osmolarity glycerol pathway[56–58], which involves Pbs2 and Hog1, which triggers osmoresponsive genes and other general stress responsive genes containing STRE elements (Fig. 1). Among the genes induced are *GPD1* (glycerol-3-phosphate dehydrogenase), *CTT1, HSP12,* and *HSP104, ALD2* (cytosolic aldehyde dehydrogenase), and others[33]. Tolerance of high salt is also dependent upon expression of other salt-responsive genes designated *HAL*[54,59].

Accumulation of glycerol is crucial to hyperosmotic response because the polyol represents a key compatible solute for baker's yeast[60,61]. Its synthesis, and importantly, its intracellular retention, is crucial to regaining turgor pressure. Following hyperosmotic exposure, as well as expressing *GPD1* as part of the HOG response, baker's yeast adjusts its metabolism to provide for increased glycerol synthesis. This entails transient increases in glycolytic enzyme activities and glycolytic intermediates[31–33,60]. In addition, cells adjust to reduce the efflux of glycerol. Normally, glycerol leaks from *S. cerevisiae,* but under hyperosmotic conditions a specific glycerol facilitator (Fps1) closes to allow for intracellular accumulation[33,62].

Clearly, the survival and environmental adaptation (and, indeed, industrial performance) of yeast depends on the severity of stresses imposed, the physiological condition of yeast at the time of stress imposition (i.e., its degree of intrinsic robustness), genetic (strain-dependent) potential, and prevailing conditions following physiological adjustment by cells. It is clear that stress response is central to survival and growth of cells in natural environments and industrial applications. For this reason a greater understanding of this process is critical to strain improvement for industrial applications. While our knowledge of this area is already considerable, we are some way from fully understanding the details. Moreover, most reports concentrate on the response of yeast subjected to specific single stresses following exponential fermentative growth under ideal physiological circumstances. Although this has intrinsic fundamental value, it disregards the broader issues for industrial yeast users, i.e., that yeast is used under nonideal physiological conditions, and multiple and sequential stresses often are encountered when yeast is in lag, fermentative, respiratory, or stationary phases. More work is needed in all of these growth phases to fully understand the molecular impact of stress types and the nature of stress tolerance in the context of industrial yeast applications.

Improving baker's yeast performance

Physiological conditioning. An important means of optimizing performance of baker's yeast is physiological conditioning during manufacture[1–5]. It is well known in the industry that there is a strong correlation between concentrations of trehalose stored by yeast and robustness. Thus, manufacturers strive for high levels of the disaccharide in final products (exceeding 10% trehalose per dry weight of yeast for cream and compressed product, and >15% for dried yeast)[63]. Interestingly, it is likely that trehalose plays a direct role in stress tolerance, protecting proteins and lipids under some physiological conditions, but that in other cases the disaccharide is more important as an energy reserve[64–66].

Certainly, the presence of high levels of trehalose correlates strongly with general induction of stress tolerance factors, making it a valuable marker for potential stress resistance of baker's yeast. Individual yeast strains behave differently with regard to storage carbohydrate metabolism (unpublished observation). All strains tend to show patterns of accumulation and diminution of trehalose and glycogen throughout batch culture as they progress through lag phase, exponential fermentative growth, diauxic lag, respiratory growth, and then a stationary phase. Some strains appear to place emphasis on trehalose manipulation, whereas others show more pronounced changes in glycogen content. Given the apparent role of trehalose in cellular protection, this behavior can influence choice of strains for use in the baking industry.

The fact that baker's yeast is forced into a stationary phase at the end of production processes also provides for higher levels of stress tolerance and general robustness. Manipulation of growth protocols, including parameters such as growth rates, addition of osmolytes, and temperature shifts predispose to good robustness of yeasts by ensuring that high levels of protectants such as trehalose and glycerol are synthesized. The optimum growth parameters for individual strains are quite specific to the genetic background of yeast, and it is possible to manipulate individual strains to suit commercial applications. For example, a baker's yeast strain will be grown differently for use as a cream or compressed product, in comparison with its use as a dried yeast[4]. In general, it is perhaps fortuitous that the most economic means of producing yeast (i.e., by respiratory metabolism on a cheap, waste carbon source containing noxious compounds) naturally provide for induction of broad stress tolerance and preconditions industrially grown yeast to withstand ongoing stresses during downstream processing and applications in baking. Indeed, yeast grown in purer sugar syrups and not exposed to raised temperature or other abuses during fed-batch growth, are less tolerant of conditions encountered in the baking industry. This may imply that inhibitors in molasses play an important role in "conditioning" yeast to become more robust. Clearly, it is a matter of balance between inducing stress tolerance and not inhibiting yeast growth and yield.

Genetic improvements. Genetic improvement of yeasts has traditionally been a somewhat empirical process of classical strain hybridization (mating haploids or protoplast fusions) and gross selection for broad traits such as fermentative activity, osmotolerance, rehydration tolerance, and organic acid resistance[1]. So far as this author is aware, all of the baker's yeast strains currently in industrial use around the world are derived through classical breeding programs. The ability to manipulate yeast by recombinant DNA techniques has been available for approximately two decades, but the true potential of this approach to strain improvement has not yet been realized. Although there are some examples of the recombinant approach being used to improve baker's yeast, these mostly deal with increasing sugar utilization or storage carbohydrate metabolism of baker's yeast[67–69]. These have been targeted as relatively simple traits to manipulate because desired changes can be achieved through a single or low number of genetic manipulations. A limitation of a broader recombinant approach is the lack of detailed knowledge of the biological bases of commercially important yeast traits. For example, what cellular functions are crucial to strong performance of yeast in rapid (vs. slower) leavening processes, or for determining good leavening in unsugared doughs, sugared doughs, or frozen doughs? Similarly what is the genetic basis of efficient biomass yield on a range of raw materials, and which genes encode drying and rehydration tolerance?

Recent developments in genetic manipulation and understanding (e.g., complete genome sequencing) of *S. cerevisiae* have been impressive. There are over 6000 open reading frame (ORF) coding regions, and the predicted number of protein coding regions is 5800 (ref. 70). Of these, only about 30% correspond to genes whose product function has been directly characterized in yeast by conventional means, including functional assay. About half of the remaining ORFs show some degree of relatedness (homologies or common motifs) to genes of yeast or other organisms whose products are functionally characterized, and as such, a role in yeast can be inferred. The functions of the remaining genes (about one third of the total ORFs) are entirely unknown[70]. Considerable effort is now going into the systematic functional analysis of the yeast ORFs, the aim being to define the role of all the genes. However, assignment of gene functions to commercially relevant traits will certainly need to go beyond the initial individual gene functional analyses. Undoubtedly, multiple genes are involved in determining physiological traits that affect yeast performance in industrial processes. There is a clear need for greater emphasis on physiological research, especially within the context of industrially relevant conditions. Major improvements in industrial yeasts will be limited severely until we develop a concerted understanding of physiological and genetic determinants, for example, of drying and rehydration tolerance, or yeast performance in frozen dough applications.

Application of recombinant DNA technology to improve yeast strains is also limited by the not insignificant matter of public perception and acceptance of its potential benefits. It is not unreasonable for the baking industry to refuse recombinant yeast unless there is an obvious benefit to the industry and its customers. To date, much of the effort in this area has gone into improving the fermentative capacity of yeast. However, for the most part classical genetic breeding programs have already produced yeasts that are rapid with regards to fermentation and quite capable of simultaneous uptake of maltose and glucose, which is ideal for instant nonsugared dough leavening. Why would a baker move to a recombinant strain with such properties if yeasts are available through more "publicly acceptable" technology? It is far more likely that recombinant strains will be accepted in those circumstances where yeast is required to perform against its natural "biological design" and where classical breeding methods are inadequate, but marked technological and commercial benefits are attainable. An example of such a case is with frozen dough yeast, where the need is to maintain resistance to long-term freezing and thawing injuries. Under the conditions of frozen dough manufacture, yeast is inoculated into doughs and there is a significant time delay at physiological temperatures where the Ras-adenylate cyclase pathway can operate and cells commit to growth, turn down stress gene expression, and lose tolerance factors. If strains could be developed that either delay the onset of the cell division cycle and/or maintain high-level expression of stress-tolerance factors, these would have an advantage in frozen dough applications. Such a phenotype goes against "biological design," and it is unlikely that classical breeding selection processes would yield useful strains. This provides a potential niche for recombinant DNA technology whereby highly specialized strains that are able to maintain high level stress tolerance despite inoculation into fermentable growth medium (such as bread dough) could be developed and would be of significant advantage to the baking industry and consumers[63]. To this end, a recombinant DNA approach may be necessary to engineer general constitutive expression of groups of stress genes governed, for example, by factors such as Hsf, Msn2/4 or Yap1/2. Additional work is needed to understand how these factors are controlled (through transcription, posttranslation, and nuclear targeting), and what effect such manipulations might have on overall robustness, growth, and yield of yeasts. Additional tempting targets for genetic manipulation are the factors listed in Table 2. Indeed, manipulation of trehalose metabolism has been the subject of attempted industrial-strain improvement using recombinant DNA approaches[63,68,69]. However, it cannot be assumed that manipulation of single enzymes or protectant levels will automatically provide greater stress tolerance in industrial conditions, not least because single stresses often have multiple cellular targets (primary and secondary), and baker's yeast is subject to more than one type of stress during its industrial life span. It is more probable that multiple manipulations of genes or modifications to controlling genes that have multiple effects, will be necessary to generate significant, physiologically relevant improvements.

Another example of where recombinant DNA techniques might be useful is in the generation of broad dough range strains. Most yeast strains for baking currently fall into two categories. Class 1 comprises the strains that ferment effectively in plain doughs, but that cannot leaven high-sugar doughs adequately because they have relatively poor glycerol production and retention, and have high invertase levels that predispose to too rapid a hydrolysis of sucrose in doughs, leading to even greater hyperosmotic stress[10]. Class 2 comprises strains that adequately leaven doughs containing high sugar concentrations, but that cannot leaven unsugared doughs efficiently because they have poor maltose metabolism. There is a logistical and economic need to

limit the numbers of individual strains manufactured in single plants or used in specific bakeries. One way to do this is to combine the leavening activity of yeast for unsugared and sugared doughs within one strain. The recombinant approach of improving maltose metabolism of class 2 strains has been applied[67], but commercial strains have not appeared in the marketplace. An alternative route for achieving broad dough range strains would be to enhance the high sugar leavening of class 1 strains by improving their glycerol synthesis and retention[10]. The production of glycerol, coupled with adaptation of the cell membrane to allow its intracellular retention (under nonhyperosmotic conditions glycerol leaks freely from baker's yeast), is critical to performance of yeast in sugared doughs and frozen doughs. Indeed, leavening of sugared doughs can be enhanced markedly by preloading of baker's yeast with glycerol[71], which can be taken up rapidly by yeast through the Fps1 facilitator and via simple diffusion[10,33,62].

There are undoubtedly niches for application of recombinant DNA technology to industrial yeast strain improvement[72,73]. These will become more numerous as our knowledge of the biological bases of industrial performance improves, and as baking processes and products become increasingly sophisticated. The unravelling of the functions of all genes of *S. cerevisiae* and ongoing studies of consequences of stress damage and cellular responses to this, combined with an in-depth analysis of the molecular basis of industrially important physiological traits, will provide a firm basis for improving baker's and other industrial yeasts. The knowledge gained from yeast research will surely have impact in other areas of biotechnology, agriculture, and medicine. Many yeast genes are homologous to genes in other organisms including humans, and some human cDNA copies can complement mutations in corresponding yeast genes[74,75]. The work being undertaken in yeasts should provide a firm basis for improving our understanding of stress tolerances of plants and processes of animal cellular responses involved in aging, cardiac disease, immunity and autoimmunity, infections, and cancer[11,12].

Conclusions

Industrial production and applications of yeasts expose these microorganisms to multiple stressful conditions. If they are to be of commercial use, baker's yeast strains must be able to survive and adapt to unfavorable environmental conditions that change steadily or rapidly. *S. cerevisiae* displays a complex set of stress responses involving numerous aspects of genetic, physiological, and metabolic control. These responses may be enhanced during production of yeast for baking by manipulating growth conditions to "condition" yeast to better stress tolerance. Traditional genetic breeding programs can only provide for limited improvement of strain robustness, the major problem being that the natural response of yeast when inoculated into doughs is to begin growth and thereby turn down stress-tolerance factors. Nevertheless, there is a requirement to maximize stress-tolerance factors in yeast, particularly during drying and during application in manufacture of frozen or high-sugar-concentration doughs. This is most likely to be achieved through recombinant DNA technology. However, despite the ever-increasing knowledge of the yeast genome and gene functions, we do not yet know enough about how commercially relevant traits are governed genetically and physiologically to permit relevant, controlled genetic manipulations. The application of recombinant DNA technology will have to offer sufficiently recognizable benefits to producers, bakers, and customers before it is likely to be accepted. Manipulation of stress tolerance to improve robustness and commercial performance of baker's yeast could provide worthy targets for this technology.

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