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REVIEW

Overview on Brewing Yeast Stress Factors

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Abstract

The environmental changes directly affect cellular activity, the ones interfering with their optimal activity or jeopardizing their life are known as stress factors. Both prokaryotes and eukaryotes are able to respond such changes through a complex network of reception and signaling which determines adaptation of growth and multiplication, gene expression modeling, metabolic a ctivity as well as other cellular changes. In the brewing industry, the conditions used for industrial fermentation impose a variety of stresses upon the inoculum. Moreover, the modern brewing techniques like high gravity brewing or the use of dried yeast as inoculum increase the magnitude of stresses imposed to brewing yeast cells. Knowledge on yeast capacity to respond effectively to the continuously changing conditions is essential for both beer quality and maintenance of yeast fermentation performance.

Keywords: *Saccharomyces*, beer, oxidative stress, ethanol stress, thermal stress, osmotic stress, high hydrostatic pressure stress, mechanical stress, nutritional stress.

1. Introduction

The utilization of yeasts in brewing industry presumes their exposure to severe environmental changes throughout yeast propagation, fermentation and yeast storage processes. At the beginning of fermentation yeasts experience temperature shock, hyper osmotic challenge caused by high solute concentrations and oxidative stress due to aerobiosis. As the fermentation progresses they are exposed to anaerobiosis, hydrostatic pressure inside the fermenters, an increase in acetaldehyde and ethanol concentration, internal acidification and starvation (1, 2). The array of stresses brewing yeast is subjected during the brewing process will be discussed further on, considering the order of appearance in the brewing process (figure 1).

2. Oxidative stress is the cellular response to damage produced either by accumulation of intracellular reactive oxygen species (ROS) -superoxid anion ($O_2^{\cdot -}$), hidrogen peroxide (H_2O_2), hydroxy radical (OH^-)- or by changes of the cellular redox state (3).

Destructive cellular effects of oxidative stress

During mitochondrial respiration, due to high oxygen concentration dissolved in the culture medium, proteins, lipids or DNA of different cellular components can suffer oxidative damages. Lipid peroxidation can lead to decreased membrane fluidity, membrane receptors and enzyme inactivation, as well as decreased specific ion permeability. Protein oxidative damage can lead to hidrogen peroxide formation, changes in molecular weight through protein aggregation or protein fragmentation through peptide bond breakage, changes of electrical charge and can increase susceptibility to proteolytic changes. ROS can also damage DNA structures through reactions determined to the carbohydrate components or to nitrogen

bases. Mitochondrial DNA is more prone to oxidative damage than the nuclear DNA possible due to the fact that former is not protected and is localized close to the production place of ROS, the electron transporter chain (3). When cellular mechanisms are not able to repair effectively the damage caused to the cellular compounds DNA oxidative destruction can determine punctual mutations, deletions, insertions, intrachromosomial recombination or crossing- over.

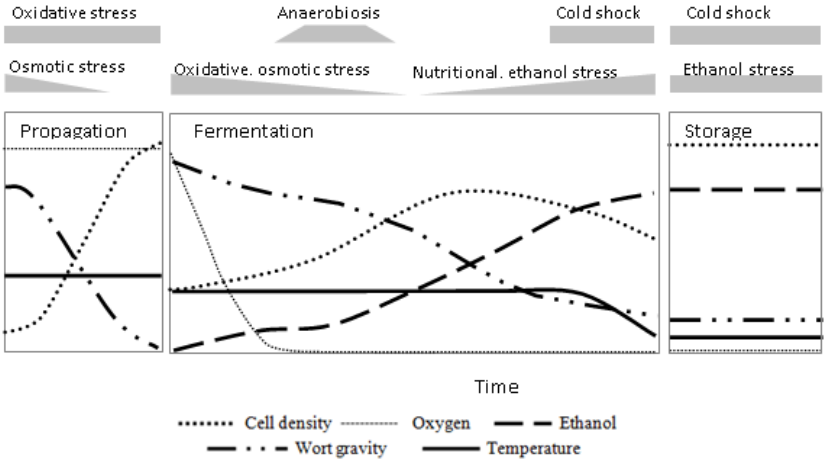

Figure 1. Types of stress during brewing fermentation process (15)

Cellular defense mechanisms, antioxidants

To counteract the negative effects of oxidative stress yeast cells use non- enzymatic or enzymatic antioxidant mechanisms. The non-enzymatic systems are usually small molecules, hydro- or lypo- solubile, that act by ROS binding: glutathione, polyamines, erithroascorbic acid, metallothioneines, flavohemoglobines (4). Primary defenses are provided by the enzymatic antioxidant mechanisms using one or more enzymes to anihilate ROS. *Saccharomyces cerevisiae* has two genes for catalase (EC 1.11.1.6): CTA1, codifying peroxizomal catalase A and CTT1 for cytosolic catalase T (3). As well, yeast have two forms of superoxide dismutase (SOD, EC 1.15.1.1): cytoplasmic SOD, with Cu and Zn atoms (Cu/Zn Sod), codified by SOD1 gene and the mitochondrial SOD, with Mn (MnSOD), codified by SOD2 gene. Yeast cells can rely on enzymatic antioxidant defense mechanisms as glutathione- peroxidase (EC 1.11.1.9), glutathione- reductase (EC 1.8.1.7), thioredoxin-peroxidase (EC 1.11.1.15) and on the most recent discovered enzymatic antioxidant, peroxiredoxins (EC 1.11.1.15), present in yeast as a family with five members, located in different cellular compartments and performing different functions (5, 6).

Involvement of the master regulator of oxidative stress, Yap1, was reported in the stress responses during fermentation process (7). Yap1 belongs to the YAP (Yeast AP-1 like) family of bZIP transcription factors, which modulate the activation of specific genes in response to various stress conditions (8). In the model budding yeast *Saccharomyces cerevisiae* ROS accumulation induces Yap1-dependent expression of the antioxidant machinery and Yap1 accumulation in the nucleus (9, 10).

Factors Affecting Yeast Oxidative Stress

Oxidative stress in yeast is directly dependent on the duration and the intensity of the stress factor (viability decreases with the duration of exposure and concentration of hydrogen peroxide), oxidant source (effect of the exogenous hydrogen peroxide is stronger and more destructive than the endogenous one), yeast cells developing phase (stationary phase cells are

more resistant to oxidative stress than the exponential phase cells), the type of brewing yeast strain (ale yeast strains are more sensitive to oxidative stress than the lager strains), cultivation media (stationary brewing yeast cells inoculated on wort exhibit a lower tolerance than the ones inoculated on yeast peptone dextrose medium) (3) and Cu ions concentration (1).

Oxidative stress in the brewing industry

Oxidative stress can appear not only as result of the internal metabolic reactions, but also it may be the result of the changes in cellular environment. Even though fermentation is performed in anaerobiosis, brewing yeast cells can be exposed to oxidative stress during yeast propagation, inoculation or during yeast storage.

The **propagation step** is performed under aerobiosis in order to obtain optimum developed yeast cells and to stimulate synthesis of fatty acids, sterols and a consistent level of reserve carbohydrates. Oxidative stress experiments during propagation showed increased catalase activity and glycogen and trehalose concentration 100h after inoculation into the propagation vessel (3).

During **storage** between fermentation rounds brewing yeast cells consume endogenous reserves for maintaining cellular functions (reduced consumption of trehalose when yeast is stored at 5°C and over-consumption of the storage carbohydrates when storage is performed at 20°C) (3). Sometimes, storage of cells as dry yeast is preferred, due to obvious reasons: longer shelf- life, significant weigh and volume reduction with implications on transport and storage, as well as higher resistance to unfavorable environmental conditions. The main disadvantage of this storage method is represented by the structural and metabolic changes in yeast, which dramatically affect viability. When obtaining dry yeast, cells are subjected to dehydration, which negatively affects cellular membrane, reduces cytoplasmic and intracellular transport, determines cytoplasmic pH changes and accumulation of both inorganic and organic ions. Any of these changes can temporary disable the enzymes activity leading to free radical formation (12).

3. Thermal stress Temperature exhibits a fundamental influence upon the metabolic processes, being able to act either as activator or as an inhibitor of microorganism development, with lethal implications sometimes. Yeast is generally considered a psychotropic microorganism, being capable of development at minimum temperatures of 1-3ºC, with optimum between 25- 30ºC and a maximum development temperature of 40ºC (13). When yeasts are exposed to temperatures outside the optimum interval, they activate the response mechanisms for maintaining homeostasis, process known as thermal shock response. If yeast are subjected to temperatures below optimum development temperature cell undergoes a cold shock, while yeast exposure to temperatures higher than the maximum optimum temperature leads to heat shock (14).

Thermal stress induced cellular changes

Thermal destruction in yeast cells results from broken hydrogen bonds and hydrophobic interaction that determine a generalized denaturation of proteins and nucleic acids. Yeast does not possess any internal mechanism for temperature adjustment and for this reason the higher the temperatures, the more extended are the cellular damages. The high temperatures determine an exponential increase of the death rates, atypical budding, cycle arrest in G1 phase, increased fluidity of plasmatic membrane and reduced permeability for essential nutrients, reduction of cellular pH, appearance of respiratory mutants (*petites*). Low temperature stress induces shrinking of yeast cell, increase of the membrane unsaturated fatty acids determining a slow transport of solute into cells, compromised membrane integrity due

to a transitory gel-like phase of the membrane's fatty acids/ sterols, destruction of the vacuolar membranes followed by vacuole breaking and growth arrest (15).

Yeast cellular response to thermal stress

Physical or chemical stress factors induce direct or indirect changes of proteins, mostly protein aggregation, which triggers malfunctions in all cellular compartments. Luckily, not all structural cellular damages are irreversible. Stress factors with reduced intensity increase synthesis of the Heat Shock Proteins (HSP), supporting microorganisms for adaptation to stress (table 1).

Table 1. Proteins involved in thermal stress in *Saccharomyces cerevisiae* (14, 16, 17)

Heat Shock Protein	Physiological function
Hsp104	Essential for thermotolerance acquisition. It is expressed constitutively in respiring cells, that do not ferment, entering stationary phase.
Hsp 100	Involvement in solubilization of protein aggregates and degradation of proteins
Hsp 90	Similar function to chaperonin and Hsp 70
Hsp83	Chaperone function
Hsp70 family	Interact with denatured proteins, helps with their solubilization and simultaneous refolding, having a chaperone function Implication in post- translational import
Hsp60	Functions similar to Hsp70, facilitates post-translational protein assembly
Reduced size Hsp	
Hsp30 Hsp26 Hsp12	Cellular role is not entirely known; it seems they are involved in the initiation of stationary phase and in induction of sporulation Hsp30 may regulate plasma membrane ATP– ase
Other proteins	
Ubiquitin	Implicated in the turnover of stress- degraded proteins
Part of glycolitic enzymes	Enolase (Hsp48), glyceraldehide 3 – phosphate dehydrogenase (Hsp35) and phosphoglycerate kinase
Catalase	Antioxidant defence
GP400 and P150	Implicated in HSP secretion.

In *Saccharomyces cerevisiae*, Hsp 100/Clp protein family was mostly studied, Hsp 104 having a decisive role for acquisition of tolerance to high temperatures and other types of stressors. Hsp 104 together with Hsp 70 and Hsp 40 form a proteic complex, which facilitates reactivation of the partially denatured proteins by high temperatures, being thus involved in maintenance of essential cellular processes under stress conditions. Synthesis of HSP can be also induced by hypertonic conditions, ethanol, as well as other stressors (18). Besides initiation of Hsp synthesis, yeasts respond to the thermal stress by accumulation of protecting compounds such as trehalose and glycerol or enzymes- catalase, mithocondrial superoxide- dismutase (19). Stimulation of the antioxidant enzymes by thermal shock can allow bonding of superoxide radicals, preventing thus the oxidative damages that would be amplified otherwise by the elevated temperatures. Poliamines like spermine and spermidine have a crucial role in thermal protection of *Saccharomyces cerevisiae.* Polyamines have a similar mode of action with Mg^{2+} ions in terms of thermal stress adaptation by improving yeast membrane integrity during stress (20). Recent studies showed a certain functional overlapping between thermal stress and oxidative stress response (21).

Factors affecting thermal stress tolerance

Yeast ability to grow and perform its metabolic activity under different temperatures depends not only on genetic heritage, but also on culture medium composition and other extrinsic physical parameters of the cellular growth. Thermotolerance can be defined as the cellular capacity of surviving the exposure to high temperatures, usually having a lethal effect. Intrinsic tolerance can be observed in yeast cells exposed to a sudden thermal sudden shock (by exposure to 50ºC for example), while induced thermotolerance appears after the cells are exposed to a moderate thermal shock (maintenance at 37ºC, 30 min.) followed by exposure to a severe thermal shock. Besides the moderate thermal shock there are other factors influencing thermotolerance: certain chemicals ($Ca^{2+,}$ trehalose reserves improves thermal resistance), osmotic dehydration (22), reduced external pH (optimum thermotolerance at pH 4), nutrient concentration and cellular growth phase (exponential phase cells are more sensitive than the stationary phase cells).

In the case of cold shock, *Saccharomyces cerevisiae* viability can be substantial improved if prior to cold shock (immersion in liquid nitrogen) yeast cells are exposed to moderate heat stress at 43ºC for 30min. This result lead to the hypothesis that heat shock proteins protect yeast cells subjected to cold shock by stabilizing the molecules and by increasing the hydrophobic interactions in yeast cells.

Thermal stress during beer fabrication

Yeast propagation

Even though brewing yeast have optimum growth temperature around 30ºC, only rarely the propagation step is performed at this temperature. Usually propagation is performed at temperatures higher than the ones used for fermentation in the first vessel (20–25ºC), followed by step-by-step temperature reduction in the following propagation stages, until reaching temperatures equal to the fermentation ones. (23) This way high yeast growth rates are attained when higher temperatures are used for propagation, the whole duration of the process being shortened.

Wort fermentation

Through conventional methods it can be performed as cold fermentation (inoculation at temperatures ranging from 5 to 6ºC, with maximum temperatures of 8 – 9ºC) or hot fermentation (wort inoculation with yeast is performed at 7 – 8ºC, maximum attained temperature being of 10 – 12ºC). The temperature does not dramatically vary during fermentation and the process does not take place very quickly, so that yeast have time to adapt to the new conditions.

Yeast storage

After beer fermentation has ended, brewed yeast biomass can be reused for up to 10-12 fermentation rounds (24). Until it is again used as inoculum yeast cells have to be stored in conditions that insure the maintenance of its viability and vitality. There are several storage possibilities that take into account storage duration: a) for short periods (two days- one week) yeast cream is first cold acid washed followed by storage in 4 – 5ºC water; b) for longer periods (maximum two- three weeks) yeast cream is stored in 0 – 2ºC beer; c) for few months storage yeast cell lyophilisation is available; d) yeast storage for years is best to be performed by keeping cells in liquid nitrogen (–196ºC). Considering the low temperature at which storage is carried out, so that the metabolism is stopped, the use of the yeast after storage needs an adaptation period to restore metabolic functions.

4. Mechanical stress Mechanical stress is also known as physical or shear stress and it appears whenever yeast is physically moved within the brewery, either naturally (e.g. driven by a convection current within the fermentation vessel) or artificially (e.g. pumping, centrifugation) (25). Yeast are generally considered resistant to physical stress, especially due to cell form and dimensions, as well as due to the rigid cell walls.

Yeast cellular changes during mechanical stress

Yeast response to shear stress overlaps partially with the classical stress response, triggering glycogen consumption, trehalose levels variation (26), viability and vitality reduction, increased slurry (which is a highly concentrated cell suspension) pH and leakage of intracellular proteases (27).

Yeast mechanical stress response

The particularity of shear stress response resides in the impact on the cell wall and its functionality. It has been reported that cell wall enzymes (invertase and melibiase) and cell wall polysaccharides (mannan and glucan) were released in slurry supernatant (27, 28) when increasing exposure times to shear stress.

Mechanical stress in the brewing industry

It is due to the repeated use of brewing yeast for 10-12 fermentation rounds. During brewing yeast reuse severe mechanical stress can appear, during yeast suspension pumping through pipes or stirring in propagation vessels. Using mechanically stressed yeast cells for beer fermentation determines the release of mannans and glucans, which generate haze in yeast slurry supernatant and beer (27, 28), impaired flocculation performance and reduction of viable cell number. Moreover, pitching sheared stressed yeast leads to extended lag phase of fermentation process (29) poor fermentation performance and off-flavors formation, together with the dramatic reduction of the brewing cycle lifespan of the yeast culture.

5. Osmotic stress

Osmotic pressure refers to the hydrostatic pressure necessary for stopping the water passing through a membrane that separates two solutions with different concentrations. When the concentration of one solution increases the water activity is reduced and an osmotic shock appears. *Saccharomyces cerevisiae* are osmotolerant microorganisms, growing in culture medium with a_w= 0.90 – 0.94, depending on temperature, nutrient content and the nature of the substance that induced a_w reduction. Yeast are known to be xerotolerant as well, meaning that cells can adapt to grow on nutritional medium with high osmolarity determined by intense water evaporation (30).

Cellular changes triggered by a_w variation and osmotic stress

Yeast have developed perception, response and adaptation mechanisms to face the frequent osmotic changes of the external environment. When the hydric potential of the growth medium is diminished, yeast face hiperosmotic stress. Hipertonic medium, with high osmotic pressure and low free water content, in contact with yeast make the water leave the cell, determining thus a reduction of the cellular volume, phenomenon known as plasmolisis. The normal cell response that appears within seconds of exposure to hiperosmotic stress is intracellular water extrusion (18).

During the adaptation step, yeast cell experience several changes, like restructuring of the actinic cytoskeleton, temporary arrest of life cycle and metabolisms' reprogramming. In the same time the mechanisms implicated in stress resistance are activated: intracellular glycerol concentration increase controlled by High Osmolarity Glicerol pathway

(23), toxic ions elimination, induction of genes expression responsible for redox metabolism and antistress proteins, as well as vacuolar fragmentation (31). The other possible extreme osmotic situation is when the hidric potential of the growth environment is high, triggering hipoosmotic stress. Under such circumstances, water invading cells determines their swelling (turgescence), which can lead to cell lyses (11).

Factors influencing yeast osmotic tolerance

As in the case of other types of stress, brewing yeast tolerance depends on the physiological state (stationary yeast are more tolerant to osmotic stress than the ones in exponential phase) (32). A possible explanation could be the induction of Stress Responsive Elements (STRE), which takes place in the beginning of the stationary phase. Tolerance and response to osmotic stress is also solute dependent.

Osmotic stress in brewing industry

Transfer of brewing yeast from the nutritional medium used for pure culture growth to the yeast growth medium is a potential source of osmotic stress. Both hipo- and hyper-osmotic stress can appear during beer fermentation, but hyperosmotic stress is the predominant one.

Wort fermentation in HGB (High Gravity Brewing) and VHGB (Very High Gravity Brewing) systems is a relatively newly developed brewing technology that uses more efficiently the invested energy and time. While traditional brewing is performed on 12ºPlato (12ºP, i.e. 12g extract per 100g liquid) worts for obtaining 5% (v/v) ethanol beers, 18ºPlato (18ºP) concentrated worts can increase the brewing capacity with 50% without making further investments, reducing the energy and personnel costs and improving beer stability (33). Nonetheless, the use of such systems is limited by the yeast' stress resistance. *Saccharomyces cerevisiae* used as ale yeast resist much better to the osmotic stress than the lager yeast strains. The use of concentrated worts has several negative aspects: most of the time they are only partially fermented, yeast viability is lost much faster compared to the traditional brewing processes and off flavors can appear (34). The main causes for sluggish fermentation are the high osmolarity of the medium from the beginning of fermentation, the high content of ethanol in the end of the process and the lack of nutrients in the final stages of fermentation. The disadvantages of concentrated worts use can be overcame by using osmotic resistant strains, tolerant to the types of stress associated with these conditions. Strains must be capable to ferment rapidly the concentrated worts and be more efficient than the usual strains. In the end of the fermentation process yeast viability and vitality must be high enough so that it could be used for several fermentation batches. Regarding beer profile, selected yeast must form the same aroma compounds and they must have the same flocculating capacity with the usual yeast strains.

6. High pressure stress Brewing fermentation is performed in large capacities fermenters, where yeast can be subjected to considerable stress, represented both by hydrostatic pressure and by gaseous pressure, the last one being due to carbon dioxide endogenously produced by yeast during fermentation. Yeast cells are the most sensitive microorganisms, a pressure of 300 - 400MPa for few minutes at 20°C induces a viability reduction of more than 6 logs (30). Regarding the effect of gaseous pressure the literature indicates that the partial pressures higher than 50kPa have an inhibitory effect upon the enzymatic system of *Saccharomyces cerevisiae* yeast (35). During the industrial fermentation process the pressure determined by the CO_2 formed can act as yeast stressor, especially combined with ethanol stress.

Effects of high pressure upon yeast

Cellular changes due to the effect of high pressure are similar with the ones of thermal and oxidative stress. Lethal effect of high hydrostatic pressure is the result of several simultaneous biological processes: cellular membrane deterioration, inactivation of enzymes implicated in DNA replication and transcription, protein denaturation leading to inactivation of enzyme activity and transcription of stress genes (35, 36). Reduction of transmembrane enzymes activity triggers malfunctions of cellular membrane permeability, determining appearance of areas that favor leaking of the cytoplasmic material. The negative effect of hydrostatic pressure exerted mostly upon cellular membranes could be an effect of the lipids' high sensitivity to high pressure, which are one order of magnitude more compressible than proteins. High hydrostatic pressure, as well as low temperatures, affects phospholipidic bilayers, determining compaction of the fatty acid chains, reducing membrane fluidity. Yeast can perform *homeoviscous adaptation*, which refers to the increase of the membrane fatty acid unsaturation, maintaining thus a functional membrane (35). Other pressure- affected organelles are vacuoles that suffer acidification due to proton dissociations. DNA stability is also negatively affected by high hydrostatic pressure, its conformation being changed from the normal B, double- helix structure, to a Z, zig-zag-ed structure. To protect cells from the stress effect of hydrostatic pressure yeast cells generally accumulate trehalose.

Factors affecting brewing yeast high pressure stress resistance

Stationary phase yeast cells exhibit an increased resistance to high hydrostatic pressure compared to exponential phase cells. Research studies indicated the profound cellular changes triggered by high pressure stress, which are maintained even after the stress has ended. For example, in yeast cells pressurized at 50MPa for 30 min. cellular recovery appears only after 120min. Comparing yeast cell response to high pressure with the one after thermal stress (30min at 40ºC) showed that the pressure stressed cells need a longer period of time for recovering after stress (37). Recent studies indicated an advantage of high pressure stress for alcoholic fermentation: at 10 MPa the fermentation of glucose to ethanol by *Saccharomyces cerevisiae* proceeded three times faster and gave a slightly increased yield when compared with the same fermentation at ambient pressure (38). Resistance to high hydrostatic pressure can be attained either by a moderate pressure treatment or by exposure to moderate thermal shock. Most probably, high pressure tolerance results from a combination between membrane fluidity preservation (rich in cholesterol) and trehalose accumulation.

High hydrostatic pressure stress in the brewing industry

Most yeast resist to hydrostatic pressures stress, but the brewing yeast strains cannot withstand hydrostatic pressures higher than 10MPa and gaseous pressure up to 50kPa. The simultaneous effect of high gaseous pressure and ethanol stress increase the negative effects upon brewing yeast cells. High gaseous pressure negatively affects cellular membrane integrity and cellular division cycle, which further influences the aroma of the final product, beer.

7. Ethanol stress Ethanol is the primary product of beer fermentation. Nonetheless it is a chemical stress factor that inhibits cellular growth and determines metabolic changes leading to the reduction of yeast fermentation performance.

Ethanol stress generated cellular effects

First signs of ethanol stress are the increase of membrane fluidity and permeability, followed by deficient transport system of essential compounds such as amino acids and glucose. Furthermore, ethanol accumulation compromises a range of cellular functions

leading to the reduction of growth, cellular metabolic rate and viability (39, 15). The pleiotropic effects of ethanol stress include the down regulation of the genes involved in RNA metabolism, protein biosynthesis, cellular biogenesis and cell growth, reflecting a growth arrest also observed under various stress conditions (39). Besides the repression of many cellular pathways, ethanol stress responses include the activation of genes involved in the cell integrity pathway and ergosterol synthesis, which are required to recover membrane rigidity. The induction of the general stress response pathway controlled by Msn2/Msn4 and HSF1-mediated signal transduction pathway (40) leads to the increase of HSP and control trehalose cytoplasm contents to stabilize and prevent aggregation of denatured and misfolded proteins (41, 42).

Moreover, ethanol stress responses involve the induction of stress proteins under the control of the stress transcription factors Crz1 and Asr1 (1, 43, 44) and affect many other cellular functions such as ionic homeostasis, energy metabolism (45).

Yeast response to ethanol stress

Yeast ethanol tolerance can be described as strain capacity to withstand high ethanol concentrations without affecting its viability and metabolism. As consequence to ethanol stress vacuolar acidification appears (46), reduction of saturated fatty acids (palmitic acid), increase of the unsaturated fatty acids (oleic acid) (47), speeding of squalen and ergosterol biosynthesis as well as phosphatidilinositol biosynthesis leading to a high ratio of phospholipids/ proteins, increased superoxiddismutase activity, high level of trehalose, stimulation of stress protein biosynthesis and thermotolerance acquisition, increased cytocrom P450 synthesis and activated ethanol metabolism.

Factors affecting yeast resistance to ethanol stress

Yeast resistance to ethanol stress increases when membrane fatty acids have longer carbon chains (24). High temperature, nutrient depravation, reduced pH and low water activity amplify the negative effects of ethanol (48).

Ethanol stress during the brewing process

Although brewing yeasts are able to tolerate reasonably high levels of ethanol, its accumulation during fermentation and persistence during storage process is nonetheless a health hazard frequently associated to sub-optimal yeast fermentation performance. Employment of serial repitching in brewing industry (24, 49), the term given to the harvesting and recycling of yeast biomass on completion of fermentation after a storage period under refrigeration and in the presence of ethanol, aggravates the undesirable conditions yeasts must deal with.

Ethanol stress is even more aggressive when **high gravity brewing (HGB)** is employed, which is an industrial practice involving fermentation of concentrated wort followed by dilution to produce beer with desired alcohol content. Therefore, maintenance of yeast fermentation capacity during beer production and yeast storage process becomes a challenge in beer industry and many efforts are made by companies and researchers to minimize the effects of ethanol stress in these processes.

During the final fermentation stages ethanol can inhibit brewing yeast activity and for this reason fermentation is usually interrupted when ethanol reaches 4-9% (24). Ethanol concentrations over 6% inhibit most of yeast strains, even though there are selected brewing strains that can tolerate well higher ethanol concentrations, over 10% or even 20%, in the case of sake yeast strains (50, 51).

Yeast cream storage is another technological step favoring ethanol stress. High temperature-ethanol combination dramatically decreases yeast viability and for this reason

yeast is usually washed with 4–5ºC water before storage and the storage tanks have cooling jackets, so that temperature is maintained at refrigeration values (24).

There are at least two technological negative consequences of using ethanol stressed yeast. The first one is the pH increase that determines the augmentation of yeast cream viscosity which makes the transport of yeast suspension more difficult, intensifying mechanical stress. The second one is the protease release in the medium, which intensifies foaming, making thus more difficult bottle filling.

8. Nutritional stress Cellular survival is possible only when an energy source is available. When the nutritional needs are not satisfied cells undergo nutritional stress.

Yeast response to nutritional stress

Survival of yeast during starvation has been shown to depend on the nature of the missing nutrient(s). In general, starvation for "natural" nutrients such as sources of carbon, phosphate, nitrogen, or sulfate results in low death rates, whereas starvation for amino acids or other metabolites in auxotrophic mutants results in rapid loss of viability (52). Depletion of fermentable sugar or assimilable nitrogen from wort determine yeast entering the stationary phase in which proliferation ceases (23). When yeasts lack certain essential aminoacids or are treated with protein synthesis inhibitors (e.g. cycloheximide) a stringent stress response appears. In *Saccharomyces cerevisiae* starvation is related to a rapid inhibition of ribosomal RNA synthesis (14) and variations in intracellular content of the two storage carbohydrates, trehalose and glycogen (53). Recent studies indicated the involvement of Nitrogen Catabolite Repression (Dal80, Gln3, Gat1) transcription factors in yeast response to ammonium limitation (40).

The existence of a gene responsible of the life cycle duration was recently revealed to be present in yeast as well. *Sir 2* extends yeast life cyle by maintaining stability of ribosomal DNA. In non-stressed yeast cells Sir2 longevity protein uses NAD as cofactor for nicotinamide production, which exerts negative feedback for Sir2. (54). When yeast cells are exposed to nutritional, thermal or osmotic stress, nicotinamide is transformed in nicotinic acid by the newly activated *PNC1*. As a result, with no inhibition factor of Sir2 it becomes more active and yeast extend their life cycle. For example a yeast with five copies of PNC1 gene has a life cycle 70% longer than a wild yeast (55, 56). Quorum Sensing is a recently discovered signaling mechanism, related to gene expression in correlation to high cellular density. Recent studies indicated the not only bacteria possess such a signaling mechanism, *Saccharomyces cerevisiae* yeast being added to the list (57). Quorum signaling is an adaptation mechanism to nutritional stress, governing filamentous growth as response to reduced nitrogen, which uses as extracellular signals aromatic alcohols.

A more dramatic adaptation mechanism to nutrient limitation is autophagy. This membrane transport catabolic phenomenon appears as response to environmental depletion of nutrients, nitrogen and carbon sources for example (58).

Nutritional stress during the brewing process

Wort is a complex substrate that yeast can use almost entirely for fermentation (exception: galactose, pentoses). After an accommodation period, necessary for induction of specific enzymes, yeast are able to ferment maltose, the main wort carbohydrate. Therefore, fermentation is not usually considered a stress technological step. Nevertheless, special nutrient availability needs attention. *Saccharomyces cerevisiae* cells exposed to nitrogen limited conditions form through aminoacid catabolism isoamilic alcohols, known off - flavor compounds of beer (59). If the concentration of assimilable wort nitrogen drops below 150 mg/L, fermentation is prematurely interrupted (24). As well, too little aminoacids determine

formation of too many higher alcohols, which negatively affects beer quality (15), while zinc depletion in beer fermentation leads to "sluggish" fermentation (60).

Yeast cream storage is a technological step when nutritional stress can appear. Yeast stored for longer periods undergoes nutritional stress and when subsequently used for fermentation processes negativelly affects beer quality. Nutritional stressed brewing yeast exhert an impared floculation capacity due to changes of the cellular surface and malfunction of the genes responsable of intercellular adhesion (61) . For example, *Saccharomyces cerevisiae* genes responsible of intercellular adhesion (*FLO1, FLO5, FLO9, FLO10)* are not constitutively expressed. Their activity is dictated by environmental signals, like carbon or nitrogen limitation, pH changes or ethanol concentration. When nitrogen source is depleted, *FLO11* gene responsible for yeast cell adhesion to abiotic surfaces is activated in search of new nitrogen source (62, 63). The unpredictible yeast flocculation capacity and the delayed flocculation onset makes very difficult the yeast separation in the end of fermentation and negatively affects beer quality and its commercial production.

9. Conclusions

The modern fermentation technologies are incredible stressful processes, brewing yeast being subjected to more than one type of stress at a time, with synergic action. Brewing yeast cells possess a variety of defense mechanisms matching the complex variety of stresses that they are exposed to during the industrial beer fabrication. The stress response in *Saccharomyces* may be general or specific: in case of heat stress yeast biosynthesize HSP, decrease the unsaturation degree of membrane fatty acids, change the cellular pH; cold stress triggers accumulation of large quantities of trehalose and increase in polyunsaturated fatty acids of the membrane; for osmoprotection yeast accumulates compatible solutes (glycerol, trehalose), increase potassium absorption and sodium efflux; as enzymatic antioxidant defense yeast use superoxid dismutase, catalase, cytochrom-peroxidase together with non-enzymatic defenses like glutathione, thioredoxins, metallothioneins and polyamines; in case of ethanol stress yeast synthesize ethanol stress proteins, change the membrane transport system and implicate mitochondrial superoxide dismutase.

Understanding the causes and effects of the numerous types of stress is useful for technological reasons, allowing establishment of improving methods for yeast fermentation performance and beer quality. These studies will also support the understanding of the mechanisms of stress processes in higher eukaryotes, like humans. For a complete mapping of brewing yeast stress factors it is compulsory to determine specific biomarkers to each type of stress.

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