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***REVIEW ARTICLE***

# THE IMPORTANCE OF TREHALOSE IN BREWING YEAST SURVIVAL

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**Abstract**

Originally, trehalose was believed to function only as a reserve carbohydrate similar to glycogen. It is now known that the degradation of trehalose yields little energy and whilst it can serve as a source of carbon skeletons for biosynthesis, it mainly functions as a membrane stabiliser and protectant for yeast cells under stress. (O'Connor-Cox *et al,* 1996). The significance of trehalose as an endogenous protectant is referred to in numerous literature sources.

***Keywords***: glycogen synthesis, trehalose biosynthesis, brewing yeast strain

## 1. Introduction

The increasing demand of brewing industry for high gravity wort as well as the desire of many breweries to use dried brewing yeast as an alternative to conventional yeast propagation, revealed the importance of a less studied carbohydrate which is trehalose. Both processes involve harsh conditions for the yeast cells.

The present paper emphasizes the major role trehalose plays in yeast cells survival, as well as the correlation between trehalose and another storage carbohydrate - glycogen. There are also pointed out few aspects of the mechanism through which trehalose acts as a protectant for the yeast cells under stress conditions.

The life cycle of yeast follows four phases, which are somewhat arbitrary because all of the phases may overlap in time:

**Lag Phase -** Glycogen, an intracellular carbohydrate reserve, is essential as an energy source for cell activity since wort sugars are not assimilated early in the lag phase. Stored glycogen is broken down into glucose, which is utilized by the yeast cell for reproduction. Low glycogen levels produce abnormal levels of vicinal diketones (especially diacetyl) and result in longer fermentations.

**Growth Phase -** The growth phase, often referred to as the respiration phase, follows the lag phase once sufficient reserves are built up within the yeast. This phase is evident from the covering of foam on the wort surface due to the liberated carbon dioxide.

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**Fermentation Phase -** The fermentation phase quickly follows the growth phase when the oxygen supply has been depleted. During this time period, yeast is mostly in suspension, allowing itself dispersal and maximum contact with the beer wort to quickly convert fermentable substances. Most beer yeasts will remain in suspension from 3 to 7 days, after which flocculation and sedimentation will commence.

**Sedimentation Phase -** The yeast begins to undergo a process that will preserve its life as it readies itself for dormancy, by producing glycogen. Glycogen is necessary for cell maintenance during dormancy and, as mentioned, it is an energy source during the lag phase of fermentation (Goldammer, 2000; Smagalski, 2007).

Several cell components, for example glycogen, trehalose and sterol, vary in concentration in response to changes in physiological condition. The concentrations of many of these cells components are known to be influential on fermentation performance. Variation from the norm in the concentrations of some of these cellular components is indicative of inappropriate yeast management (Briggs *et al*., 2004).

By definition having been recovered from a previous fermentation, pitching yeast is in stationary phase. On pitching into a single batch of air-saturated wort, the critical cell cycle events on which successful fermentation depends is developing step by step (Boulton and Quain, 2001).

As Werner-Washburne *et al*. (after Boulton and Quain, 2001) mentioned,"shutting down metabolism and entry into stationary phase is the cells' strategy for long-term survival for months or perhaps even years". Cells' response to starvation is so substantial, that a great deal of changes can be seen in physiology, genetics and morphology.

Much of what is known about stationary phase in brewery fermentations relates to the consequences of yeast storage and its subsequent fitness for a new fermentation. In gross terms, stationary phase cells are under threat and metabolism is focused on survival in the hope that the environment in which yeast cells are located will eventually improve (Boulton and Quain, 2001).

There are two classes of storage carbohydrates involved in the yeast survival process, which have important roles in brewery fermentation: **glycogen** (fig. 1 *a*) and **trehalose** (fig. 1 *b*).

**Glycogen** is a polymer of α-D-glucose. It has a branched structure containing chains of 10-14 residues of α-D-glucose joined by 1→4 linkages. Approximately 1 in 12 glucose residues also makes -1,6 glycoside bond with a second glucose, which results in the creation of a branch (Wikipedia: glycogen).

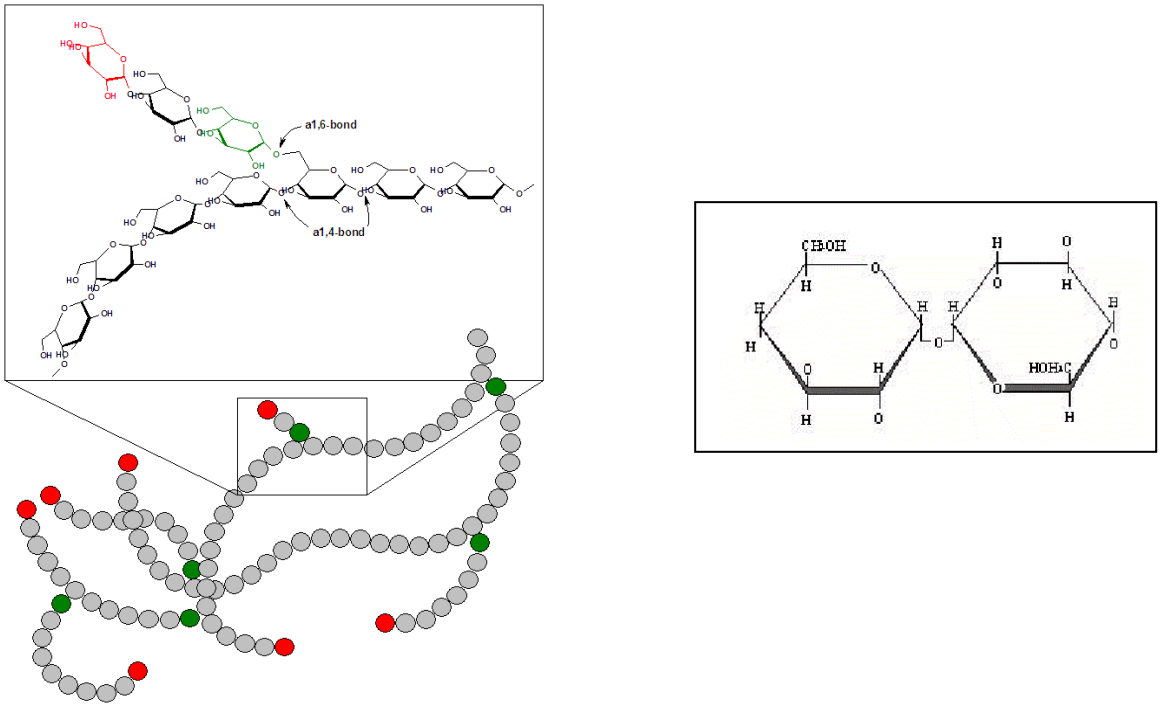
## 2. Glycogen synthesis pathway

Glycogen is synthesised from glucose, via glucose 6-phosphate and glucose 1-phosphate (fig. 2).

The pathway uses uridine diphosphate (UDP) as a carrier of glucose units. Glycogen synthase (UDP-glucose: glycogen 4-α-D-glucosyltransferase) catalyses chain elongation by successive transfer of glucosyl units from UDP-glucose to the growing α-(1→4)-linked polyglucose polymer. A second enzyme, branching enzyme (1,4–α-D-glucan 6-α-D-(1,4-α-glucano)-transferase) forms the α-(1→6)-glucosidic bonds which form the branch points in the growing polymer.

## 3. Glycogen degradation

Dissimilation of glycogen uses another enzyme system. Glycogen phosphorylase, in the presence of phosphoric acid, repeatedly removes successive glucose molecules from the non-reducing ends of glycogen chains, liberating molecules of glucose 1-phosphate. A debranching enzyme, which is a hydrolytic amylo-α-(1→6)-glucosidase cleaves the α-(1→6)-glucosidic bonds such that in combination with the phosphorylase permits complete utilisation of glycogen (Boulton and Quain 2001).




(a) (b)

***Fig. 1.*** *The structure of storage carbohydrates involved in brewing yeast survival a – glycogen; b – trehalose (O'Connor-Cox et al, 1996, Wikipedia: glycogen; trehalose)*

Glycogen is the major storage polysaccharide in brewing yeasts, accounting for up to 40% (w/w) of the dry weight of cells. The amount accumulated is strain specific and depends on many fermentation and yeast storage variables. (O'Connor-Cox *et al*, 1996)

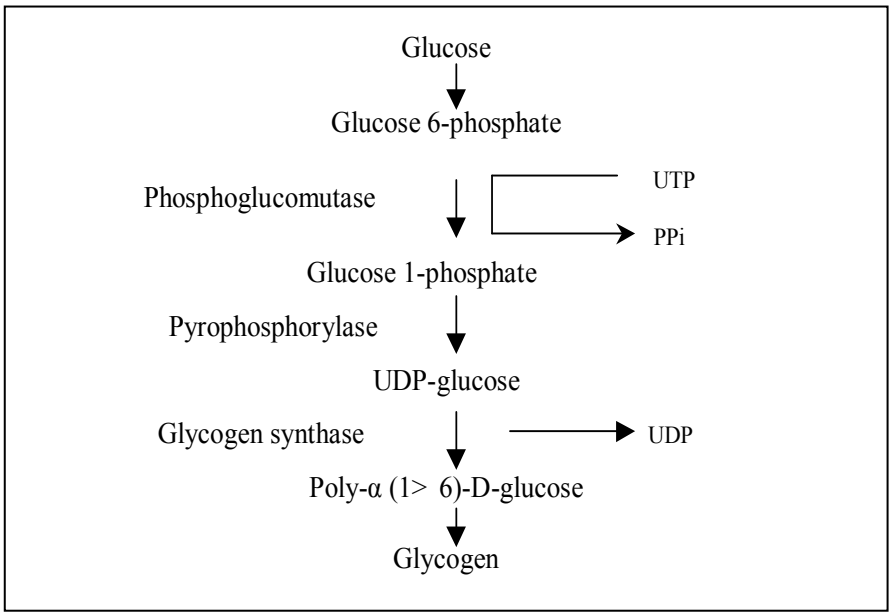



***Fig. 2****. Glycogen biosynthetic pathway (Boulton and Quain 2001)*

In the context of brewery fermentation it has been suggested that glycogen fulfils **two vital** roles:

***First:*** It provides the carbon and energy for synthesis of sterols and unsaturated fatty acids during the aerobic phase of fermentation (Boulton and Quain, 2001). Because these substances often determine the ultimate amount of yeast growth, their concentrations are usually related to fermentation performance.

Therefore, the initial glycogen content available to fuel the biosynthesis of unsaturated lipids is an indirect indicator of the potential to form these critical compounds (O'Connor-Cox *et al*, 1996)

***Second:*** It provides energy for cellular maintenance functions during the stationary phase of fermentation and in the storage phase between cropping and re-pitching (Boulton and Quain 2001).

When the yeast is pitched, glycogen reserves are mobilized for sterol synthesis. Oxygen triggers glycogen dissimilation, as soon as the pitch is done, being used as an energy and carbon source for sterol synthesis, because the exogenous carbohydrates can not yet be used due to lack of cellular membrane functionality.

During the last phase of active fermentation, glycogen assimilation is promoted by a high levels of sugars present in the wort in comparison with other components.

Nutrients consumption, others than sugars, will gradually reduce yeast growth and favour progressive glycogen accumulation, while fermentation process proceeds. As soon as fermentable sugars are not available anymore, glycogen degradation starts in order to allow yeast overcome the starvation period (Steward and Russell, 1993).

Since glycogen is utilised for maintenance functions during storage it is important to remove yeast crops from fermenter as soon as the primary fermentation is over in order to prevent excessive glycogen degradation. A useful procedure in this respect is cooling the content of the fermenter. In storage vessels low temperatures also reduce yeast metabolic activity and conserves glycogen stores.

Even so, the need to have sufficient glycogen to fuel sterol synthesis during subsequent fermentation serves to limit the time for witch pitching yeast can be safely stored without compromising subsequent fermentation performance. In practice, this is usually no longer than 3 days at 2…4°C (Boulton and Quain 2001).

Within the yeast cell there are 2 pools of glycogen. The first one is soluble, and its concentration is modulated by the changes in the physiological state of the yeast cell.

The second one can only be solubilised by acidic treatment. It has a structural role, being covalently linked to β-glucans from the cell wall (Steward and Russell, 1993)

**Trehalose** is a non-reducing disaccharide in which the two glucose units are linked in an α–1,1-glycosidic linkage (Elbein *et al*., 2003). Like glycogen it is also synthesised in reactions which utilise uridine diphosphate as a carrier of glucose molecules (Boulton and Quain 2001).

Trehalose is present in a wide variety of organisms, including bacteria, yeasts, fungi, insects, invertebrates and lower and higher plants, where it may serve as a source of energy and carbon.

In yeast and plants, it may also serve as a signalling molecule to direct or control certain metabolic pathways (Elbein *et al*., 2003).

### *Pathway for the biosynthesis of trehalose*

The key enzyme is trehalose phosphate synthase (TPS) which catalyses the transfer of a glucose residue from uridine diphosphate glucose to glucose 6-phosphate.

A phosphatase liberates the phosphate group forming trehalose (Boulton and Quain, 2001).

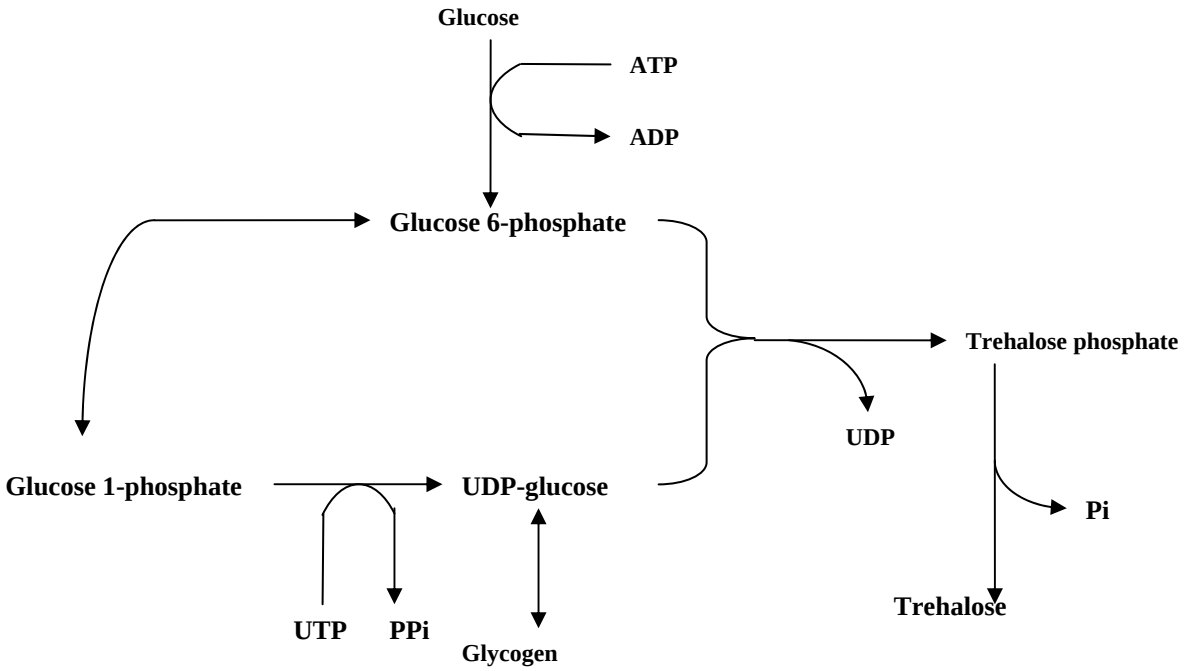



***Fig. 3.*** *Trehalose biosynthetic pathway (Boulton and Quain, 2001).*

Within the yeast cell, TPS is part of a trehalose synthase complex made of 4 subunits. One of these subunits is trehalose-6-phosphate synthase (TPS1), another one is trehalose-6-phosphate phosphatase. They convert glucose 6-phosphate plus UDP-glucose to trehalose. In addition to the TPS1 and TPS2 gene products, this complex also contains regulatory subunits: TSL1 and TPS3 (a homologue of TSL1 identified by systematic sequencing) (Bell *et al*., 1998)

In order to explain the relation between trehalose metabolism and glycolysis, scientists proposed the following possible hypothesis:

- TPS has a regulative function that restricts glucose influx by interacting with the glucose transportation system.
- Trehalose metabolism can prevent exces glycolisis, by using or deviating the sugars which carry phosphate groups (sugar-P) into trehalose synthesis process. Through this pathway, anorganic phosphate is produced, neccessary for the activiy of glyceraldehyde-3-P dehydrogenase, involved in glycolisis.
- Trehalose-phosphate exerts a restrictive control on the influx of sugars into glycolisis by restricting hexokinase activity (* * * Yeastgenome).

Synthesis of trehalose may derive from glucose or from glucosyl residues derived from degradation of glycogen (Boulton and Quain, 2001). This is the most widely distributed pathway for the byosynthesis of trehalose (Elbein *et al*., 2003).

There are also other pathways described for the biosynthesis of trehalose:

- A pathway that has been reported in a few unusual bacteria involves the intramolecular rearrangement of maltose (glucosyl - α 1,4 – glucopyranoside) to convert the 1, 4-linkage to the 1,1-bond of trehalose. This reaction is catalyzed by the enzyme called trehalose synthase and gives rise to free trehalose as the initial product.
- Another pathway involves several different enzymes, the first of which rearranges the glucose at the reducing end of a glycogen chain

to convert the α1,4-linkage to an α,α–1,1-bond. A second enzyme then releases the trehalose disaccharide from the reducing end of the glycogen molecule.

- In mushrooms there is a trehalose phosphorylase that catalyzes the phosphorolysis of trehalose to produce glucose-1-phosphate and glucose. This reaction is reversible in vitro and could theoretically give rise to trehalose from glucose-1-P and glucose (Elbein *et al*., 2003)

## 4. Trehalose accumulation in the yeast cell

The amount of trehalose in *Sacch. cerevisiae* can constitute up to 23% or more of the dry weight of the cell, depending on the growth conditions and stage of life cycle (Kim *et al*., 1996)

Low trehalose concentration in yeast during exponential phase is the result of glucose repression (Boulton and Quain, 2001).

It was demonstrated that trehalose concentration accumulated within the yeast cell is proportional to the gravity of the wort. Thus trehalose content of the yeast harvested from an 11°Plato wort accounted for no more than 2-3% of the yeast dry weight, whereas this increased to 20-25% of the cell dry weight in yeast removed from 25°Plato (Boulton and Quain, 2001).

Trehalose starts to accumulate during the transition from the exponential phase to the stationary phase, when the glycogen reserves are being utilised. The repressive effects of glucose are quantitatively higher in the trehalose synthesis pathway than in glycogen synthesis pathway. Trehalose accumulates only after glycogen was produced, the latter one being used as glucose residue supplier for trehalose synthesis (Steward and Russell, 1993).

## 5. Trehalose functions

There is plenty of evidences that prove high tolerance to stress is associated with trehalose accumulation.

Yeast, in common with other eukaryotes, exhibits a heat shock response. This phenomenon is triggered when cells are exposed, for a short period, to a higher but non-lethal temperature at which growth is not permitted. Such cells returned to a growth-permitting temperature exhibit an increased but transient tolerance to subsequent exposure to lethal temperatures (Boulton and Quain, 2001).

It is well documented that the acquisition of thermotolerance is closely linked to the synthesis of a small set of proteins, the stress or heat shock proteins (hsp) (Hottiger *et al*., 1994).

It has been demonstrated that the presence of $Ca^{2+}$ ions together with a heat shock on yeast cells, may lead to increaseing activity of the enzymes involved in trehalose synthesis (trehalose-6-phosphate synthase and trehalose-6-phosphate phosphatase) and decreaseing the activity of trehalases (Neves and Francois, 1992).

More recent studies have established a stronger correlation between the trehalose content of yeast cells and their resistance to temperature extremes, dehydration and freezing-thawing cycles. From these data it has been proposed that the primary function of trehalose in yeast is not as an energy reserve, but as protectant of cell membranes and proteins under conditions that deplete the activity of intracellular water (Neves and Francois, 1992).

There is general agreement that proteins are among the cell components mostly prone to heat-induced damage an it has been proposed that protein denaturation is the actual cause of thermal killing (Hottiger *et al*., 1994).

Because of nonreducing sugar, trehalose does not show Maillard reaction with amino compounds such as amino acids or proteins (Neves and Francois, 1992), reactions that denaturates proteins.

Moreover, it is supposed that trehalose has a suppressive effect on the auto oxidation of unsaturated fatty acids. The formation of hydroperoxide (HPOD), which is the initial reaction product of linoleic acid oxidation, was suppressed by trehalose. So, it is considered that trehalose might directly interact with linoleic acid. As a result, the formation of hydroperoxide is suppressed.

The results of the experiments performed by Higashiyama (2002), suggest the existence of an interaction between 3, 6 positions of two trehalose

molecules and 9, 10, 12, 13 positions of the linoleic acid molecule (Higashiyama T., 2002).

***Trehalose functions***

- Trehalose has the ability to stabilise protein structure such that in the presence of this disaccharide many enzymes exhibit resistance to heat and desiccation.
- Trehalose protects cells from temperature extremes by stabilising membrane structure.
- Trehalose is a most effective agent for preventing damage to membranes by its ability to prevent phase transition events in lipid bi-layers.
- Trehalose synthesis requires metabolic energy, whereas no ATP is generated in its dissimilation (Boulton and Quain, 2001).

## 6. Trehalose behaviour

Spectroscopic studies indicate that the hydroxyl groups of trehalose form hydrogen bonds with the polar head groups of the lipid bi-layer and stabilize the membrane against phase transitions which could otherwise result in displacement of membrane proteins and/or leakage of cytosolic contents (Kim *et al*., 1996).

Several theories have been proposed as to why trehalose exerts far greater protective effects than other disaccharides like sucrose and maltose. These include suggestions that its special properties are due to a higher glass transition temperature or that it forms direct hydrogen bonds with lipids in cells, replacing similar bonds with water molecules At the University of Messina, Italy, it was used a specialized spectroscopic technique to examine interactions between molecules of trehalose and water. The beam of neutrons produced by a specialized spectrometer was used to measure vibration in the bonds formed between the sugars and water molecules.

The data show that trehalose creates a more crystalline formation with neighbouring water molecules than that created between water molecules and the two similar disaccharides. Trehalose modifies the structural and dynamic properties of water, forming a unique entity with water molecules which makes it better able to protect biological structures.

The trehalose-water complex is more rigid than other sugar complexes. This rigidity would protect against high temperatures. But looking at the water itself, the water molecules next to trehalose are more flexible than bulk water. This would protect biological molecules against cold, because it would be harder to form ice (* * * *Trehalose puts life on hold*).

For maximum effectiveness, trehalose requires to be present at both the inner and outer surfaces of the membrane. In order for trehalose to exert its protective effects it must be transported from the site of synthesis in the cytosol to the membrane. In *Sacch. cerevisiae* it was demonstrated the presence of a transporter which rendered the cells capable of taking up exogenous trehalose. Eleutherio *et al*. (after Boulton and Quain, 2001) postulated that the same carrier was responsible for transporting intracellular trehalose to the periplasm and inner membrane.

Thus, mutant strains with no carrier could not withstand dehydration although trehalose accumulation was unimpaired. The same mutants could be afforded protection from dehydration by the addition of exogenous trehalose (Boulton and Quain, 2001).

The transporter is inactive during the exponential phase of cell growth when the supply of fermentable sugars is abundant and cellular trehalose levels are low. During respiratory and stationary phase of the life cycle, the trehalose transporter is synthesized in response to the exhaustion of fermentable sugars in the growth medium, which also coincides with an increase in cellular trehalose levels (Kim *et al*., 1996).

Cellular levels of trehalose are controlled by a balance between its synthesizing and hydrolyzing enzymes (Kim *et al*., 1996).

Two enzymes are capable of hydrolyzing trehalose:

- A neutral cytosolic trehalase (NTH): most active at *p*H 6.7 to 7.0;

- An acidic vacuolar trehalase (ATH): most active at *p*H 4.0 to 5.0.

The role of NTH in protecting cells against heat shock has recently been demonstrated (Kim *et al*., 1996**).**

ATH and NTH show opposite patterns of activity during the yeast life cycle.

The activity of NTH is high when cells are growing exponentially on fermentable sugars and decays rapidly as cells enter respiratory and stationary phases.

By contrast, ATH activity is detected only when cells enter respiratory and stationary phases or when they are grown on a respiratory substrate such as ethanol or glycerol. Therefore, high ATH activity corresponds to stages of the yeast life cycle when its substrate, trehalose, accumulates to appreciable levels, while NTH is active only during the exponential growth phase, when trehalose levels are low .

Kim *et al*. (1996) described their studies on different mutants: strains lacking the activity of ATH, strains lacking the activity of NTH and strains lacking both enzymes. The purpose of these assays was to discern differences in their growth characteristics under various nutrient conditions.

Both in rich glucose media and in lower glucose media, mutants lacking ATH accumulated substantially higher trehalose levels than mutants lacking NTH. In all cases, however, trehalose levels began to slowly decrease as the cells continued in stationary phase, presumably due to the mobilization of trehalose as a reserve energy source (Kim *et al*., 1996).

Since abolishing ATH activity appears to result in a hardier yeast strain, an obvious question remains concerning the role of this enzyme in yeast physiology.

When stationary cells are re-inoculated into rich medium at lower cells densities, they rapidly degrade accumulated trehalose and resume growth in an exponential manner. ATH may be necessary for this rapid degradation of accumulated trehalose. If ATH is inactive, this transition out of stationary phase may be retarded.

Although the accumulation of trehalose is critical for yeast survival under various stress conditions, it may have undesirable effects if it is not degraded. For example, depositing trehalose on the plasma membrane increases the rigidity of the membrane, thus potentially inhibiting various physiological functions for which a more fluid membrane is necessary**.**

NTH is necessary for hydrolizing the intracellular trehalose. ATH hydrolysis the extracellular trehalose (Kim *et al*., 1996).

Trehalose metabolism was studied in the yeast strain *Saccharomyces cerevisiae* var. *uvarum* (strain AJL 2036) during fermentation in normal gravity, high-gravity (HG), and very-high-gravity (VHG) worts. The maximum cellular trehalose concentration was found to be proportional to original wort gravity. This effect was independent of the ethanol concentration in fermenting wort. An increase in wort gravity resulted in both higher cellular trehalose contents and increased glycerol concentrations, irrespective of the carbohydrate used to increase the gravity. As wort gravities increased, the cellular trehalose levels rose more than did the glycerol concentrations. This behaviour was found to be a general osmotic stress response, but the effect was more marked in glucose-supplemented fermentations. Trehalose metabolism was confirmed to be stronger under nutrient control. This yeast strain's metabolism of trehalose was different when under glucose repressed or de-repressed conditions. In VHG worts, a significant accumulation of trehalose occurred very early in the fermentations. Yeast in these worts behaved as if depressed despite the presence of significant amounts of glucose. It is proposed that trehalose is an important osmoprotectant and stress indicator compound for brewing yeast fermenting HG or VHG worts (Majara *et al*., 1996 a).

Carbonation of non-growing slurry resulted in trehalose accumulation indicating that decarbonation of pitching yeast may prevent cell deterioration (Majara *et al*., 1996 b).

Heat shock, in particular, has been used to great effect to understand the underlying mechanism behind trehalose formation (Quain, 1991).

During yeast handling and storage the yeast trehalose content should be preserved at the levels measured at the time of yeast harvesting (Majara *et al*., 1996 b).

The means to accomplish this include:

- Short storage time
- Low storage temepartures
- Lack of oxygen (Boulton and Quain, 2001; Majara *et al*., 1996 b).

An alternative to the usual practice of supplying wort with oxygen at the start of fermentation, is direct oxygenation of pitching yeast. This process promotes sterol synthesis at the expense of glycogen dissimilation (Boulton and Quain, 2001).

Callaerts *et al*. (1993) reported that the oxygenation process also resulted in trehalose accumulation in a pattern which showed a positive correlation with sterol formation. Presumably this was also a response to stress, in this case due to oxygen (Boulton and Quain, 2001). Trehalose behaves – metabolically – in total contrast to glycogen, being accumulated in the cell under starvation (be it carbon, nitrogen or phosphate) conditions instead of conditions of ”plenty”, as glycogen does. Its formation is frequently fuelled by the breakdown of glycogen and its degradation can – in some cases – be triggered by the addition of glucose (Quain, 1991).

## 7. Trehalose determination

Trehalose determination has been carried out for many years using the anthrone (10H-Anthracen-9-one) colorimetric method, which may be subject to interferences. Alternatives to this problem seem to be the use of HPLC techniques or specific enzymatic assays. Each rate method showed correlations over 0.97 with the others.

However, only HPLC and the enzymatic method were statistically identical. The results showed that, in some cases, the anthrone method may, in fact, produce inflated results although presenting consistency within the set of data (Ferreira *et al*., 1997; Wikipedia, Anthrone).

## 8. Conclusions

Trehalose is either hydrolised, being used as a carbon source, or synthesised being used as a stabilizer and protectant of cellular membrane. Trehalose accumulation appears to be a general response to stress such as freezing, dehydration, ethanol shock, oxygenation, and carbonation. In these instances, trehalose is believed to act as a membrane stabilizer and protecting agent against the applied stresses.

In conclusion, in the brewing industry, care must be taken when yeast is used to a subsequent fermentation, as high levels of trehalose detected in the pitching yeast offer a signal that yeast was submitted to stress factors during storage and handling.

## 9. Commercial applications

Trehalose accumulation in the yeast cells has important consequences for different commercial applications. The fact is already accepted that high trehalose levels are important for producing active dry yeast. In this case, trehalose offers protection during drying process. It also offers cryoprotection when yeast is used in frozen dough (Higashiyama, 2002).

In addition, yeast strains that yield higher levels of ethanol during fermentation may be exploited to produce ethanol as an alternative fuel resource

Having the ability to precisely manipulate resistance to stress conditions may allow the generation of strains having combinations of useful features, stress resistance and particular sensory characteristics included (Kim *et al*., 1996).

Trehalose found commercial applications as a food supplement, being in accordance with GRAS in USA and EU food specifications.

Although less soluble and less sweet than sucrose( its relative sweetness being 45% of sucrose, trehalose is used in the food industry as a sweetener (Higashiyama, 2002).

**Abreviations**: VHG: very high gravity, NTH: neutral trehalose, ATH: acidic trehalose, hsp: heat shock proteins, TPS: trehalose phosphate synthase.

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