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Review Article

Study of Gene Expression of *Saccharomyces cerevisiae* Under Osmotic Stress in Fermentation Processes

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Abstract

Adaptation to altered environments is a unique property of living organisms. The yeast *Saccharomyces cerevisiae*, as a model system, is an excellent microorganism for the study of the molecular biology and physiology of osmoadaptation. The purpose of this study is the understanding of gene expression in *Saccharomyces cerevisiae* under osmotic stress conditions and the consequences of its stress response in food and beverage industry. The yeast achieves long-term adaptation by stimulating a mitogen-activated protein (MAP) kinase cascade, the High Osmolarity Glycerol (HOG) pathway, with the expression of two genes, *GPD1* and *GPP2*, encoding the key enzymes glycerol-3-phosphate dehydrogenase (Gpd) and glycerol-3-phosphatases (Gpp), respectively. As a result, glycerol is produced as an osmolyte and it accumulates intracellularly to adapt to hyperosmotic conditions or the cell can control its rapid export through the transmembrane glycerol transport channel, to adapt to hyposmotic conditions. An integrated understanding of osmoadaptation in *S. cerevisiae* will be beneficial for practical applications and improvements in food and beverage industry, especially taking advantage of biotechnology and its tools, such as genetic engineering, for glycerol biosynthesis or for more robust strains construction.

***Keywords**: Yeast; HOG Pathway; Glycerol; Fermentation*

Introduction

Saccharomyces cerevisiae is a microorganism used both in the classical food and beverage industry and technology as well as in modern biotechnology and scientific research. The tolerance and adaptability of *S. cerevisiae* under environmental stress conditions have identified the yeast as the key microorganisms to optimal productivity in specific industrial fermentation processes, especially in baking, wine-making and beer production. Thus, studying and understanding the biochemical systems and gene regulation during osmotic stress is highly important. The significant role of the yeast in industrial biotechnology is due to the fact that it is a model organism and its whole genome has been sequenced [1]. Therefore, gene manipulation has become easier than ever before and, with the advances in genetic and metabolic engineering, new strains can be constructed having an impact in productivity in food and beverage industry.

The wide range of applications of *S. cerevisiae* is directly related to the food industry, mainly through industrial fermentation. The yeasts are grown to produce bakery dough (yeast cells) and for use in alcoholic fermentation reactions for the production of alcoholic beverages. The basis of bakery, winemaking and brewing is the production of alcohol from the yeast, which metabolises sugars, producing ethanol and carbon dioxide as an end product under anaerobic conditions. However, during all these changes, osmotic stress occurs

and glycerol formation is needed for treatment in response to osmotic stress. To respond to external high osmolality, the yeast *S. cerevisiae* activates Hog1 MAP kinase (MAPK or mitogen-activated protein kinase) via the HOG signalling pathway [2-6]. The yeast achieves long-term adaptation to hyperosmolar conditions by accumulating the conventional osmolyte which is glycerol, into cytoplasm. To succeed that, the activated Hog1 protein (as the Hog1 MAP kinase is called) is transferred from the cytoplasm to the nucleus [7], where it induces the expression of genes encoding the enzymes necessary for the synthesis of glycerol (*GPD1, GPP2*). In the cytoplasm, the activated Hog1 protein closes the glycerol channel Fps1 controlling the intracellular and extracellular concentration of glycerol [8]. Thus, Hog1 controls the production, insertion and maintenance of glycerol, as well as regulates the evolution of the cell cycle for optimal adaptation and communicates with the other yeast MAPK pathways to control cell morphogenesis [8-10]. Additionally, the disaccharide trehalose is another critical osmolyte accumulated during fermentation processes and yeast cells can deal with osmotic stress.

The aim of this review was to present recent published data extracted from scientific databases (*Saccharomyces* Genome Database, PubMed, ScienceDirect, Google Scholar) studying the genetic and biochemical mechanism of osmoadaptation of the yeast *Saccharomyces cerevisiae* and the applications of this mechanism in the Food and Beverage industry.

Mechanism of osmoadaptation

The HOG pathway is a signal transduction pathway that perceives environmental changes *via* phosphorylation and gene activation [11,12] in the following order: a) a change in the osmolarity of the environment e.g. increase in the concentration of NaCl, is recognised by special transmembrane proteins in the plasma membrane of the yeast, called osmosensors which form the upstream part of the HOG pathway, b) the signal induced by the osmotic stress is transferred from the osmosensors to the downstream part of the pathway called "Hog1 MAPK cascade" and the key protein Hog1 MAPK is activated (by phosphorylation), c) Hog1 MAPK is transported into the nucleus where it regulates transcription of glycerol metabolism genes and other stress-related genes and d) glycerol is produced and can either remain intracellularly or be transferred extracellularly for osmoadaptation, its transport being controlled by glycerol channel Fps1 (Figure 1).

The signal is perceived by osmosensors in the plasma membrane: The upstream part of the high osmolarity glycerol (HOG) MAP kinase pathway (the part that receives the signal for osmo-adaptation) comprises of two branches: the Sln1 (Synthetic Lethal of N-end rule) and Sho1 (Synthetic, High Osmolarity-sensitive). Sln1 and Sho1 are both transmembrane proteins that act as osmosensors that recognise the extracellular osmotic change and then transfer the signal intracellularly [13-15]. The Sho1 branch uses two similar, but separate, signalling mechanisms, namely HKR1 (Hansenula mrakii Killer toxin Resistant) and MSB2 (Multicopy Suppression of a Budding defect) branches. A signalling response in the Sho1 branch is initiated by osmosensors Hkr1 and Msb2, which are highly glycosylated single-path transmembrane proteins. Through an interaction between the Msb2/Hkr1 osmosensors and the Sho1 co-osmosensor, this response leads to activation of specific enzymes (p21 activated kinase or PAK)-like kinases named Ste20 (Sterile 20 protein) and Ste50 (Sterile 50 protein), a function that requires the binding of the small enzyme Cdc42 (Cell Division Cycle 42) which is a GTPase [1,16,17]. These enzymes function as upstream activators. The other branch use Sln1p which is a transmembrane autophosphorylating histidine protein kinase that acts as osmosensor transferring the signal to intermediate sensor proteins Ypd1 (tyrosine Phosphatase Dependent 1) and Ssk1 (Suppressor of Sensor Kinase 1). It is a negative regulator of the HOG signalling pathway, since it is the unphosphorylated form of Ssk1 that interacts with and activates the downstream MAP kinase cascade [18]. Under normal environmental conditions, Ssk1 must be maintained in a stably phosphorylated state and deletion of Sln1 is lethal because of pathway overactivation [9,19,20].

The Hog1 MAPK cascade: The central core of the HOG pathway is the signalling unit called "Hog1 MAPK cascade", composed of three sequentially activating kinases. The first kinase (MAPKKK or mitogen-activated protein kinase kinase kinase) is either Ste11 (Sterile 11 protein) via the Sho1 branch or Ssk2/Ssk22 (Suppressor of Sensor Kinase) via the Sln1 branch which they are activated (phosphorylated) by an upstream kinase (Ste20, Ste50) or by binding of an activator protein (Ssk1). Under conditions of high osmolarity, the histidine kinase Sln1 is no longer active and the unphosphorylated form of Ssk1 interacts with and activates Ssk2 and Ssk22, two MAPKKKs that further stimulate downstream effectors.

The second kinase (MAPKK or mitogen-activated protein kinase kinase) is Pbs2 (Polymyxin B Sensitivity) MAPKK which is common for both branches. A signal emanating from either branch converges on MAPKK, Pbs2. The third kinase (MAPK) is the key protein Hog1 MAPK and is activated by Pbs2. The second and third kinases (MAPKK and MAPK) are activated by dual phosphorylation of the conserved Threonine (Thr) and Tyrosine (Tyr) residues within the activation loop [7,21-25].

Hog1 controls gene expression by regulating transcription factors: The activated Hog1 MAPK is transported from the cytoplasm to the nucleus where it modulates gene expression by regulating several unrelated transcription factors responsible for controlling the expression of a subset of osmo-responsive genes, including genes that control the biosynthesis of glycerol, either directly or in collaboration with other factors. These Hog1-regulated transcription factors include the transcription activators Hot1 (High-Osmolarity-induced Transcription), Smp1 (Second MEF2-like Protein 1), Msn1, Msn2, and Msn4 (Msn=Multicopy suppressor of SNF1 mutation) and the transcription repressor Sko1 (Suppressor of Kinase Overexpression). These factors can act independently or in combination at specific promoters to elaborate a fast and dynamic transcriptional response to osmotic stress [26-32]. Direct phosphorylation of promoter-specific transcription factors is the mechanism by which the Hog1 MAPK modulates initiation of transcription. *In vivo* coprecipitation and phosphorylation studies showed that Smp1 and Sko1 interact with, and are directly phosphorylated by, Hog1. Regulation of Sko1 function has an extra layer of complexity: while Sko1 acts as a transcription activator in the presence of stress, it acts as a transcriptional repressor in the absence of stress [7].

Hog1 induces production of Glycerol: In yeast *S. cerevisiae* glycerol is synthesized from an intermediate in the glycolysis, dihydroxyacetone phosphate (DHAP), by two-step enzymatic reactions. In the first reaction dihydroxyacetone phosphate (DHAP) is converted to Glycerol-3-phospate, in presence of NADH, catalysed from the key enzyme Glycerol-3-phosphate dehydrogenase (isoenzymes Gpd1 and Gpd2). The second reaction includes the dephosphorylation of Glycerol-3-phospate and the final glycerol production which is catalysed from the key enzyme Glycerol-3-phosphate phosphatase (isoenzymes Gpp1 and Gpp2). The genes *GPD1* and *GPP2* are expressed in response to hyperosmotic stress, and induce glycerol production under aerobic conditions while the genes *GPD2* and *GPP1* are expressed during anaerobic conditions. Their transcription is activated by the presence of activated Hog1 in the nucleus. Lack of these genes severely impairs growth at high osmolarity. Expression of sugar transporters and genes involved in sugar metabolism are also upregulated in response to osmostress [33,34].

Glycerol transport through Fps1 channel: Glycerol is rapidly accumulated in response to osmo stress, starting within the first minute, and there is significant accumulation of glycerol after 30 minutes of exposure to high osmolarity. This rapid increase in glycerol production cannot be attributed only to an increase in the transcription of particular genes, and therefore additional mechanisms other than transcriptional regulation must exist that permit such a rapid response. There are two main mechanisms to achieve such a rapid initial increase in glycerol concentration: changes in carbon metabolism and changes in glycerol transport [7]. The intracellular concentration of glycerol in *S. cerevisiae* is partly regulated by the Fps1 glycerol channel (Fps or fdp1 Suppressor 1 is a aquaglyceroporin) of the cytoplasmic membrane. An increase in external osmolarity, e.g. increased salt concentration, induces the closure of the channel Fps1, while an osmotic reduction causes the channel to open. These reactions occur within seconds of change in external osmolality in order e.g. to extract glycerol rapidly to prevent cellular burst in a hypotonic environment that creates a sub-osmotic shock. The control of Fps1 channel activity as a response to hyperosmolar shock includes an additional pair of regulatory factors, Rgc1 (glycerin channel regulator 1) and Rgc2 as well as MAPK Hog1. Thus, Hog1 causes the closing of Fps1 channel protein under hyperosmolar shock conditions by phosphorylation and elimination of Rgc2 (and possibly Rgc1) from the C-terminal cytoplasmic domain of Fps1 [4,7,35-37].

As osmotic balance is re-established, Hog1 activity decreases, and Hog1 is exported back to the cytoplasm. Thus, there are mechanisms that control Hog1 nuclear import/export, as well as downregulation of Hog1 activity.

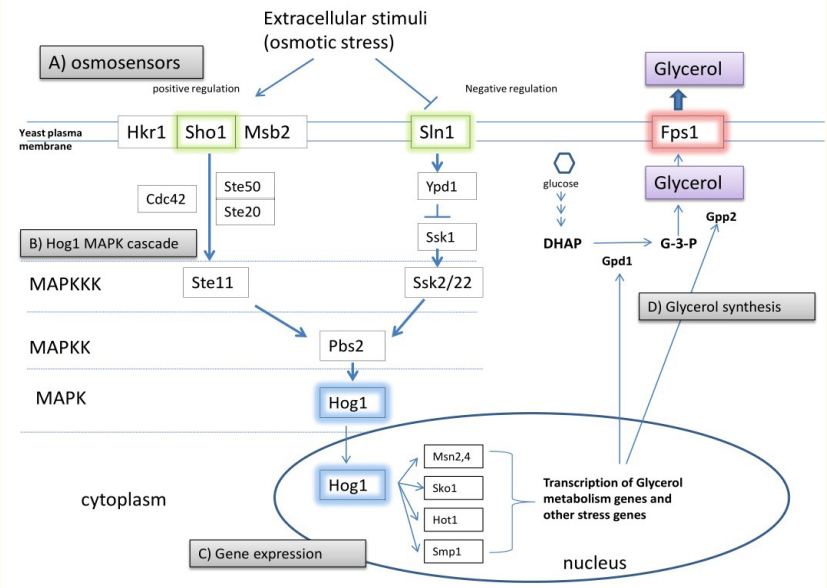

Figure 1: *A simplified diagram of osmoadaptation in yeast: In response to an increase in extracellular osmolarity, the Hog1 MAPK is activated. The HOG pathway in S. cerevisiae consists of two upstream branches comprising of A) osmosensors Sln1 and Sho1 and B) a downstream MAPK cascade (consisting of 3 consecutive MAP kinases: Ssk2/Ssk22 and Ste11 MAPKKKs, Pbs2 MAPKK, and Hog1 MAPK). Osmotic stress reduces Sln1 activity (negative regulation) resulting in unphosphorylated Ssk1 (the active form), which in turn activates Ssk2/22. Osmotic stress also activates the Sho1 branch (positive regulation) via two osmosensor mucins, Msb2 and Hkr1. Activation of the HOG pathway leads to rapid translocation of the protein Hog1 into the nucleus, which in turn stimulates expression of osmo-responsive genes (C) via several transcription factors, including the synthesis of the glycerol-producing enzymes Gpd1 and Gpp2 and other stress-induced proteins (D). Glycerol efflux is controlled by the activity of the Fps1p channel protein [adapted from 4,7,34,36,38].*

Applications in Food and Beverage Industry

Baking

During fermentation of yeast cells (*Saccharomyces cerevisiae*), and baker's yeast production, cells are exposed to many environmental stresses such as freeze-thaw, high sugar or salt concentrations, air- drying and oxidative stress [39-41]. Cellular macromolecules, including proteins, nucleic acids and membranes, are damaged under stress conditions, leading to inhibition of cell growth and decreased cell viability and fermentation. To avoid lethal damage from osmotic stress, yeast cells activate stress-tolerant mechanisms via stress-triggered signal-transduction pathways, such as the HOG pathway that leads to the production of glycerol. Glycerol is a conventional osmotic agent capable of balancing the difference in osmotic pressure without interfering with other metabolic reactions. 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 [42]. A characteristic example of osmotic stress during yeast biomass production is the growth of yeast cells in a closed molasses fermentation system that is highly variable, containing different amount of sugars, salt and other substrates. As a result, cells are exposed to low water activity and excess salt and sugars. The later stages of the process also include hyperosmolar stress. Hyperosmotic stress is also involved when 0.5% of salt is added as a widely used pre-treatment in the preparation of compressed dough

pieces for transport and storage, when yeast cells are stored cells as a cream or dry yeast due to dehydration and when ice forms outside of cells in frozen dough, causing a reaction in water activity [33]. Another important factor of osmotic stress during the baking process is the exposure of yeast cells in the high sugar concentration of most bakery products which contain over 30% of added glucose or fructose syrup, as a sweetener, which reduces dough water activity [33] and can exert a severe osmotic stress [43].

Glycerol production is a crucial property of baker's yeast and a transcriptomic analysis of fermenting baker's yeast confirmed the activation of HOG pathway and the induction of glycerol production after the inoculation in bread dough [44]. Furthermore, it is suggested that trehalose, as an osmolyte, has an important role as well in the production of 'instant active dry yeast' and in the maintained viability in frozen dough Finally, many strategies have been focused on glycerol metabolism for the improvement of baker's yeast viability in sweet dough. Glycerol overproduction can be achieved with hyperactivation of HOG pathway by mutant MAP kinase under the control of appropriate promoters or with controlling the Fps1p channel to increase intracellular levels of glycerol [45].

Wine-making

From the ancient years until now, the transformation of grape must into wine is based on the vital role of *Saccharomyces cerevisiae*. The selection of proper culture can influence the fermentation process because it depends on grape cultivar, must composition, general conditions of fermentation and the final required product. Nowadays, specific and pure yeast culture is inoculated to the grape must for the initiation of fermentation [46]. The production of commercial dry yeast strains exposes them to a variety of osmotic stress. Yeasts are stored frozen in -80°C in a high glycerol solution, usually between 15 - 40%. After thawing, the strains are placed in a high growth medium at 30°C in bioreactors. At the end of the process, when the biomass has reached the desired level, the dough lacks oxygen and carbon sources, so it can cause stress response mechanisms. As a result, intracellular glycerol and trehalose will be increased and stress-resistant proteins will be stimulated. This stage is significant since the response to stress will protect the cells during the stressful post-production stages such as centrifugation, washing and drying and guarantee their survival. Finally, the dough is packaged under nitrogen and sold for sale to the winemakers as active dry yeast [47].

Due to the fact that wine yeast strains have an unusual genetic context and properties different from those of non-industrial strains, the formation of by-products may have an impact on the organoleptic characteristics of wine [48]. Glycerol, as a sugar alcohol and by-product of ethanol fermentation, does not contribute to the aroma of wine although it does contribute to its smoothness. Glycerol production during sugar catabolism plays an important biological role in maintaining the redox cytoplasm balance, especially during anaerobic growth. Since sugar ethanol formation is redox neutral, cytosolic NADH, produced in excess during other cellular processes such as biomass production and excretion of oxidized metabolites, is reoxidized by Gpd in the formation of glycerol [47,49]. Most significantly, glycerol plays the role of an osmo-protective conventional substrate in hyperosmotic stress [50]. Furthermore, it is argued that under controlled conditions, the yeast strain strongly influences the amount of glycerol that it is produced [48].

Turning grape must into wine, is a complex biochemical and microbiological complex [51]. The grape must have a high content of osmotically active substrates, mainly glucose and fructose. Inoculation of active dry yeast into grape must causes a series of new challenges in yeast that are related to low pH as well as hyperosmotic stress due to high sugar content [52]. Therefore, yeast cells must adapt to low pH (2.9-3.8) and high osmolarity before fermentation begins [46]. After inoculation, osmotic stress causes the removal of water from yeast cells and leads to a decreased water availability and decreased turgor pressure. The response of cells in these changes is the modification of the cell wall and the cytoskeleton and the synthesis of glycerol, to create an osmotic equilibrium [53].

During alcoholic fermentation, the transfer of the cells in a glucose-enriched medium leads to an increased gene transcription related to protein synthesis, affecting more than 2000 genes [54]. It has been observed that much of the transcription response happens during the lag phase due to the presence of glucose in the medium. Initial stresses do not lead to a permanent stress and only from stationary phase, when yeast integrates numerous stress signals (osmotic, ethanol), a general stress response is established [52]. Data recently obtained demonstrate that accurate expression of stress response genes may be important for the fermentative behaviour of yeast strains. Strains that cannot complete vinification show lower expression levels during vinification than strains capable to complete the process. Additionally, high and maintained levels of some stress response genes have been identified in strains that consume very low amounts of glucose [55] and therefore have higher yields. Overproduction of glycerol has also been the target of genetically modified wine dough for two reasons: (i) glycerol improves the body of wine, and (ii) reduced ethanol formation is a valuable strategy for producing low-ethanol beverages [56].

Brewing

Ethanol production in alcoholic beverages, especially in beers, is of great commercial interest due to their increased consumption. Beers are produced from grains, which do not contain fermented sugars. Beer production mainly uses barley in Europe, and rice in Japan. The barley selected for this purpose must be low in protein and high in starch. Since yeast does not contain amylase and cannot ferment starch, barley starch must first be converted into sugar. Barley undergoes a series of processes that gradually turn it into beer. These processes, in turn, are the malting of barley for the production of malt (from which the water-sweetened wort is produced), the brewing of the wort, after the addition of hops and, finally, the alcoholic fermentation of the sugars of the wort by the yeast, to create beer. To ensure the quality of their products, brewers cultivate their own yeast strains in pure cultures that originate from an authentic cell. In this way, each cubic centimetre of yeast contains 20 million cells having the same properties [47].

Saccharomyces cerevisiae is the dominant species that is used worldwide in brewing industry. Particular strains of this species, known as "brewer's yeasts", have a profound influence on the flavour and aroma characteristics [57]. Therefore, selecting the proper yeast strain with the required fermentation and recycling features is crucial due to the fact that the strain will affect the rate and extend of fermentation, the flavour characteristic and the overall quality and stability of the finished beer, and most importantly, the economic viability of the brewery [58]. During the total process of brewing, yeast cells are exposed to various stresses throughout pre-fermentation (acid-washing, oxidative stress, cold shock and nutrient starvation), primary and secondary fermentations (osmotic stress, ethanol toxicity, pH, temperature fluctuations, and CO_2/hydrostatic pressure) or post-fermentation (mechanical shear, cold shock and nutrient starvation) [59]. Also, the physiological state of brewing yeast determines its tolerance to stresses, so stationary yeasts are more tolerant to osmotic stress than the ones in exponential phase [60].

However, the two most significant sources of osmotic stress is the acid-washing and the inoculation of yeast cells into wort. Acid-washing is used for the removal of bacterial contaminants as well as to fluidize yeast slurries, and involves submersion of yeast slurries in food-grade acids to achieve low pH (2.2 - 2.4) and that cause osmotic stress due to abundance of dissociated H^+ ions. On the other hand, yeast inoculation into wort is by far the most important source of osmotic stress due to the exposure of cells in a complex medium containing high concentrations of sugars [61]. Glycerol, which is produced via the High Osmolarity Glycerol (HOG) pathway, counterbalances the external pressure acting as a compatible solute while trehalose can stabilize proteins, internal membrane structure and the plasma membrane [62].

Recent methods of producing low-alcohol beer with fermentation manipulation or post-fermentation removal of ethanol have resulted either in loss of beer malt flavour or loss of aromatic ingredients. In addition, elimination of ethanol based on distillation or refining is costly and difficult. An alternative technique, for producing beer with reduced ethanol content, could be created by providing breweries with a genetically modified yeast strain that produce less ethanol during the full fermentation of malt sugars. The reduction in ethanol production could be achieved by the metabolic engineering of carbon flow in yeast resulting in an increased formation of other fermentation products such as glycerol. However, only by-products that do not affect the taste of beer are acceptable. A shift of carbon to glycerol flow at the expense of ethanol formation in the dough was achieved by using a brewing dough overexpressing *GPD1* which resulted in a 18% reduction in ethanol content in beer. The future target of metabolic engineering will be to combine the optimization of ethanol reduction and by-product formation, thereby facilitating direct use of the modified brewery dough for the production of low-alcohol beer [56].

Conclusion

Application of yeasts in traditional biotechnologies such as baking, brewing and wine making, involves their exposure to numerous environmental stresses. 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. The economic significance of *S. cerevisiae* is underlined by the fact that its annual world production exceeds the combined production of all other industrial microorganisms by about two orders of magnitude. The economic values of fermented beverages and foods involving yeasts are enormous and they have wide-ranging fundamental and industrial importance [41]. In bakery, the dough is exposed to environmental stresses such as freeze-thawing, high sugar concentration, air drying and oxidative stress. The mechanisms developed by *Saccharomyces cerevisiae* for the treatment of stress are mainly the production and accumulation of glycerol and other substances such as trehalose. Therefore, with the use of genetic engineering, the transfer of heterologous genes in baker's yeast has the potential of construction of new strains that can modify dough performance in both fresh and frozen sweet doughs and bread quality [45].

In winemaking, the main problem is the degree of cell survival during freezing. Glycerol acts as a protective substance for the survival of cells during freezing. When the yeast is inoculated into the must, the cells are experiencing hyperosmolar shock leading to the elimination of water from the cell and the decrease in the availability of water. Thus, glycerol is synthesized to restore osmotic balance. The main biotechnological objective is the overproduction of glycerol from genetically engineered wine dough due to the improvement of the body of the wine, but mainly to reduce the formation of ethanol, which currently has a great commercial interest in low-alcoholic beverages.

Finally, in brewing, during alcoholic fermentation, glycerol production plays an important biological role as it retains the redox balance of the cytoplasm especially during anaerobic growth. Also, since sugar alcohol formation is redox neutral, cytosolic NADH is reoxidised through Gpd in the formation of glycerol. The main objective is the production of low-alcohol beer with fermentation manipulation and the development of strains with improved fermentation capacity by overexpression or deletion of transcription factors. This was achieved by using a bakery dough overexpressing *GPD1* and reducing the ethanol content of beer by 18%. However, although many genetically engineered yeast strains have been produced in various labs, there is no commercially available genetically modified yeast yet in EU to be used in the food industry.

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