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## Recent Advances in Yeast Biomass Production

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### 1. Introduction

Yeasts have been used by humans to produce foods for thousands of years. Bread, wine, sake and beer are made with the essential contribution of yeasts, especially from the species *Saccharomyces cerevisiae*. The first references to humans using yeasts were found in Caucasian and Mesopotamian regions and date back to approximately 7000 BC. However, it was not until 1845 when Louis Pasteur discovered that yeasts were microorganisms capable of fermenting sugar to produce CO<sub>2</sub> and ethanol. Ancient practices were based on the natural presence of this unicellular eukaryote, which spontaneously starts the fermentation of sugars. As industrialisation increased the manufacture of fermented products, the demand of yeast grew exponentially. At the end of the 19th century, addition of exogenous yeast biomass to produce bread and beer started to become a common practice. Wineries were more reluctant to alter traditional practices, and started using exogenous yeast inocula in the 1950's, especially in countries with less wine tradition (USA, South Africa, Australia and New Zealand). In the 1960's, yeast biomass-producing plants contributed to the technology of producing large amounts of active dry yeast (ADY), and its use rapidly spread to European countries (Reed and Nagodawithana, 1988).

Nowadays, modern industries require very large amounts of selected yeasts to obtain high quality reproducible products and to ensure fast, complete fermentations. Around 0.4 million metric tonnes of yeast biomass, including 0.2 million tonnes baker's yeast alone, are produced each year worldwide. Efficient and profitable factory-scale processes have been developed to produce yeast biomass. The standard process was empirically optimised to obtain the highest yield by increasing biomass production and decreasing costs. However in recent years, several molecular and physiological studies have revealed that yeast undergoes diverse stressful situations along the biomass production process which can seriously affect its fermentative capacity and technological performance.

In this chapter, we review the yeast biomass production process, including substrates, growth configuration, yield optimisation and the particularities of brewing, baker- or wine-yeasts production. We summarise the new studies that describe the process from a molecular viewpoint to reveal yeast responses to different stressful situations. Finally, we

highlight the key points to be optimised in order to obtain not only high yields, but also the best biomass fermentative efficiency, and we provide future directions in the field.

## 2. Molasses: A suitable substrate

Beet or cane molasses are the main substrate used in yeast production plants. These materials were selected for two main reasons: first, yeasts grow very well using the sugars present in the molasses and second, they are economically interesting since they are a waste product coming from sugar refineries without any other application. Usually, molasses contain between 65% and 75% of sugars, mainly sucrose (Hongisto and Laakso, 1978); but the composition is highly variable depending on the sucrose-refining procedure and on the weather conditions of that particular year. Sucrose is extracellularly hydrolysed by yeasts in two monosaccharides, glucose and fructose, which are transported to and incorporated into the yeast metabolism as carbon sources. However, molasses are deficient in other essential elements for yeast growth. One of them is nitrogen since its molasses content is very poor (less than 3%). Yeasts can use some of the amino acids present in molasses, but addition of nitrogen sources is needed, generally in the form of ammonium salts or urea. Magnesium and phosphate elements are also supplemented in salt forms. Finally, three vitamins (biotin, thiamine and pantothenic acid), required for fast growth, must be supplemented since their content in molasses is also very low (Oura, 1974; Woehrer and Roehr, 1981). Another negative aspect of molasses being used as a substrate to produce yeasts is the presence of different toxics that can affect yeast growth. Variable amounts of herbicides, insecticides, fungicides, fertilizers and heavy metals applied to beet or cane crops can be found in molasses and in different stocks. Moreover bactericides, which are added during sugar production in refinery plants, can be found (Reed and Nagodawithana, 1988). All these toxics can decrease yeast performance by inhibiting growth (Pérez-Torrado, 2004). In fact, a common practice in yeast plants is to mix different stocks to dilute potential toxics.

The effects of molasses composition on yeast growth have been recently analysed at molecular level by determining the transcriptional profile of yeast growing in beet molasses and by comparing it to complete synthetic media (Shima *et al.*, 2005). The results revealed that yeast displays clear gene expression responses when grown in industrial media because of the induction of *FDH1* and *FDH2* genes to detoxify formate and the *SUL1* expression as a response to low sulphate levels. Thus it can be concluded that molasses are far from being an optimal substrate for yeast growth. Another interesting conclusion drawn is that molecular approaches can be especially suited to gain insight into the yeast biomass production process.

In the last years, the price of molasses has increased because of their use in other industrial applications such as animal feeding or bioethanol production (Arshad *et al.*, 2008; Kopsahelis *et al.* 2009; Xandé *et al.*, 2010), thus rendering the evaluation of new substrates for yeast biomass propagation a trend topic for biomass producers' research. New assayed substrates include molasses mixtures with corn steep liquor (20:80), different agricultural waste products (Vu and Kim, 2009) and other possibilities as date juice (Beiroti and Hosseini, 2007) or agricultural waste sources, also called wood molasses, that can be substrate only for yeast species capable of using xylose as a carbon source.

### 3. Scaling up: Batch and fed-batch

Nowadays, yeast biomass propagation of wine, distiller's and brewer's yeasts are usually produced in baker's yeast plants. The procedure is designed as a multistage-based fermentation, previously defined for the production of baker's yeast (Chen and Chiger, 1985; Reed and Nagodawithana, 1991) using supplemented molasses as growth media. The first stage (F1) is initiated with a flask culture containing molasses, which is inoculated with the selected yeast strain. Production cultures may be periodically renewed from the stock cultures maintained under more stringent control procedures in a central quality control laboratory. Then, the initial culture is used to inoculate the first fermentor, and cells grow in various transient stages during the batch (F2-F4) and fed-batch (F5-F6) phases of the process. In a sequence of consecutive fermentations, the yeast biomass grown in small fermentors is used to inoculate larger tanks (Reed, 1982; Chen and Chiger, 1985; Reed and Nagodawithana, 1991; Degre, 1993).

In the initial batch phase (F2), cells are exposed to the high sugars concentration present in molasses. All the other nutrients are also present in the fermentor, and pH must be adjusted to 4.5-5.0 after sterilisation to be then monitored during batch fermentation. Once the batch phase has started, the only controllable parameters are temperature and aeration. Yeast propagation typically involves continuous aeration or oxygenation, but a relatively short aeration period has been suggested to suffice (Maemura *et al.*, 1998). However the presence of O<sub>2</sub> from the beginning of the process allows yeast cells to synthesise lipids, thereby revitalising the sterol-deficient cell population and ensuring that fermentation can proceed efficiently. Besides, those propagation experiments carried out in non-oxygenated media considerably reduce yeast growth and increase internal oxidative stress (Boulton, 2000; Pérez-Torrado *et al.*, 2009).

During batch fermentation (F2-F4), a growth lag phase takes place in which cells synthesise the enzymes involved in gluconeogenesis and the glyoxylate cycle (Haarasilta and Oura, 1975). During the subsequent exponential phase, a very small amount of glucose is oxidised in the mitochondria, but when the sugar concentration drops below a strain-specific level or the specific growth rate in aerobic cultures exceeds a critical value ( $\mu_{crit}$ ), a mixed respiro-fermentative metabolism occurs. This phenomenon has been described as the "Crabtree effect" (De Deken, 1966; Pronk *et al.*, 1996) and was originally considered a consequence of the catabolite repression and limited respiratory capacity of *S. cerevisiae* (Postma *et al.*, 1989; Alexander and Jeffries, 1990). It has also been suggested that there is no limitation in the respiratory capacity, as can be deduced from the increased respiratory capacity displayed by a PGK-overproducing mutant, indicating that the activity of respiration itself is not saturated and suggesting that it is not the main cause triggering ethanol production and inducing the long-term Crabtree effect (Van der Aar *et al.*, 1990). However, more recent works have showed that Crabtree effect is derived from the limited mitochondrial capacity to absorb the NADH produced in the glycolysis (Vemuri *et al.*, 2007).

Alcoholic fermentation leads to a suboptimal biomass concentration because the ATP yield is much lower than the yield obtained during respiratory carbohydrate degradation (Verduyn, 1991; Rizzi *et al.*, 1997). However, pre-adaptation to large amounts of glucose during the batch phase is necessary to ensure the produced biomass' optimal fermentative capacity by accumulating several necessary reserve metabolites to be used in the fed-batch phase (Dombek and Ingram, 1987; Rizzi *et al.*, 1997; Pérez-Torrado *et al.*, 2009). In

addition, prolonged growth in aerobic, glucose-limited chemostat cultures of *S. cerevisiae*, avoiding the batch phase, causes a partial loss of glycolytic capacity (Jansen *et al.*, 2005). The presence of O<sub>2</sub> during the process also allows yeast to oxidise alcoholic fermentation-produced ethanol when sucrose is exhausted, which triggers the metabolism to change from fermentation to respiration, and eliminates ethanol from the media. When ethanol is exhausted, the fed-batch phase starts (F5-F6). In the transition to the respiratory phase, an increase in the cAMP levels triggers the breakdown of storage carbohydrates and an increased influx of glucose into the glycolytic pathway. The resulting increase in the NAD<sup>+</sup>/NADH ratio stimulates respiration in combination with a drop in the ATP level, which is consumed mainly during biomass formation (Pérez-Torrado, 2004; Xu and Tsurugi, 2006; Pérez-Torrado *et al.*, 2009). In some industrial wine yeast production plants, fed-batch phases are initiated without consuming ethanol from the growth media, which considerably reduces the biomass yield.

Optimisation of biomass productivity requires an increase in both the specific growth rate and the biomass yield during the fed-batch phase to the highest values possible under sugar-limited cultivation. Generally, the growth rate profile during fed-batch cultivation is controlled primarily by the carbohydrate feedstock feed rate (Beudeker *et al.*, 1990). The control of optimum dissolved oxygen during the fed-batch phase is also essential to obtain a high biomass yield, and important studies have been done to optimise aeration control (Blanco *et al.*, 2008). Therefore sugar-limited cultivation in the presence of O<sub>2</sub> allows the full respiratory growth of *S. cerevisiae*, achieving much higher biomass yields than during the batch phase (Postma *et al.*, 1989). If the only objective is to maximise the biomass concentration starting with a sufficiently concentrated inoculum from the batch phase, it is necessary to grow cells at a rate as close to the critical growth rate as possible ( $\mu_{crit}$ ), which depends exclusively on the yeast strain (Valentinotti *et al.*, 2002), avoiding ethanol and acetate formation. Many of the parameters that have an impact on yeast's metabolic activities have to be controlled (Miskiewicz and Borowiak, 2005). The pH and temperature are important parameters to be controlled during this phase: maintaining pH constantly at around 4.5 by adjusting the pH automatically with acid/base solutions, and maintaining temperature at 30°C. Properly designed final fed-batch fermentations should also permit yeast cells maturation. This can be accomplished by stopping the feeding of nutrients at the end of fermentation, but allowing slight aeration to continue for an hour (Oura *et al.*, 1974). During this period, the substrate is completely assimilated and allows ripened cells to become more stable and avoids autolysis.

Many research efforts have focused on optimising fed-batch processes for baker's yeast production with different aims (productivity, yeast quality, or energy saving) and most have been commonly done under laboratory conditions (Van Hoek *et al.*, 1998; Van Hoek *et al.*, 2000; Jansen *et al.*, 2005; Henes and Sonnleitner, 2007; Cheng *et al.*, 2008), but rarely under pilot plant conditions (Di Serio *et al.*, 2001; Lei *et al.*, 2001; Gibson *et al.*, 2007; Gibson *et al.*, 2008). They have all been designed to mainly analyse the fed-batch phase without considering the whole process. The first published study on the complete industrial process was the simulation of wine yeast biomass propagation by performing batch and fed-batch phases in only one bioreactor (Pérez-Torrado *et al.*, 2005). This simplification of the process enabled the study of yeast physiology from a molecular point of view with a bench-top design (Fig. 1), whose results display a good correlation with those obtained from pilot plants and this set of parameters for further investigation.



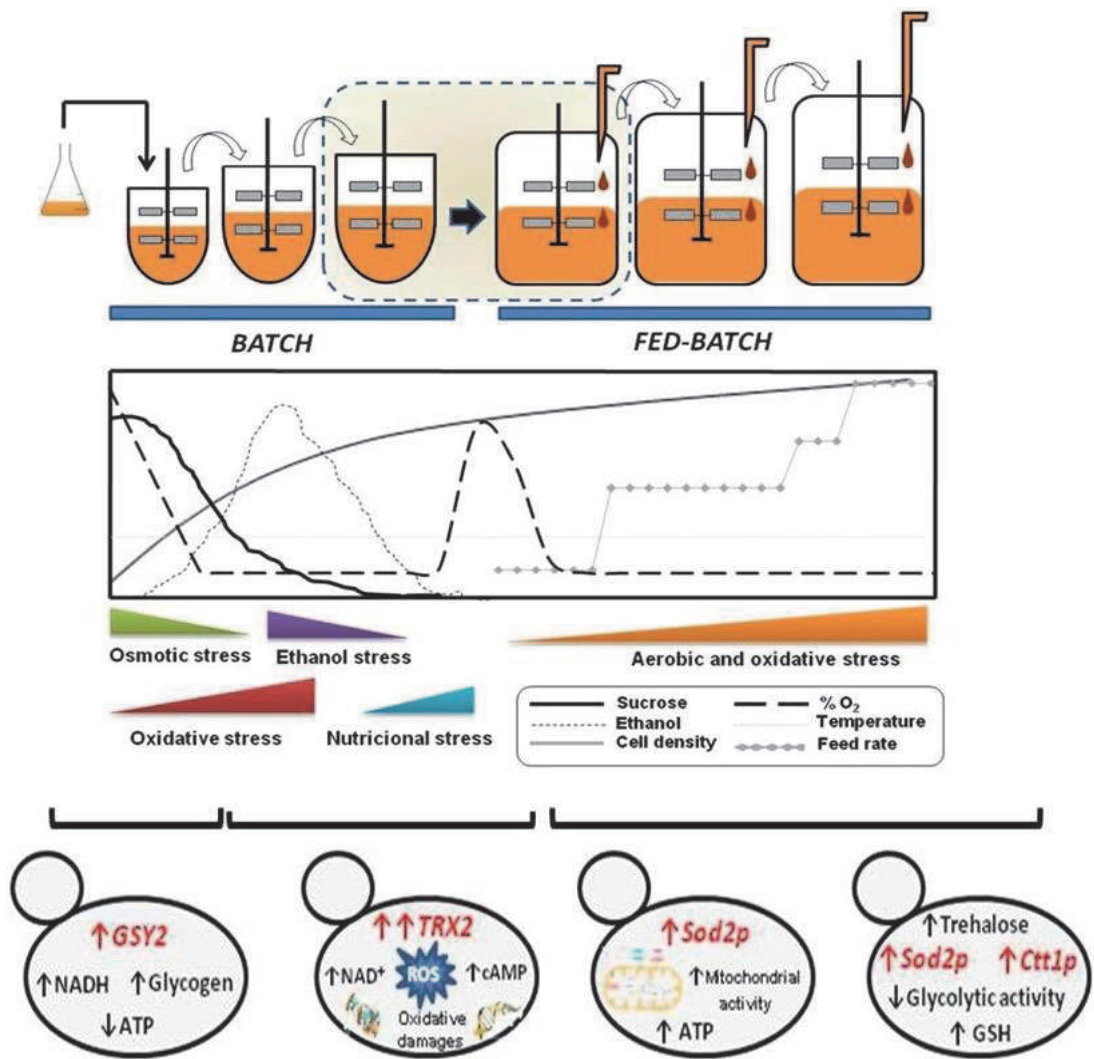


Fig. 1. Diagram of the different stages in the industrial yeast biomass propagation process. The parameters employed throughout the process (sucrose and ethanol production / consumption, dissolved O<sub>2</sub>, cell density and feed rate) have been adapted from Gómez-Pastor *et al.*, 2010b. The lower panel shows representative cellular states, along with the most relevant metabolites, proteins and gene expressions throughout biomass propagation.

#### 4. Desiccation of wine yeasts

In contrast to baker's and brewer's yeast, seasonal wine production requires the development of highly stable dry yeast products. At the end of biomass propagation, wine yeast cells are recovered and dehydrated to obtain ADY (Chen and Chiger, 1985; Degre, 1993; Gonzalez *et al.*, 2005). After the maturation step, yeast cells are separated from fermented media by centrifugation, and are subjected to washing separations to reduce non-yeast solids, a necessary step because they affect the proper rehydration process of ADY for must fermentation. The separation process yields a slightly coloured yeast cream containing up to 22% yeast solids. After this step, the yeast cream can be stored at 4°C after adjusting the pH to 3.5 to avoid microbial contaminations. The cream yeast is further dehydrated to 30-35% solids by means of rotary vacuum filters or filter presses. The filtered yeast is usually

mixed with emulsifiers prior to its extrusion into yeast strands. The yeast cake is extruded through a perforated plate, while particles are loaded into the dryer and dehydrated to obtain a product with very low residual moisture. Although several types of dryers exist (roto-louvre, belt dryers, spray dryers), the one most commonly used in industry is the fluidized-bed dryer. In this dryer, heated air is blown from the bottom through yeast particles at velocities which keep them in suspension. Air is treated to reduce its water content and to ensure that the yeast temperature does not exceed 35°C or 41°C during drying. Drying times may vary from 15 to 60 min depending on the mass volume and the used conditions. Finally, ADY with less than 8% residual moisture is vacuum-packaged or placed in an inert atmosphere, such as nitrogen and CO<sub>2</sub>, to reduce oxidation. Depending on the strain, loss of viability is estimated at between 10% and 25% per year at 20°C. For this reason, manufacturers recommend storing ADY at 4°C in a dry atmosphere for a maximum 3-year period.

In order to produce an ADY product with acceptable fermentative activity and storage stability, several factors must be taken into account. The drying temperature and rate can be critical for yeast resistance to dehydration and rehydration (Beney *et al.*, 2000; Beney *et al.*, 2001; Laroche and Gervais, 2003). Some studies have shown that cell death during desiccation is strongly related to membrane integrity loss, leading to cell lysis during rehydration (Beney and Gervais, 2001; Laroche *et al.*, 2001; Simonin *et al.* 2007; Dupont *et al.*, 2010). A gradual dehydration kinetics, which allows a slow water efflux through the plasmatic membrane and homogenous desiccation, followed by a progressive rehydration during the starter preparation, have been related with high cell viability (Gervais *et al.*, 1992; Gervais and Marechal, 1994, Dupont *et al.*, 2010). The amount of cell constituents leaked during rehydration can also be reduced by adding emulsifiers, such as sorbitan monostearate (Chen and Chiger, 1985). Moreover, biomass propagation conditions have a major influence on yeast resistance to dehydration-rehydration. Several cultivation factors can affect cell resistance to desiccation, such as the substrate, growth phase and ion availability (Trofimova *et al.*, 2010).

## 5. Yeast stress along biomass production

Several classic studies have evaluated the energy, kinetic and yield parameters of the yeast biomass production process (Reed, 1982; Chen and Chiger, 1985; Reed and Nagodawithana, 1991; Degre, 1993). However, the biochemical and molecular aspects of yeast adaptation to adverse industrial growth conditions have been poorly characterised. In recent years, a substantial effort has been made to gain insight into yeast responses during the process. It was believed that industrial conditions were optimised to obtain the best performing yeast cells, but now we know that yeast cells endure several stressful situations that induce multiple intracellular changes and challenge their technological fitness (Attfield, 1997; Pretorius, 1997; Pérez-Torrado *et al.*, 2005). With wine yeast, moreover, the biomass is concentrated and dehydrated at the end of the process to obtain ADY yeasts that can be stored for long periods of time (Degre, 1993). Subsequently in a period of several hours during maturation and final drying processing, cells undergo nutrient limitation and a complex mixture of different stresses (thermic, osmotic, oxidative, etc.) (Garre *et al.*, 2010). As a result, these dynamic environmental injuries seriously affect biomass yield, fermentative capacity, vitality, and cell viability (Attfield, 1997; Pretorius, 1997; Pérez-Torrado *et al.*, 2005; Pérez-Torrado *et al.*, 2009).

Eukaryotic cells have developed molecular mechanisms to sense stressful situations, transfer information to the nucleus and adapt to new conditions (Hohmann and Mager, 1997; Estruch, 2000; Hohmann, 2002). Protective molecules are rapidly synthesised in stressful situations and transcriptional factors are activated, thus changing the transcriptional profile of cells. Many stress response genes are induced under several adverse conditions through sequence element STRE (stress-responsive element), which targets the main transcriptional factors Msn2p and Msn4p (Kobayashi and McEntee, 1993; Martinez-Pastor *et al.*, 1996). This pathway, also known as the “general stress response pathway”, increases the expression of many different genes, including the well-studied *HSP12* and *GSY2* genes involved in protein folding and glycogen metabolism, respectively (Boy-Marcote *et al.*, 1998; Estruch, 2000). Furthermore, yeast cells have been seen to respond specifically to certain stresses. During thermal stress, transcriptional factor Hsf1p activates the transcription of genes, such as *STI1*, which code for those proteins that counteract protein denaturation and aggregation (Lindquist and Craig, 1988; Sorger, 1991). Aerobic growth during biomass propagation and pro-oxidants also generate reactive oxygen species (ROS), leading to several types of oxidative damage to cells (Gómez-Pastor *et al.*, 2010a). To neutralise the harmful effects of oxidative stress, proteins are generated, and they participate in two major functions: antioxidants (such as *GSH1*, *TRX2*, *CUP1*, and *CTT1*) to reduce proteins and eliminate ROS damage, and metabolic enzymes (such as *PMG1* and *TDH2*) that redirect metabolic fluxes to synthesise NADPH by slowing down catabolic pathways like glycolysis (Godon *et al.*, 1998). Another well-known specific stress response is the high-osmolarity glycerol response pathway (Brewster *et al.*, 1993), which induces the genes involved in glycerol synthesis (*GPD1*, *GPP2*) and methylglyoxal detoxification (*GLO1*). Intracellular accumulation of glycerol counteracts hyperosmotic pressure to avoid water loss (Hohmann, 2002). There are other stress response pathways that remain poorly understood, such as those involved in the adaptation to nutrient starvation. Large groups of well-known stress response genes and other genes with unknown functions, such as *YPG1*, are induced after exposure to one kind of stress, and are also involved in the protective mechanism against other different stresses, a phenomenon known as cross-protection (Coote *et al.*, 1991; Piper, 1995; Trollmo *et al.*, 1988; Varela *et al.*, 1992; Bauer and Pretorius, 2000). The molecular responses of laboratory *S. cerevisiae* strains to different stresses have been thoroughly studied, and a large body of knowledge is available (Gasch and Werner-Washburne, 2002; Hohmann and Mager, 2003). In addition, several approaches for the characterisation of stress responses under industrial conditions have been carried out for wine and lager yeasts (Pérez-Torrado *et al.*, 2005; Gibson *et al.*, 2007), and some correlations have been found between stress resistance of several yeast strains and their suitability for industrial processes (Beudeker *et al.*, 1990; Ivorra *et al.*, 1999; Aranda *et al.*, 2002; Pérez-Torrado *et al.*, 2002; Zuzuarregui *et al.*, 2005; Pérez-Torrado *et al.*, 2009; Gómez-Pastor *et al.*, 2010a). For these reasons, the study of stress responses under industrial conditions has become an important research field to improve our knowledge of not only complex industrial processes, but of yeast capabilities.

Given the antiquity of yeast fermentation processes, these microorganisms have evolved in natural stressing environments, which have favoured the selection of “domesticated” yeast that displays high stress resistance (Jamieson, 1998). Studies of brewing yeast under industrial fermentations have demonstrated the suitability of the marker gene expression as a tool to study yeast stress responses in industrial processes (Higgins *et al.*, 2003a). Monitoring stress-related marker genes, such as *HSP12*, *GPD1*, *STI1*, *GSY2* and *TRX2*,



during bench-top growth trials of wine yeast biomass propagation have demonstrated that osmotic (*GPD1*) and oxidative stresses (*TRX2*) are the main adverse conditions that *S. cerevisiae* senses during this process (Pérez-Torrado *et al.*, 2005). Afterwards, a genome-wide expression analysis of the same process established stress-critical time points throughout the process based on the profiles of different oxidative stress response genes (Gómez-Pastor *et al.*, 2010b). Three relevant stressful points have been defined during biomass propagation: the first during the metabolic transition from fermentation to respiration in the batch phase; the second critical point is the end of the batch phase when previously produced ethanol is completely consumed; the third interesting point is the end of the fed-batch phase, after a long period under respiratory metabolism. Among these set points, metabolic transition during the batch phase is the most relevant as several genes relating to cell stress, especially those related to oxidative stress (*TRX2*, *GRX2* and *PRX1*), protein degradation, aerobic respiration and NADPH production, are induced while ribosomal proteins are dramatically repressed (Gómez-Pastor *et al.*, 2010b). Similar results have been observed in a genome-wide expression analysis during biomass propagation of brewer's yeasts, which also displays a strong induction of the genes involved in ergosterol biosynthesis and oxidative stress protection in initial industrial lager fermentation stages (Higgins *et al.*, 2003b; reviewed in Gibson *et al.*, 2007; Gibson *et al.*, 2008). However, while osmotic stress plays a role in initial biomass propagation stages as a result of the large amount of sugar in molasses, oxidative stress takes place throughout the process as a result of aeration (reviewed in Gibson *et al.*, 2007).

As mentioned earlier, an oxygen supply is necessary to generate yeast biomass and to ensure optimal physiological conditions for effective fermentation (Chen and Chiger, 1985; Reed and Nagodawithana, 1991; Hulse, 2008). Oxygen is required for lipid synthesis, which is necessary to maintain plasma membrane integrity and function, and consequently for both cell replication and the biosynthesis of sterols and unsaturated fatty acids. Despite its potential toxicity, eliminating oxygen in the first part of the batch phase diminishes biomass yield (Boulton *et al.*, 2000; Pérez-Torrado *et al.*, 2009) and avoids the expression of those genes related to oxidative stress response, such as *TRX2* and *GRE2*, which significantly increases oxidative cellular damage, such as lipid peroxidation, when the bioreactor is re-oxygenated to oxidise ethanol (Pérez-Torrado *et al.*, 2009). Clarkson *et al.* (1991) demonstrated that cellular antioxidant defences, such as Cu/Zn superoxide dismutase, Mn superoxide dismutase and catalase activities of brewing yeast strains, also change rapidly after adding or removing O<sub>2</sub> from fermentation.

During an industrial-scale propagation of wine and brewing yeasts, catalase and Mn superoxide dismutase activities increase as propagation proceeds (Martin *et al.*, 2003; Gómez-Pastor *et al.*, 2010a), indicating the importance of oxidative stress response throughout the process, whereas Sod1p (Cu/Zn superoxide dismutase) transiently accumulates at the end of the batch phase when ethanol is consumed (Gómez-Pastor *et al.*, 2010a). A study of different types of oxidative damage during wine yeast biomass propagation has revealed that lipid peroxidation considerably increases during the metabolic transition from fermentation to respiration, which decreases to basal levels during the fed-batch phase (Gómez-Pastor *et al.*, 2010a). Besides, the protein carbonylation analysis, one of the most important oxidative damages (Stadtman and Levine, 2000), has revealed different protein oxidation patterns during biomass propagation, which reach maximum global carbonylation levels at the end of the batch phase (Gómez-Pastor *et al.*, 2010a). As

protein oxidation causes the loss of catalytic or structural integrity, further research into the specific oxidised proteins during biomass production should be done to correlate the detriment in fermentative capacity with specific damaged proteins. In addition, reduced glutathione, an important antioxidant molecule, varies during the process as is lowered during the metabolic transition, while oxidised glutathione increases. Then, reduced glutathione increases constantly in different stages of the process (Gibson *et al.*, 2006; Gómez-Pastor *et al.*, 2010a). Whether glutathione is directly affected by O<sub>2</sub> during biomass propagation remains unknown and requires further investigation.

The fed-batch phase is characterised by the accumulation of other important antioxidant molecules, such as trehalose and thioredoxin (Trx2p) (Pérez-Torrado, 2004; Gómez-Pastor, 2010), although the mRNA levels for the *TRX2* gene significantly increase during the batch phase metabolic transition (Pérez-Torrado *et al.*, 2009). On the other hand, glycogen, a secondary long-term energy storage molecule which has been related to adaptation to the respiratory metabolism (Francois and Parrou, 2001), also accumulates at the end of the fed-batch phase (Pérez-Torrado, 2004). Studies using different dilution rates during the continuous cultivation of baker's yeast have shown that the accumulation of trehalose and glycogen has a negative effect as it increases dilution rates, which is also detrimental for fermentative capacity and cellular responses to heat stress during dehydration (Ertugay and Hamaci, 1997; Garre *et al.*, 2009). Despite a high biomass yield and the accumulation of several beneficial metabolites obtained during the fed-batch phase, *S. cerevisiae* dramatically diminished fermentative capacity after prolonged glucose-limited aerobic cultivation due to several glycolytic enzymes' diminished activity (Jansen *et al.*, 2005).

Proteomic studies have also been carried out to gain a better understanding of the fluctuations in the stress-related gene mRNA levels during biomass propagation and to correlate glycolytic enzyme activities with their corresponding protein levels. However, the proteomic data available from industrial processes are very limited and usually centre on bioethanol production (Cot *et al.*, 2007; Cheng *et al.*, 2008) or wine and beer fermentations (Trabalzini *et al.*, 2003; Zuzuarregui *et al.*, 2006; Salvadó *et al.*, 2008; Rossignol *et al.*, 2009). Recent proteomic studies performed by 2D-gel electrophoresis during wine yeast biomass propagation have revealed that several glycolytic enzyme isoforms increase during biomass production. This is probably due to the post-translational modifications after oxidative stress exposure (Gómez-Pastor *et al.*, 2010b; Costa *et al.*, 2002). Trabalzini *et al.* (2003) suggested that some specific isoforms of glycolytic/gluconeogenic pathway enzymes in wine strains of *S. cerevisiae* are involved in the physiological adaptation to different fermentation stresses. There have also been reports of the differential stress regulations of several proteins (Arg1p, Sti1p and Pdc1p) among different industrial strains possibly having important industrial implications for strain improvement and protection (Caesar *et al.*, 2007). It is interesting to note that biomass propagation experiments using a *trx2* deletion strain have shown a low number of several glycolytic enzyme isoforms and, consequently, an increase in oxidative cellular damage, such as lipid peroxidation and global protein carbonylation (Gómez-Pastor, 2010). During the metabolic transition in the batch phase, several proteins relating to oxidative stress are expressed (Prx1p, Ahp1p, Ilv5p, Pdi1p, Sod1p and Trr1p), which directly correlates with their mRNA levels observed for this growth stage (Gómez-Pastor *et al.*, 2010b). This scenario indicates adaptation to the new condition. In contrast, the genes coding for most of the heat shock proteins, chaperons (Mge1p, Hsp60p, Ssb1p and Ssc1p) and proteins related to ATP metabolism are specifically

induced during the metabolic transition, but their protein levels decline throughout the process. The proteins with the highest expression levels at the end of the biomass propagation include Tdh1p, which codifies for glyceraldehyde-3-phosphate dehydrogenase, and Bmh1p and Bmh2p, homologues to the mammalian 14-3-3 proteins involved in global protein regulation at the post-translational level (Bruckmann *et al.*, 2007). The expression of these proteins at the end of biomass propagation is important as they control the translation of several glycolytic proteins (Fba1p, Eno1p, Tpi1p, Pck1p, Tdh1p, Tdh3p and Gpm1p), as well as the levels of those proteins involved in amino acid biosynthesis and heat shock proteins translation (Bruckmann *et al.*, 2007). This may explain the lack of correlation between the transcriptomic and the proteomic analyses for glycolytic enzymes during biomass propagation. Under oxidative stress, some glycolytic proteins (Tdh3p, Pdc1p, Ad1p and Eno1p) have been described to be specifically modified by oxidation (Le Moan *et al.*, 2006). This oxidation process could explain the loss of fermentative capacity observed in some commercial wine yeast industrial strains at the end of the biomass propagation process (Gómez-Pastor *et al.*, 2010a, b). Regarding this hypothesis, it is worth noting that the overexpression of the *TRX2* gene in industrial yeasts significantly increases the obtained biomass' fermentative capacity by improving the oxidative stress response during propagation, and by decreasing lipid and protein oxidation (Pérez-Torrado *et al.*, 2009; Gómez-Pastor *et al.*, 2010a, c). Figure 1 summarizes the different stresses affecting yeast cells during the biomass propagation process, especially those encountered during the batch phase, and shows the different cellular states with the most relevant metabolites, genes and proteins expressed in each propagation stage.

The industrial yeast biomass dehydration process also involves damaging environmental changes. As the biomass is being concentrated, water molecules are removed and temperature increases, all of which affect the viability and vitality of cells (Matthews and Webb, 1991). Dehydration is known to cause both cell growth arrest and severe damage to membranes and proteins (Potts, 2001; Singh *et al.*, 2005). Removal of water molecules causes protein denaturalisation, aggregation, and loss of activity in an irreversible manner (Prestrelski *et al.*, 1993). Additionally at the membrane level, desiccation is associated with an increased package of polar groups of phospholipids, and with the formation of endovesicles leading to cell lysis during rehydration (Crowe *et al.*, 1992; Simonin *et al.*, 2007). Yeasts have several strategies to maintain membrane fluidity (Beney and Gervais, 2001). One of them is to accumulate ergosterol, this being the predominant sterol in *S. cerevisiae*. Sterols have been proposed to maintain the lateral heterogeneity of the protein and lipid distribution in the plasma membrane because of the putative role they play in inducing microdomains, the so-called lipid rafts (Simons and Ikonen, 1997). Ergosterol synthesis has been related with yeast stress tolerance (Swan and Watson, 1998), and its beneficial role in the different processing steps of industrial yeast has been documented. Its synthesis during biomass production is critical to ensure suitable yeast ethanol tolerance in its later application in wine fermentation (Zuzuarregui *et al.*, 2005). Moreover, the addition of oleic acid and ergosterol during wine fermentation mitigates oxidative stress by reducing not only the intracellular content of reactive oxygen species, but oxidative damage to membranes and proteins, and enhancing cell viability (Landolfo *et al.*, 2010). Recently, experiments with a *erg6Δ* mutant strain, deficient in the ergosterol biosynthetic pathway and which accumulates mainly zymosterol and cholesta-5,7,24-trienol instead of ergosterol, have shown that the nature of sterols affects yeast survival during dehydration, and that

resistance to dehydration-rehydration cycles can be restored with ergosterol supplementation during the anaerobic growth of the *erg6Δ* mutant (Dupont *et al.*, 2010). Recent phenomic and transcriptomic analyses during the desiccation of a laboratory strain have indicated that this process represents a complex stress involving changes in about 12% of the yeast genome (Ratnakumar *et al.*, 2011). Under these conditions, the induction of 71 genes grouped into the “environmental stress response” category was observed, suggesting a role of the general stress transcription factors Msn2p and Msn4p in the desiccation stress response. Furthermore, the phenomic screen looking for genes that are beneficial to desiccation tolerance has identified several of the transcriptional regulators or protein kinases involved in oxidative (*ATF1*, *SKN7*) and osmotic (*HAL9*, *MSN1*, *MSN2*, *MSN4*, *HOG1*, *PBS2*, *SSK2*) stress responses. Although studies with lab strains generate interesting information about the desiccation process, an analysis of stress marker genes during dehydration in ADY production has revealed that inductions of gene expressions in wine yeast T73 are generally moderate, although statistically significant, in some steps, such as hot air drying and final product (Garret *et al.*, 2010). One such example is the induction of osmotic stress marker *GPD1* due to water loss. However, despite the yeast biomass losing approximately 95% of water content during this dehydration process, *GPD1* induction is not as important as previously observed in lab yeast strains under osmotic stress (Pérez-Torrado *et al.*, 2002). These data are in agreement with the robustness of industrial yeasts strains compared to laboratory strains (Querol *et al.*, 2003), and also with the well-known relevance of biomass propagation conditions to confer resistance to subsequent suboptimal conditions (Bisson *et al.*, 2007). One interesting aspect in the same study carried out by Garre and coworkers (2010) is that the highest induction is displayed by oxidative stress marker *GSH1* that codes for  $\gamma$ -glutamylcysteine synthetase activity. This observation is supported by: i) significant inductions of the other genes involved in oxidative stress response, such as *TRR1* and *GRX5*, ii) rise in the cellular lipid peroxidation level, iii) increased intracellular glutathione accumulation, and iv) a peak of its oxidized form GSSG during the first minutes of drying. In addition, a genomic analysis of an oenological-dried yeast strain has shown a strong induction of the other genes related with oxidative stress response, such as *CTT1*, *SOD1*, *SOD2*, *GTT1* and *GTT2* (Rossignol *et al.*, 2006). Currently, free radical damage is emerging as one of the most important injuries during dehydration. Several studies with laboratory yeast strains have shown considerable ROS accumulation during dehydration that results in protein denaturation, nucleic acid damage and lipid peroxidation (Espindola *et al.*, 2003; Pereira *et al.*, 2003; França *et al.*, 2005, 2007). Antioxidant systems appear to be interesting targets affecting yeast's desiccation tolerance. Several examples using lab strains have been shown. Overexpression of antioxidant enzymes genes, such as *SOD1* and *SOD2*, increases yeast survival after dehydration (Pereira *et al.*, 2003), whereas a mutant without cytosolic catalase activity is more sensitive to water loss (França *et al.*, 2005). Glutathione seems to play a significant role in the maintenance of intracellular redox balance because glutathione-deficient mutant strains are much more oxidised after dehydration than the wild-type strain, and they show high viability loss (Espindola *et al.*, 2003). Furthermore, addition of glutathione to *gsh1* cells restores survival rates to control strain levels. Remarkably, the overexpression of the *TRX2* gene in wine yeast has proved a successful strategy to improve fermentative capacity and to produce lower levels of oxidative cellular damage after dry biomass production than its parental strain (Pérez-Torrado *et al.*, 2009; Gómez-Pastor *et al.*, 2010a).



The accumulation of some metabolites has been related to yeasts' resistance to drying and subsequent rehydration. One of them is the amino acid proline. This amino acid exhibits multiple functions *in vitro*: it enhances the stability of proteins, DNA and membranes, inhibits protein aggregation, and acts as a ROS scavenger; but its functions *in vivo*, particularly as a stress protectant, are poorly understood. Although *S. cerevisiae* cells do not accumulate this amino acid in response to stresses, it has been recently shown with laboratory strains that proline-accumulating mutants are more tolerant than wild-type cells to freezing, desiccation, oxidative, or ethanol stress (reviewed in Takagi, 2008; Kaino and Takagi, 2009). Self-cloning has been used to construct the baker's yeasts that accumulate proline by carrying the disruption of the *PUT1* gene involved in the degradation pathway, and expressing a mutant *PRO1* gene that encodes a less sensitive  $\gamma$ -glutamate kinase to feedback inhibition in order to enhance biosynthetic activity. The engineered yeast strain shows enhanced freeze tolerance in doughs (Kaino *et al.*, 2008). A recent transcriptomic analysis of air-dried cells has suggested activated transport and metabolic processes to increase the intracellular concentration of proline during yeast desiccation (Ratnakumar *et al.*, 2011).

Interestingly, wine yeasts accumulate large amounts of disaccharide trehalose, usually in the 12-20% range of cell dry weight (Degre, 1993) although higher percentages have been detected in industrial stocks (Garre *et al.*, 2010). Trehalose content has been proposed as one of the most important factors to affect dehydration survival. Baker's yeasts with 5% of trehalose are 3 times more sensitive to desiccation than those cells accumulating 20% of trehalose (Cerrutti *et al.*, 2000). The main function of this metabolite is to act as a protective molecule in stress response. This effect can be achieved in two ways: by protecting membrane integrity through the union with phospholipids (reviewed in Crowe *et al.*, 1992); by preserving the native conformation of proteins and preventing the aggregation of partially denatured proteins (Singer and Lindquist, 1998a). The indispensability of this metabolite to survive dehydration is a controversial subject. Some studies have suggested that its presence is essential and needed in both sides of the membrane to confer suitable protection (Eleuterio *et al.*, 1993; Sales *et al.*, 2000). However, these results are argued alongside the *tps1* mutant's dehydration resistance, which is unable to synthesise trehalose, as other authors have indicated (Ratnakumar and Tunnacliffe, 2006). On the other hand, dehydration tolerance conferred by trehalose seems to be also related to its ability to protect cellular components from oxidative injuries (Benaroudj *et al.*, 2001; Oku *et al.*, 2003; Herdeiro *et al.*, 2006; da Costa Morato *et al.*, 2008; Trevisol *et al.*, 2011). The addition of external trehalose during dehydration reduces intracellular oxidation and lipid peroxidation and increases the number of viable cells after dehydration (Pereira *et al.*, 2003). Moreover, the compensatory trehalose accumulation observed in *hsp12Δ* mutants confers a higher desiccation tolerance than the parent wild-type cells, which is the result of increased protection by mutant cells against reactive oxygen species (Shamrock and Lindsey, 2008). Some studies have proved the applicability of this metabolite to improve industrial yeast tolerance to dehydration. A clear and simple example is that of Eleuterio and co-workers (1997), where the trehalose accumulation induced by osmotic stress in the species *Saccharomyces uvarum* var. *carlsbergensis* before dehydration is enough to achieve survivals of up to 60% after drying, whereas the stationary cells presenting low trehalose levels are unable to survive. The construction of trehalose-overaccumulating strains by removing

degradative activities emerges as a useful strategy for industrial yeasts (Kim *et al.*, 1996). Studies done with laboratory strains have shown that the deletion of genes *ATH1* and *NTH1*, respectively encoding acid and neutral trehalase activity, improve yeast cells viability after dehydration, which is provoked by hyperosmotic stress (Garre *et al.*, 2009). Similar approaches using baker's yeast have also been successful, and defective mutants in neutral or acid trehalase activities exhibit higher tolerance levels to dry conditions than the parent strain, as well as increased gassing power of frozen dough (Shima *et al.*, 1999).

## 6. Conclusions

In the last few decades, the yeast biomass production industry has contributed with many advanced approaches to traditional technological tools with a view to studying the physiology, biochemistry and gene expression of yeast cells during biomass growth and processing. This has provided a picture of the determinant factors for the commercial product's high yield and fermentative fitness. Cell adaptation to adverse industrial conditions is a key element for good progress to be made in biomass propagation and desiccation, and towards the characterisation of specific stress responses during industrial processes to clearly indicate the main injuries affecting cell survival and growth. One major aspect of relevance in the complex pattern of molecular responses displayed by yeast cells is oxidative stress response, a network of mechanisms ensuring cellular redox balance by minimising structural damages under oxidant insults. Different components of this machinery have been identified as being involved in cellular adaptation to industrial growth and dehydration, including redox protein thioredoxin, redox buffer glutathione and several detoxifying enzymes such as catalase and superoxide dismutase, plus protective molecules like trehalose which play a relevant role in dehydration.

## 7. Future prospects

In spite of the sound knowledge available on molecular responses to exogenous oxidants, the endogenous origin of oxidative stress in yeast biomass production, given the metabolic transitions required for growth under the described multistage-based fermentation conditions and desiccation, makes it challenging to search for the specific targets undergoing oxidative damage during both biomass propagation and desiccation, and to correlate this damage with physiologically detrimental effects. Based on the currently global data available and the use of potent analytical and genetic manipulation tools, further research has to be conducted to (i) define specific oxidised proteins and to know how this oxidation affects fermentative efficiency, (ii) identify new key elements in stress response, which can be manipulated to improve it and can be also used as markers to select suitable strains for biomass production, (iii) analyse the effects of potential beneficial additives, such as antioxidants, on yeast cells' ability to adapt to stress, and then yeast biomass' yield and fermentative fitness in industrial production processes.

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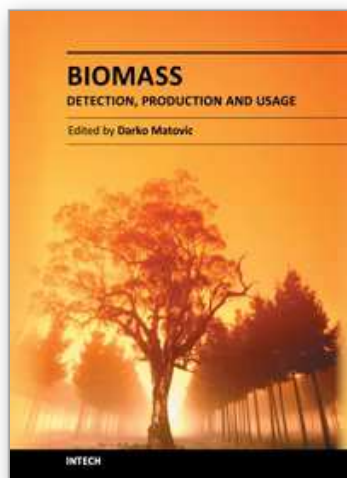
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## **Biomass - Detection, Production and Usage**

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Biomass has been an intimate companion of humans from the dawn of civilization to the present. Its use as food, energy source, body cover and as construction material established the key areas of biomass usage that extend to this day. Given the complexities of biomass as a source of multiple end products, this volume sheds new light to the whole spectrum of biomass related topics by highlighting the new and reviewing the existing methods of its detection, production and usage. We hope that the readers will find valuable information and exciting new material in its chapters.

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