Different fermentation different yeast biology essay

Science, Biology

Yeasts are unicellular fungi and one of the most important groups of eukaryotic microorganisms. Historically they were the first microorganisms to be domesticated by humans, and although they were only identified as the microorganisms responsible for fermentation in 1860, evidence for fermented beverages and foods goes back thousands of years. Initially it is likely that fermentations took place by autochthonous yeasts and exchanges of yeasts between beverages and food are thought to be common. At some unknown point in time, humans started selecting specific yeasts for each process of fermentation (Sicard and Legras 2011; Donalies et al. 2008). Nowadays the production of yeast biomass is the biggest production of single-celled microorganism worldwide with high demand from modern industries. In the food/beverages industry, besides the fresh pressed yeasts for baking, new ways of commercializing yeasts have been developed such as, active dry yeasts and immobilized yeasts. These new forms of yeasts allow storage for a longer period of time, which is particularly relevant for wine industry due its seasonal use. Furthermore, yeast applications are far more diverse, as they are also used in chemical industries, health care and biomedical and environmental research (Kosseva 2011; Pérez-Torrado et al. 2009)

Different fermentation, different yeast?

Among the fermenting microorganisms, yeasts are the most important group of eukaryotic microorganisms used for fermenting beverages and foods. Although more than 600 yeast species are known, the most important, involved in brewing, wine making and baking are part of the Saccharomyces sensu stricto complex and are very similar to each other (Donalies et al.

2008; Sicard and Legras 2011). Despite their similarities, yeasts differ in terms of fermentation properties and byproduct formation depending on their use. For instance, baker’s yeast should efficiently ferment maltose, and present high tolerance to osmotic stress, drying and freezing. Brewer’s yeast should also be able to ferment maltose and maltotriose, but differently from the previous they are usually used freshly propagated, ideally in the late logarithmical growth phase under conditions similar to brewing. Wine yeasts on the other hand, are used in dried form, and besides stress tolerance mentioned before other traits are desirable, such as, high ethanol tolerance, and in some cases reduction of wine acidity (Donalies et al. 2008). Given the different yeast characteristics and the different needs from industry, it is of extreme importance to carefully select the strain that better suits the end product and process.

Importance of strain selection in wine industry

Commercial wine yeast strains of Saccharomyces cerevisiae have, over time, been selected to fulfill highly different and specific oenological requirements and, consequently more than 200 strains with significantly diverging phenotypic traits are now produced globally (Rossouw et al. 2009). Originally, wine was produced by spontaneous fermentation performed by the autochthon microflora without resorting to inoculation of starter cultures, giving high-quality wines with a unique regional character with an added commercial value. However, the drawbacks of spontaneous fermentations are the lower predictability and the inconsistencies in wine quality, due to the existence of several uncontrollable variables. Nowadays it is generally

accepted, at least in large-scale productions, that yeast inoculation offers more advantages than spontaneous fermentations although there is no consensus among smaller wine makers in using starter cultures. The advantages of yeast inoculation are different depending on the vinification process and yeast strain characteristics, but, usually rely on the fact that fermentations initiate and progress faster and importantly is associated with a greater quality consistency in final product (González et al. 2011; Pretorius 2000). Over the years, different criteria have been purposed for yeast strain selection; these criteria can be considered under three categories: (i) properties that affect the performance of the fermentation process (fermentation power, strain resistance to various stress factors and nitrogen demand); (ii) properties that determine wine quality and character (positive or negative influence on the sensory qualities of the wine produced) and (iii) properties associated with the industrial production (the yeasts must be adaptable to large-scale propagation and needs to be tolerant of the stresses of drying, packaging, storage and rehydration and reactivation by the winemaker) (Fleet 2008, González et al. 2011).

Yeasts for sparkling wine - selection

Sparkling wines belong to a category of " special wines" due to the process involved in their production process (wines whose CO2 is derived exclusively via fermentation). Although sparkling wines are produced on a smaller scale than still wines, their economic impact for the enology industry is high because of its added value. This type of wine results from a second fermentation of a base wine, which can take place in a sealed bottle

(traditional method or methode champenoise) or in a hermetically-sealed tank, under isobaric conditions (bulk, or Charmat process) (Pozo-Bayón et al. 2009; Torresi et al. 2011). Secondary fermentation occurs in very specific conditions since the base wine already has a considerable percentage of alcohol (near 10%) in which only some strains of S. cerevisiae can grow and ferment. Low temperatures (10 – 18ºC) and increasing pressure are also factors that can limit or slow down the fermentation process. Even if conditions are not ideal for yeasts, a good base wine, controlled temperature and a well selected strain is usually enough to assure a good second fermentation process. Therefore it is essential to carefully select appropriate strains to use for sparkling wine's second fermentation since it is one of the most important factors that influences the final characteristics of the sparkling wine produced (Torresi et al. 2011; Carrascosa et al. 2011). Yeasts strains selected for sparkling wine production must satisfy a series of additional characteristics compared to those used in the first fermentation of base wine production: they should tolerate the ethanol concentration of the base wine, have fermentation activity at low temperatures (the temperature in cellars can be lower than 12ºC), be resistant to the pressure caused by carbon dioxide, be easily separated from the final product and finally they should not produce unpleasant aromas (Carrascosa et al. 2011). A relevant aspect in sparkling wine production is the sedimentation and yeast removal process (remuage), which are labor intensive and time-consuming. The sediment is removed by freezing the neck of the bottle and the ice plug is squeezed out the bottle by the internal pressure (dégorgement). The use of immobilized yeast instead of free cells may advantageously facilitate and

shorten the remuage and dégorgement processes. For this reason their use has been studied over the last decades and they are now widely used in wine industry (Martynenko and Gracheva 2003; Fumi et al. 1987; Fumi et al. 1988; Gòdia et al. 1991). The main concern regarding immobilized yeasts in the beverage industry is the possibility of a negative influence in flavour profile. Gòdia et al. (1991) found organoleptical properties of sparkling wine to be improved when immobilized yeast cells were used. Later on, Yokotsuka et al. (1997) compared the process of sparkling wine production with encapsulated and non-encapsulated yeast concluding that: (i) there were no significant chemical differences between both processes; (ii) the sparkling wines produced both ways were very similar in terms of taste and bouquet and (iii) the use of immobilized yeasts within alginate beads was practical for commercial/industrial production.

Industrial production of immobilized yeasts

Batch and Fed-Batch yeast biomass propagation

In the standard process of wine yeast propagation, selected yeast strain is inoculated and this initial culture is used to inoculate the batch fermenter. During the initial batch phase, cells are exposed to high sugar concentration and, although under aerobic conditions, most likely processing sugars through a mixed respiro-fermentative metabolism. This phenomenon is known as " Crabtree effect", meaning that if sugar concentration exceeds a certain threshold, even in presence of oxygen, fermentative metabolism also happens. Fermentative metabolism results in ethanol production; this byproduct is unwanted in high concentration, since it can compromise the

growth rate of cells and thus final yield. Fermentation metabolism is also less efficient in matters of ATP production in comparison with respiratory metabolism and promotes a lower biomass yield, but this initial phase is essential so yeasts can accumulate reserve metabolites to be used in the fed-batch phase. Avoiding this phase causes a partial loss of glycolytic capacity (Pérez-Torrado et al. 2009; Gómez-Pastor et al. 2011). During the final stage of the batch phase, when sugars are exhausted, cells start to oxidize the ethanol in the medium triggering a change from fermentative metabolism to respiration and consuming the ethanol present so that fed-batch can be initiated (if fed-batch is initiated before this step, biomass yield would be considerably lower) (Gómez-Pastor et al. 2011). Fed-batch propagation of microbial strains has become a very important method in the field of biotechnology from research to industry and is commonly used for different purposes and products due to their simplicity, scalability and flexibility (Wlaschin and Hu 2006). It consists in controlling the feed rate at which nutrients are added in order to control cell growth and/or product formation. There are several ways of controlling the feed rate, usually involving a feedback system, of which the one that seems to work better is the regulation of nutrient entrance by the respiration quotient (reviewed in González et al. 2011; Lee et al. 1999). One can consider two types of fed-batch processes: an intermittent-harvest fed-batch, and the traditional fed-batch, both of them are similar in some ways to a batch process. Cells are inoculated at a lower density in a medium (not very different from a usual batch medium) and allowed to grow exponentially with basically no external manipulation (the only controlled parameters are temperature and aeration)

until some of the nutrients are almost consumed and cells start to reach stationary phase. By this time if in an intermittent-harvest fed-batch process, a fraction of cells and products are collected and the fermenter is refilled with medium. On the other hand, if in a traditional fed-batch process while cells are still in exponential phase but nutrients are becoming depleted, concentrated medium is added continuously or intermittently allowing a higher cells concentration to be achieved. Since neither cells nor medium are removed from the fermenter the fed-batch culture must be started in a volume near half the maximum capacity of the fermenter (Wlaschin and Hu 2006). For wine yeast propagation fed-batch is initiated under aerobic conditions and controlling the input of sugars in order to minimize ethanol formation. During fed batch, with limited sugar and presence of oxygen it is possible to achieve a higher biomass yield than during batch phase thanks to cells growing on respiratory metabolism. For maximizing final cell concentration it is advantageous to grow cells as close to critical growth rate as possible without inducing ethanol or acetate formation, the critical growth rate is a characteristic of each strain. A well designed fed-batch process should also allow yeast cell maturation where the feed is stopped (Gómez-Pastor et al. 2011). Maturation phase consists mainly in the consumption of residual sugars, in this phase cells enter in stationary phase and expression of certain genes is induced, some compounds are accumulated and yeast resistance is enhanced (Garre et al. 2010). It is advisable that cells destined for preservation and later use should be collected in stationary phase rather than exponential growing phase, since the latter ones would have higher viability losses (Cheong et al. 2008). After maturation they enter in a final

stage of production where they are centrifuged for separation of cells from the medium, immobilized (on the chosen matrix), dried and packed.

Encapsulation and dehydration

In the last years there has been new advances and a trend in the application of immobilized cell technology to implement innovation in food and beverage industry associated with processing, preservation and storage of the products. Yeasts as a commercial product have different formulations that can be grouped into three main types: (i) compressed yeast (fresh yeast), ready for immediate use; (ii) dried yeast (active – ADY and instant – IDY) and (iii) immobilized yeast. ADY and immobilized yeasts are usually sold in airtight packages, vacuum seal or filled with an inert gas. Cell immobilization can be classified into four types based on the mechanism and the nature of support material: (i) attachment or adsorption to a support surface of an inert solid matrix (porous glass, diatomaceous earth, particles of gluten); (ii) entrapment within a porous matrix (e. g. gels of calcium alginate in the form of beads about 2 mm in diameter); (iii) containment behind or within a barrier (membrane) and (iv) self-aggregation of highly flocculent cells (Verbelen et al. 2006; Nedovic et al. 2011). Each immobilization method has its specific advantages and drawbacks and the nature of the application often dictates the choice. For the application to the food and beverage industry, whatever the support used for immobilization, it must fulfil certain requirements: (i) allowing to achieve a high surface / volume ratio which translates to a higher cell density, (ii) having mechanical and chemical stability, (iii) allow for maintenance of the cell viability; (iv) not to interfere

with the physiological and metabolic activity of immobilized cells, (v) does not adversely affect the final product and have consumer acceptance (Nedovic et al. 2011). The encapsulation in natural polymer matrices (alginate, chitosan and agar) is regarded as one of the most promising techniques for immobilization, because it allows polymerization to occur in very mild conditions, preserving the structural integrity and functionality of the cells. Such natural polymers also offer the advantages of being non-toxic, biocompatible and cheap (Kosseva 2009; Martynenko and Gracheva 2003). Alginate is a naturally derived polysaccharide extracted from various species of algae. The composition of the polymer chain depends on the source and this influences alginate functional properties.

The use of encapsulated yeasts in wine industry presents some drawbacks such as diffusion limitation of nutrients, metabolites and oxygen imposed by the porosity of the matrix and the high cell density in the gel beads. However, it also offers several advantages such as improved fermentation yields, higher tolerance to ethanol (the matrix provide a protective environment against ethanol toxicity) and protection from other stresses (immobilized yeasts seem to be in general in lower stress during fermentation than free cells in the same conditions) (Verbelen et al. 2006).

Stresses during the manufacturing process

In recent years, several physiological and molecular studies have revealed that yeasts are exposed to stressful conditions along biomass propagation process, which can affect its fermentative capacity and performance (Pérez-Torrado et al. 2005, Gómez-Pastor et al. 2010, 2011). Each different stage

comprises a distinctive combination of stress factors. At the start of the batch fermentation, yeasts are submitted to a pre-adaptation to high sugars concentrations to promote the accumulation of reserve metabolites to be used in the next step of fed-batch. Consequently, yeasts are primarily exposed to an osmotic stress, which tends to decrease gradually as the sugars are consumed. Also present is an ethanol stress, which increases during the alcoholic fermentation and decreases thereafter, due to the metabolic transition from fermentation to respiration. Another important stress present during this initial phase is oxidative stress. Oxidative stress is caused by the presence of reactive oxygen species (ROS) that results mainly from yeast metabolism. Aeration in this phase of the process is necessary as shown by Pérez-Torrado et al. (2009) whose work revealed that eliminating aeration during the beginning of batch phase diminishes biomass yield. Major oxidative stress-critical points in this phase were identified as the metabolic transition from fermentation to respiration, the end of batch phase (Goméz-Pastor et al. 2010) Near the end nutritional stress may be present once sugars and ethanol are completely or almost completely consumed. Along the fed-batch phase the main stress situation is an increasing oxidative stress due to a long period of oxidative metabolism (Goméz-Pastor et al. 2011; Gibson et al. 2007). As before, the end of this phase has also been identified as a critical point for oxidative stress (Goméz-Pastor et al. 2010). Oxidative stress is present throughout the whole process, but it is a necessary stress factor. For obtaining the maximum process yield possible is necessary the presence of oxygen (needed for lipid synthesis and consequently for cell replication and biosynthesis of sterols and unsaturated

fatty acids) and a sugar limited feed rate. Furthermore, this stress has been shown to be useful since it triggers expression of certain genes and molecules that protect the cells and positively affect fermentative capacity (Pérez-Torrado et al. 2009). During fed-batch it is not unfamiliar to alter conditions in benefit of some features. Nutrient limitation is often used for this end. If triggered at right time, in the right conditions it can induce the production/accumulation of certain compounds (such as storage carbohydrates) that can be advantageous during less favorable fermentations (Jørgensen et al. 2002; Albers et al. 2007). Accumulation of compounds triggered by limited availability of a given nutrient will vary depending on the nutrient and on the conditions of aerobiose/anaerobiose. (Thomsson et al. 2005; Nilsson et al. 2001). Prior to immobilization yeasts still undergo maturation. Maturation phase consists on the consumption of residual sugars, so the primary stress factor present is nutrient limitation. Oxidative stress continues to be present, and resulting from changes of temperatures thermic stress arises. It is important that before the next phase cells enter stationary phase (active dividing cells are less resistant). This state allows cells to develop several adaptations that can enable them to survive adverse conditions (Walker and Djick 2006). Furthermore, cells that have not entered this phase are less likely to survive the following stages (González et. al 2011). Features acquired this far will reflect undoubtedly in the ability of yeasts to survive final stages of production. The final stage, in this type of production process, consists in immobilization and drying. A large combination of stress factors is present during this stage. Yeasts are still under nutrient limitation conditions, and osmotic stress is

expectedly relevant as a consequence of gradual dehydration. Temperatures of drying are also not optimal causing cells to be under thermic stress. Finally, oxidative stress is also still present and seemingly one of the most important during this stage (Garre et al. 2010; Goméz-Pastor et al. 2011). Stress imposed to yeasts during an industrial production process is a major factor to understand. Yeasts' resistance to stress has been related to fermentative capacity. Resistant strains are less likely to give rise to a stuck fermentation (Ivorra et al. 1999) and commercial/industrial strains are usually more tolerant to stress than lab strains (Carrasco et al. 2001).

Characterisation of a Production Process / Study Outline

The characterisation of a production process of industrial strains of Saccharomyces cerevisiae is relevant since they are widely used and because the quality of fermentation process is directly related to the quality and characteristics of the commercialized yeasts, which depends upon the production process they went through. Several aspects should be taken in account during biomass propagation. Since it is possible (and quite frequent) to change conditions of growth in order to obtain some specific characteristics that will be useful later on, it is imperative to control how those variables influence the characteristic in question and if they influence any others. Using a parametric analysis is possible to do an integrated evaluation of yeasts' physiology during the production process at an industrial scale. This analysis allows an optimization of the process in order to produce more efficient and more stress resistant yeasts. The production processes addressed in this thesis concern the production of immobilized

yeasts for sparkling wines at Proenol. Proenol, in partnership with research teams from the Centre National Recherche Scientifique (Toulouse, France) and several Portuguese Institutions of Science and Technologie has developed unique production process for encapsulation and dehydration of microorganisms. Presently, Proenol holds a production facility for encapsulated/dried yeast and bacteriaBesides fed-batch, maturation, immobilization and final product were also characterized. Different characteristics thought to be important for the process and final product were studied and are explained next.

Viability, Vitality and Cell Number

During an industrial production process of yeast viability, vitality and cell number are three key factors to control. Viability and vitality are two ways of evaluating quality of yeasts during and after production, and also a way to predict (to some extent) their performance in wine. Cell number is relevant so it is possible to assess growth rate, and predict if final biomass yield is going to be as expected. Viability represents the percentage of live cells in a given sample and during a production process is used as a quality control procedure. Viability is also one of the most important parameters to control during a production process since if there is a decrease on viability that would mean that conditions are somehow not optimal and that would compromise not only the final yield but also viability after immobilization/drying and storage and ultimately it could influence yeasts fermentation capacity. Depending upon method, viability can be consider ability to replicate (Colony Forming Units - CFUs), membrane integrity

(propidium iodide, methylene blue) or metabolic activity (Methylene blue reduction, enzymatic activity). Also, relevant to monitor yeast vitality, that is, their capacity to achieve complete metabolic activity. This so called vitality is related with the time fermentation takes to reach maximum rate (Aranda et al. 2011), this parameter is usually more suitable for predicting future performance of yeasts.

Trehalose and Glycogen – Strictly storage carbohydrates?

Glycogen and trehalose are storage carbohydrates present in yeast cells. The cell content of these two compounds differs greatly upon environmental changes indicating the existence of a complex regulatory system. Both these molecules have energetic functions in yeast cells. Glycogen is known to accumulate when glucose is still present in the medium, and is mobilized when there is no other exogenous carbon source available. Trehalose on the other hand does not fit the energy store concept that well, since it is known to accumulate after glucose has been consumed and while using ethanol as a carbon source, and it is used much later under non-stressed conditions (François & Parrou 2001; François et al. 2012). Several works have been published, showing that different stressful/unfavorable conditions induce accumulation of these two molecules (Walker and Djick 2006; Jørgensen et al. 2002). Accumulation of both these carbohydrates is usually promoted at lower growth rates rather than at higher ones. If there is an increase of growth rate these molecules can be rapidly mobilized and are thought to supply extra ATP to cells (required for budding process) (François et al. 2012). Nutritional stress usually affects positively carbohydrates levels in

yeasts. Nitrogen depletion seems to induce both trehalose and glycogen accumulation, while carbon starvation appears to induce trehalose accumulation (to a smaller scale) at the expense of glycogen (Jørgensen et al. 2002). Phosphate depletion on the other hand, by itself, does not induce trehalose nor glycogen accumulation (Ertugay et al. 1997). Temperature-related stress has an impact on trehalose and glycogen levels as well. Low temperatures induce the expression of genes involved in the metabolism of glycogen and trehalose and accumulation of both these carbohydrates (Aguilera et al. 2007), high temperatures to some extend also induce accumulation of trehalose (Trevisol et al. 2011). Growth conditions of high levels of ethanol have been implicated in accumulation of trehalose in alcohol-resistant yeasts (Ding et al. 2009; Alexandre et al. 1998) Accumulation of both these storage carbohydrates is usually promoted at lower growth rates rather than higher ones. Trehalose and glycogen have been implicated as factors of resistance to several adverse conditions. Glycogen is thought to be used as an energy source when cells are adapting to new conditions, and to have a role in maintaining viability during storage (Deshpande et al. 2011). Regarding ethanol tolerance, trehalose has been implicated, by some authors, in yeast protection due to its function of stabilizing proteins, in alcohol-tolerant yeasts. However there is a lack of consensus whether a correlation between trehalose and resistance to ethanol exists or not, since some authors have had contradictory results (Ding et al. 2009; Alexandre et al. 1998) On matters of stress related to temperature it is generally accepted that trehalose has an important role as a cell protectant both in low or high temperatures. Higher levels of trehalose

have also been correlated with freezing resistance (Aguilera et al. 2007). At high temperatures trehalose enables cells to survive (Trevisol et al. 2011). Moreover, from an industrial point a view, experiences done in laboratory strains and baker’s yeast with defective mutants in neutral or acid trehalase activities have proven successful, since these mutants accumulate more trehalose than the parent strain and appear to have a higher tolerance to drying (higher viability) (reviewed in Goméz-Pastor et al. 2011). Trehalose is thought to protect yeasts for desiccation preserving the integrity of the plasma membrane binding to phospholipids (substituting water), protects proteins from denaturation in hydrated cells and prevents denaturated proteins aggregation (Cerruti et al. 2000). However this model has been questioned, Ratnakumar & Tunnacliffe (2006) work showed that on S. cerevisiae mutant with a TPS1 (trehalose-6-phosphate synthase) deletion, no influence was observed to desiccation tolerance when comparing with wild type. However it has been suggested that adding certain amounts of disaccharides, such as trehalose, to cell suspension may improve viability outcome after freeze drying (Cerruti et al. 2000). In a later work Rodríguez-Porrata et al. (2011) reach similar conclusions by demonstrating that, extracellular trehalose if added in the suspension (prior to desiccation), has beneficial impact on yeast survival. Recently, it has also been suggested that trehalose is necessary for improving fermentation performance longevity, ability to withstand stressful industrial conditions (François & Parrou 2001; Trevisol et al. 2011). Therefore, it is strongly recommended that wine yeast strains (sparkling wines’ included) with higher trehalose and glycogen accumulation ability are developed (Pretorius 2000) but taking in account

that other key characteristics such as viability should not be compromised, when promoting accumulation of these two compounds.

Neutral Lipids

Neutral lipids are important storage molecules in eukaryotic cells, Saccharomyces cerevisiae being no different. Neutral lipids are mainly triacylglycerols (TGA) and steryl esters (STE), and are usually a part of intracellular structures called lipid particles (lipid bodies or droplets). Like the majority of storage compounds they are synthesized during stress conditions and not during exponential growth (Schlee et al. 2006). Lipid particles are formed by a hydrophobic core of TGA and STE surrounded by a phospholipid monolayer with some specific proteins embedded (Daum et al. 2007). When needed neutral lipids can be hydrolyzed into ergosterol, fatty acids and diacylglycerol, which can be used as building blocks for membrane synthesis and/or for producing energy. The presence of these degradation products and more specifically the proportion in which they are present in the cellular membrane is closely related with membrane fluidity and permeability, essential for stress tolerance. For instance, brewer’s yeast ability to ferment and tolerate ethanol is directly related to the nature of unsaturated fatty acids and sterols on their membranes. They are also important for membrane proliferation under certain conditions such as anaerobiosis (pool of neutral lipids increases survival capacity under anaerobiosis) (Schlee et al. 2006), long time survival in stationary phase and for viability fermentative capacity and stress tolerance in fermentative S. cerevisiae. Cultivation/propagation conditions also seem to influence neutral lipids

content qualitative and quantitatively (Rupčić & Jurešić 2010). Neutral lipids are important to monitor for their energy storage function their accumulation is related to high survival capacity in anaerobic conditions (Müller et al. 1997).

Total Protein Levels

The importance of determining the protein content is that it usually reflects the metabolic status of the yeasts. Since most cellular proteins are enzymes, high protein content is generally correlated with high metabolic activity. Accordingly, it is known that exponential growing cells present higher protein levels than cells in a stationary phase (Majara et al. 1998). Growth rate, temperature and carbon sources and/or culture medium are also factors affecting protein content (Verduyn 1991). Protein content is a good indicator for predicting fermentative capacity since this capacity is directly related to the amount and activity of glycolytic and fermentative enzymes. Therefore, it is important to produce cells with high protein content (Jørgensen et al. 2002), since decreases from usual values of protein levels tend to be indicative of quality deterioration (Majara et al. 1998). After production cells usually have a total protein content between 40-60% of their dry weigh depending upon the above mentioned factors (Larsson et al. 1997; van Hoek et al. 2000; Jørgensen et al. 2002; Albers et al. 2007). Nutritional stress also seems to influence protein content, especially nitrogen starvation which has been related with a decrease on total protein content in yeast cells (Larsson et al. 1997; Jørgensen et al. 2002; Albers et al. 2007). Decreases of 30-50% of total protein content in yeast cells during nitrogen limitation have been

reported by several authors (van Hoek et al. 2000; Jørgensen et al. 2002; Albers et al. 2007). In all studies the protein content was calculated on a dry weight basis, and as Jørgensen et al. (2002) suggests, part of this decrease could be due to an increase of dry weight that results from the accumulation of other molecules, such as storage carbohydrates (known to happen when in nitrogen limitation) which are also reported in the studies mentioned.

Glutathione

Glutathione is a tripeptide (L-γ-glutamyl-L-cysteinylglycine), non-protein thiol compound found in high concentrations in most - living cells. This thiol can occur in cells mainly under two forms, its oxidized form (GSSG) or its reduced form (GSH), the latter being usually predominant. GSH/GSSG ratio in conditions of aerobic growth (on rich YEPD media) is about11-16: 1. In the presence of reactive oxygen species (ROS), such as H2O2, the GSH/GSSG ratio tends to decrease (Grant 2001). Glutathione has been implicated in several processes of yeast cell protection against stressful situations like nutritional, environmental and oxidative stresses (Penninckx 2000). Perhaps, the most relevant role of this molecule is it’s the protective function regarding oxidative stress, as it is a major antioxidant it protects cells from reactive oxygen species (ROS) and toxic electrophiles reducing their damaging effects (Suzuki et al. 2011). Furthermore, S. cerevisiae can increase its glutathione levels as a response to oxidative stress by increasing its synthesis (through Gsh1, ■-glutamylcysteine synthetase and Gsh2, glutathione synthetase) or by recycling GSSG, reducing it by the action of glutathione reductase (Jamnik et al. 2006; Grant, 2001). Furthermore, an

overlap between oxidative and osmotic stress response is known to exist in yeasts and it has been demonstrated that glutathione plays an important role in protecting yeast cells from stress caused by exposure to NaCl (Jamnik et al. 2006). In a situation of sulphur starvation GSH can also be used as an endogenous sulphur source, since glutathione cycle plays a key role in the regulation of sulphur fluxes. Nitrogen starvation triggers accumulation of GSH in the vacuoles where a vacuolar enzyme catalyzes a reaction, which later results in its amino acids release, acting like an endogenous source of amino acids for growth and maintenance (Penninckx 2002). During industrial process of biomass propagation Gomez-Pastor et al. (2011) reported that the GSH content varied during the process, lowering during metabolic transition (from fermentation to respiration) while GSSG increases. from metabolic transition on GSH tends to increase through the different stages of the process. It is still unknown if glutathione levels are directly affected by oxygen during propagation (Gomez-Pastor et al. 2011). . It has been shown that during dehydration, in the final stages of the production process, there is a strong accumulation of ROS and consequentially cells are under oxidative stress. Antioxidant systems have, therefore, an important role on yeast’s desiccation tolerance (Gomez-Pastor et al. 2011; Espindola et al. 2003). Defective mutants of yeast cells lacking GSH1 present a high sensitivity to dehydration (Espindola et al. 2003). It is usually of positive influence the production (and later use) of yeast with higher content of glutathione for wine fermentation purposesdue to glutathione role as a preserver of wine aromas and because it delays the organoleptical ageing. For production process glutathione is a key feature to monitor for its

influence in final viability. Yeasts glutathione content is usually near between 0. 5-1% of its dry weight (Li et al. 2004), and recently commercial yeasts (ADY) with increased glutathione content (3%) have been commercialized with the argument that it is advantageous for avoiding organoleptical ageing by preventing the appearance of free radicals (Fermentis 2008).

Relative Gene Expression

There are several studies that evaluate kinetic parameters, energy and process efficiency of yeast production, which has been optimized to obtain the highest biomass yield. However, very little is known about the molecular mechanisms for adaptation to harsh conditions of industrial production. This results mainly from the difficulty in simulating a process on industrial scale in laboratory conditions, although more recently some authors have made an approach by using micro-fermenters in bench-top trials (González et al. 2011; Pérez-Torrado et al. 2005). During the process of encapsulated yeast production, yeast cells experience several stress situations and they respond to these environmental changes by extensively altering their gene expression. In these conditions, in which protective molecules must be synthesized by cells in order to survive, some transcriptional factors are activated and yeasts' transcriptional profile is altered. (Pérez-Torrado et al. 2005). Although not much information is available on yeast responses in our specific conditions, several works have been published on yeasts' molecular stress response (Estruch 2000; Gasch 2003; Pérez-Torrado et al. 2005; Garre et al. 2010). Yeast cells exposed to conditions of mild stress develop tolerance not only to higher doses of the same stress but also to other

stressors. This phenomenon is called cross-protection and suggests the existence of a general stress response (GSR). Given the necessity, GSR would induce the expression of many different genes through a common element in the promoter, which coordinates the induction of several genes, the stress response element (STRE). Associated with STRE are the transcription factors Msn2p and Msn4p. These factors were purposed to be activated by stress inducing a set of genes in response (Gasch 2003; Estruch 2000). Nevertheless, STRE regulation is far from simple and STRE-containing genes do not show a uniform pattern of expression, pointing to the existence of other regulatory sequences involved in stress-induced transcription (Pérez-Torrado et al. 2005; Estruch 2000). In a very comprehensive work Gash et al. (2000) showed that yeast cells under different environmental stress factors responded not only through STRE containing genes, but approximately 900 genes showed similar response to different conditions. This response that involves induced/repressed genes is known as environmental stress response (ESR). Not invalidating GSR, ESR is a much more broad approach for describing alterations at a molecular level in response to stress. Rather than a general mechanism of regulation Gasch et al. (2000) showed the existence of signaling pathways that depend upon condition. In yeasts, stress response seems to be tailored to the amount of stress, meaning that more stress usually corresponds to larger and more prolonged alterations of gene expression but, as for less stressful conditions, this response is transient (Gasch et al. 2000; Gasch 2003). An exception to these transient responses is the case of nutrient starvation where cells enter a quiescent state until nutrients are available and likewise involves a

persistent gene expression response that ends with the end of starvation (Gasch et al. 2000). However ESR existence does not mean that there is an equal response to all stresses. ESR is initiated when conditions are suboptimal in order to prepare cells to a rapid response. For example, it is known that genes involved in response to oxidative stress are induced in ESR, but in response to oxidative stress specifically they are super induced (Gasch 2003). This response modulation through a very complex system of signaling pathways, transcription factors and post translational regulation is what allows a specific response to each set of conditions. Monitoring stress marker genes is a commonly use approach for evaluating changes in stress response related genes and assess/identify the stress yeasts undergo and their response to it. A good stress marker gene is usually one that responds specifically to a given stress. In a situation of mixed stresses, as in an industrial propagation process, genes that respond strongly to more than 2 stresses are not adequate to monitoring purposes, and are thus unsuitable to be a stress gene marker (Pérez-Torrado et al. 2005). Selected genes for this work were: TRX2 which was shown to strongly respond to oxidative stress, GPD1 involved in glycerol synthesis and part of the specific high-osmolarity glycerol response pathway, HSP12 which is part of the previous mentioned GSR involving STRE sequence and transcription factors Msn2p/Msn4p. All of the above were used in a similar work monitoring stress marker genes in a yeast propagation process by Pérez-Torrado et al. (2005) and shown to respond strongly to the respective stress and poorly to others usual present in this kind of process. GSH1 was discarded in that case for not presenting a strong response during the whole process but included by us, since we

intended to follow the process after propagation phase (maturation, immobilization and drying) and other work reported a strong response by GSH1 during drying (Garre et al. 2010). GSH1 and TRX2 are both stress marker genes for oxidative stress but their regulation is different and involves different transcription factors. In a bench top trial of wine yeast biomass propagation it was demonstrated that the main adverse conditions that yeasts face in this particular process are osmotic and oxidative stress, GPD1 and TRX2 respectively (Pérez-Torrado et al. 2005; Goméz-Pastor et al. 2011). Other studies have identified oxidative critical stressful timepoints during the propagation process: the metabolic transition from fermentation to respiration, the end of the batch phase when ethanol is totally consumed and the end of the fed-batch (Goméz-Pastor et al. 2011; Goméz-Pastor et al. 2010). Under this oxidative stress, genes such as GSH1, TRX2 are usually induced. Furthermore, Goméz-Pastor et al. (2010) work also showed that overexpression of TRX2 had a positive impact on biomass yield. Osmotic stress, specifically high-osmolarity stress, results in a well known response called the high-osmolarity glycerol response pathway (HOG pathway), in these conditions genes involved in glycerol synthesis (such as GPD1 and GPP2) and others from the same pathway are induced in order to glycerol accumulate inside the cells and avoid water loss. Regarding nutrient starvation, there is a poorly understood stress response, although it is known that some genes of unknown function are induced, such as YGP1 (Pérez-Torrado et al. 2005).