Letters in Applied Microbiology

Letters in Applied Microbiology ISSN 0266-8254

REVIEW ARTICLE

Biotechnological impact of stress response on wine yeast

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Keywords
fermentation biotechnology, gene expression, stress response, wine, yeast.

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2017/1547: received 15 July 2016, revised 9 September 2016 and accepted 29 September 2016

doi:10.1111/lam.12677

Abstract

Wine yeast deals with many stress conditions during its biotechnological use. Biomass production and its dehydration produce major oxidative stress, while hyperosmotic shock, ethanol toxicity and starvation are relevant during grape juice fermentation. Most stress response mechanisms described in laboratory strains of *Saccharomyces cerevisiae* are useful for understanding the molecular machinery devoted to deal with harsh conditions during industrial wine yeast uses. However, the particularities of these strains themselves, and the media and conditions employed, need to be specifically looked at when studying protection mechanisms.

Introduction

Grape juice fermentation is a complex biotechnological process that produces a final product full of subtle flavours and aromas. Alcoholic fermentation, which transforms the sugars present in grapes (an equimolar mixture of free glucose and fructose) into ethanol, is a central metabolic pathway shared by many fungi. However, the yeasts of the genus *Saccharomyces*, in particular the strains of the species *S. cerevisiae*, carry out the bulk of fermentation and are, therefore, called wine yeasts by default (Ribéreau-Gayon *et al.* 2006). *Saccharomyces* are not among the epiphytic yeasts present on the surface of grapes, e.g. as is the case of the genera *Hanseniaspora* (*Kloeckera*), *Candida*, *Pichia* and *Hansenula* (Jackson 2000). *Saccharomyces* is very easily found in winery equipment and seems to be the usual source for spontaneous inoculation. Why is *Saccharomyces* imposed on the other yeasts present in primordial grape juice? The key factors involved are its high fermentative power and its better adaptation to this harsh changing environment due to its ability to sense, react and adapt its physiology to the sequential stress conditions that yeast cells encounter during their biotechnological use (Fig. 1). Therefore, increasing stress tolerance is a suitable way to improve yeast industrial performance (Pretorius 2000). The aim of this review is to analyse both the main stress conditions that wine yeasts encounter during wine production and the molecular mechanisms devoted to deal with them.

Stress conditions that wine yeasts face during wine production

The grape surface is not the ancestral habitat of *S. cerevisiae,* and traditional fermentations rely on the yeasts present in the cellar to be inoculated by chance (Jackson 2000). This implies that yeasts have to be in a dormant, but viable, state for a whole year and must tolerate seasonal temperature changes. Another kind of yeast reservoirs are insect guts. Wine strains of *S. cerevisiae* have been found in social wasp guts, and these fungal cells are passed to the progeny (Stefanini *et al.* 2012). Obviously, understanding the physiology of the cells left out in the cellar or in a digestive tract is difficult, but studies with laboratory strains could provide us with some insights. When nutrients are scarce, cells enter a quiescent state called the stationary phase (Herman 2002; De Virgilio 2012). This implies that the cell cycle arrests at a special point called G_0, where several molecular adaptations occur, such as reduced transcription and translation,

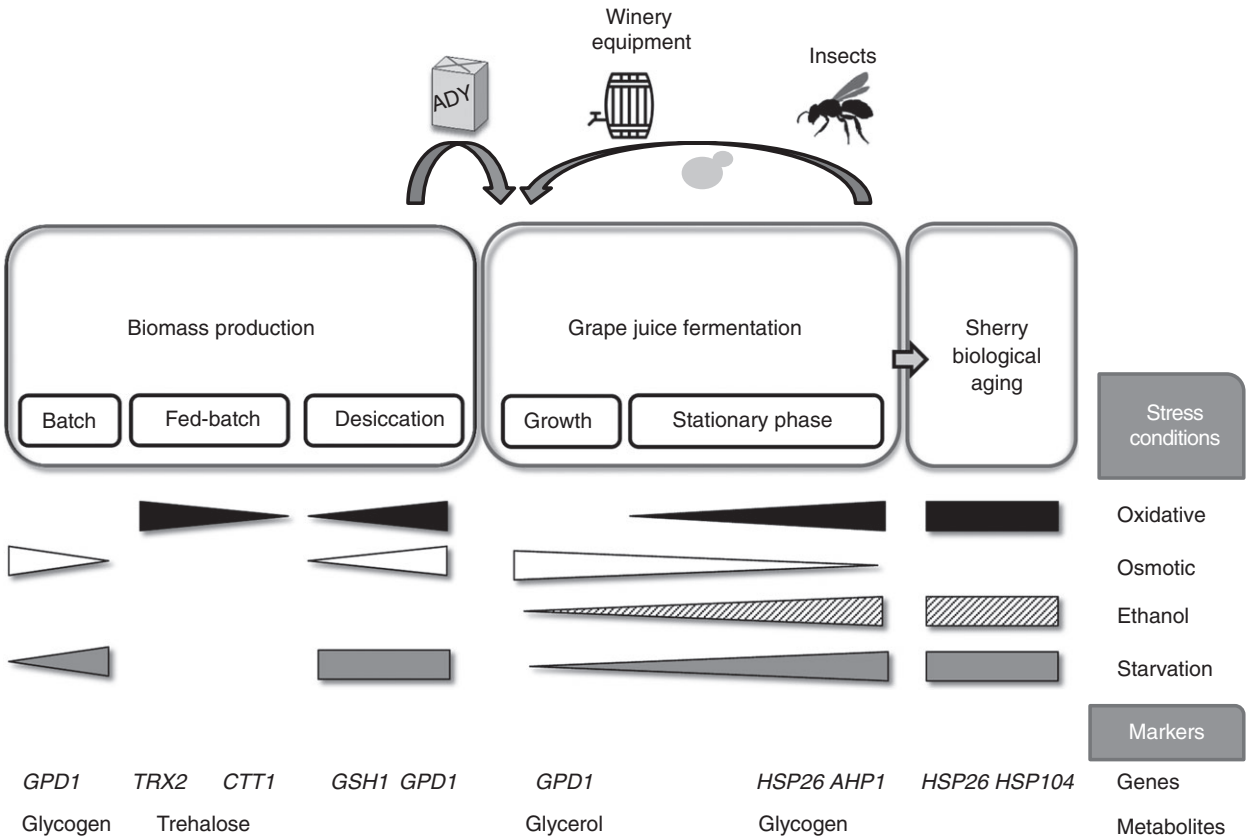

Figure 1 Stress response during the biotechnological use of wine yeast strains. The main winemaking stages, from biomass production, grape juice fermentation and Sherry ageing (when required), are indicated by boxes. Arrows show the transitions of yeasts from different steps of the process. The profiles of the main stress conditions are indicated and show their increase or decrease in the aforementioned stages. The genes relevant to stress tolerance that can be used as stress markers are shown, together with the main protective metabolites. ADY, active dry yeast.

increased stress tolerance and glycogen accumulation as an energy source. All these responses may be present in winery yeasts when nutrients in grape juice are exhausted. Afterwards, cells are probably dried during most of the year, so resistance to desiccation must also be relevant.

This tolerance to drying has proven biotechnological utility. Spontaneous traditional fermentations are still carried out, but most fermentations on an industrial scale rely on a selected yeast inoculum (Pérez-Torrado *et al.* 2015). This makes the process more reliable as yeast with good fermentative and organoleptic behaviour can be selected to perform fermentation. As winemaking is a seasonal event, the production of such starters in the form of active dry yeast (ADY) is the chosen way to market these starters (Fig. 1). Yeasts are propagated first in batch and then in scaled-up fed-batch growth in fermenters using diluted cane or beet molasses as a growth substrate. A respiratory metabolism is then imposed, so more biomass is produced. However, these respiratory conditions cause internal oxidative stress (Perez-Torrado *et al.* 2005). Molasses are poor in nitrogen sources, so starvation stress is present from the beginning. To prevent this scenario, a nitrogen source is usually added. The produced biomass is then dehydrated on a hot air bed. Therefore, thermal stress is relevant in the process, plus the obvious rise in osmolarity due to the efflux of water, and also an internal oxidative insult, has been detected (Pérez-Torrado *et al.* 2015).

Active dry yeast is usually rehydrated in warm water (around 37°C) and causes both hyposmotic stress and mild thermal stress (Rossignol *et al.* 2006). The environment that a rehydrated commercial yeast or an indigenous yeast encounters is a highly hyperosmotic medium (over 200 g l^{-1} and up to 250 g l^{-1} of sugars), with a low pH (3·0–3·5), plus the usual addition of sulphite as an antioxidant and disinfectant. These conditions may select against many bacteria and filamentous fungi that could be present in grapes (Fleet 1993). When the oxygen dissolved in juice is consumed and the carbon dioxide produced by fermentation displaces it, low oxygen

conditions restrain the survival of aerobic micro-organisms. Although rich in sugars, grape juice is poor in nitrogen sources and vitamins, which are the nutritional limitations that mark the end of cell proliferation. A nitrogen source, usually in the form of diammonium phosphate, is often added (Ribéreau-Gayon *et al.* 2006). *Saccharomyces* yeasts will impose on other yeasts because they can adapt their metabolism to fast alcoholic fermentation given their tolerance to the combination of the above-mentioned stressful conditions. The strategy of *Saccharomyces* has been described as make-accumulate-consume (Piskur *et al.* 2006). As a crabtree-positive yeast, ethanol is produced quickly by *Saccharomyces* and is not consumed until sugars have been depleted. Relative tolerance to ethanol, compared with apiculate yeast species present in grapes, contributes to the fact that *Saccharomyces* outcompetes all the other initial fungi and dominates. Lack of nutrients, high ethanol concentration and cellular oxidative stress all lead to the progressive death of the yeast population. Such ageing of nondividing cells is called chronological ageing (Longo *et al.* 2012; Orozco *et al.* 2012a).

This is the end of the process for most wines and their yeasts, but some postfermentative processes lead to specific wines, which are carried out by specialized yeasts that face extreme stress conditions. Sherry fino wines derive from postfermentation biological ageing. Ethanol is added to wine up to 15%. Under this condition, a biofilm (vellum) of a specific kind of yeasts (flor yeasts) forms on the surface. This ability to form a biofilm and to gain access to oxygen enables flor yeasts to adopt a respiratory metabolism that consumes ethanol to produce energy (Alexandre 2013), which leads to the accumulation of acetaldehyde, a specific stressor of this stage (Aranda *et al.* 2002). High ethanol and acetaldehyde, together with poor nutrients and high oxygen concentrations, are the marks of biological wine ageing. Sparkling wines also undergo a second fermentation inside bottles after adding sugar. This environment is poor in nutrients other than sugars, high in ethanol and involves high CO_2 pressure. Under these conditions, lysis of death cells contributes to final product characteristics (Jackson 2000; Cebollero and Gonzalez 2007).

As a free micro-organism, most stress conditions and the responses they trigger are related to metabolism, so metabolic stresses must be considered. For instance, ethanol and acetaldehyde are normal metabolites that become toxic when a threshold is surpassed. Oxidative stress, the main stress condition during biomass production, is not caused by an exogenous oxidant but by the endogenous reactive oxygen species (ROS) produced mainly in the mitochondria due to unbalances that take place during either metabolic transitions or normal respiratory metabolism. Even hyperosmotic shock at the very beginning of grape juice fermentation is produced by sugars, the main nutrient of yeast. Therefore, the stress response is not easily distinguished from metabolic adaptation, and the pathways that deal with stress are tightly controlled by nutrient availability, as explained in the next section.

Yeast stress response

Saccharomyces cerevisiae response to environmental stress is complex and relies on multiple pathways that have been extensively studied in laboratory strains (Hohmann and Mager 2003). Most are based on kinases that end up remodelling transcription through stress-specific transcription factors. However, an ever-growing body of evidence suggests that other gene expression mechanisms, such as translation initiation or mRNA stability, act as tools to quickly react to a stimulus (de Nadal *et al.* 2011). An intertwined response exists between different pathways, which are all influenced by nutrient-sensing pathways (Conrad *et al.* 2014; Rodkaer and Faergeman 2014). When nutrients are present, the protein kinase A (PKA), AMPK Snf1 and TOR/Sch9 pathways promote cell growth and division by inhibiting general stress transcription factors Msn2/4. These factors bind to a DNA sequence element, STRE (Stress Response Element), which is present in most of the gene promoters induced by stress. Heat shock factor Hsf1 is regulated similarly to regulate heat shock proteins (De Virgilio 2012). Protein kinase A and Snf1 sense abundance of sugars and TOR are devoted mainly to signalling when nitrogen sources are abundant. When nutrients are scarce, these pathways rearrange yeast physiology to enter the stationary phase (Winderickx *et al.* 2003; De Virgilio 2012). Their impact on stress response is channelled mainly through kinase Rim15, which stimulates Msn2/4 transcription factors.

Specific stress responses sometimes rely on specific signalling pathways. For instance, the HOG mitogen-activated protein kinase (MAPK) pathway is a signalling system that controls osmoregulation (Hohmann 2015). When cells are exposed to hyperosmotic shock, they produce glycerol as a compatible osmolyte. The enzymes devoted to its production are controlled by the HOG pathway. Hog1 kinase activates the Hot1 transcription factor which, in turn, activates glycerol synthesis from glycolytic intermediate dihydroxyacetone phosphate by up-regulating glycerol-3-phosphate dehydrogenase and glycerol phosphatase genes *GPD1* and *GPP1/2,* respectively. Besides, it induces a pump called Slt1, which introduces glycerol from the environment.

The response to oxidative damage triggers synthesis on the enzymes that are able to detoxify ROS. Catalases

degrade H_2O_2, superoxide dismutases transform the superoxide anion, and various peroxiredoxins degrade a variety of peroxides (Herrero *et al.* 2008; Ayer *et al.* 2014). Apart from eliminating oxidative insult, oxidative stress response systems control the redox status of cells by preventing and repairing damage of molecules prone to oxidation, such as the thiol residues of proteins. There are two basic redox-controlling systems: one is based on tripeptide glutathione and the other on small proteins called thioredoxins (Ayer *et al.* 2014). The final reducing power comes from NAPDH obtained through metabolic activity, mainly via the pentose phosphate pathway. The transcription of all these genes is controlled by specific transcription factors Yap1 and Skn7, which are prone to the redox changes that control their activity.

Needless to say, all these pathways work in a coordinated fashion and stress response genes are usually regulated by more than one of the above-mentioned transcription factors. This causes the phenomenon called cross-protection: previous exposure to a kind of stress protects against another kind of environmental insult. The cores of stress response genes are labelled as environmental stress response (ESR), a set of around 300 genes whose expression is induced, with some 600 genes whose expression is repressed when exposed to stress conditions, such as heat shock, oxidative or reductive stress, osmotic shock, nutrient starvation, DNA damage and an extreme pH (Gasch *et al.* 2000; Causton *et al.* 2001). General stress transcription factors Msn2/4 mediate most of the common transcriptional activation caused by these different stress conditions.

Stress response during the biotechnological use of wine yeast and its improvement

Tolerance to environmental stress conditions is a key factor to achieve the biotechnological success of *Saccharomyces* yeasts. A correlation between high tolerance to stress and good fermentative capacity has been found (Ivorra *et al.* 1999), and stress tolerance is a good criterion for selecting enologically interesting yeasts (Zuzuarregui and del Olmo 2004). The most tolerant yeasts better adapt to their environment and thrive in it. For instance, the strains that better tolerate ethanol are more abundant in a sherry cellar (Aranda *et al.* 2002). The previously depicted stress pathways have been described in laboratory strains of *S. cerevisiae* grown in laboratory media. Industrial strains of wine yeasts display genetic differences with laboratory strains. They are prototrophic, their ploidy is variable, and most are roughly diploid (Carrascosa Santiago *et al.* 2011). Sequencing commercial wine strains reveals differences compared with other yeasts, which affect discrete genomic regions and rearrangements that contain genes that confer biotechnological advantages, but conserve basic genetic information (Novo *et al.* 2009; Borneman *et al.* 2016). As explained below, molecular studies of wine yeast under biotechnological conditions have been proven in accordance with mostly the expected behaviour observed in laboratory strains. However, some specific genetic variations may explain different responses to specific stresses; for instance, the differences found in the response to weak acids between wine yeast strains (Brion *et al.* 2013).

ADY production

Regarding biomass production, the expression of selected stress genes was studied in the batch and fed-batch stages in industrial yeast strains (Perez-Torrado *et al.* 2005). The high sugar concentration of initial batch growth induced typical osmogene *GDP1*, while the induction of cytosolic thioredoxin *TRX2*, a typical oxidative stress gene, took place in the fed-batch stage. A detailed transcriptomic analysis during fed-batch growth in molasses has confirmed the induction of many oxidative stress genes (thioredoxins, peroxiredoxins, glutaredoxins, etc.) during the process, particularly during the diauxic shift caused by sugar consumption (Gomez-Pastor *et al.* 2010a). A similar up-regulation of the proteins used to deal with oxidative stress has been observed in a proteomic analysis (Gomez-Pastor *et al.* 2010a). Therefore, oxidative stress would appear to be the main negative condition that operates against the yeast cells that grow in fermenters. In fact, the overexpression of the *TRX2* gene leads to a wine strain with increased biomass production (Gomez-Pastor *et al.* 2010b).

The drying process also causes internal oxidative stress as the markers of oxidative damage, e.g. lipid peroxidation and glutathione levels, increase (Gomez-Pastor *et al.* 2010b). Induction of genes that perform an antioxidant function, such as that of the thioredoxin reductase *TRR1* gene, is observed. High levels of protective disaccharide trehalose, and strong catalase and glutathione reductase activities, have been related with good drying performance in wine (Gamero-Sandemetrio *et al.* 2014). The overexpression of hydrophilin *SIP18* in industrial strains decreases ROS after oxidative stress and increases viability after desiccation (Lopez-Martinez *et al.* 2013). Once again, this proves that increased stress tolerance can improve ADY production. The non-*Saccharomyces* yeasts present at the start of fermentation contribute to the final organoleptic structure of wine. This is why the co-inoculation of *Saccharomyces* with some of these yeasts may improve the process (Fleet 2008; Jolly *et al.* 2014). However, these yeasts are more stress sensitive and are difficult to produce as ADY (Pereira Ede *et al.* 2003). Antioxidant

enzymes, such as catalase and superoxide dismutase, are good markers for studying the stress tolerance of non-*Saccharomyces* yeasts as their activity correlates to fermentative performance (Gamero-Sandemetrio *et al.* 2013). Different stress tolerances exist, even among *Saccharomyces* species of enological interest. Using hybrids, it has been proven that *S. cerevisiae* mitochondria better protect against dehydration than *Saccharomyces uvarum* mitochondria (Picazo *et al.* 2015a). Therefore, the oxidative stress response is a potential target for wine yeast improvement, and using natural antioxidants like argan oil is a feasible way to improve biomass production (Gamero-Sandemetrio *et al.* 2015).

Rehydration

When warm water is added to ADY, a sudden change in yeast physiology takes place, as revealed by a transcriptomic analysis carried out in the rehydration step (Rossignol *et al.* 2006; Novo *et al.* 2007). Compared with ADY, general stress response genes (e.g. those that code for heat shock proteins) and oxidative stress genes are down-regulated during rehydration (Rossignol *et al.* 2006). This indicates that dehydration triggers a very strong stress response, due to the highly damaging effects of water loss, which are much more stressful than rehydration conditions. However, acid stress response genes (e.g. pump *PDR12*, which deals with transporting organic acids) and the genes involved in proton homeostasis (e.g. H^+ pump *PMA1*) are induced. This indicates that ion unbalances are the main stress condition to occur during quick rehydration.

Grape juice fermentation

When yeast cells come into contact with grape juice, a sudden change in the stress gene expression takes place. *GPD1*, an osmotic stress gene, is activated within the first hour after inoculating grape juice from a stationary preculture (Perez-Torrado *et al.* 2002). Induction of heat shock protein gene *HSP104* takes 7 h and *HSP12* expression does not change. Yet when using ADY as the inoculum, *GPD1* is not induced at the start of fermentation (Rossignol *et al.* 2006), like most stress responsive genes. A proteomic analysis at the very beginning of fermentation matches this profile, with stress proteins, including Gpd1, being repressed after inoculation (Salvado *et al.* 2008). These results indicate that the physiological status of the starter is a determinant in the stress response during vinification. The HOG pathway acts during fermentation, but *GPD1* regulation is only partially dependent on the HOG pathway. Therefore, further regulatory mechanisms are expected to take place during grape juice fermentation (Remize *et al.* 2003). Posttranscriptional mechanisms also happen, such as the translation of Gpd1, which is also modulated during winemaking through the action of mRNA-binding protein Pub1 (Orozco *et al.* 2016).

A global analysis run throughout fermentation has indicated that the bulk of ESR stress genes are induced in later fermentation stages when cells enter the stationary phase (Rossignol *et al.* 2003; Varela *et al.* 2005). Environmental stress response genes have been defined as being responsive to transient stress (Gasch *et al.* 2000). Yet, when the stress conditions are sustained throughout fermentation, a novel set of 223 additional genes defined the more specific fermentation stress response (FSR)(Marks *et al.* 2008). This new set contains canonical stress genes, but 62% of them have not yet been related to stress response. Ethanol stress may contribute to this stress response, but nutrient starvation seems a key factor as most of these genes are regulated by general stress transcription factors Msn2/4, whose action is relieved when nutrients are scarce (Rodkaer and Faergeman 2014). Indeed, Msn2 overexpression in wine yeast improves stress response and increases the fermentative rate (Cardona *et al.* 2007), which reinforces this point. When comparing the transcriptomes of different yeast strains, relevant differences in gene expression can be linked to specific phenotypic differences, although no clear stress response pathways are differentially regulated (Rossouw and Bauer 2009).

At the proteomic level, most chaperones are repressed in later fermentation stages, but some stress proteins, such as Hsp26 and peroxiredoxin Ahp1, are induced at the protein level as they are transcriptionally up-regulated (Varela *et al.* 2005; Rossignol *et al.* 2009). Hsp26, together with Hsp12, is also induced at the protein level when low-temperature wine fermentation is carried out (Salvado *et al.* 2012). Therefore, chaperones like Hsp26 are good molecular markers that mark stress under many biotechnological conditions.

At the end of fermentation, yeast cells age and die. Chronological longevity reflects the viability of a yeast culture in a nondividing state, such as the stationary phase (Longo *et al.* 2012). As most grape juice fermentation takes place with no cell division, cells start dying when fermentation is incomplete (Ribéreau-Gayon *et al.* 2006). Such ageing is biotechnologically relevant. A good stress response delays the cellular ageing process. Heat, a low pH and two carbon metabolites produced by fermentation (mainly ethanol, but also acetaldehyde and acetic acid) shorten the wine yeast life span (Orozco *et al.* 2012b). A transcriptional analysis of industrial strains with different longevities has indicated that the oxidative stress response is necessary to achieve a full life span

(Orozco *et al.* 2012a). Damage caused by ROS with high sugar fermentation is known to be prevented by superoxide dismutase and protective disaccharide trehalose (Landolfo *et al.* 2008). Starvation is an important stress condition that occurs at the end of fermentation, but works in an unexpected manner as regard ageing. Reducing nutrient intake, i.e. dietary restriction, extends the life span by lowering mainly the activity of nutrient-sensing pathways and, therefore, by increasing the stress response (Longo *et al.* 2012). This is also the case with wine yeasts. Low nitrogen or TOR inhibition extends the chronological life span (Orozco *et al.* 2012c; Picazo *et al.* 2015b). High PKA activity also shortens life spans in wine yeasts, probably due to its control of the general stress response (Orozco *et al.* 2012a).

An artificial stress condition that takes place during wine fermentation is imposed by adding sulphite to grape juice to prevent spoilage and oxidation (Ribéreau-Gayon *et al.* 2006). Adaptation of wine yeasts to this particular harmful agent is not based directly on gene expression regulation, but on chromosomal rearrangement, which creates a novel allele of sulphite efflux pump *SSU1* with a constitutively higher expression, which is only present in *S. cerevisiae* wine strains (Perez-Ortin *et al.* 2002).

Postfermentative processes

After fermentation, the main stress condition is a high ethanol concentration that is constantly present. An increased expression of heat shock protein (*HSP*) genes, particularly *HSP26* and *HSP104*, correlates with ethanol tolerance in flor yeasts (Aranda *et al.* 2002). Acetaldehyde also brings about the induction of a similar set of *HSP*s (Aranda and del Olmo 2004). Overexpression of superoxide dismutases *SOD1* and *SOD2*, and of *HSP12* genes, improves vellum formation and cell viability in flor yeasts (Fierro-Risco *et al.* 2013). This indicates that oxidative stress is highly relevant during this mainly aerobic process. During sparkling wine second fermentations, nutritional stress conditions activate autophagy, which eventually leads to yeast cell death and lysis (Cebollero and Gonzalez 2006).

Concluding remarks

The increasing amount of data produced by the global analysis of the transcriptome, proteome and metabolome of wine yeast under industrial conditions, together with the knowledge at the genomic level of both *Saccharomyces* and non-*Saccharomyces* yeasts by using next-generation sequencing tools, will provide a complete view of the biotechnological behaviour of these organisms in the near future. Systems biology approaches will unveil a full picture of these industrial processes, while synthetic biology will enable us to engineer the yeasts of the future, each with its optimal performance for every process.

Acknowledgements

This work has been funded by a grant from the Spanish Ministry of Economy and Competitiveness (AGL2014-52984-R).

Conflict of Interest

The authors declare that they have no conflict of interest.

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