



WINE YEAST: THE CHALLENGE OF LOW TEMPERATURE

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Wine yeast: The challenge of low temperature

Doctoral Thesis

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DECLARATION

I, the undersigned, hereby declare that the work contained in this dissertation is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

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Preface

Yeast has been used by humans for thousands of years, mainly for produce food products as beer, wine, bread and the like. This ancient empirical know-how was transmitted over the time during generations to handle all those biotechnological processes without knowing what or why those food products change their physical properties such as flavor, texture, consistency, longevity, etc. In XIV century first optical microscope was invented by Anton van Leeuwenhoek and whole new microscopic life forms were discovered allowing the microbiology and biotechnology to start as relevant sciences. A steady development of biological knowledge and science followed and start to unravel the know-why and know-what behind all those ancient biotechnological processes. Pioneers on these fields were eminent personalities such as Louis Pasteur, Robert Koch, and Alexander Fleming among many others.

Nowadays modern biotechnology has become a complex science field in which convergence of various, previously distinct, biological disciplines occurs: microbiology, genetics, biochemistry, molecular biology, genetic engineering, etc. Yeast has played a central role on development of this new scientific field. Furthermore these microorganisms, and mainly the species *Saccharomyces cerevisiae*, has become a model organism of eukaryotic cell studies in modern biotechnology.

In the last 30 years, explosion of genetic engineering and high throughput techniques like genomics, proteomics and metabolomics in combination with so-called classical techniques have produced a knowledge-based transition of traditional food products elaboration to high technological food production all over the world.

Wine industry has been also involved in this technological revolution that encompasses all oenological practices from field to glass. One of the key points in wine production is the biocatalized reaction that transforms grape sugars (glucose and fructose) into ethanol and carbon dioxide. Yeasts are the main responsible of this phenomenon but also responsible of production of diverse secondary metabolites (glycerol, acetate, succinate, piruvate, and esters) that greatly contribute to the wine sensorial properties. Due to great impact of yeast to final wine quality, big efforts on wine biotechnology, in which this thesis may be included, have been dedicated to study yeast on winemaking process.

Introduction and outline of the thesis

What are *Saccharomyces* yeasts?

Yeasts are unicellular fungi widespread in natural world. They can be easily found in flowers, plant leaves, fruits, soil, etc. Yeasts are also found in animal skin or intestinal tracts of warm-blooded animals usually as innocuous flora but occasionally as a pathogen organism. However, for humanity yeasts have had a special relevance in human driven food production. Yeasts are true fungi of the phylum *Ascomycetes*, class *Saccharomycetes* (also called *Hemiascomycetes*). Yeasts multiply as single cell that divides by budding (e.g. *Saccharomyces*) or direct fission division, (e.g. *Schizosaccharomyces*), or they may grow as simple irregular filaments (mycelium).

Historically, taxonomy used morphological, reproductive and physiological characters to classify yeasts. This methodology was based mainly in binary characteristics with rapid evolution and subjected to rampant homoplasy.

These inherent limitations led to a chaotic mass of synonyms and misclassifications. In the case of *Saccharomyces* genus this situation was extremely confusing, so was the case that old genera names seem to be almost randomly assigned. Fortunately, multilocus and genome-scale phylogenetics are bringing light to yeast nomenclature and to *Saccharomyces* phylogeny (Hittinger, 2013). *Saccharomyces* genus has solved numerous challenges and controversies, to date, new DNA sequencing techniques has solved them and it is now clear that *Saccharomyces* sensu stricto is formed by seven naturally occurring members and some industrial hybrids (Figure 1).

Recently the considerable controversy around *Saccharomyces bayanus* species complex have been solved by Libkind et al. (2011), built in the discovery of *Saccharomyces eubayanus* sp. nov. New derived phylogenetic tree include *Saccharomyces uvarum* and *Saccharomyces eubayanus* ssp. nov. within the seven sensu stricto *Saccharomyces* spp. (Figure 1). Finally, *Saccharomyces cariocanus* is reproductively isolated from *Saccharomyces paradoxus* by multiple reciprocal translocations (Liti et al., 2006; Naumov et al., 2000). However, both known strains are

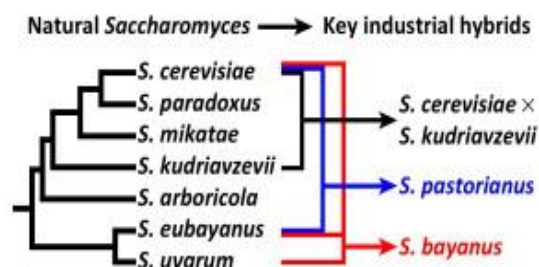


Figure 1 *Saccharomyces* sensu stricto phylogenetic tree and key industrial hybrids. (Hittinger, 2013)

phylogenetically circumscribed within the diversity of interfertile *S. Paradoxus* (Liti et al., 2009, 2006), suggesting *S. cariocanus* strains may be mutants rather than an eighth species of the genus (Hittinger, 2013).

Saccharomyces life cycle and population genomics

Budding yeast are present in nature either as heterothallic or homothallic strains (Figure 2). Both forms are able to sporulate in adverse conditions as low nutrient availability. During sporulation, the diploid yeast cell produce four haploid spores by meiosis that remain within an envelope called ascus. Sporulation ability is variable as function of studied strain ranging from near 100% to no sporulation.

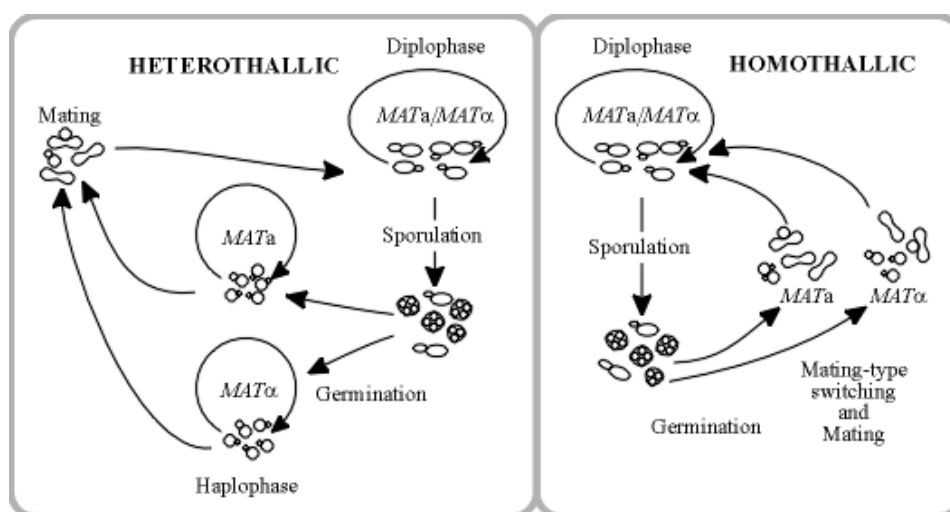


Figure 2 Heterothallic and homothallic life strains cycles in *S. cerevisiae*.

Wine yeast do not sporulate easily and when they do it, spore viability remains rather low (Codón et al., 1995; Mortimer et al., 1994). Usually asci contain four haploid ascospores, nonetheless many strains produce asci with three or less spores. Budding yeast have two mating types “a” and “ α ”. In heterothallic strains those mating types are under control of a pair of $MATa/MAT\alpha$ heterozygous alleles. So when sporulation occurs each asci contains two $MATa$ and two $MAT\alpha$ haploid ascospores. When spores germinate, due to favorable conditions, vegetative growth starts and mating of the $MATa$ and $MAT\alpha$ can occur. Nonetheless, if the haploid spores are mechanically separated by micromanipulation, the haplophase of heterothallic strains can be stably maintained (Figure 2, left panel). In homothallic strains the presence of the HO allele causes switching of the mating type in growing haploid cells, such that mother cell switch mating type once mitosis occurred. Therefore, mating occurs and there is only a transient haplophase in homothallic strains (Figure 2, right panel).

As illustrated in Figure 3, just one out of 1000 effective generations in *Saccharomyces* are meiotic divisions and 99% of this sexual cycles correspond to self fertilization (Ruderfer et al., 2006; Tsai

et al., 2008). Studies performed in population genomics have provided data that unequivocally show that sexual reproduction and outcrossing are rare but extremely important features in *Saccharomyces* life cycle (Hittinger, 2013; Liti et al., 2009; Schacherer et al., 2009). *Saccharomyces* have an unusually high recombination rate that allows them to make the most of the scarce outcrossing opportunities (Magwene et al., 2011). The history of the genus, dominated by clonal reproduction, suggest that natural populations are remnants of repeated population bottlenecks in essentially clonal lineages (Dujon, 2010) and implies a strong evolutionary influence of genetic drift and predicts that trait variations largely defined by the genetic history of each population (Warringer et al., 2011).

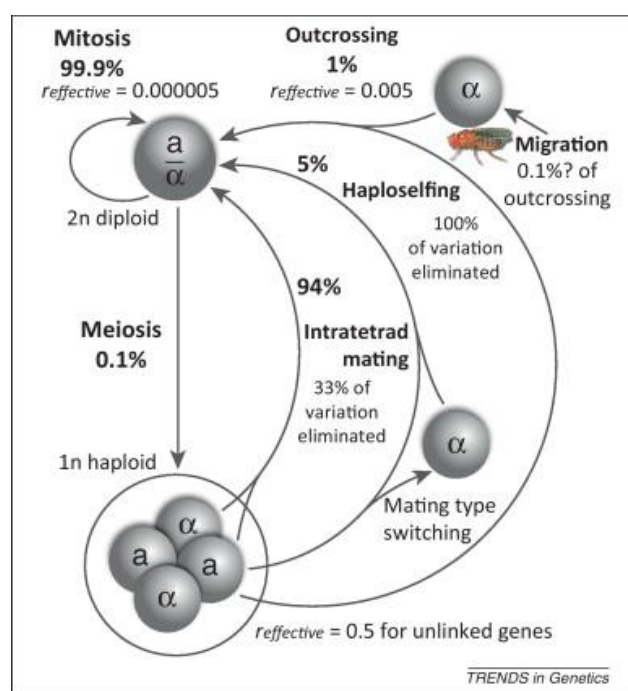


Figure 3 The *Saccharomyces* life cycle Adapted from Hittinger (2013).

***Saccharomyces* central carbon metabolism, taking advantage of inefficiency**

Metabolic pathways of the central carbon metabolism are highly conserved along the whole living beings and practically identical between yeast species. However the presence of different isoenzymes, nutrient uptake mechanisms and in particular the regulation of respiration and fermentation differ substantially (Flores et al., 2000), creating a wide universe of metabolism strategies and behaviors in yeast.

Energy and carbon metabolism are tightly linked in yeast as in other heterotrophic organisms, i.e. anabolism is coupled with catabolism. ATP is produced by oxidation of carbon sources that may also be used as biosynthetic precursors, and ultimately it is used in any cellular function that requires it.

Yeasts are able to utilize a broad range of carbon sources as sugars, amino acids, alcohols, organic acids, etc. Nonetheless all those different compounds may support yeast growth, main and preferred carbon sources for yeast growth are sugars (mainly hexoses and disaccharides). The metabolism of hexoses and disaccharides differ only in the initial basic steps of the metabolism

and share the same metabolic central blocks derived from glycolysis, the tricarboxylic acid cycle (TCA) and the pentose phosphate pathway.

More efficient way to metabolize carbon sources for yeast is aerobic or respiratory metabolism. In the case of *S. cerevisiae*, glucose respiratory metabolization generates 38 ATP molecules (2 from glycolysis and 2 from GTP formed in the TCA cycle and 34 ATP molecules from oxidative phosphorylation). During aerobic metabolism, both mitochondrial and cytosolic NADH is reoxidized by the respiratory chain. This energetic yield is 19-fold higher than the glucose dissimilation via alcoholic fermentation. Moreover, this energetic efficiency is also reflected in biomass yield of carbon limited continuous cultures where the respiring cell cultures show a 5-fold higher biomass yield than fermentative cultures (Verduyn et al., 1991). Alcoholic fermentation is known as the metabolization of glucose into ethanol. This catabolic process provides the cell with 2 ATP molecules per glucose converted into ethanol. However, alcoholic fermentation is a redox-neutral process and so alternative catabolism of glucose, mainly into glycerol, must be utilized to maintain the redox balance during growth.

Alcoholic fermentation is a clear inefficient way to obtain energy from carbon sources. Nevertheless anyhow *Saccharomyces* and especially *S. cerevisiae* has found the way to take advantage of this inefficient carbon catabolism in certain environments, like wine fermentation, as will be discussed hereafter.

Where are *Saccharomyces* found and what are they doing in nature?

Saccharomyces were first isolated and studied from fermentative environments and related habitats such as vineyards (Vaughan-Martini and Martini, 1995). Main species of this genus isolated from those environments were *S. cerevisiae*, *S. bayanus* and hybrids harboring different contribution from those species as well as from *S. kudriavzevii* and *S. eubayanus*. One of the most famous *Saccharomyces* is the allopolyploid hybrid (*S. cerevisiae* x *S. eubayanus*) *S. pastorianus* (syn. *Saccharomyces carlsbergensis*) responsible for the multimillionaire global market of lager-brewing industry (Dunn and Sherlock, 2008; Libkind et al., 2011). Other species of *Saccharomyces* genus have been found to be more related to processes such as Belgian-style brewing and cold-fermenting wine strains, this was the case of *S. kudriavzevii* hybrids. *S. uvarum* was mainly found to contribute in several champagne fermentations and the case of triple-hybrid (*S. cerevisiae* x *S. uvarum* x *S. eubayanus*), named *S. bayanus* (Figure 1), was associated to several brewing processes (Kurtzman et al., 2011; Libkind et al., 2011).

The collection of *sensu stricto* yeasts from natural environments began in the late 1950s in Japan, when *S. paradoxus* was firstly isolated from the bark and surrounding soil of oak (*Quercus*) and pine (*Pinus*) species (Yoneyama, 1957). From these first studies, *Saccharomyces* species have

been isolated in a wide range of forest microhabitats: oak bark, exudates, acorns, leaves, dung or soil. Although understanding *Saccharomyces* ecology of natural environments remains a challenge, it seems clear that there are strong and perhaps specific associations with oaks and other plants that produce excess sugars, and wind, water and insects probably facilitate dispersal (Stefanini et al., 2012; Taylor and Berbee, 2006).

The rise of *Saccharomyces* as a model

The complete genome sequence of the baker's yeast *Saccharomyces cerevisiae* by Goffeau et al., (1996) was the origin of a whole bunch of systematic functional genomics initiatives. The abundance of novel genes coupled with emerging functional genomics strategies represented a gold mine for studying basic molecular mechanisms of eukaryotic cells. Nowadays more than 80% of yeast ~5,780 protein coding genes have been functionally characterized (Peña-Castillo and Hughes, 2007).

Saccharomyces cerevisiae is one of the most widely used eukaryotic model organisms. It has been used as a model to study aging (Murakami and Kaerberlein, 2009), regulation of gene expression (Biddick and Young, 2009), signal transduction (Hohmann et al., 2007), cell cycle (Nasheuer et al., 2002), metabolism (Brocard-Masson and Dumas, 2006; López-Mirabal and Winther, 2008), apoptosis (Owsianowski et al., 2008), neurodegenerative disorders (Miller-Fleming et al., 2008), and many other biological processes. Currently, up to 30% of genes implicated in human disease have orthologs in the yeast proteome (Foury, 1997).

All those cited applications and findings have been based on the technologies and scientific fields emerged from the genome sequencing occurred in mid 90's. Probably the most important contribution of yeast as a model system have been the "Gene-Protein-Function" association, in other words, unravel the connection of a gene with a protein and which function it provides to the cell. At this time just ~15% of the ~5,780 genes remain without annotation. As in many other processes, i.e. genome sequencing itself, annotate this remaining 15% may take longer than the first 85% and completion of our understanding of the function of each and every gene will be an asymptotic process (Botstein and Fink, 2011).

Saccharomyces cerevisiae, as a model, is in the boundaries of the knowledge resulting from knowing the genome sequence and the derived tools and technologies that allow its study and analysis. From genome expression to human diseases study, passing by regulatory networks, data bases, gene ontology, gene and protein interaction networks, integration of co-expression and protein-gene interaction networks. *Saccharomyces cerevisiae* has become the organism in which limits of modern biotechnology first overtake.

Due to all this enormous quantity of information and also thanks to the new tools and techniques developed in *Saccharomyces*, it has become the most important model organism for molecular genetics, genomics, proteomics, interactomics, metabolomics, etc. Thanks to recent work characterizing and describing its diversity and genetics, the *Saccharomyces* genus is ready to become also one of the most powerful models for evolution (Hittinger, 2013). As evolutionary model, baker's yeast is important in two senses. One is its use in studies of evolutionary adaptation to selective pressure, mainly due to yeast amenability to be manipulated, its short doubling time and the simplicity and low cost in the analysis of its genome. The second is to infer in the course of evolution of the species and perform experiments to validate these inferences. This thesis has worked at this level obtaining new phenotypic data and evidences about how temperature preferences differ along *Saccharomyces* genus and how temperature influenced the evolution of the genus, as explained in chapter I of this document.

Yeast growth and predictive microbiology

As mentioned before in this document, yeast growth occurs mainly by mitosis. This reproductive strategy generate division of one piece of yeast in two daughter cells and causes the duplication of initial cell number and, as a consequence, produce the exponential growth of the population. When yeasts are grown in batch culture its growth may be divided in four different phases: Lag phase (1), exponential growth (2), stationary phase (3) and dead phase (4). This typical progression of phases draws a characteristic curve when population (Number of cells) is plotted versus Time (Figure 4).

1. During lag phase yeast adapt to the media and prepare cellular machinery to grow. In this stage cells are not able to divide yet.
2. Exponential phase (log phase) is characterized by cell duplication. This phase may be characterized mathematically by doubling time (time necessary to double population) or by growth rate (which is a measure of the number of divisions per cell per unit time).
3. During stationary phase population remain stable and happens due to a limiting nutrient or an inhibition factor. Stationary phase results from a situation in which growth rate and death rate are equal.
4. In dead phase growth media nutrients become exhausted or toxic compounds reach critical concentration and cells die.

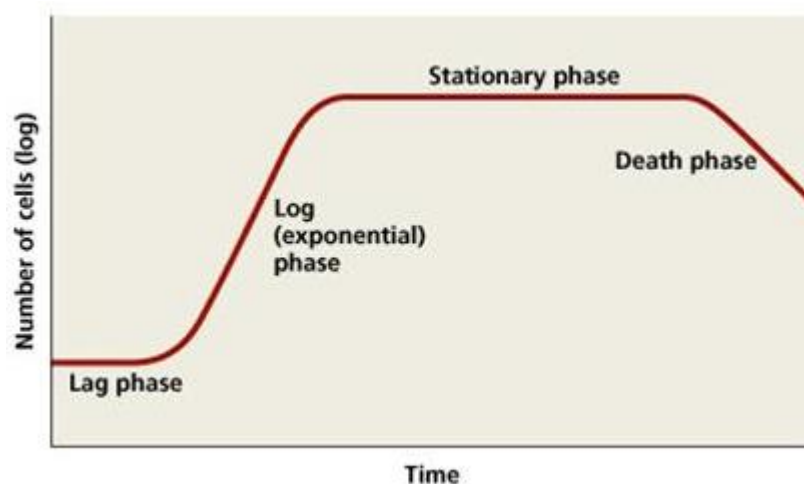


Figure 4 Yeast growth curve characterization.

Predictive microbiology is a field of food microbiology based on mathematical models that tries to describe microorganism behavior at different physical and chemical conditions. Along the chapters that compose this document different models have been used in order to characterize yeast growth as function of temperature and ethanol concentration in the media. Equations and models used will be explained and discussed below in the different chapters.

***Saccharomyces* in biotech industry: The winemaking**

Biotechnological applications of *Saccharomyces* occur since thousands of years ago, mainly related to food products such as beer, wine, bread, etc. Modern biotechnology has allowed scientific community and industry to use microbes, and in particular *Saccharomyces*, this ancient partner of humanity, in more complex and modern uses as enzymes and protein synthesis, biofuel production, bioremediation of waste-water, etc. Nonetheless, the most economically relevant applications within yeast biotechnology still remain being the same: beer, bread and wine production. The most well-known and commercially significant yeast are the related species and strains of *Saccharomyces cerevisiae*. These organisms have long been utilized to ferment the sugars of rice, grapes, wheat, barley, and corn to produce alcoholic beverages and in baking industry to expand, or raise, dough. *Saccharomyces cerevisiae* is commonly used as baker's yeast and for some types of fermentation.

Yeast: in winemaking

Winemaking is an ancient art allowing the fermentation of grape must into wine. With a history of more than 8000 years, wine production is one of the world's oldest biotechnological processes. Wine quality is the result of a complex network of interactions, established along different steps in the winemaking process, from the agronomic management of grapevine culture to the conditions in which wine is finally served to the consumer (Pretorius, 2000). Alcoholic

fermentation is one of the most important processes that take place during winemaking. Yeasts are the main microorganism responsible for this fermentation process, metabolizing must sugars (glucose and fructose) into ethanol. Moreover during the process they produce diverse secondary metabolites (glycerol, acetate, succinate, pyruvate, higher alcohols and esters) that greatly contribute to the sensorial properties of wine (Fleet, 1993). Both yeast growth and metabolism are influenced by a series of abiotic (osmotic pressure, pH, ethanol, temperature, etc.) and biotic factors (microorganism species, killer factors, grape variety, etc.) as well as by nutrient availability.

Several yeast genera are related to wine fermentation process such as *Candida*, *Pichia*, *Cryptococcus*, *Brettanomyces*, *Rhodotorula*, *Metschnikowia*, *Torulaspota* and *Kluyveromyces* (Fleet, 2003; Romano et al., 2006). However, *Saccharomyces* genus and specifically *S. cerevisiae* is the main responsible species for wine fermentation, that's why this species is known as "the wine yeast" (Bisson, 2005; Querol and Fleet, 2006).

S. cerevisiae has developed mechanisms that allow them to overcome the specific conditions of growth that they must face during alcoholic fermentation. Grape must composition presents culture conditions that are far from optimal for most microorganisms. In addition, it undergoes continuous transformations during alcoholic fermentation, due to changes in environmental factors as well as biological activity of the fermenting microorganisms. Therefore, wine yeasts have developed mechanisms allowing them to sense environmental changes in order to maintain the integrity of the cell and its metabolic activity throughout the whole winemaking process (Bauer and Pretorius, 2000). First stage of winemaking concerns lag phase, in which yeast cells must adapt to low pH (2.9-3.8), high osmolarity (sugar concentration up to 300 g/l) and high SO₂ content (40-100 mg/l) of the new environment before cell proliferation occurs. After lag phase, yeast biomass starts growing exponentially. The biological activity of yeast causes various stress conditions throughout the fermentation process, which include rapid nutrient limitation and starvation (nitrogen, which is available in quantities of just 50-600 mg/l at the start of the fermentation, is soon depleted), temperature variations and ethanol toxicity. One-third of the ethanol and the main fraction of glycerol present in wine are obtained in this phase. In the following phase (stationary in terms of biomass yield) the remaining two-thirds of the ethanol (up to 11-16%) and the wine aromatic compounds which determine the quality of the final product are produced (Salmon and Barre, 1998). All of these stages impose important stress conditions upon the yeast cell. Wine fermentation ends when the concentration of residual sugars is below 2 g/l.

Yeast strain factor is recognized as having a major influence in the quality of wines (Querol and Ramon, 1996). In the practice this observation led to the use of selected yeast strains that are usually commercialized as active dry wine yeast (ADWY). Commercial wine yeast strains have

been selected to meet specific requirements of wine producers with regard to phenotypic traits. These criteria have experienced an evolution from the first wine yeast strains commercialized, that were simply expected to ensure complete fermentation with rapid kinetics; to somewhat sophisticated properties, more specifically influencing wine quality, including production or release of primary and secondary aroma compounds, ability to release enzymes or mannoproteins, other sensory properties, tolerance to difficult fermentation conditions due to chemical composition of musts (sugar content, SO₂, antifungal compounds) or to external factors like too high or too low temperatures, killer phenotype, chemical stability, or technological properties (Pretorius and Bauer, 2002). As a result, more than 200 different yeast strains, almost exclusively of the species *S. cerevisiae*, are currently produced and sold in the global market of wine industry.

Yeast ecology in wine fermentation

In natural or spontaneous fermentations a sequential succession of microorganisms occurs spontaneously as must conditions evolve with time (Beltran et al., 2002; Fleet and Heard, 1993; Garde-Cerdán and Ancín-Azpilicueta, 2006; Pretorius, 2000). Along the process metabolites of some species become inhibitors or substrates for others.

Hanseniaspora spp. are often the predominant yeast genus on the surface of grape berries, accounting for the 50 – 75 % of the total population, although other *non-Saccharomyces* species of the genera *Candida*, *Pichia*, *Cryptococcus*, *Brettanomyces*, *Rhodotorula*, *Metschnikowia*, *Torulaspota* and *Kluyveromyces* are also usually isolated from freshly extracted grape juices, reaching population levels between 3 and 6 log₁₀ CFU/ml (Fleet, 2003; Romano et al., 2006; Torija et al., 2001).

Contrary to popular belief, fermentative species of the *Saccharomyces* genus occur at extremely low populations on healthy, undamaged grapes and are rarely isolated from intact berries and vineyard soils (Pretorius, 2000; Xufre et al., 2006). However, as the fermentation progresses, they displace the *non-Saccharomyces* species and occupy their fermentative niche. Within *Saccharomyces* genus, fermentative species of *sensu stricto* group, in most of the cases, become predominant at final stages of spontaneous winemaking process. However, in industrial winemaking environments, *Saccharomyces cerevisiae* is the main species responsible of fermentation (Bisson, 2005; Querol and Fleet, 2006). This species vigorously ferments the grape sugars (glucose and fructose) into ethanol and carbon dioxide, even in presence of oxygen (the Crabtree effect). Environmental changes may modify microorganism succession and even the contribution of them into the wine.

The process in which an organism modify or alter the environment, often in favor of increase the own ecological success, it's known as niche construction. Grape must fermentation has been

considered a clear example of niche construction performed by *Saccharomyces* species and specifically by *S. cerevisiae*. During wine fermentation, a strong selective pressure favors the *Saccharomyces*' strategy of making life difficult for other microorganism and contributes to its own survival. *Saccharomyces* performs a rapid conversion of the available sugars into ethanol, a toxic compound, which can be later respired by itself (Piskur et al., 2006; Thomson et al., 2005; Woolfit and Wolfe, 2005). Niche construction via ethanol production has been a widely accepted strategy to explain *Saccharomyces* ecological success in fermentative environments. Nonetheless, Goddard (2008) recently proposed that temperature may also plays an important role in niche construction via the metabolic activity of *S. cerevisiae* during fermentation. Due to the vigorous fermentation of sugars carried out by this species, a non negligible thermal energy estimated at 104.43 kJ/mol is released during fermentation (Williams, 1982). This energy is responsible for the temperature rise (up to 6 °C) that occurs during vinification (Goddard, 2008). Incidentally, *S. cerevisiae* performs better at higher temperatures than other yeast species (Arroyo-López et al., 2009).

For example when the fermentation is performed at temperatures lower than 15-20°C, *Hanseniaspora* and *Candida* species may produce a significant contribution to final wine flavor (Erten, 2002), mainly due to their growth performance at low temperatures that become closer to those showed by *Saccharomyces* species (Chapter I of this Thesis: Salvado et al., 2011). Torija et al. (2001) also showed that non-*Saccharomyces* species are more prevalent at the beginning of white wine fermentations than in red wine ones, as a consequence of lower fermentation temperatures.

The predominant species and the diversity of species are also changed with ripeness degree of grape berries. The ripeness of the grape increases the predominance of *Candida stellata* (currently identified as *C. zemplinina*) in the must. Other apiculate yeasts such as *H. osmophila* overcame *H. uvarum* and *C. stellata* in some fermentation conditions. There was a direct relationship between high sugar content in the must (higher ripeness) and the presence of *H. osmophila* (Hierro et al., 2006).

Traditionally wine fermentation has been carried out spontaneously by microbiota present both grape must and winery. However, nowadays, the possibility of inoculating selected active dry wine yeast (ADWY) have been a valuable tool to standardize wine quality and enhance the control of fermentation process. Use of this methodology reduces lag phase, minimizes contribution of undesirable microorganisms, ensures a rapid and complete fermentation of must and allows to minimize the variations on the so called secondary or fermentative aromas among different seasons (Fleet and Heard, 1993).

Nonetheless, ADWY inoculation also provides some disadvantages as the diminution of the organoleptic complexity of final product. Inoculation of selected ADWY reduces the number of

indigenous *Saccharomyces* strains in favor of the starter. This reduction in diversity of natural strains have been observed during several seasons even when spontaneous fermentations take place in cellars which have previously been inoculated (Beltran et al., 2002; Constantí et al., 1997).

Currently there is an increasing demand of new yeast strains with specific characteristics and performances to overcome in a suitable and efficient way the requirements of the modern winemaking.

Modern winemaking: the challenge of low temperature

In modern winemaking, vinification process is mainly driven by single strain inoculum. Currently, selected strains of *S. cerevisiae* are used in winemaking industry in order to obtain predictable and reproducible products and minimize the risk of spoilage by undesirable microbiota. There are hundreds of commercially available wine yeast strains that may accomplish with much more than vigorous and reliable consumption of available sugars and its transformation into ethanol. Wine yeast is responsible of synthesis and metabolic release of whole bunch of aromatic compounds which greatly affect the sensory properties of wine (Swiegers and Pretorius, 2005). Therefore differences in yeast metabolism, either by genetic divergences present in natural diversity or variations within a strain, produced incidentally or by human directed evolution, may generate a strong variability in wine organoleptic properties. Nowadays, wineries demand to selected yeast not only a complete set of characteristics to ensure a reproducible high quality product but also yeast-induced variations that introduce specific and singular personality to their wine.

Modern biotechnology and its tools are crucial for the understanding of the genetic and molecular bases that may allow overcoming all those challenges emerged from industry needs and consumers demands. Wine biotechnology is now dealing with a complete set of research lines to obtain strains able to cover the most of the desirable characteristics that a wine yeast strain must have on XXI century wine industry (Table 1).

There are many strategies to obtain new yeast strains with these desirable properties, such as selection of natural isolates, hybridization, induced mutagenesis and generation of genetic modified organisms (GMO). Nonetheless most of those strategies are time consuming or even unable to export to industry due to legal restrictions, as in the case of GMO.

Intense research and development is currently being performed to overcome industry requirements such as enhance aromatic precursors release (Howell et al., 2005; Swiegers et al., 2005), lowering ethanol yield (Cadière et al., 2011), improvement of autolysis and bioadsorption properties (Chassagne et al., 2005; Gonzalez et al., 2003), improvement glycosidase and carbon

sulfur lyase activity, acquirement of metabolization (oxidation/reduction) of phenolic acids, enhancement macromolecular hydrolysis activity, etc (Bisson and Karpel, 2010).

<p>Fermentation properties</p> <ul style="list-style-type: none"> •Rapid initiation of fermentation •High fermentation efficiency •High ethanol tolerance •High osmotolerance •Low temperature optimum •Moderate biomass production
<p>Flavour characteristics</p> <ul style="list-style-type: none"> •Low sulphide/DMS/thiol formation •Low volatile acidity production •Low higher alcohol production •Liberation of glycosylated flavour precursors •High glycerol production •Hydrolytic activity •Enhanced autolysis •Modified esterase activity
<p>Technological properties</p> <ul style="list-style-type: none"> •High genetic stability •High sulphite tolerance •Low sulphite binding activity •Low foam formation •Flocculation properties •Compacts sediment •Resistance to desiccation •Zymocidal (killer) properties •Genetic marking •Proteolytic activity •Low nitrogen demand
<p>Metabolic properties with health implications</p> <ul style="list-style-type: none"> •Low sulphite formation •Low biogenic amine formation •Low ethyl carbamate (urea) potential

Table 1 Adapted from Pretorius (2000)

Enhance of aromatic compounds in wine have been one of the fields in which scientific community have put more efforts. Numerous studies addressed this field focused on detect and induce gene networks involved in aromatic compound synthesis and enhance enzyme production to improve the release of aromatic molecules present as precursors in must and wine (Howell et al., 2005; Lilly et al., 2006a, 2006b; Swiegers et al., 2009). In last decades, several studies have also explored the influence of non-*Saccharomyces* yeast on wine flavor and aroma and their possible use on modern winemaking industry. Wine-related genera such as *Hanseniaspora*, *Metschnikowia*,

Candida, and *Pichia* have been described to be able to produce higher levels of esters and other aroma components than *Saccharomyces* does (Fleet and Heard, 1993; Fleet, 1993; Fleet et al., 2002; Howell et al., 2006; Swiegers et al., 2005). Some studies have shown that *H. uvarum* contributes positively to aroma and wine complexity due to its enhanced production of glycerol, esters and acetoin (Romano and Suzzi, 1996; Romano et al., 1997). In order to explore non-*Saccharomyces* potential to enhance wine aroma, co-inoculation fermentations have been studied and characterized and its industrial use seems to be a promising strategy to obtain wines with improved aroma profiles and with special and singular characteristics.

Other interesting strategy already used in wineries is lowering the fermentation temperature in order to prevent primary aroma volatilization and also to obtain higher production of desirable active aroma compounds derived of the wine yeast metabolism as well as a reduction in undesirable secondary ones (Beltran et al., 2008; Llauradó et al., 2002; Torija et al., 2003)

The challenge of low temperature fermentations

From a technology standpoint, one of the greatest advances in the wine industry in recent years is as simple as controlling the temperature in winemaking. The temperature control avoids temperature increases (above 30°C) that result in stuck fermentations. The alcoholic fermentation process is highly exothermic, and toxicity by ethanol significantly increases at higher temperatures, affecting the viability of yeasts. But the temperature control was not only useful to ensure a more efficient fermentation process but also to obtain products with better sensory quality. The wines fermented at low temperature (10-15 °C) prevent the volatilization of primary or varietal aromas and increase the synthesis of secondary aromas produced by yeasts (Beltran et al., 2008). In general, it is considered that low temperatures increase the fruity aromas typical of some esters (isoamyl acetate) and decrease some lower quality compounds (acetic acid, acetaldehyde and ethyl acetate) in white wines and rosé wines (it is needed higher temperatures in the case of the red wine fermentation for the extraction of color) (Beltran et al., 2008; Llauradó et al., 2002; Torija et al., 2003). However, the differences in the chemical composition of wine find its true significance when it is reflected at the sensory level. Triangular test identification, with professional and non-professionals tasters, showed a clear preference for wines from fermentations at low temperatures (13 °C). The wines fermented at low temperature were always better qualified by both professional tasters and non-professionals ones (Novo et al., 2003).

Despite these qualitative benefits that provide the low temperature of fermentation to the finished product, one must bear in mind the drawbacks associated with this type of fermentation. In fact, all the negative effects are the result of the difficulty that the wine yeast *Saccharomyces cerevisiae* has to grow at non-optimal temperature. The optimum temperature for wine

fermentation of *Saccharomyces* is among the 25-28 ° C. Therefore, to the inherent difficulty of a wine fermentation (high concentration of sugars, low pH, presence of ethanol, nutrient deficiency, etc.), it should be added a sub-optimal temperature for development of the primary fermentation agent. The main consequence of low temperature on the kinetics of yeast growth is a very long latency phase of up to a week, once inoculated into the must. Keep in mind that, at the industry level, it is generally used dry yeast rehydrated in water at 37 ° C and inoculated in the must after half an hour. If must have been previously cooled to low temperature, this represents a thermal shock that decreases the viability of yeast and requires a longer acclimation (lag phase). Some studies propose protocols for rehydration in order to promote better adaptation of the inoculated yeasts to the must (Novo et al., 2003; Rodríguez-Porrata et al., 2008). However, it is difficult to avoid very long fermentations. Therefore, both the energy expenditure to keep musts refrigerated and the problem of long occupation of fermenters that prevents a rapid product turnover, limit the industrial use of cold fermentation. It would therefore be very interesting from an industrial point of view to know the global mechanisms of adaptation and acclimation to low temperature of the wine yeast *S. cerevisiae*. This knowledge would allow the selection or breeding of strains for use in cold fermentation minimizing mentioned disadvantages. Currently there are a large number of commercial yeasts in the global wine market, over 200 (Rossouw et al., 2009), however, very few have been selected based on a good fermentative capacity at low temperatures.

As extensively exposed in this document *S. cerevisiae* is the main species responsible for alcoholic fermentation, especially in industrial winemaking, but closely related species *Saccharomyces uvarum* and natural hybrids within *Saccharomyces* genus (*S. cerevisiae* × *S. bayanus* and *S. cerevisiae* × *S. kudriavzevii*) have been also found to conduct low temperature vinification processes (González et al., 2006; Sipiczki and Ciani, 2002; Sipiczki, 2008). Some studies have shown that those natural hybrids of *Saccharomyces* seem to perform better than *S. cerevisiae* itself at low temperature wine fermentations (Gangl et al., 2009; González et al., 2007). Despite of already mentioned negative aspects of low temperature during alcoholic fermentations, mainly in terms of industrial management and risk of sluggish and stuck fermentations, these drawbacks may be overcome by selection of cryotolerant wine yeast. At this point both *S. uvarum* and *Saccharomyces* natural hybrids are promising options not only due to their good performance at low temperature but also due to their capacity to contribute into wine quality with higher production of secondary aroma compounds and glycerol (Antonelli et al., 1999; Gangl et al., 2009; Giudici et al., 1995; González et al., 2007; Sipiczki and Ciani, 2002; Tosi et al., 2009)

Yeast adaptation and acclimation to low temperature

According to Tai et al., (2007) we must distinguish between adaptation and acclimation for a particular environmental stress. The difference basically lies in the exposure time to which the

cells are subjected against this stress. A sudden environmental change, what we mean by shock, triggers a rapid and dynamic response in the cell that is intended to preserve the viability of the cell. This entails an adaptation to this change and that is what we mean by "stress response". By contrast, prolonged exposure to a nonlethal stimulus leads to an acclimation, which allows to the cells grow under these sub-optimal conditions. Even if the stimulus is maintained for multiple generations, mutational changes in the cell will be fixed that will result in an evolutionary adaptation of the genome (Dunham et al., 2002). According to Tai et al. (2007), the transcriptional reprogramming that occurs after a short exposure to cold or acclimation to this sub-optimal temperature is completely different. Most published works have focused on the study of adaptation to temperature by heat shock (Homma et al., 2003; Murata et al., 2005; Sahara et al., 2002; Schade et al., 2004). In these works it was analyzed the global transcriptional response after lowering the temperature of the growth medium. In wine fermentation, it is interesting to unravel not only the ability of yeast cells to adapt to fermentation medium but also the acclimation process that let the cells grow and ferment the sugars of the must. To this end, global transcription of commercial wine yeast at different stages of an industrial fermentation at low temperature were conducted along the whole fermentation process by Beltran et al. (2006). A comparison of fermentations at 13°C and at 25°C showed: a response to stress in the first days of fermentation (similar to that described by Schade as Late Cold Response mediated by transcriptional factors Msn2/Msn4) and an over-expression of genes involved in lipid metabolism, nitrogen metabolism genes and proteins and permeases involved in nutrient transport into the cell. This study also finds a remarkable activation of genes involved in ribosome synthesis and transcription (RNA metabolism). The activation of these genes reflects a compensatory mechanism due to the decrease in the rate of translation and the increased amount of inactive ribosomes previously described by Al-Fageeh and Smales (2006). Recently a metabolomic approach has observed that main differences between the metabolic profiling of *S. cerevisiae* growing at 12°C versus 28°C were in lipid metabolism and redox homeostasis (López-Malo et al., 2013). With the same aim of improving understanding mechanisms of adaptation and acclimatization of yeast to low temperature, evolution of the proteome of a commercial wine yeast strain in the first 24 hours after inoculation is showed in this thesis in the 3rd chapter (Salvadó et al., 2008). The most significant protein changes also included enzymes related to nitrogen and carbon metabolism, and proteins related to stress response. The construction of mutants and over-expressing strains in some of those genes and its influence in fermentative behavior and performance during low temperature fermentations will be presented in 4th chapter of this doctoral thesis (Salvadó et al., 2012).

Outline of the thesis

This thesis aims to study the influence of temperature in wine yeast. Different aspects and disciplines are involved to unravel the effects of low temperature in *Saccharomyces* genus and specifically in *Saccharomyces cerevisiae*. The main objective of this thesis is to generate new and original data to pave the way to understand temperature role in wine fermentation. The results enclosed in the first and the second chapters of this thesis were developed in order to understand how temperature influences both *Saccharomyces* genus evolution and the ecology of wine yeasts during wine fermentation. To achieve this objective, we study the temperature influence in wine yeasts by combining the description of their biological temperature range by mathematical modeling with their growth capacity as function of temperature. These results were published in two scientific international journals:

- Salvadó, Z., Arroyo-López, F.N., Guillamón, J.M., Salazar, G., Querol, A., Barrio, E., 2011. Temperature Adaptation Markedly Determines Evolution within the Genus *Saccharomyces*. *Applied and Environmental Microbiology*. 77, 2292–2302.
- Salvadó, Z., Arroyo-López, F.N., Barrio, E., Querol, A., Guillamón, J.M., 2011. Quantifying the individual effects of ethanol and temperature on the fitness advantage of *Saccharomyces cerevisiae*. *Food Microbiology*. 28(6), 1155-1161.

These articles have revealed that temperature has played an important role in *Saccharomyces* genus evolution and show quantitative data of competitiveness of different wine yeast species along their biological temperature range.

Other objective of this thesis has been to improve the knowledge on how *S. cerevisiae* deal with fermentation performance at low temperature by studying its mechanisms of adaptation and acclimation. Proteomic analysis of wine yeast in the first 24 hours after inoculation into must was done, in order to complement the already published omic-studies in this field and to identify protein changes that could be relevant in this process. Additionally, a functional genomics approach, issue from our proteomic study, was developed in order to identify single gene influence at low temperature fermentations. From those results two more articles, enclosed in chapter 3rd and 4th, were published in scientific international journals:

- Salvadó, Z., Chiva, R., Rodríguez-Vargas, S., Rández-Gil, F., Mas, A., Guillamón, J.M., 2008. Proteomic evolution of a wine yeast during the first hours of fermentation. *FEMS Yeast Research*. 8, 1137–1146.

- Salvadó, Z., Chiva, R., Rozès, N., Cordero-Otero, R., Guillamón, J.M., 2012. Functional analysis to identify genes in wine yeast adaptation to low-temperature fermentation. *Journal of Applied Microbiology*. 113, 76–88.

As this thesis is a collection of manuscripts published in different scientific journals, following chapters include the already mentioned publications.

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Chapter 1: Temperature adaptation markedly determines evolution within the *Saccharomyces* genus

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Temperature Adaptation Markedly Determines Evolution within the Genus *Saccharomyces*[†]

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The present study uses a mathematical-empirical approach to estimate the cardinal growth temperature parameters (T_{min} , the temperature below which growth is no longer observed; T_{opt} , the temperature at which the μ_{max} equals its optimal value; μ_{opt} , the optimal value of μ_{max} ; and T_{max} , the temperature above which no growth occurs) of 27 yeast strains belonging to different *Saccharomyces* and non-*Saccharomyces* species. *S. cerevisiae* was the yeast best adapted to grow at high temperatures within the *Saccharomyces* genus, with the highest optimum (32.3°C) and maximum (45.4°C) growth temperatures. On the other hand, *S. kudriavzevii* and *S. bayanus* var. *uvarum* showed the lowest optimum (23.6 and 26.2°C) and maximum (36.8 and 38.4°C) growth temperatures, respectively, confirming that both species are more psychrophilic than *S. cerevisiae*. The remaining *Saccharomyces* species (*S. paradoxus*, *S. mikatae*, *S. arboricolus*, and *S. cariocanus*) showed intermediate responses. With respect to the minimum temperature which supported growth, this parameter ranged from 1.3 (*S. cariocanus*) to 4.3°C (*S. kudriavzevii*). We also tested whether these physiological traits were correlated with the phylogeny, which was accomplished by means of a statistical orthogram method. The analysis suggested that the most important shift in the adaptation to grow at higher temperatures occurred in the *Saccharomyces* genus after the divergence of the *S. arboricolus*, *S. mikatae*, *S. cariocanus*, *S. paradoxus*, and *S. cerevisiae* lineages from the *S. kudriavzevii* and *S. bayanus* var. *uvarum* lineages. Finally, our mathematical models suggest that temperature may also play an important role in the imposition of *S. cerevisiae* versus non-*Saccharomyces* species during wine fermentation.

The estimation of the temperature range in which microorganisms are able to grow is very important for the food industry, to guarantee food safety or optimize fermentative conditions, for example, but also in ecological and taxonomic studies to classify and identify the different species of microorganisms. In this way, several works have shown the marked importance of temperature for the growth of industrial yeasts (1, 4, 22, 24), as well as the influence of this environmental factor in determining the natural distribution of wild species (12, 21, 23, 25). Specifically, there is an increasing interest in determining the influence of temperature in the adaptation of *Saccharomyces* species to both wild and fermentative environments (8, 21, 25).

The *Saccharomyces* genus includes several species associated only with natural habitats (*S. cariocanus*, *S. kudriavzevii*, *S. mikatae*, *S. paradoxus*, and *S. arboricolus*) and others that are present in both fermentative and wild habitats (*S. cerevisiae* and *S. bayanus*). The ability of the latter to ferment a broad range of beverages (cider, beer, and wines) and foods (bread, vegetables, etc.) (19) has unconsciously favored their selection by humans for thousands of years. It is thought that tempera-

ture could play an important role in the imposition and presence of *S. cerevisiae* in human activities (8). Several studies have shown that *S. cerevisiae* is well adapted to grow at higher temperatures, while other species, such as *S. bayanus* and *S. kudriavzevii*, are better adapted to grow at lower temperatures (2, 3, 11, 15, 22). However, there is a lack of quantitative information on this respect, and many of these studies do not include the whole biological temperature range in which *Saccharomyces* yeasts are able to grow. Thus, a more detailed study of the influence of temperature on *Saccharomyces* growth is necessary, including a larger number of strains isolated from different origins.

In this endeavor, predictive microbiology could be a very useful tool. This discipline uses mathematical models to quantitatively describe the behavior of microorganisms as a function of environmental variables (14). In the specific case of temperature, a primary model (usually a sigmoidal function) is first required to estimate the yeast growth parameters under diverse isothermal conditions. Then, a secondary model is necessary to appropriately describe the effects in the whole biotemperature range assayed (dynamic conditions). Fortunately, temperature has been a factor widely studied, and diverse secondary mathematical models are available in the references (14, 18, 29). Specifically, we have used in the present study the cardinal temperature model with inflection (CTMI) developed by Rosso et al. (20), because of the simplicity of its use and the easy and direct biological interpretation of results.

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Temperature adaptation markedly determines evolution within the *Saccharomyces* genus

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Running title: Effects of temperature on *Saccharomyces* growth

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Abstract

The present study uses a mathematical-empirical approach to estimate the cardinal growth temperature parameters (T_{\min} , T_{opt} , T_{\max} and μ_{opt}) of 27 yeast strains belonging to different *Saccharomyces* and non-*Saccharomyces* species. *S. cerevisiae* was the yeast best adapted to grow at high temperatures within the *Saccharomyces* genus, with the highest optimum (32.3°C) and maximum (45.4°C) growth temperatures. On the contrary, *S. kudriavzevii* and *S. uvarum* showed the lowest optimum (23.6 and 26.2°C) and maximum (36.8 and 38.4 °C) growth temperatures, respectively, confirming that both species are more psychrophilic than *S. cerevisiae*. The remaining *Saccharomyces* species (*S. paradoxus*, *S. mikatae*, *S. arboricolus* and *S. cariocanus*) showed intermediate responses. With respect to the minimum temperature which supported growth, this parameter ranged from 1.3 (*S. cariocanus*) to 4.3°C (*S. kudriavzevii*). We also tested if these physiological traits were correlated with the phylogeny, which was accomplished by means of a statistical orthogram method. The analysis suggested that the most important shift in the adaptation to grow at higher temperatures occurred in the *Saccharomyces* genus after the divergence of *S. arboricolus*, *S. mikatae*, *S. cariocanus*, *S. paradoxus* and *S. cerevisiae* from *S. kudriavzevii* and *S. uvarum* lineages. Finally, our mathematical models suggest that temperature may also play an important role in the imposition of *S. cerevisiae* versus non-*Saccharomyces* species during wine fermentation.

Keywords: Cardinal temperature model; Orthogram method; Statistical modeling techniques; *Saccharomyces*; non-*Saccharomyces*.

INTRODUCTION

The estimation of the temperature range where microorganisms are able to grow is very important for the food industry, for example, to guarantee food safety or optimize fermentative conditions, but also in ecological and taxonomic studies to classify and identify the different species of microorganisms. In this way, several works have shown the marked importance of temperature on the growth of industrial yeasts (Arroyo López et al., 2006; Charoenchai et al., 1998; Serra et al., 2005; Sorensen and Jakobsen, 1997), as well as the influence of this environmental factor in determining the natural distribution of wild species (Lachance et al., 2003; Sampaio and Goncalves, 2008; Sniegowski et al., 2002; Sweeney et al., 2004). Specifically, there is an increasing interest to determine the influence of temperature in the adaptation of *Saccharomyces* species to both wild and fermentative environments (Goddard, 2008; Sampaio and Goncalves, 2008; Sweeney et al., 2004).

The *Saccharomyces* genus includes several species associated only with natural habitats (*S. cariocanus*, *S. kudriavzevii*, *S. mikatae*, *S. paradoxus* and *S. arboricolus*) and others present in both fermentative and wild habitats (*S. cerevisiae* and *S. bayanus*). The ability of the latter to ferment

broad types of beverages (cider, beer, wines) and foods (bread, vegetables, etc.) (Querol and Fleet, 2006), has unconsciously favored their selection by humans for thousands of years. It is thought that temperature could play an important role on the imposition and presence of *S. cerevisiae* in human activities (Goddard, 2008). Several studies have shown that *S. cerevisiae* is well adapted to grow at higher temperatures, while other species as *S. bayanus* and *S. kudriavzevii* are better adapted to grow at lower temperatures (Arroyo-López et al., 2009; Belloch et al., 2008; Kishimoto and Goto, 1995; Naumov, 1996; Serra et al., 2005). However, there is a lack of quantitative information on this respect, and many of these studies do not include the whole biological temperature range where *Saccharomyces* yeasts are able to grow. Thus, a more detailed study on the influence of temperature on *Saccharomyces* growth is necessary, including a larger number of strains isolated from different origins.

In this way, predictive microbiology could be a very useful tool. This discipline uses mathematical models to quantitatively describe the behaviour of microorganisms as a function of environmental variables (McMeekin, 1993). In the specific case of temperature, firstly a primary model (usually a sigmoid function) is required to estimate the yeast growth parameters under diverse isothermal conditions. Then, a secondary model is necessary to appropriately describe the effects in the whole bio-temperature range assayed (dynamic conditions). Fortunately, temperature has been a factor widely studied, and diverse secondary mathematical models are available in the bibliography (McMeekin, 1993; Oscar, 2002; Zwietering et al., 1991). Specifically, we have used in the present study the cardinal temperature model with inflection (CTMI) developed by (Rosso et al., 1993) because the simplicity of its use and the easy and direct biological interpretation of results.

The study of the evolution of a determined phenotype (in our case yeast response *versus* temperature changes) within a taxon (*Saccharomyces* genus), is essential to understand the evolutionary history of the group. A meaningful approach for a comprehensive understanding is to first generate a well supported molecular phylogenetic tree of the group and then interpret the evolution of morphological traits in the light of this phylogeny. Evolution of the trait may be plastic and stochastic or, on the contrary, may evolve according to a trend tightly linked to the phylogeny. Recent methods have been developed to detect phylogenetic dependence in comparative data (Covain et al., 2008; Ollier et al., 2006). These tests have the advantage of the only use of the topological structure of the tree. Specifically, the orthogram method developed by (Ollier et al., 2006) represents a relevant approach because it detects and characterizes phylogenetic dependence of quantitative data, and, at the same time, highlights different patterns of evolution along a phylogenetic tree.

The main goal of the present study is to determine the whole biological temperature range where the different *Saccharomyces* species are able to grow. For this purpose, primary (re-parameterized Gompertz equation) and secondary (CTMI) models are used to fit experimental data collected at different isothermal conditions. We also evaluate the phylogenetic dependence of the CTMI parameters by means of a canonical procedure, which allows variance decomposition along the phylogenetic tree (orthogram method). Finally, CTMI models are also used to describe the effect of temperature on a hypothetical sympatric association between *S. cerevisiae* and the rest of *Saccharomyces* species.

MATERIALS AND METHODS

Yeast strains and inocula preparation. A total of 27 yeast strains, belonging to different *Saccharomyces* and non-*Saccharomyces* species, were used in the present study. Yeasts were selected to obtain representative isolates from natural (10 strains) and fermentative (17 strains) habitats, where possible. Their origins and designations are listed in Table 1.

Inocula were prepared by introducing one single colony from pure cultures of each strain into 5 ml of YM broth medium (Difco™, Becton and Dickinson Company, USA). After 48 h of incubation at room temperature (25±2°C), 1 ml of each tube was centrifuged at 9000 x g for 10 min, the pellets washed with sterile saline solution (9 g/L), centrifuged and re-suspended again in 0.5 ml of sterile saline solution to obtain a concentration of about 7.3 log₁₀ cfu ml⁻¹, which was confirmed by surface spread on YM agar plates. These yeast suspensions were used to inoculate the different experiments as described below.

Table 1. Origin and designation of the 27 yeast strains used in this study.

Species	Strains	Origin/Country	Designation†
<i>S. cerevisiae</i>	CECT 10131	<i>Centaurea alba</i> flower (Spain)	<i>Sc 10131</i>
	T73 ^L	Wine fermentation (Spain)	<i>Sc T73</i>
	PE35M	Masato fermentation (Peru)	<i>Sc PE35M</i>
	CPE7	Sugarcane fermentation (Brazil)	<i>Sc PE7</i>
	KYOKAI = CBS 6412	Sake fermentation (Japan)	<i>Sc KYOKAI</i>
	TEMOHAYA-MI26	Agave fermentation (Mexico)	<i>Sc TEMO</i>
	Qa23 ^L	Wine fermentation (Portugal)	<i>Sc Qa23</i>
	TTA ^M	Wine fermentation (France)	<i>Sc TTA</i>
	PDM ^M	Wine fermentation (France)	<i>Sc PDM</i>
	RVA ^M	Wine fermentation (Spain)	<i>Sc RVA</i>
<i>S. paradoxus</i>	CECT 1939 ^{NT} = CBS 432 ^{NT}	Tree exudate (Russia)	<i>Sp 1939</i>

	120M		Pulque fermentation (Mexico)	<i>Sp</i> 120M
	K54		Wine fermentation (Croatia)	<i>Sp</i> K54
<i>S. uvarum</i> ^{††}	NCAIM 789		<i>Carpinus betulus</i> exudate (Hungary)	<i>Su</i> NCAIM
	BM58 ^L		Wine fermentation (Spain)	<i>Su</i> BM58
<i>S. kudriavzevii</i>	CA111		<i>Quercus ilex</i> bark (Spain)	<i>Sk</i> CA111
	CR85		<i>Quercus ilex</i> bark (Spain)	<i>Sk</i> CR85
	CR89		<i>Quercus faginea</i> bark (Spain)	<i>Sk</i> CR89
	CR90		<i>Quercus faginea</i> bark (Spain)	<i>Sk</i> CR90
<i>S. mikatae</i>	NBRC 1815 ^T = CBS 8839 ^T		Soil (Japan)	<i>Smik</i>
<i>S. arboricolus</i>	CBS 10644 ^T		<i>Quercus fabri</i> bark (China)	<i>Sarb</i>
<i>S. cariocanus</i>	CBS 8841 ^T		Fruit fly (<i>Drosophila</i> sp) (Brazil)	<i>Scar</i>
Non- <i>Saccharomyces</i>	<i>Hanseniaspora uvarum</i>		Wine fermentation (Spain)	<i>Hu</i>
	CECT 10389 <i>Candida zemplinina</i>		Wine fermentation (Spain)	<i>Cs</i>
	CECT 11108			
	<i>Torulaspota delbrueckii</i>		Wine fermentation (Spain)	<i>Td</i>
	CECT 11199			
	<i>Kluyveromyces marxianus</i>		Wine fermentation (Spain)	<i>Km</i>
	CECT 10585			
	<i>Pichia fermentans</i> CECT 10064		Wine fermentation (Spain)	<i>Pf</i>

[†] Strain designations used in the present study, ^{††} The term *S. uvarum* was adopted to designate the strains of *S. bayanus* var. *uvarum* used in this work, ^T Type strain, ^{NT} Neotype strain, ^L Commercial strains from Lallemand Inc., ^M Commercial strains from Mauri yeast Australia.

Yeast growth conditions. The basal culture medium selected for all experiments was Yeast Nitrogen Base broth (YNB, DifcoTM), supplemented with 20 g/L of glucose as carbon source. Medium was sterilized by filtration (0.2 µm) and stored at 4°C until its use. The final pH of the medium was 5.4 ± 0.1.

Growth was monitored at 600 nm in a SPECTROstar Omega instrument (BMG Labtech, Offenburg, Germany) at different temperatures (4, 8, 10, 14, 22, 29, 33, 37, 40, 42, and 46°C). Measurements were taken every hour for 3 days after a pre-shaking of 20 s for temperatures included between 22 and 46°C. However, for lower temperatures (from 4 to 14°C), microtiter plates (96 wells) had to be incubated outside the SPECTROstar spectrophotometer and then transferred into it to take the measurements every 8 h for 14 days. The wells of the microplate were filled with 0.01 ml of inoculum and 0.25 ml of YNB medium, reaching always an initial OD of approximately 0.2 (inoculum level of ~ 6.0 log₁₀ cfu ml⁻¹). The inocula were always above the

detection limit of the apparatus, which was determined by comparison with a previously established calibration curve. OD measurements could be used to estimate growth parameters because there was always a linear relationship between OD and yeast plate counts within the range studied (data not shown). Un-inoculated wells for each experimental series were also included in the microtiter plates to determine, and consequently subtract, the noise signal. All experiments were carried out in triplicate. Therefore, a total of 891 growth curves (11 levels of temperature x 27 strains x 3 replicates) were obtained and analysed.

Primary modeling. Growth parameters were obtained from each treatment by directly fitting OD measurements *versus* time to the re-parameterized Gompertz equation proposed by (Zwietering et al., 1990), which was originally introduced in predictive microbiology by (Gibson et al., 1987). It has the following expression:

$$y = D \times \exp \left\{ -\exp \left[\frac{\mu_{\max} \times e}{D} (\lambda - t) + 1 \right] \right\} \quad (1)$$

where $y = \ln(\text{OD}_t / \text{OD}_0)$, OD_0 is the initial OD and OD_t is the OD at time t ; $D = \ln(\text{OD}_{\max} / \text{OD}_0)$, being OD_{\max} the maximum optical density reached, μ_{\max} is the maximum specific growth rate (h^{-1}), and λ the lag phase period (h). Growth data from each temperature and strain were fitted by a non-linear regression procedure, minimizing the sum of squares of the difference between experimental data and the fitted model, i.e., loss function (observed-predicted)². This task was accomplished using the non-linear module of the Statistica 7.0 software package (StatSoft Inc, Tulsa, OK, USA) and its Quasi-Newton option.

Secondary modeling. The cardinal temperature model with inflection (CTMI) (Oscar, 2002; Rosso et al., 1993) was used to describe the μ_{\max} changes of yeasts as a function of temperature (T , °C). CTMI is a descriptive model purely based on empirical observations, and includes the three cardinal temperature values often used in microbiology. It has the following expression:

$$\begin{aligned} \mu &= 0 && \text{if } T \leq T_{\min} \text{ or } T \geq T_{\max} \\ \mu &= \mu_{\text{opt}} (D/E) && \text{if } T_{\min} < T < T_{\max} \\ D &= (T - T_{\max}) (T - T_{\min})^2 \\ E &= (T_{\text{opt}} - T_{\min}) [(T_{\text{opt}} - T_{\min})(T - T_{\text{opt}}) - (T_{\text{opt}} - T_{\max})(T_{\text{opt}} + T_{\min} - 2T)] \end{aligned} \quad (2)$$

where T_{\max} is the temperature above which no growth occurs, T_{\min} is the temperature below which growth is no longer observed, and T_{opt} is the temperature at which the μ_{\max} equals its optimal value (μ_{opt}). As in the previous case, CTMI parameters were estimated by a non-linear regression procedure using Statistica 7.0 software package. Fit adequacy was checked by the proportion of variance explained by the model (R^2) respect to experimental data.

ANOVA analyses. Firstly, strains were checked for significant differences among them by means of an analysis of variance using the one-way ANOVA module of Statistica 7.0 software. Dependent variables introduced for the analysis were the CTMI parameters obtained from secondary modeling. The post-hoc comparison was carried out using the Scheffé test, which is considered to be one of the most conservative post-hoc tests (Winer, 1961). An alternative advantage of the Scheffé test is that it can also be used with unequal sample sizes.

A second ANOVA analysis was then performed by grouping the different *Saccharomyces* strains as a function of their respective species. In this way, CTMI average parameters for species *S. cerevisiae*, *S. paradoxus*, *S. uvarum* and *S. kudriavzevii* were estimated from data of 10, 3, 2 and 4 strains isolated from diverse origins, respectively. This way, the biological temperature range obtained for each species represents a general behavior rather than a single-strain trait.

Identification of phylogenetic dependence. We use the orthogram method developed by (Ollier et al., 2006) to determine the existence of dependence among the CTMI model parameters (T_{opt} , T_{max} and μ_{opt}) and *Saccharomyces* phylogeny. This method, based on the variance decomposition along the phylogenetic tree, considers as null hypothesis the complete absence of phylogenetic dependence, and uses diverse statistics ($R2Max$, $SkR2k$, D_{max} and SCE) to corroborate this hypothesis (Ollier et al., 2006). The method consists essentially of three steps. Firstly, phylogenetic relations between the units of the tree are described by means of dummy variables, taking values equal to 1 (for descendent tips within a node) or 0 (for the rest of tips). In a second step, these dummy variables are order in decreasing phylogenetic dissimilarity. Finally, since these dummy variables are not linearly independent, an orthonormal decomposition is necessary to obtain a matrix of linearly independent vectors (orthonormal vectors) which are used as regressors against CTMI model parameters. The method provides two graphical tools, called orthogram and cumulative orthogram, obtained by plotting the squared coefficients and the cumulative squared coefficients against the orthonormal vectors, respectively, very useful to interpret and identify the possible phylogenetic dependence. A complete description of the procedure and interpretation of statistics can be found in (Ollier et al., 2006) and (Covain et al., 2008). Orthograms and associated tests were conducted using *ade4* package (Chessel et al. 2004) in R 2.4.0 software (Ihaka and Gentleman, 1996).

The topology of the phylogenetic tree of *Saccharomyces* genus (Figure 1) was obtained from (Wang and Bai, 2008), who used sequences of the ITS-5.8S rDNA region and 26S rDNA D1/D2 domains to determine the phylogenetic relationships among *Saccharomyces* species. The phylogenetic position of *S. paradoxus* populations from America (in our case strain *Sp 120M*) and Europe (strains *Sp 1939* and *Sp K54*) were obtained from (Liti et al., 2006). *Torulasporea delbrueckii* was included as outgroup.

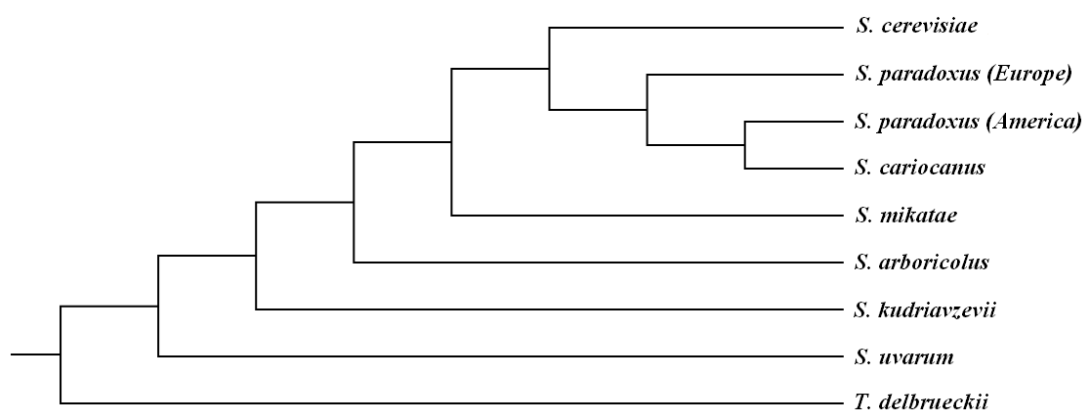


Figure 1. Phylogenetic tree of the *Saccharomyces* genus. Topology was obtained from (Liti et al., 2006) and (Wang and Bai, 2008). *Torulopsis delbrueckii* was used as outgroup.

RESULTS

Estimation of the temperature range where yeasts are able to grow. In this work, we have studied and compared the response of 27 yeast strains in a wide range of temperature values (from 4 to 46°C). For this purpose, yeasts were monitored by means of OD measurements and their respective biological growth parameters (μ_{\max} and λ) estimated at each value. A total of 891 growth curves were fitted using a non-linear regression procedure with the re-parameterized Gompertz equation proposed by (Zwietering et al., 1990), which represents an empirical approach for the estimation of the growth parameters. In all cases the fit was good, with a R^2 ranging from 0.95 to 0.99. Although the lag phase duration was also calculated for all experiments, it was not possible to model appropriately this parameter as a function of this environmental variable. Even at low temperatures, the lag phase of the different yeast species was very short, always below 15 h (data not shown). However, changes in μ_{\max} as a function of temperature could be well fitted by means of the CTMI secondary model.

As an example, Figure 2 shows the fit of the CTMI model to μ_{\max} experimental data obtained for the wild strains *Sc 10131* and *Sk CR85*. Clearly, a considerable difference was noticed between both microorganisms, especially evident with respect to the optimum and maximum growth temperatures. In this way, *Sc 10131* was able to grow up to 45°C and had its optimum growth around 33°C, with an estimated μ_{\max} at this temperature of 0.45 h⁻¹. On the contrary, *Sk CR85* was not able to grow at 37°C and showed an optimum growth around 24°C, with a μ_{\max} of 0.29 h⁻¹. Below 20°C, we can see that both fits are practically overlapped. The minimum temperature to support growth was around 4°C in both cases (Figure 2).

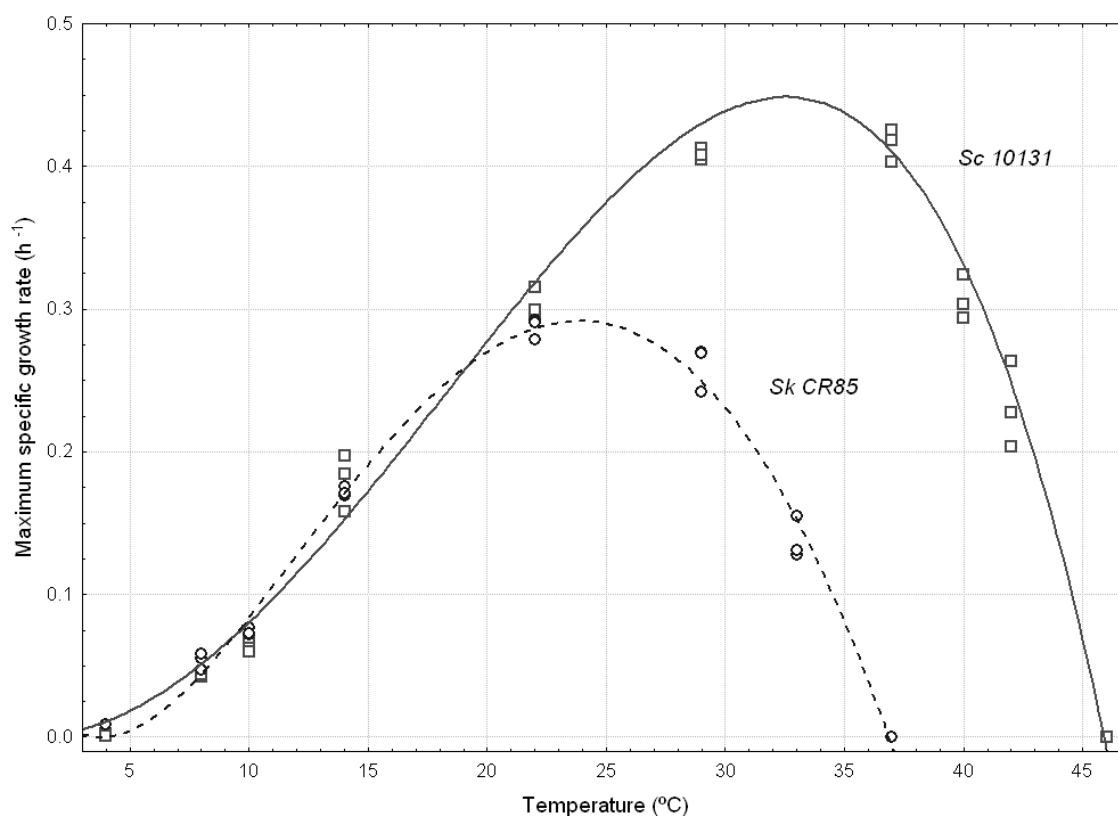


Figure 2. Fit of the cardinal temperature model to experimental data obtained for strains *S. cerevisiae* CECT10131 (squares) and *S. kudriavzevii* CR85 (circles). The quantitative parameters of both fits are shown in Table 2.

An advantage on the use of the CTMI model is that all its parameters have biological meanings, so the interpretation of this type of model is very easy and direct. Table 2 shows the temperature-dependent parameters obtained with the CTMI model for the 27 yeasts strains assayed in this work. For each strain, average values were obtained from 3 independent experiments. The Scheffé post hoc comparison test was used to distinguish significant differences among strains for each parameter. A total of 13, 11, and 5 different

Table 2. Estimated parameters (μ_{opt} , T_{max} , T_{min} and T_{opt}) of the cardinal temperature model for the 27 yeast strains assayed in this work. Standard deviations for each parameter (in parentheses) were obtained from 3 independent non-linear fits.

Strain designation	μ_{opt} (h ⁻¹)	T_{opt} (°C)	T_{max} (°C)	T_{min} (°C)
<i>Sc 10131</i>	0.449 (0.009) ^m	32.86 (0.61) ^{ij,k}	45.90 (0.10) ^{d,e}	0.74 (0.10) ^a
<i>Sc T73</i>	0.425 (0.043) ^{lm}	31.42 (0.59) ^{gh,ij,k}	45.51 (0.11) ^d	4.11 (1.65) ^a
<i>Sc PE35M</i>	0.375 (0.008) ^{ijklm}	29.99 (0.22) ^{efgh,i}	45.48 (0.12) ^d	5.04 (0.23) ^a
<i>Sc PE7</i>	0.298 (0.010) ^{c,d,e,f,g,h,i}	31.62 (0.39) ^{hij,k}	45.50 (0.14) ^d	4.38 (0.61) ^a
<i>Sc KYOKAI</i>	0.284 (0.014) ^{c,d,e,f,g}	34.75 (0.76) ^k	42.00 (0.01) ^c	4.02 (2.45) ^a
<i>Sc TEMO</i>	0.377 (0.006) ^{ijklm}	34.19 (0.35) ^{jk}	45.96 (0.01) ^{d,e}	0.76 (0.44) ^a
<i>Sc Qa23</i>	0.358 (0.020) ^{hij,k,l}	31.72 (0.95) ^{hij,k}	45.79 (0.09) ^{d,e}	3.55 (0.85) ^a
<i>Sc TTA</i>	0.384 (0.024) ^{kl,m}	32.71 (0.75) ^{ij,k}	46.06 (0.12) ^{d,e}	0.43 (0.33) ^a
<i>Sc PDM</i>	0.347 (0.003) ^{f,g,h,ij,k}	31.33 (0.43) ^{g,h,ij}	45.77 (0.09) ^{d,e}	3.72 (0.69) ^a
<i>Sc RVA</i>	0.382 (0.020) ^{ijklm}	32.09 (0.69) ^{hij,k}	45.99 (0.16) ^{d,e}	1.69 (1.23) ^a
<i>Sp 1939</i>	0.313 (0.008) ^{e,f,g,h,ij,k}	29.32 (0.50) ^{e,f,g,h}	42.20 (0.13) ^c	3.15 (0.14) ^a
<i>Sp 120M</i>	0.357 (0.009) ^{g,h,ij,k,l}	30.18 (0.82) ^{f,g,h,i}	40.36 (0.51) ^b	1.05 (0.14) ^a
<i>Sp K54</i>	0.288 (0.007) ^{c,d,e,f,g,h,i}	30.27 (0.65) ^{f,g,h,i}	42.10 (0.14) ^c	0.89 (1.21) ^a
<i>Su NCAIM</i>	0.307 (0.012) ^{d,e,f,g,h,i,j}	26.78 (0.23) ^{b,c,d,e}	37.02 (0.10) ^a	0.93 (0.39) ^a
<i>Su BM58</i>	0.283 (0.007) ^{c,d,e,f}	25.70 (0.51) ^{a,b,c,d}	39.71 (0.05) ^b	3.81 (0.44) ^a
<i>Sk CA111</i>	0.198 (0.002) ^b	23.14 (0.11) ^a	36.75 (0.03) ^a	4.94 (0.15) ^a
<i>Sk CR85</i>	0.291 (0.004) ^{c,d,e,f,g,h,i}	23.88 (0.09) ^{a,b,c}	36.87 (0.08) ^a	3.77 (0.40) ^a
<i>Sk CR89</i>	0.258 (0.015) ^{b,c,d,e}	23.68 (0.34) ^{a,b}	36.69 (0.06) ^a	4.07 (0.73) ^a
<i>Sk CR90</i>	0.284 (0.004) ^{c,d,e,f,g,h}	23.74 (0.28) ^{a,b}	36.91 (0.15) ^a	4.37 (1.12) ^a
<i>Smik</i>	0.318 (0.002) ^{e,f,g,h,ij,k}	29.20 (1.46) ^{e,f,g,h}	40.20 (0.10) ^b	1.79 (0.95) ^a
<i>Sarb</i>	0.328 (0.009) ^{e,f,g,h,ij,k}	28.14 (0.61) ^{d,e,f,g}	39.80 (0.04) ^b	2.26 (0.99) ^a
<i>Scar</i>	0.329 (0.006) ^{e,f,g,h,ij,k}	28.81 (0.73) ^{d,e,f,g,h}	41.79 (0.06) ^c	1.31 (1.10) ^a
<i>Hu</i>	0.198 (0.013) ^b	24.51 (0.30) ^{a,b,c}	36.87 (0.07) ^a	4.71 (2.81) ^a
<i>Cs</i>	0.096 (0.004) ^a	24.77 (0.54) ^{a,b,c}	37.00 (0.01) ^a	2.81 (1.02) ^a
<i>Td</i>	0.235 (0.006) ^{b,c,d}	27.08 (0.48) ^{c,d,e,f}	40.19 (0.07) ^b	4.42 (1.63) ^a
<i>Km</i>	0.362 (0.004) ^{ijkl}	38.68 (0.36) ^l	46.28 (0.03) ^e	2.43 (1.26) ^a
<i>Pf</i>	0.232 (0.008) ^{b,c}	25.51 (0.82) ^{a,b,c,d}	40.38 (0.28) ^b	1.25 (0.81) ^a

Note: Values followed by different superscript letters, within the same column, are significantly different according to a Scheffé post hoc comparison test.

Groups were obtained for parameters μ_{opt} , T_{opt} , and T_{max} , respectively. On the contrary, no significant differences were found among strains in the case of T_{min} . These parameters ranged from 0.096 (*Cj*) to 0.449 h⁻¹ (*Sc 10131*) for μ_{opt} , from 23.1 (*Sk CA111*) to 38.7°C (*Km*) for T_{opt} , from 36.7 (*Sk CR89*) to 46.3°C (*Km*) for T_{max} , and finally from 0.4 (*Sc TTA*) to 5.0°C (*Sc PE35M*) for T_{min} . Figure 3 provides a graphical representation of the biological temperature range where yeast strains were able to grow. In general, the *S. cerevisiae* strains exhibited the highest maximum and optimum growth temperatures within the *Saccharomyces* genus, with the exception of strain *Sc PE35M* which had a lower T_{opt} (~30°C). However, *S. kudriavzevii* strains clearly showed the lowest maximum and optimum growth temperatures, although very similar to those values obtained for the *S. uvarum* strains. With the exception of the species *K. marxianus*, which resulted to be the most thermotolerant microorganisms assayed in this work (see Table 2), the rest of non-*Saccharomyces* yeasts exhibited optimum growth temperatures ranging from 24 to 27°C, and maximum growth temperatures from 36 to 40°C.

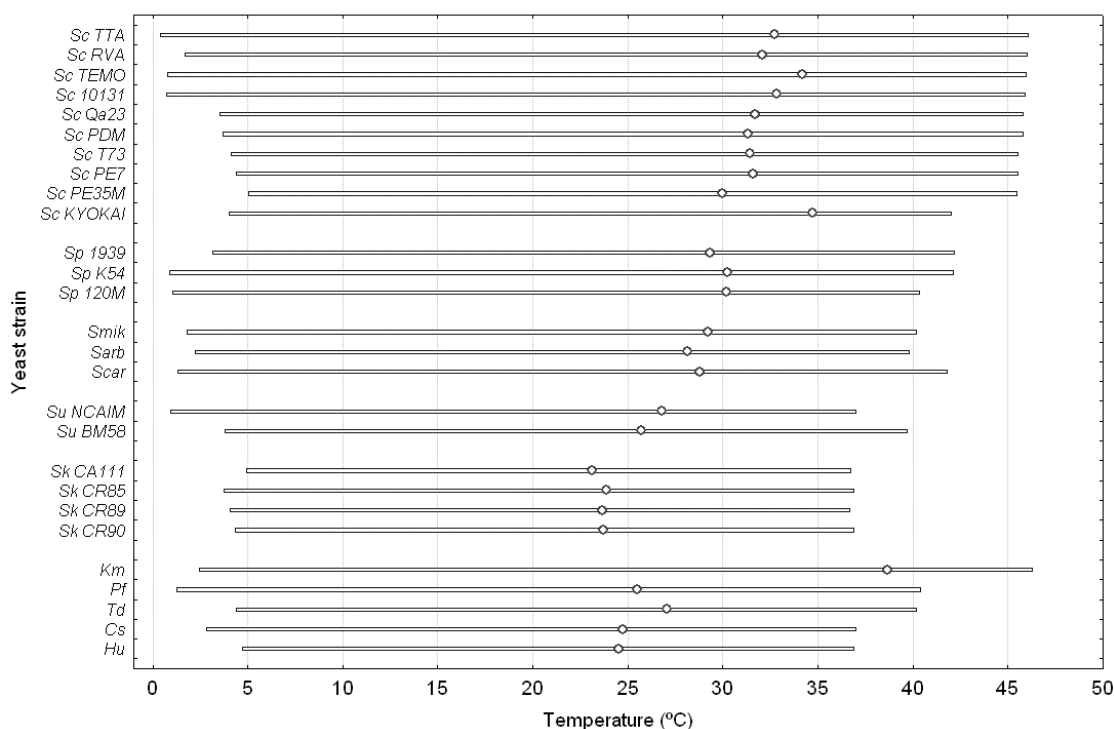


Figure 3. Temperature range where the 27 yeast strains assayed in this work were able to grow. Optimum growth temperatures are marked with a circle on the rectangles.

The second ANOVA analysis, grouping strains according to their respective *Saccharomyces* species, showed that *S. cerevisiae* was the species with the statistically highest T_{opt} and T_{max} values (average of 32.3 and 45.4°C, respectively) (Table 3). On the contrary, *S. kudriavzevii* exhibited the lowest T_{opt} and T_{max} values (significantly different with respect to the rest of *Saccharomyces* species in the case of T_{opt}), with 23.6 and 36.8°C, respectively. Again, no significant differences were found among yeasts for their T_{min} values.

Table 3. ANOVA analysis for the parameters of the cardinal temperature model (μ_{opt} , T_{max} , T_{min} and T_{opt}) grouping strains according to their respective *Saccharomyces* species.

Species	Num. of strains/cases	μ_{opt} (h ⁻¹)	T_{opt} (°C)	T_{max} (°C)	T_{min} (°C)
<i>S. cerevisiae</i>	10 / 30	0.368 (0.051) ^b	32.27 (1.45) ^d	45.39 (1.17) ^d	2.84 (1.91) ^a
<i>S. uvarum</i>	2 / 6	0.295 (0.016) ^a	26.24 (0.69) ^b	38.36 (1.47) ^{b,c}	2.37 (1.61) ^a
<i>S. kudriavzevii</i>	4 / 12	0.258 (0.039) ^a	23.61 (0.35) ^c	36.80 (0.12) ^c	4.29 (0.74) ^a
<i>S. paradoxus</i>	3 / 9	0.319 (0.031) ^{a,b}	29.92 (0.73) ^a	41.55 (0.93) ^a	1.69 (1.25) ^a
<i>S. mikatae</i>	1 / 3	0.318 (0.002) ^{a,b}	29.20 (1.46) ^a	40.20 (0.10) ^{a,b}	1.79 (0.95) ^a
<i>S. arboricolus</i>	1 / 3	0.328 (0.009) ^{a,b}	28.14 (0.61) ^{a,b}	39.80 (0.04) ^{a,b}	2.26 (0.99) ^a
<i>S. cariocanus</i>	1 / 3	0.329 (0.006) ^{a,b}	28.81 (0.73) ^{a,b}	41.79 (0.06) ^a	1.31 (1.10) ^a

Note: Values followed by different superscript letters, within the same column, are significantly different according to a Scheffé post hoc comparison test.

A clear and more intuitive interpretation of the overall response of the *Saccharomyces* species as a function of temperature can be obtained by the graphical representations of the models, which are shown in Figure 4. *S. cerevisiae* clearly appears as the most thermotolerant *Saccharomyces* species, exhibiting the highest μ_{max} , T_{opt} and T_{max} values. The species *S. paradoxus*, *S. mikatae*, *S. cariocanus* and *S. arboricolus* formed a group with very similar responses (no significant differences among them). On the contrary, *S. kudriavzevii* and *S. uvarum* showed the lowest μ_{opt} , T_{opt} and T_{max} values. However, both species had the highest μ_{max} when temperature was included in the interval 10-20°C. The minimum temperatures to support growth were very similar among all *Saccharomyces* species, although *S. kudriavzevii* showed a slight, but not significant, higher T_{min} value. We also studied a possible relationship among the CMTI model parameters obtained for the diverse *Saccharomyces* species.

This way, we found a linear correlation (R^2 always above 0.90) between T_{opt} and T_{max} , μ_{opt} and T_{max} , and μ_{opt} and T_{opt} . However, there was no correlation between T_{opt} and T_{min} or μ_{opt} and T_{min} . This means that species which supported the highest growth temperatures, had also the highest T_{opt} and μ_{opt} values, but on the contrary, species with the lowest T_{opt} and μ_{opt} values did not exhibited the minimum growth temperatures. This fact can also be graphically observed in Figure 4.

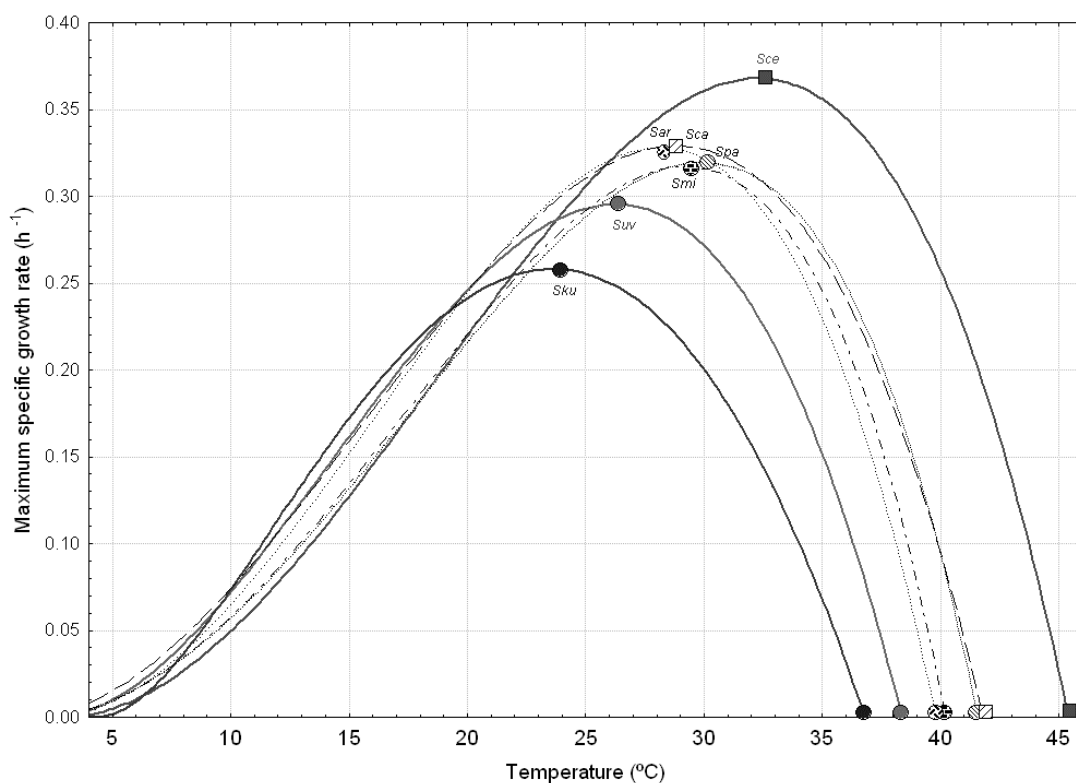


Figure 4. Changes of the maximum specific growth rates of the yeast species *S. cerevisiae*(Sc), *S. paradoxus*(Sp), *S. mikatae* (Sm), *S. arboricolus* (Sa), *S. cariocanus* (Sc), *S. uvarum* (Su) and *S. kudriavzevii* (Sk) as a function of temperature. Graph was built using the average *Saccharomyces* parameters of the cardinal temperature model shown in Table 3.

Figure 5 shows the theoretical evolution of the μ_{\max} ratios of the *Saccharomyces* species as a function of temperature. This parameter was obtained dividing the μ_{\max} of *S. cerevisiae* between the μ_{\max} of the rest of *Saccharomyces* species and vice versa. A ratio of 1 is indicative of both yeasts growing with similar μ_{\max} at a specific temperature value. On the contrary, a ratio of 2 means that one yeast grows two-fold faster than the other. Thus, this parameter provides valuable information on the effects of temperature on a hypothetical sympatric association between *S. cerevisiae* and the rest of *Saccharomyces* species. In all cases, *S. cerevisiae* resulted to be the most competitive yeast at high temperatures. Models show that *S. cerevisiae* grows faster than *S. uvarum* above 24°C, and at 35°C, its μ_{\max} almost doubles to *S. uvarum* (Figure 5a). In the rest of comparisons, *S. cerevisiae* grows faster than *S. kudriavzevii*, *S. paradoxus*, *S. mikatae*, *S. arboricolus* and *S. cariocanus* above 22, 19, 20, 26 and 25°C, respectively (Figures 5b, 5c, 5d, 5e, and 5f). Below these values, *S. cerevisiae* progressively grows slower than the other species and is less competitive. In fact, at 5°C, the μ_{\max} of *S. uvarum* doubles the μ_{\max} of *S. cerevisiae* (Figure 5a).

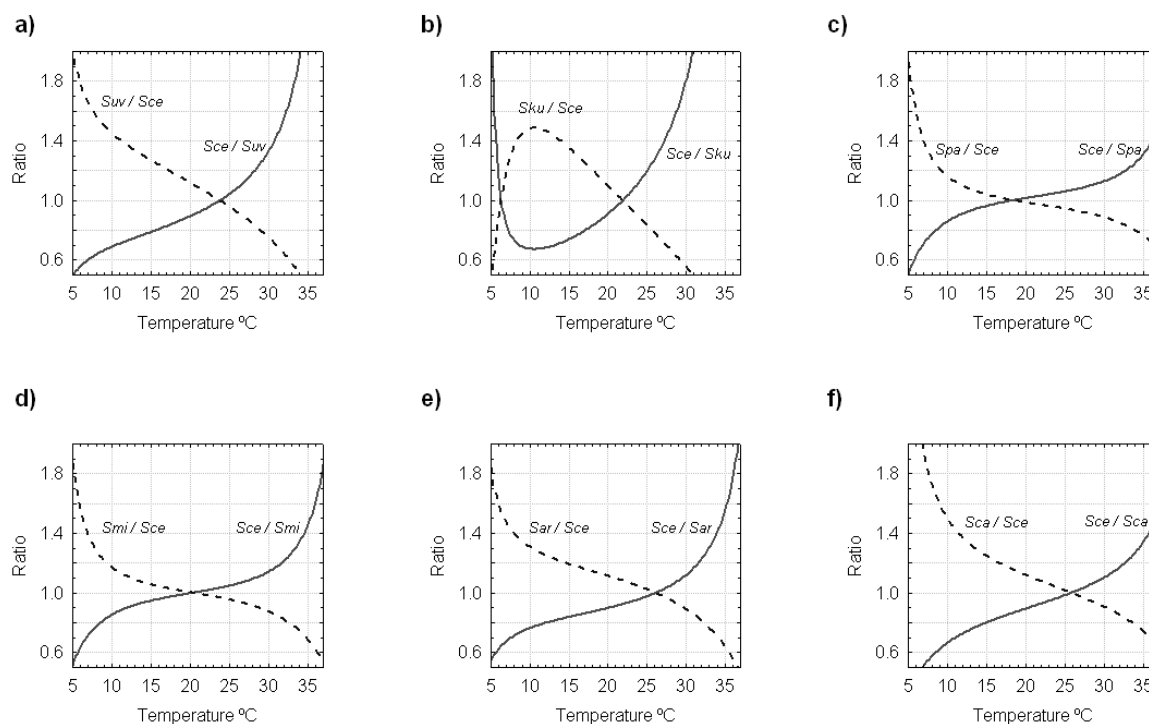


Figure 5. Model predictions for the ratios of the maximum specific growth rates of *S. cerevisiae*(*Sce*) versus a) *S. uvarum* (*Suv*), b) *S. kudriavzevii* (*Sku*), c) *S. paradoxus* (*Spa*), d) *S. mikatae* (*Smi*), e) *S. arboricolus* (*Sar*), and f) *S. cariocanus* (*Scs*) as a function of temperature.

We also determined the influence of temperature on the growth of *S. cerevisiae* and non-*Saccharomyces* strains isolated from wine fermentations. Figure 6 shows the μ_{\max} evolution of 5 commercial *S. cerevisiae* strains and 4 non-*Saccharomyces* wine yeasts as a function of temperature. The *S. cerevisiae* strains were able to grow up to 45°C, and they had their T_{opt} around 32°C with a μ_{opt} ranging from 0.35 to 0.42 h⁻¹ (see Table 2). However, the strains of *T. delbrueckii* (*Td*) and *Pichia fermentans* (*Pf*) were unable to grow above 40°C, whilst this value was even lower (around 36°C) for species *Hanseniaspora uvarum* (*Hu*) and *Candida zemplinina* (*Cz*). The optimum growth temperatures of these 4 strains were included in the interval 24-27°C, with a μ_{opt} always lower than *S. cerevisiae* strains (between 0.096 and 0.235 h⁻¹). As can be deduced from Figure 6, above 20°C the difference between the μ_{\max} of *S. cerevisiae* and the μ_{\max} of non-*Saccharomyces* strains progressively increased. relationship with *Saccharomyces* phylogeny. We did not analyze T_{\min} because no significant differences were observed among strains. The topology of the phylogenetic tree with node identifications (left side), and the corresponding values for each tested variable and strain (right side) are shown in Figure 7a. The orthogram analysis showed that orthonormal vector representing node 3 (which differentiates *S.arboricolus*, *S. mikatae*, *S. cariocanus*, *arboricolus*, *S. mikatae*, *S. cariocanus*, *S. paradoxus* and *S. cerevisiae* from *S. kudriavzevii* and

Analysis of phylogenetic dependence. CTMI model parameters (μ_{opt} , T_{opt} and T_{max}), which are quantitative variables, were tested using the orthogram approach (17) to determine a possible

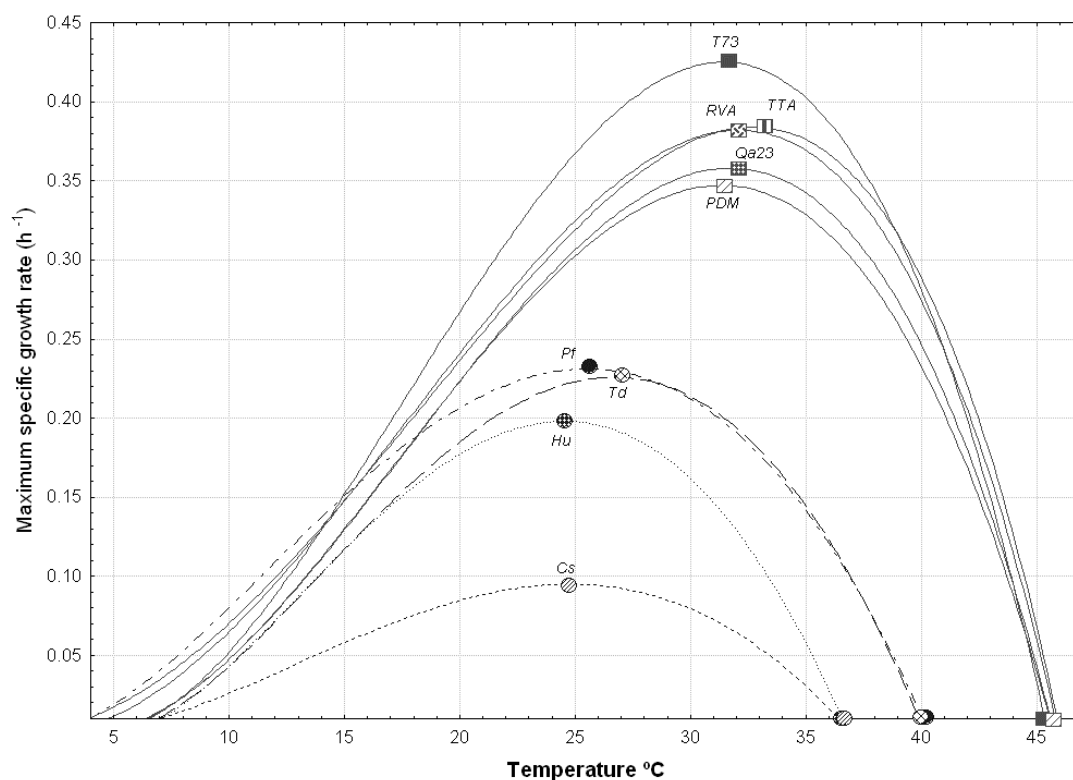


Figure 6. Changes of the maximum specific growth rates of the non-*Saccharomyces* species *H. uvarum* (*Hu*), *C. zemplinina* (*Cs*), *T. delbrueckii* (*Td*) and *P. fermentans* (*Pf*) and different *S. cerevisiae* wine strains (*Sc T73*, *Sc Qa23*, *Sc TTA*, *Sc RVA*, *Sc PDM*) as a function of temperature.

S. uvarum) explained the greatest part of the variance for the three analyzed parameters (Figures 7b, 7c and 7d). This vector peaked in all cases outside of the confidence limits (represented by dashed lines in Figures 7b, 7c and 7d: upper panels), and showed a strong departure from the expected value under the hypothesis of absence of phylogenetic dependence (represented by a solid straight line). Cumulative variance representation confirmed the preponderance of the orthonormal vector 3 in the variance distribution, and again a significant departure from the null hypothesis was registered for parameters μ_{opt} , T_{max} and T_{opt} (solid line in Figures 7b, 7c and 7d: lower panels). The maximum deviation from the expected values was given for the sum of the first three and six orthonormal vectors for T_{opt} and T_{max} parameters, respectively (vertical arrows in Figures 7c and 7d; lower panels). Phylogenetic dependence was also confirmed by the statistical tests R2Max, SkR2k, D_{max} and SCE. For T_{opt} and T_{max} parameters, all tests were significant, showing p-values lower than 0.0001. In the case of μ_{opt} , 3 of 4 tests displayed significant values (0.042, 0.030 and 0.017 for R2Max, SkR2k and D_{max} , respectively). Only the SCE test was not able to indicate significant departure from H_0 , with a p-value of 0.061. For the three CTMI

parameters, the R2Max test indicated that a significant part of the variance was explained by a single vector contribution, indicative of a single punctual modification event occurred in the evolutionary history of the genus. Relating this result with the orthogram plots, we identified that the significant change of the variables occurred after the divergence of *S. arboricolus*, *S. cariocanus*, *S. mikatae*, *S. paradoxus* and *S. cerevisiae* from *S. kudriavzevii* and *S. uvarum* lineages (node 3, Figure 7a). Moreover, the decomposed variance plot and the SkR2k statistics also indicated that a significant variance explanation occurred in nodes towards the root. However, according to the orthogram plot and the cumulative orthogram plot of parameters T_{\max} and T_{opt} (Figures 7c and 7d), a secondary modification of parameters could also have occurred at node 6, after the divergence of *S. paradoxus* and *S. cerevisiae* lineages, although the statistics tests did not support the presence of this second event. In any case, these results suggest that these three parameters have been modeled during the evolution of the *Saccharomyces* genus.

DISCUSSION

The influence of temperature on microorganism growth has widely been studied by microbiologists, and different mathematical models have been developed to quantify and predict its effects. (Rosso et al., 1993), and more recently (Oscar, 2002), compared several temperature secondary models on the basis of different criteria (simplicity and biological significance of parameters, applicability, quality of fit and ease of determination of parameters), concluding that the CTMI model was better than its competitors to fit a total of 48 data sets belonging to different species of microorganisms. In the present study, the CTMI model also resulted very useful to fit the experimental growth data of 27 yeast strains in the whole biological temperature range.

S. uvarum and *S. kudriavzevii* are considered as the most psychrotrophic species of the *Saccharomyces* genus (Belloch et al., 2008; Kishimoto and Goto, 1995; Serra et al., 2005). Our results also corroborate these observations, and *S. uvarum* and *S. kudriavzevii* were the yeasts with the lowest T_{opt} and T_{\max} values. Unfortunately, scarce information is available in the literature to carry out a quantitative comparison with data obtained from this paper. Serra et al., (2005) mentioned that the optimum growth temperature for the wine strain *S. uvarum* P3 was attained around 28°C, close to the value obtained from this work for the average of the two *S. uvarum* strains assayed (26.3°C). In the case of *S. kudriavzevii*, (Arroyo-López et al., 2009) estimated, using response surface methodology, that the optimum growth temperature of the type strain IFO 1802^T was attained at 24°C, while in this paper the average of four *S.*

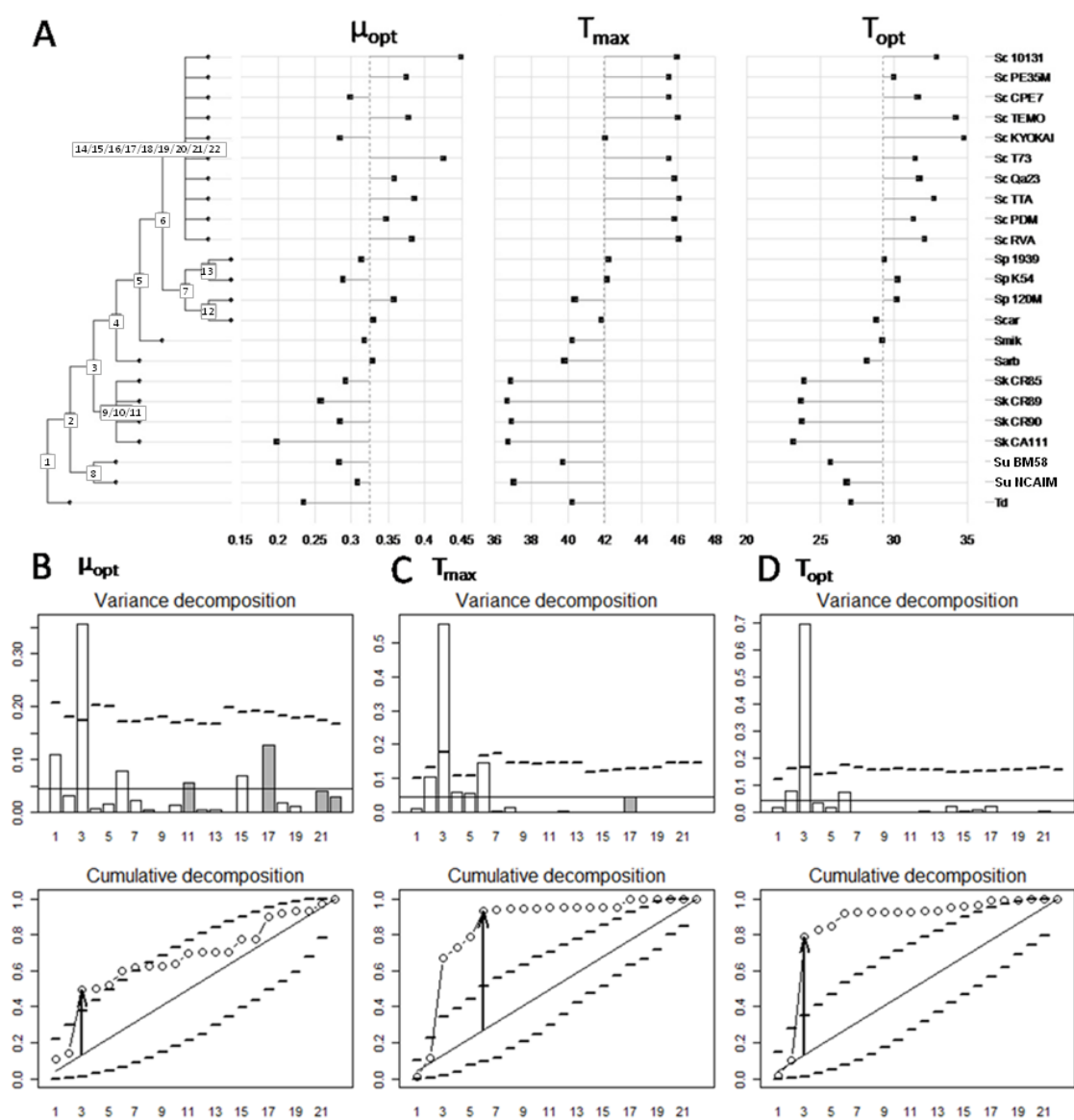


Figure 7. Phylogenetic dependence analysis for the cardinal temperature model parameters (μ_{opt} , T_{max} and T_{opt}) of the 22 *Saccharomyces* strains. *Torulasporea delbrueckii* was used as outgroup. A) Topology of the phylogenetic tree (on the left), dot plot of parameters (center) and strain designations (on the right). Variance decomposition using orthograms (upper panels) and cumulative orthograms (lower panels) for μ_{opt} (B), T_{max} (C) and T_{opt} (D). In the orthogram plots, abscise gives the number of vectors associated to nodes, while ordinate shows the contribution of the vector to the variance of the parameter by the squared regression coefficients (white for positive and grey for negative coefficients). Dashed line corresponds to upper confidence limits at 95% deduced from Monte Carlo permutations. Solid line represents the mean value. In the cumulative orthogram plots, ordinate shows the cumulated contribution of successive vectors to the variance. Diagonal solid line represents expected value under absence of phylogenetic dependence. Dashed lines correspond to the bilateral 95% confidence intervals. Circles show the observed value of cumulated squared regression coefficients. Vertical arrows mark the maximum deviation from the expected value (diagonal line).

kudriavzevii strains was obtained at 23.6°C. (Belloch et al., 2008) mentioned that both species were able to grow at 30°C, but not at 37°C. (Sampaio and Goncalves, 2008) reported that the maximum growth temperature for wild *S. kudriavzevii* and *S. uvarum* strains were 35°C and 36°C, respectively. In this work, the T_{\max} values estimated were slight higher, 36.8°C for *S. kudriavzevii* and 38.3°C for *S. uvarum*.

On the contrary, *S. cerevisiae* was the most thermotolerant species within *Saccharomyces* genus, with the highest optimum (32.3°C) and maximum (45.4°C) growth temperatures. Several authors (Arroyo-López et al., 2009; Serra et al., 2005) have reported that the optimum growth temperature of *S. cerevisiae* wine strains was around 34°C. The association of *S. cerevisiae* with human fermentations is well known, and preliminary data suggest that temperature could play an important role on the predominance of this species. (Heard and Fleet, 1988) observed that *S. cerevisiae* dominated traditional grape juice fermentations when they were carried out at higher temperatures. Recently, (Goddard, 2008) also showed that temperature resulted to be an important factor on the imposition of *S. cerevisiae* versus non-*Saccharomyces* yeasts during wine fermentations. An increase of temperature from 16 to 23°C as a consequence of the highly vigorous respiro-fermentative consumption of sugars (Crabtree effect), favored the rapid growth of *S. cerevisiae* cells and its final imposition, although the initial frequency of this species was very low (<1%) (Goddard, 2008). Such data clearly correlated with our results. We found that above 20°C, the μ_{\max} of the *S. cerevisiae* wine strains increased faster than the μ_{\max} of the non-*Saccharomyces* wine species. These differences were especially evident at 32°C. Even in the optimum growth temperature conditions of the non-*Saccharomyces* species (~25°C), the μ_{\max} of the *S. cerevisiae* wine strains were always significantly higher.

Unfortunately, our knowledge of the ecology and distribution of the *Saccharomyces* species in wild environments is still very limited, but the present study could shed some light on the influence of temperature on the ecological interactions among *Saccharomyces* species. Diverse studies have shown that *S. cerevisiae* and its sibling species *S. paradoxus* occupy the same ecological niches (oak exudates, oak bark and oak-associated soils) in widely separated woodland sites (Naumov et al., 1998; Sniegowski et al., 2002). (Sweeney et al., 2004) reported that the growth temperature profiles of diverse *S. paradoxus* and *S. cerevisiae* wild strains, isolated from a single natural site, were different. *S. paradoxus* wild isolates exhibited T_{opt} around 30°C (similar to those presented in this work), while for *S. cerevisiae* T_{opt} was above 37°C. (Sampaio and Goncalves, 2008) also carried out an interesting study on the influence of temperature on the sympatric association of four *Saccharomyces* species (*S. cerevisiae*, *S. paradoxus*, *S. uvarum* and *S. kudriavzevii*) isolated from oak bark samples. Their study showed that temperature played a fundamental role in the interactions among the *Saccharomyces* species. They suggested that circadian temperature changes

could provide a range of temperatures allowing the sympatric association, involving a species more adapted to grow at high temperatures (*S. cerevisiae* or *S. paradoxus*) and another species more adapted to grow at low temperatures (*S. uvarum* or *S. kudriavzevii*). Our results also support the hypothesis that adaptation to grow at different temperatures could be a very important factor in the ecology of *Saccharomyces* species. Mathematical models developed in the present study reveal that *S. cerevisiae* grows faster than the rest of *Saccharomyces* species at high temperatures, but exhibits a loss of competitiveness at low temperatures. Our models estimate the limits of temperature at which *S. cerevisiae* grows faster than the other species. These temperature values are 24°C for *S. cerevisiae* with respect to *S. uvarum*, 22°C with respect to *S. kudriavzevii*, 19°C to *S. paradoxus*, 20°C to *S. mikatae*, 26°C to *S. arboricolus* and 26°C to *S. cariocanus*. In this way, in a hypothetical sympatric association between the species *S. cerevisiae* and *S. kudriavzevii*, if temperature were the only limiting factor, the growth of *S. cerevisiae* would be selectively favored over *S. kudriavzevii* at temperatures above 22°C. However, circadian temperature changes around this value, would favor the growth of both species even in the same ecological niche. It is worth noticing that this is a hypothetical case based on theoretical models. However, these predictions confirm previous studies of relative fitness tests carried out by (Sampaio and Goncalves, 2008) with the species *S. cerevisiae* and *S. kudriavzevii* at 10 and 30°C. If the culture was incubated at 10°C, at the end of the fermentation only the species *S. kudriavzevii* was found, while *S. cerevisiae* dominated the fermentations at 30°C. On the contrary, both species coexist in nature in the same microhabitats (oak barks), where a circadian cycle of temperatures is present. Another additional argument presented by (Sampaio and Goncalves, 2008) for the sympatric association of *Saccharomyces* species is the absence of overlapping in the geographic distribution of *S. kudriavzevii* and *S. uvarum*, the two species more adapted to low temperatures.

According to the competitive exclusion principle, niche differentiation is necessary for closely-related species sympatric coexistence, and the different temperature growth profile exhibited for the *Saccharomyces* species could explain this phenomenon. The analysis of the phylogenetic dependence of the CTMI parameters showed that a single event, occurred after the divergence of *S. arboricolus*, *S. mikatae*, *S. cariocanus*, *S. paradoxus* and *S. cerevisiae* from *S. kudriavzevii* and *S. uvarum* lineages, favored the adaptation of the former species to grow at higher temperatures. The analysis also suggests that a second event could now be occurring in the *S. cerevisiae* lineage after its divergence from *S. paradoxus* and *S. cariocanus*, which would explain the higher thermotolerance exhibited by this species. Another interesting point, although it was not revealed by the phylogenetic dependence analysis, is the progressive adaptation of *S. kudriavzevii* to grow at lower temperatures. This was evidenced because *S. uvarum*, the first species to diverge within the *Saccharomyces* genus, exhibited higher T_{opt} and T_{max} values than *S. kudriavzevii*. In light of these

results, temperature has influenced the evolution of the *Saccharomyces* genus, favoring the adaptation of some species to grow at lower (*S. kudriavzevii*) and higher (especially *S. cerevisiae*) temperatures.

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Chapter 2: Quantifying the individual effects of ethanol and temperature on the fitness advantage of *Saccharomyces cerevisiae*

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Quantifying the individual effects of ethanol and temperature on the fitness advantage of *Saccharomyces cerevisiae*

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ABSTRACT

The presence of *Saccharomyces cerevisiae* in grape berries and fresh musts is usually very low. However, as fermentation progresses, the population levels of this species considerably increase. In this study, we use the concept of fitness advantage to measure how increasing ethanol concentrations (0–25%) and temperature values (4–46 °C) in wine fermentations affects competition between *S. cerevisiae* and several non-Saccharomyces yeasts (*Hanseniaspora uvarum*, *Torulopsis delbrueckii*, *Candida zemplinina*, *Pichia fermentans* and *Kluyveromyces marxianus*). We used a mathematical approach to model the hypothetical time needed for *S. cerevisiae* to impose itself on a mixed population of the non-Saccharomyces species described above. This approach also took into consideration the influence of environmental factors and the initial population levels of *S. cerevisiae* (0.1, 1.0 and 10.0%). Our results suggest that *Saccharomyces* niche construction via ethanol production does not provide a clear ecological advantage (at least not until the ethanol concentration exceeds 9%), whereas a temperature rise (above 15 °C) does give *S. cerevisiae* a considerable advantage. The initial frequency of *S. cerevisiae* considerably influences the time it needs to impose itself (until it reaches a final frequency of 99% in the mixed culture), the lowest time values being found at the highest initial frequency. In light of these results, the application of low temperatures in the wine industry could favor the growth and survival of non-Saccharomyces species for a longer period of time.

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1. Introduction

Nowadays wine production is an industrial process influenced by a complex set of abiotic factors (osmotic pressure, pH, ethanol, temperature, etc.) and biotic factors (microorganism species, killer factors, grape variety, etc.). Among the biotic factors, yeasts are very important because they are the main microorganisms responsible for the fermentation process, during which they produce diverse secondary metabolites (glycerol, acetate, succinate, pyruvate, higher alcohols and esters) that greatly contribute to the sensorial properties of wine (Fleet, 1993). *Hanseniaspora* is often the predominant yeast genus on the surface of grape berries, accounting for the 50–75% of the total population, although other non-Saccharomyces species of the genera *Candida*, *Pichia*, *Cryptococcus*, *Brettanomyces*, *Rhodotorula*, *Metschnikowia*, *Torulopsis* and *Kluyveromyces* are also usually isolated from freshly extracted grape

juices, reaching population levels between 3 and 6 log₁₀ CFU/ml (Torija et al., 2001; Fleet, 2003a; Romano et al., 2006).

Contrary to popular belief, fermentative species of the *Saccharomyces* genus occur at extremely low populations on healthy, undamaged grapes and are rarely isolated from intact berries and vineyard soils (Pretorius, 2000; Xufre et al., 2006). However, as the fermentation progresses, they displace the non-Saccharomyces species and occupy their fermentative niche. *Saccharomyces cerevisiae* is the main species responsible for this phenomenon (Bisson, 2005; Querol & Fleet, 2006). This species vigorously ferments the grape sugars (glucose and fructose) into ethanol and carbon dioxide, even in presence of oxygen (the Crabtree effect). A strong selective pressure favors the *Saccharomyces*'s strategy of making life difficult for other microorganisms by rapidly converting the available sugars into ethanol, a toxic compound, which can be later respired by *Saccharomyces* (Thomson et al., 2005; Woolfit and Wolfe, 2005; Piskur et al., 2006).

Niche construction theory is based on the hypothesis that an organism's activities modify its environment to give it an ecological advantage over its competitors. In wine fermentations, niche

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Quantifying the individual effects of ethanol and temperature on the fitness advantage of *Saccharomyces cerevisiae*

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Abstract

The presence of *Saccharomyces cerevisiae* in grape berries and fresh musts is usually very low. However, as fermentation progresses, the population levels of this species considerably increase. In this study, we use the concept of fitness advantage to measure how increasing ethanol concentrations (0-25 %) and temperature values (4-46 °C) in wine fermentations affects competition between *S. cerevisiae* and several non-*Saccharomyces* yeasts (*Hanseniaspora uvarum*, *Torulopsis delbrueckii*, *Candida zemplinina*, *Pichia fermentans* and *Kluyveromyces marxianus*). We used a mathematical approach to model the hypothetical time needed for *S. cerevisiae* to impose itself on a mixed population of the non-*Saccharomyces* species described above. This approach also took into consideration the influence of environmental factors and the initial population levels of *S. cerevisiae* (0.1, 1.0 and 10.0 %). Our results suggest that *Saccharomyces* niche construction via ethanol production does not provide a clear ecological advantage (at least not until the ethanol concentration exceeds 9 %), whereas a temperature rise (above 15 °C) does give *S. cerevisiae* a considerable advantage. The initial frequency of *S. cerevisiae* considerably influences the time it needs to impose itself (until it reaches a final frequency of 99 % in the mixed culture), the lowest time values being found at the highest initial frequency. In light of these results, the application of low temperatures in the wine industry could favor the growth and survival of non-*Saccharomyces* species for a longer period of time.

Keywords: *Saccharomyces*; non-*Saccharomyces*; fitness advantage; ethanol; temperature; wine fermentation.

1. Introduction

Nowadays wine production is an industrial process influenced by a complex set of abiotic factors (osmotic pressure, pH, ethanol, temperature, etc.) and biotic factors (microorganism species, killer factors, grape variety, etc.). Among the biotic factors, yeasts are very important because they are the main microorganisms responsible for the fermentation process, during which they produce diverse secondary metabolites (glycerol, acetate, succinate, pyruvate, higher

alcohols and esters) that greatly contribute to the sensorial properties of wine (Fleet, 1993). *Hanseniaspora* is often the predominant yeast genus on the surface of grape berries, accounting for the 50 – 75 % of the total population, although other non-*Saccharomyces* species of the genera *Candida*, *Pichia*, *Cryptococcus*, *Brettanomyces*, *Rhodotorula*, *Metschnikowia*, *Torulaspota* and *Kluyveromyces* are also usually isolated from freshly extracted grape juices, reaching population levels between 3 and 6 log₁₀ CFU/ml (Toriija et al., 2001; Fleet, 2003a; Romano et al., 2006).

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Niche construction theory is based on the hypothesis that an organism's activities modify its environment to give it an ecological advantage over its competitors. In wine fermentations, niche construction via ethanol production has been a traditionally accepted hypothesis for explaining how *Saccharomyces* imposes itself on non-*Saccharomyces* species. This hypothesis is supported by the fact that most non-*Saccharomyces* yeasts produce low amounts of ethanol and by the clear correlation between increased ethanol concentrations and reduced non-*Saccharomyces* populations (Goddard, 2008).

Recently, Goddard (2008) proposed that temperature could also play an important role in niche construction via the metabolic activity of *S. cerevisiae* during fermentation. Due to the vigorous fermentation of sugars carried out by this species, a non negligible thermal energy

estimated at 104.43 kJ/mol is released during fermentation (Williams, 1982). This energy is responsible for the temperature rise (up to 6 °C) that occurs during vinification (Goddard, 2008). Incidentally, *S. cerevisiae* performs better at higher temperatures than other yeast species (Arroyo-López et al., 2009).

In recent decades, the winemaking industry has effectively solved the problem of increased temperature caused by exothermal reactions on fermentation tanks. The use of low temperatures (10-15 °C) in the wine fermentation process can also enhance the production and retention of flavor volatiles (Torija et al. 2003; Beltran et al. 2006; Beltran et al. 2008). However, it is widely known that temperature reduction severely affects yeasts' growth performance (Sørensen & Jakobsen, 1997; Charoenchai et al., 1998; Serra et al., 2005; Arroyo-López et al., 2006). Thus, lowering the fermentation temperature changes the wine fermentation and gives non-*Saccharomyces* species a greater chance of surviving in an environment where *S. cerevisiae* have traditionally been the dominant species (Pretorius, 2000; Xufre et al., 2006).

In this study, we use an in-silico approach to individually study the effects of temperature and ethanol on the growth of an *S. cerevisiae* strain and various non-*Saccharomyces* yeasts isolated from wine fermentations. A set of mathematical models were built to quantitatively assess the influence of both factors on the fitness advantage of *S. cerevisiae* compared to the other species with which it is hypothetically competing. The results of this work could shed light on how ethanol and temperature provide *S. cerevisiae* with the ecological advantage that allows it to outcompete non-*Saccharomyces* yeasts in wine environments, and determine how lowering the fermentation temperature could affect competition between wine species.

2. Material and methods

2.1. Yeast strains and inocula preparation

Six yeast strains, one commercial *Saccharomyces cerevisiae* (Lalvin T73) and five non-*Saccharomyces* strains (*H. uvarum* IATA185, *C. zemplinina* IATA246, *T. delbrueckii* IATA296, *K. marxianus* IATA1484 and *P. fermentans* IATA167, henceforth *Hu*, *Cz*, *Td*, *Km* and *Pf*, respectively)

were used in the present study. All these strains were isolated from Spanish wine fermentations and they belong to the culture collection of the “Instituto de Agroquímica y Tecnología de los Alimentos (IATA)”.

Inocula were prepared by introducing one single colony from pure cultures of each strain into 5 ml of Yeast-malt-peptone-glucose broth medium (YM, Difco™, Becton and Dickinson Company, *S. paradoxus*, USA). After 48 h of incubation at room temperature ($25\pm 2^\circ\text{C}$), 1 ml of each tube was centrifuged at 10,000 rpm for 10 min, and the pellets were washed with sterile saline solution (9 g/l), centrifuged and re-suspended in 0.5 ml of sterile saline solution to obtain a concentration of about $7.3 \log_{10}$ CFU/ml, which was confirmed by surface spread on YM agar plates. These yeast suspensions were used to inoculate the different experiments as described below.

2.2. Growth conditions

The basal growth medium selected for all experiments was Yeast Nitrogen Base (YNB, Difco™) supplemented with 20 g/l of glucose as the carbon source. The medium was sterilized by filtration (0.2 μm), giving a final pH of 5.5 ± 0.1 .

For the ethanol assays, the medium was modified with sterile pure ethanol (assay 99.8%) (Scharlau Chemie S.A., Spain) to obtain the following concentrations: 0, 7.82, 15.64, 23.45, 39.09, 54.73, 62.54, 78.18, 93.82, 117.27, 156.36 and 194.45 g/l. These concentrations can be converted into percentages (a more commonly used unit in the wine industry) by simply dividing by 7.818, this figure being the density of ethanol at 30°C . Therefore, the ethanol percentages assayed in this work were included in the interval 0 to 25%. The temperature was set at 30°C for all ethanol tests.

For the temperature assays, the YNB medium was inoculated as described below and incubated at different temperatures (4, 8, 10, 14, 22, 29, 33, 37, 40, 42, and 46°C) in order to obtain the whole temperature range in which yeasts are able to grow. Ethanol was set at 0 % for these experiments.

2.3. Optical density measurements

Growth was monitored at 600 nm in a SPECTROstar Omega instrument (BMG Labtech, Offenburg, Germany). Measurements were made every hour for 3 days after a pre-shaking of 5 s. At low temperatures (4-14 °C), the microplates had to be incubated outside the spectrophotometer and then placed inside it before measurement (every 8 hours for 14 days). The wells of the microplate were filled with 0.01 ml of inoculum and 0.25 ml of YNB medium (modified with ethanol when required), to always ensure an initial OD of approximately 0.2 (inoculum level of $\sim 6.0 \log_{10}$ CFU/ml). The inocula were always above the detection limit of the apparatus, which was determined by comparison with a previously established calibration curve. Uninoculated wells for each experimental series were also included in the microplate to determine and therefore subtract the noise signal. All experiments were carried out in triplicate. As a result, a total of 216 ethanol growth curves (12 levels x 6 strains x 3 replicates) and 198 temperature growth curves (11 levels x 6 strains x 3 replicates) were obtained and analyzed.

2.4. Primary modeling

Growth parameters were calculated from each ethanol and temperature level by directly fitting OD measurements versus time to the reparameterized Gompertz equation proposed by Zwietering et al. (1990):

$$y = D \cdot \exp\{-\exp[\left(\frac{\mu_{\max} \cdot e}{D}\right) \cdot (\lambda - t)] + 1\} \quad (1)$$

where $y = \ln(\text{OD}_t / \text{OD}_0)$, OD_0 is the initial OD and OD_t is the OD at time t ; $D = \ln(\text{OD}_{\max} / \text{OD}_0)$ is the asymptotic maximum, μ_{\max} is the maximum specific growth rate (h^{-1}), and λ is the lag phase period (h). Growth data were fitted by a non-linear regression procedure, minimizing the sum of squares of the difference between experimental data and the fitted model, i.e., loss function $(\text{observed} - \text{predicted})^2$. This task was accomplished using the non-linear module of the Statistica 7.0 software package (StatSoft Inc, Tulsa, OK, USA) and its Quasi-Newton option.

2.5. Secondary modeling

The effect of increasing ethanol concentrations on yeast μ_{\max} could be well fitted by means of a linear regression fit:

$$y = -A \cdot x + B \quad (2)$$

where y is the μ_{\max} for each ethanol concentration (x), B is the μ_{\max} in the absence of ethanol, and A is a slope parameter (this being negative due to the inhibitory effects of ethanol). Minimum inhibitory concentration (MIC) is the minimum value of x where μ_{\max} is 0, representing the minimum ethanol concentration where yeasts are not able to grow. These parameters were calculated using the linear module of the Statistica 7.0 software package.

A cardinal temperature model with inflection (CTMI) (Rosso, et al., 1993) was used to describe the μ_{\max} changes as a function of temperature (T , °C). CTMI is a descriptive model purely based on empirical observations, and includes the three cardinal temperature values often used in microbiology. It has the following expression:

$$\begin{aligned} \mu &= 0 && \text{if } T \leq T_{\min} \text{ or } T \geq T_{\max} \\ \mu &= \mu_{\text{opt}} (D/E) && \text{if } T_{\min} < T < T_{\max} \\ D &= (T - T_{\max}) (T - T_{\min})^2 \\ E &= (T_{\text{opt}} - T_{\min}) [(T_{\text{opt}} - T_{\min})(T - T_{\text{opt}}) - (T_{\text{opt}} - T_{\max})(T_{\text{opt}} + T_{\min} - 2T)] \end{aligned} \quad (3)$$

where T_{\max} is the temperature above which no growth occurs, T_{\min} is the temperature below which growth is no longer observed, and T_{opt} is the temperature at which the μ_{\max} equals its optimal value (μ_{opt}). As with the primary models, CTMI parameters were estimated by a non-linear regression procedure using Statistica 7.0 software package. Fit adequacy was checked by the proportion of variance explained by the model (R^2) respect to experimental data.

2.6. *S. cerevisiae* fitness advantage

The fitness advantage (m , h^{-1}) between two microorganisms can be defined by the following expression: $m = r_S - r_N$, where r_S corresponds to the μ_{max} of the *S. cerevisiae* strain and r_N is the μ_{max} of the non-*Saccharomyces* strains (Goddard, 2008). Thus, this parameter represents the differences in μ_{max} for a specific environmental condition.

In the present study, secondary models for ethanol and temperature were used to predict the μ_{max} value changes throughout the whole range assayed, and, consequently, to determine the m of *S. cerevisiae* compared with the non-*Saccharomyces* strains as a function of both factors. We restricted the calculus of m exclusively to the competitive zone, which included the temperature and ethanol values in which both *Saccharomyces* and non-*Saccharomyces* strains were able to grow. Thus, in the case of ethanol, concentrations above MIC provided the limits of the competitive zone, whereas for temperature T_{min} and T_{max} were the lower and upper limits respectively.

2.7. Time for imposition of *S. cerevisiae* on mixed cultures

Differences in μ_{max} in a specific competitive environment can explain why a given species imposes itself on another. However, the initial frequencies of both populations will also influence the time for imposition (TFI) of the most competitive microorganism. Using m , we may theoretically calculate the time (t , days) taken by *S. cerevisiae* (initial frequency, p_0) to reach a determined final frequency (p_t) versus the non-*Saccharomyces* strains (with an initial frequency $q_0 = 1 - p_0$ and a final frequency $q_t = 1 - p_t$). The following equation, developed by Hartl & Clark (1997) and recently used by Goddard (2008), shows the relationship between all these parameters.

$$t = \frac{1}{m} \cdot \frac{\ln(p_t \cdot q_0)}{q_t \cdot p_0} \quad (4)$$

We used different the initial frequencies of *S. cerevisiae* to simulate the individual influence of temperature and ethanol concentration on competition between mixed cultures in real wine. These were set at: 0.1 % (usual frequency in fresh musts), 1.0 % and 10.0 % (frequency after addition of a starter culture). The initial frequencies within the non-*Saccharomyces* group were 45 % for *H. uvarum*, 45 % for *C. zemplinina* and 10 % for *T. delbrueckii*, these being based on bibliographic data (Romano et al., 2006; Torija et al., 2001). The final frequencies of *S. cerevisiae* and the non-*Saccharomyces* strains were set at 99.9 and 0.1 % respectively.

2.8. Statistical data analysis

The one-way ANOVA module of Statistica 7.0 software was used to check for significant differences between the species. The dependent variables introduced for the analysis were the parameters obtained from secondary modeling of the temperature and the ethanol. The post-hoc comparison was carried out using the Scheffé test, which is considered to be one of the most conservative post-hoc tests (Winer, 1961).

3. Results

3.1. Effects of ethanol on μ_{\max}

For the six wine yeast strains assayed in this work, μ_{\max} decreased linearly when ethanol concentrations increased from 0 to 25 %, although above 16 % no growth was observed for any yeasts (Figure 1). The fit was satisfactorily carried out by means of a linear regression procedure, with an R^2 ranging from 0.95 to 0.99 (data not shown). Table 1 shows the average parameters of the linear fit obtained from three independent experiments.

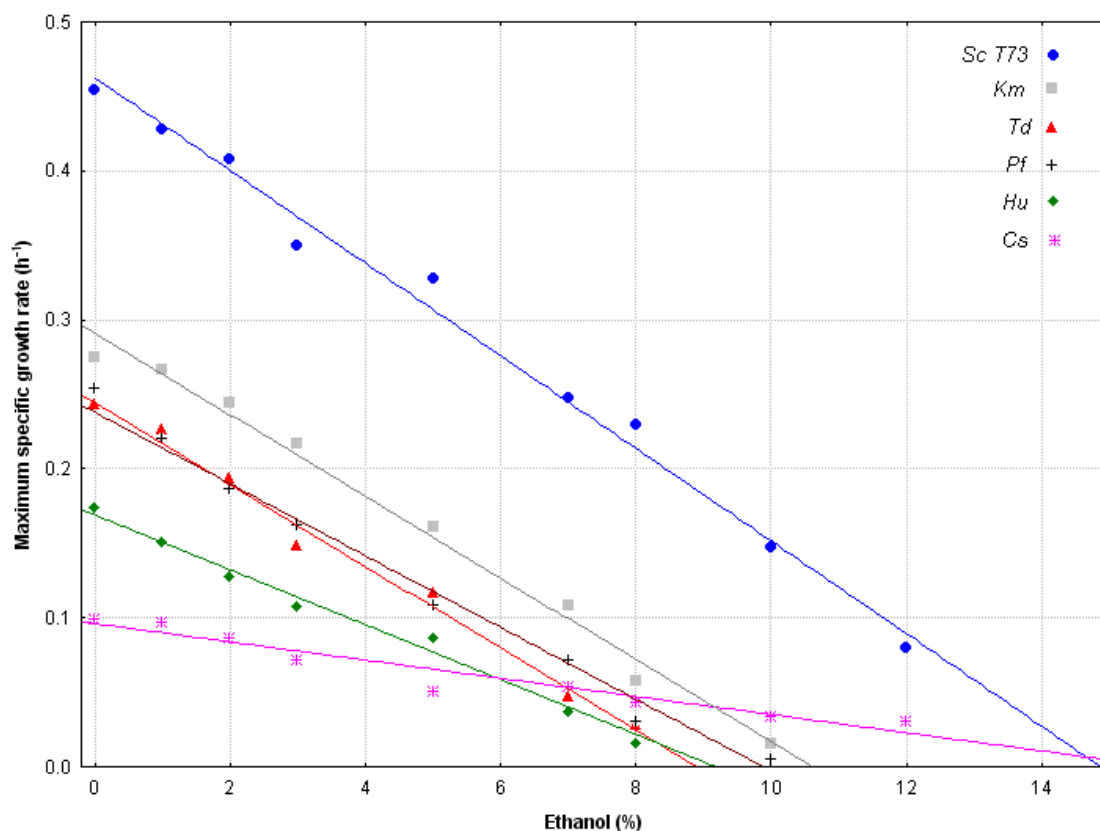


Figure 1. Changes in the maximum specific growth rates of the non-*Saccharomyces* species *H. uvarum* (*Hu*), *C. zemplinina* (*Cz*), *T. delbrueckii* (*Td*), *P. fermentans* (*Pf*) and *K. marxianus* (*Km*) and the commercial *S. cerevisiae* wine strain (*Sc T73*) as a function of ethanol concentration. Experimental data were fitted by means of a linear regression procedure. Standard deviations were intentionally omitted to improve the readability of the graphs.

The interception in the origin (B), in other words the value for μ_{\max} in the absence of ethanol at 30 °C, ranged from 0.462 (in the case of yeast *Sc T73*) to 0.096 h⁻¹ (for strain *Cz*) (Table 1). However, the effect of ethanol on μ_{\max} can be better interpreted by means of the slope parameter of the linear equation (A), which indicates the magnitude in the reduction of μ_{\max} when ethanol increases. Therefore, all the non-*Saccharomyces* strains were less influenced by the increase in ethanol than the yeast *Sc T73*, with significant differences between *Cz*, which showed the lowest absolute slope value, and *ScT73*, which showed the highest (Table 1). Obviously, this reduced the competitiveness of *S. cerevisiae* when the ethanol concentration increased in the media, this being particularly evident with yeasts *Cz* and *Hu* (Figure 2). However, the reduction was less marked for strains *Td*, *Km* and *Pf*. This trend remained until the ethanol concentration reached the MIC value for the non-*Saccharomyces* strains. Although *ScT73* suffered a marked reduction in m when ethanol increased, the higher initial μ_{\max} of this *S. cerevisiae* strain meant that

Table 1. Parameters of the linear regression fit for the inhibitory effects of ethanol on μ_{\max} .

Standard deviations for each parameter (in parentheses) were obtained from 3 independent experiments.

Yeast strain	Slope (A) [†]	Interception in the origin	MIC (%)
<i>Sc T73</i>	0.0311 (0.0019) ^d	0.462 (0.011) ^c	14.88 (0.73) ^c
<i>Cz</i>	0.0061 (0.0002) ^a	0.096 (0.002) ^a	15.57 (0.21) ^c
<i>Td</i>	0.0274 (0.0002) ^{c,d}	0.244 (0.002) ^c	8.88 (0.06) ^a
<i>Hu</i>	0.0184 (0.0028) ^b	0.169 (0.011) ^b	9.28 (0.85) ^{a,b}
<i>Km</i>	0.0274 (0.0002) ^{c,d}	0.291 (0.001) ^d	10.61 (0.23) ^b
<i>Pf</i>	0.0241 (0.0003) ^c	0.238 (0.005) ^c	9.86 (0.12) ^{a,b}

[†]Equation: $y = -A \cdot x + B$, where $y = \mu_{\max}$ (h^{-1}) and $x = \text{ethanol concentration}$ (%).

Note: Values followed by different superscript letters within the same column are significantly different according to a Scheffé post hoc comparison test.

ScT73 always had a positive m value, which indicates that it has the best fitness (higher μ_{\max}), with the exception of *Cz*, for all the ethanol conditions assayed. In this regard, MIC values ranged from 15.57 % (for strain *Cz*, the most resistant yeast to ethanol) to 8.88 % (for strain *Td*, which was the least resistant strain tested in this work). Thus, the only non-*Saccharomyces* strain with a MIC value higher than *ScT73* was *Cz* (Table 1).

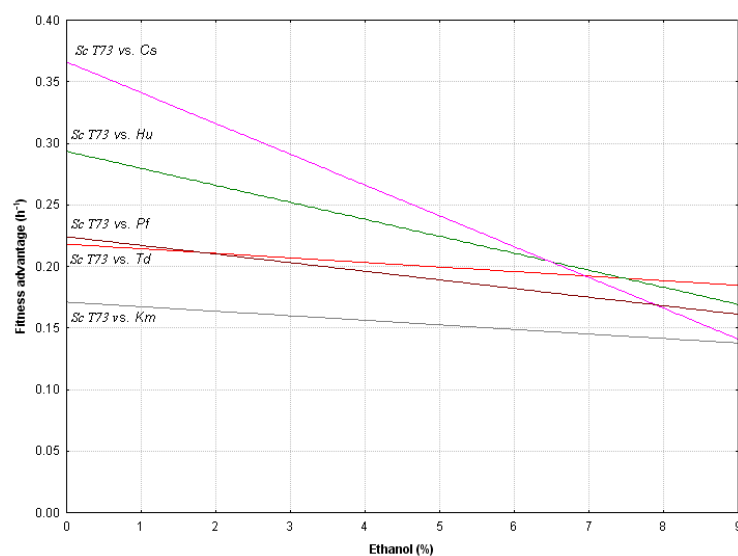


Figure 2. Model predictions for the fitness advantage (m , h^{-1}) of *Saccharomyces cerevisiae* (*ScT73*) versus *H. uvarum* (*Hu*), *C. zemplinina* (*Cz*), *T. delbrueckii* (*Td*), *P. fermentans* (*Pf*) and *K. marxianus* (*Km*) as a function of ethanol concentration.

3.2. Effects of temperature on μ_{max}

Changes in μ_{max} as a function of temperature could be well fitted by means of a CTMI secondary model, with an R^2 ranging from 0.97 to 0.99 (data not shown). This model has the advantage that it includes the three cardinal temperature values (T_{min} , T_{opt} and T_{max}), which are often used in microbiology to describe the behavior of microorganisms as a function of this environmental factor. Table 2 shows the temperature-dependent parameters obtained with the CTMI model for the six wine yeasts assayed in this work. A Scheffé post hoc comparison test was used to distinguish significant differences among species. No significant differences were observed for the T_{min} parameter, whereas differences were observed for the T_{opt} , T_{max} and μ_{opt} , obtaining until 4 different homogeneous groups (Table 2).

Table 2. Estimated parameters (μ_{opt} , T_{max} , T_{min} and T_{opt}) of the cardinal temperature model for the six wine yeast strains assayed in this work. Standard deviations for each parameter (in parentheses) were obtained from 3 independent non-linear fits.

Strain reference	μ_{opt} (h^{-1})	T_{opt} ($^{\circ}C$)	T_{max} ($^{\circ}C$)	T_{min} ($^{\circ}C$)
<i>Sc T73</i>	0.425 (0.043) ^d	31.42 (0.59) ^c	45.51 (0.11) ^c	4.11 (1.65) ^a
<i>Hu</i>	0.198 (0.013) ^b	24.51 (0.30) ^a	36.87 (0.07) ^a	4.71 (2.81) ^a
<i>Cz</i>	0.096 (0.004) ^a	24.77 (0.54) ^a	37.00 (0.01) ^a	2.81 (1.02) ^a
<i>Td</i>	0.235 (0.006) ^b	27.08 (0.48) ^b	40.19 (0.07) ^b	4.42 (1.63) ^a
<i>Km</i>	0.362 (0.004) ^c	38.68 (0.36) ^d	46.28 (0.03) ^d	2.43 (1.26) ^a
<i>Pf</i>	0.232 (0.008) ^b	25.51 (0.82) ^{a,b}	40.38 (0.28) ^b	1.25 (0.81) ^a

[†]Values followed by different superscript letters within the same column are significantly different according to a Scheffé post hoc comparison test.

These parameters ranged from 0.096 (Cz) to 0.425 h⁻¹ ($Sc T73$) for μ_{opt} , from 24.5 (Hu) to 38.7 °C (Km) for T_{opt} , from 36.9 (Hu) to 46.3 °C (Km) for T_{max} , and finally from 1.3 (Pf) to 4.7 °C (Hu) for T_{min} . Figure 3 provides a graphical representation of the μ_{max} changes for all yeasts within the whole temperature range assayed. All the non-*Saccharomyces* species had their optimal growth around 25 °C, except Km , which was around 39 °C. However, for yeast $ScT73$, the optimal growth was around 32 °C.

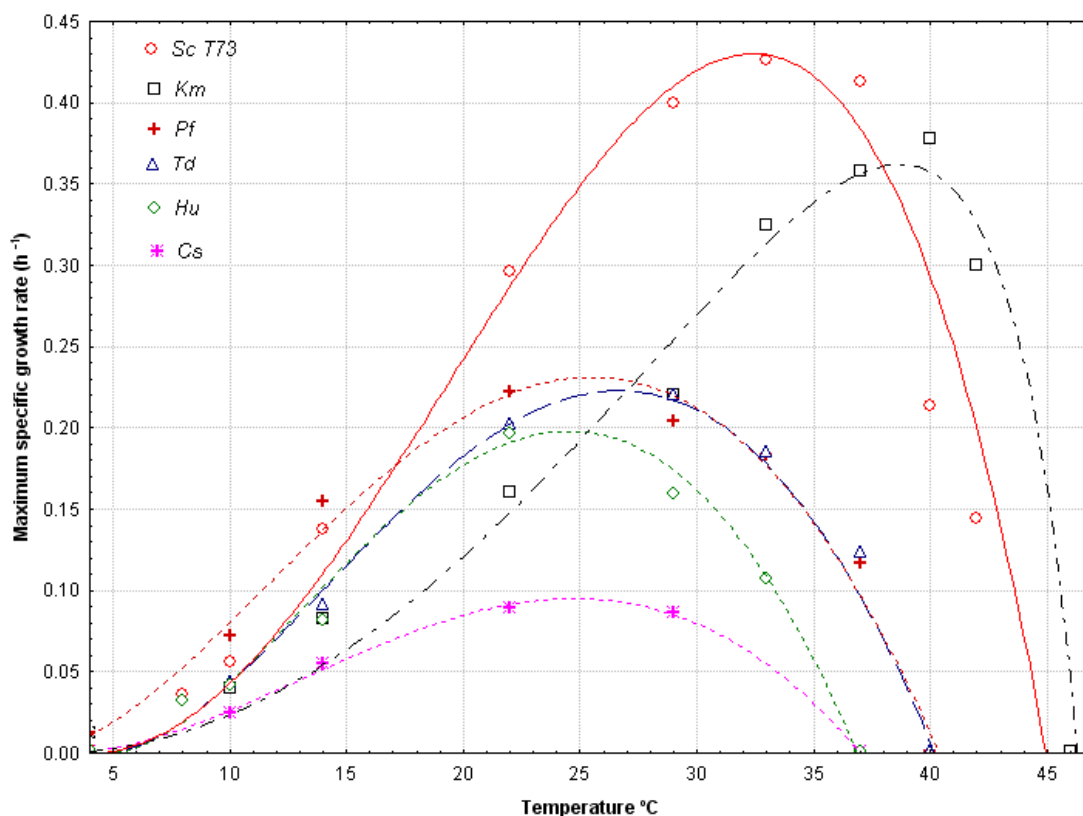


Figure 3. Changes in the maximum specific growth rates of the non-*Saccharomyces* species *H. uvarum* (Hu), *C. zemplinina* (Cz), *T. delbrueckii* (Td), *P. fermentans* (Pf) and *K. marxianus* (Km) and the commercial *S. cerevisiae* wine strain ($Sc T73$) as a function of temperature. Experimental data were fitted by means of a cardinal temperature model. Standard deviations were intentionally omitted to improve the readability of the graphs.

Figure 4 shows how the m of $ScT73$ changed as a function of temperature and compares this with the different non-*Saccharomyces* strains Hu , Cs , Td and Pf , which all showed a similar behavior for this parameter. When the temperature rose, the competitiveness (m) of *S. cerevisiae* clearly

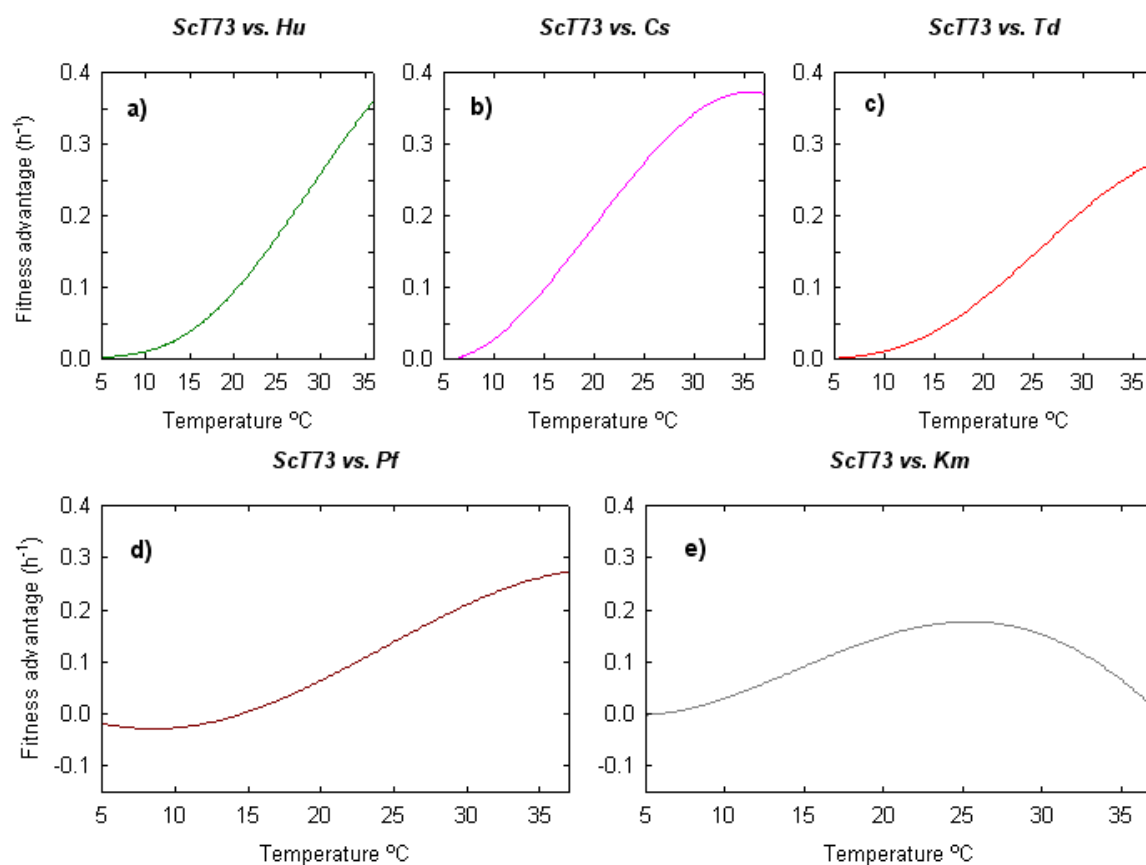


Figure 4. Model predictions for the fitness advantage (m , h^{-1}) of *Saccharomyces cerevisiae* (*ScT73*) versus a) *H. uvarum* (*Hu*), b) *C. zemplinina* (*Cz*), c) *T. delbrueckii* (*Td*), d) *P. fermentans* (*Pf*) and e) *K. marxianus* (*Km*) as a function of temperature.

increased (see Figures 4a, b, c and d). For *Hu*, *Cs*, *Td*, m never took negative values, which means that *ScT73* always showed a higher μ_{max} than its competitors. However, for *Pf*, m took negative values below 15 $^{\circ}\text{C}$, which means that the μ_{max} of *Pf* below this temperature was higher than the μ_{max} of *ScT73*. The m of *ScT73* compared with that of *Km* was different. When the temperature rose, the m of *ScT73* increased to a maximum value of around 25 $^{\circ}\text{C}$. Then, the m of *S. cerevisiae* decreased to 36 $^{\circ}\text{C}$, at which it took negative values due to the higher μ_{max} of *Km* above this temperature.

3.3. Time for imposition

The imposition of *S. cerevisiae* on non-*Saccharomyces* strains depends, among other things, on its ability to adapt to the diverse factors present in wine (referred to in this study as fitness advantage) and on the initial population levels of the species. We have shown how m changes as a function of temperature and ethanol concentration. However, in this section we evaluate how different initial population levels of *S. cerevisiae* influence this species' hypothetical TFI on a mixed population comprising diverse non-*Saccharomyces* wine yeasts. To do so, we use the mathematical approach described by Hartl and Clark (1997), which allows us to take into account different initial frequency values: 0.1, 1.0, and 10.0 %. The first two values correspond to situations commonly found in fresh grape juice (very low initial populations of *S. cerevisiae*), whereas the 10% value may correspond to a common situation that results from the inoculation practices used in wineries for industrial wine production.

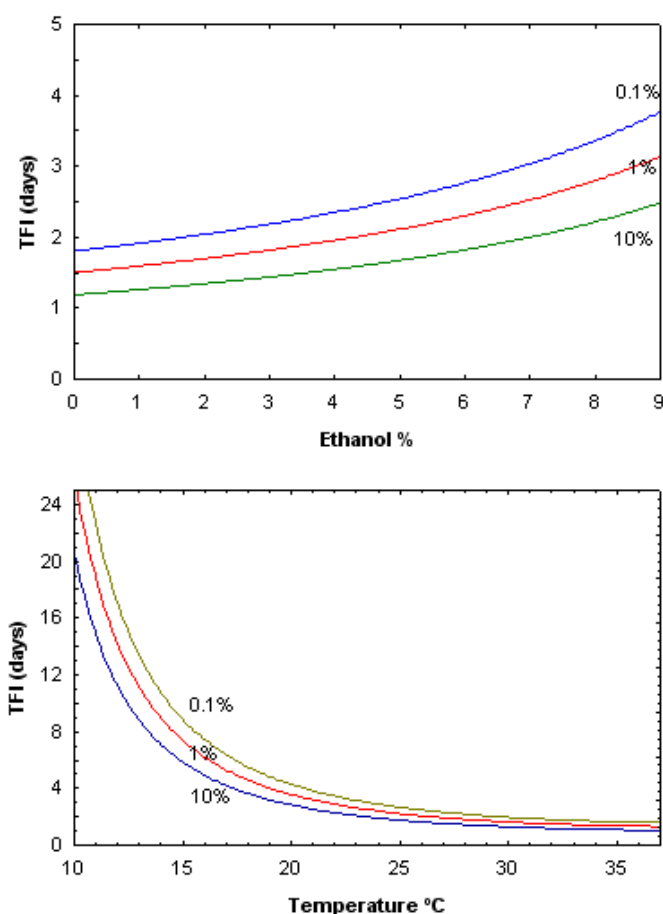


Figure 5. Model predictions for time for imposition (TFI, days) of *Saccharomyces cerevisiae* (ScT73) versus a theoretical non-*Saccharomyces* mixed population (45 % *H. uvarum*, 45 % *C. zemplinina* and 10 % *T. delbrueckii*) as a function of ethanol (upper panel) and temperature (lower panel). Solid lines represent days needed by *Saccharomyces* to reach a population of 99.9 % from different initial frequencies (0.1 %, 1 % or 10 %).

Figure 5 shows the effect of ethanol and temperature on *S. cerevisiae*'s TFI at different initial population levels. When the ethanol concentration increased, the TFI also increased (Figure 5, upper panel). On the other hand, the TFI decreased when the initial frequency of *S. cerevisiae* was higher. When the initial *S. cerevisiae* frequency was 0.1%, this time ranged from 1.7 days, in the absence of ethanol, to 3.6 days, in the presence of 9 % of ethanol. However, when the initial frequency was 10 %, the TFI was 1.2 days in the absence of ethanol and 2.4 days in the presence of 9 % of ethanol.

Comparatively, the effects of temperature on *Saccharomyces* imposition were greater than the effects of ethanol (Figure 5, lower panel). As the temperature of the process decreased, the time needed for *Saccharomyces* imposition increased exponentially. This effect was especially evident at temperatures below 15 °C, and at 10 °C, where at least 21 days were need for the imposition of *Saccharomyces*. In contrast, temperature values above 25 °C clearly favored the imposition of *Saccharomyces* at both lower or higher initial frequencies, with a final frequency of 99.9 % always being reached within 3 days.

4. Discussion

S. cerevisiae, even when it is scarcely present in the fresh grape juice (Pretorius, 2000; Xufre et al., 2006), becomes the main species during the final stages of the process. Although the key factor which gives *Saccharomyces* its competitive advantage over non-*Saccharomyces* species has been studied for several years, it has still not been completely unravel. In this study, we have modeled the fitness advantage of a commercial strain of *S. cerevisiae* and of several non-*Saccharomyces* wine species as a function of temperature and ethanol concentration. Our results show that increased ethanol reduces the competitiveness of *S. cerevisiae*, at least, until the MIC value of its competitors is reached. Once the ethanol exceeds the MIC value, the non-*Saccharomyces* lose their ability to grow and, consequently, cannot compete with the *Saccharomyces*, which could generally stand higher ethanol levels. These data suggest that the *Saccharomyces*' high ethanol tolerance could be one of the key factors to its hegemony at the end of fermentation when ethanol exceeds 9-10 %.

However, the data cannot explain the progressive imposition of this species during the earlier stages or its dominance over *C. zemplinina*, which was the most resistant yeast.

However, our data show that an increase in temperature gives *Saccharomyces* a clear advantage over non-*Saccharomyces* species. Indeed, a temperature increase of just a few degrees strongly increases the presence of *Saccharomyces* and, therefore, dramatically reduces its TFI over the most commonly found yeast species in wine environments (Figure 5). This effect was particularly evident at temperatures above 15 °C.

These data could be directly applied to industrial fermentations carried out at low temperatures (10-15 °C). This would produce wines with improved flavor volatiles because it would lead to greater retention of the primary aroma and enhance the production of secondary aromas (Torija et al. 2003; Beltran et al. 2006; Beltran et al. 2008). There is little information available about microbial evolution in low temperature fermentations. A few studies suggest that non-*Saccharomyces* species have a higher chance of survival in low temperature fermentations (Fleet, 2003b; Heard & Fleet, 1988; Sharf & Margalith, 1983), a finding which is supported by our experiments, which suggest that low temperature fermentations could reduce the imposition of *Saccharomyces*, thus giving non-*Saccharomyces* species a greater chance to contribute to the final flavor of the wine.

The data in this work were obtained in single culture experiments. This means that other factors such as competition between microorganisms for space and nutrients, oxygen affinity, or the production of toxic compounds (killer factors) were not taken into account (Nissen et al., 2003, 2004; Hanl et al., 2004; Albergaria et al. 2009). Obviously, many factors can influence population imposition in mixed cultures. However, we have proved that both factors, temperature and ethanol, can considerably influence the ecology of microorganisms during wine fermentation. Our data suggest that *Saccharomyces* niche construction via ethanol production does not provide an ecological advantage, or at least not until ethanol concentrations reach 9-10%;

however, the temperature increase produced by *Saccharomyces* during fermentation provides this species with a clear advantage over non-*Saccharomyces* species.

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Chapter 3: Proteomic evolution of a wine yeast during the first hours of fermentation



RESEARCH ARTICLE

Proteomic evolution of a wine yeast during the first hours of fermentation

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Abstract

The inoculation of active dry wine yeast (ADWY) is one of the most common practices in winemaking. This inoculation exposes the yeast cells to strong osmotic, acidic and thermal stresses, and adaptation to the new medium is crucial for successful fermentation. We have analysed the changes that occur in the ADWY protein profile in the first hours after inoculation under enological-like conditions at a low temperature. Protein changes mainly included enzymes of the nitrogen and carbon metabolism and proteins related to the cellular stress response. Most of the enzymes of the lower part of the glycolysis showed an increase in their concentration 4 and 24 h after inoculation, indicating an increase in glycolytic flux and in ATP production. However, the shift from respiration to fermentation was not immediate in the inoculation because some mitochondrial proteins involved in oxidative metabolism were induced in the first hours after inoculation. Inoculation in this fresh medium also reduced the cellular concentration of stress proteins produced during industrial production of the ADWY. The only exception was Cys3p, which might be involved in glutathione synthesis as a response to oxidative stress. A better understanding of the yeast stress response to rehydration and inoculation will lead to improvements in the handling efficiency of ADWY in winemaking and presumably to better control of fermentation startup.

Introduction

The use of active dry wine yeast (ADWY) is a widespread practice in wine technology. It replaces spontaneous fermentations in order to obtain more reproducible wines by better control of alcoholic fermentation (Ribereau-Gayon *et al.*, 2000). ADWY is obtained from selected natural wine yeast, which is propagated in molasses and then desiccated. The molecular response of yeast to propagation and desiccation has been studied (Perez-Torrado *et al.*, 2005; Singh *et al.*, 2005), and it is well established that this desiccation process causes a water deficit that leads to the arrest of cellular functions. A rehydration period is, therefore, required for these cellular functions to resume and for the membrane functionality to recover fully (Boulton *et al.*, 1995). The current process is to rehydrate the dried yeast by incubating it in water at 37 °C for a short period of time, followed by seeding the rehydrated cells into large fermenta-

tion tanks. One of the main characteristics of optimal wine fermentation is the set of physiological and metabolic changes that occur immediately on inoculating yeast cells into the musts (Bauer & Pretorius, 2000).

When ADWY is inoculated into the must, the yeast cells are subjected to different stress situations as a consequence of the high sugar concentration or the low pH of the medium. Moreover, some oenological practices increase these stressful conditions for ADWY. This is the case with low-temperature fermentations. Fermentation at low temperatures improves taste by restructuring flavour profiles with potential oenological applications (Feuillat *et al.*, 1997; Charoenchai *et al.*, 1998; Torija *et al.*, 2003). However, lowering the fermentation temperature to 13 °C or even lower has some disadvantages, including increasing the length of the process and the risk of stuck or sluggish fermentations (Meurgues, 1996; Bison, 1999). Thus, although low-temperature fermentation can be useful for

Proteomic evolution of a wine yeast during the first hours of fermentation

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Running title: Proteome changes during wine fermentation

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Abstract

The inoculation of active dry wine yeast (ADWY) is one of the most common practices in winemaking. This inoculation exposes the yeast cells to strong osmotic, acidic and thermal stresses, and the adaptation to the new medium is crucial for a successful fermentation. We have analysed the changes which occur in the ADWY protein profile in the first hours after inoculation under oenological-like conditions at low temperature. Protein changes mainly included enzymes of the nitrogen and carbon metabolism and proteins related to the cellular stress response. Most of the enzymes of the lower part of the glycolysis increased their concentration 4 h and 24 h after inoculation, indicating an increase of the glycolytic flux and of the ATP production. However the shift from respiration to fermentation was not immediate in the inoculation because some mitochondrial proteins involved in oxidative metabolism were induced in the first hours after inoculation. Inoculation in this fresh medium also reduced the cellular concentration of stress proteins produced during industrial production of the ADWY. The only exception was Cys3p which might be involved in glutathione synthesis as a response to oxidative stress. A better understanding of the yeast stress response to rehydration and inoculation will lead to improvements in the handling efficiency of ADWY in wine making and presumably to better control of fermentation start up.

Key words: wine, *Saccharomyces cerevisiae*, proteome, ADWY, lag phase, exponential phase

Introduction

The use of Active Dry Wine Yeast (ADWY) is a widespread practice in wine technology. It replaces spontaneous fermentations in order to obtain more reproducible wines by better control of alcoholic fermentation (Ribereau-Gayon *et al.*, 2000). ADWY is obtained from selected natural wine yeast which is propagated in molasses and then desiccated. The molecular response of yeast to propagation and desiccation has been studied (Perez-Torrado *et al.*, 2005; Singh *et al.*, 2005), and it is well established that this desiccation process causes a water deficit that leads to the arrest of cellular functions. A rehydration period is therefore required for these cellular functions to resume and for the membrane functionality to fully recover (Boulton *et al.*, 1995). The current process is to rehydrate the dried yeast by incubating it in water at 37°C for a short period of time, followed by seeding the rehydrated cells into large fermentation tanks. One of the main characteristics of optimal wine fermentation is the set of physiological and metabolic changes which occur immediately upon inoculating yeast cells into the musts (Bauer & Pretorius, 2000).

When ADWY is inoculated into the must, the yeast cells are subjected to different stress situations as a consequence of the high sugar concentration or low pH of the medium. Moreover some oenological practices increase these stressful conditions for ADWY. This is the case with

low temperature fermentations. Fermentation at low temperatures improves taste by restructuring flavour profiles with potential oenological applications (Feuillat *et al.*, 1997; Charoenchai *et al.*, 1998; Torija *et al.*, 2003). However, lowering the fermentation temperature to 13°C or even lower has some disadvantages, including increasing the length of the process and the risk of stuck or sluggish fermentations (Meurgues, 1996; Bisson, 1999). Thus, although low temperature fermentation can be useful for the oenological industry, it also has an adverse effect on cell growth, increasing yeast stress during wine production.

Successful ADWY adaptation involves a metabolic reorganization in order to maintain cell activity (vitality). There remains much we do not know about many aspects of this molecular response to early adaptation to musts. These mechanisms could be analysed by the whole array of “omics” currently available. In our group, we used DNA microarrays to analyse the transcriptional response of wine yeast after rehydration and inoculation in different media (Novo *et al.*, 2007). The main responses after inoculation in a fermentable medium were the activation of some genes of the fermentation pathway and of the non-oxidative branch of the pentose pathway, and the induction of a huge cluster of genes related to ribosomal biogenesis and protein synthesis. In a similar study, Rossignol *et al.* (2006) analysed the transcriptomic changes of wine yeast throughout different points of the lag phase, detecting a substantial transcriptional remodelling during this period. In addition to these global transcriptional analyses, other studies have addressed the protein modifications during lag phase and growth initiation in *S. cerevisiae* (Brejning & Jespersen, 2002; Brejning *et al.*, 2005). The changes which occur during the lag phase are characterised by an overall change in protein synthesis and reflect the physiological condition of the yeast, which affects its fermentative capacity and fermentation performance (Quain 1988, Blomberg 1997).

In this study, we have focused on the changes which occur in the ADWY protein profile after inoculation in a synthetic grape must. These changes should reflect the yeast cells' adaptation to the new medium. This adaptation is especially dramatic in fermentations at low temperature when yeasts have longer lag phases, thus increasing the length of fermentation and the risk of growing non-desirable microorganisms.

Materials and Methods

Yeast strain and growth conditions

The commercial wine yeast *Saccharomyces cerevisiae* QA23 (Lallemand S.A., Canada) was cultivated in a synthetic must (SM) prepared according to Riou *et al.* (1997), but with 200 g L⁻¹ of reduced sugars (100 g L⁻¹ glucose and 100 g L⁻¹ fructose) and without anaerobic factors. The Yeast Assimilable Nitrogen (YAN) content was 300 mg N L⁻¹: ammoniacal nitrogen (NH₄Cl) 120

mg N L⁻¹ and amino acids 180 mg N L⁻¹. This active dry wine yeast (ADWY) was rehydrated in water using the manufacturer's recommendations (30 min at 37°C). After counting under the microscope, the appropriate dilution of the rehydrated QA23 was transferred to synthetic must to get an initial cell concentration of 2 x 10⁶ cells mL⁻¹. The cell suspension was incubated at 13°C with a slight agitation to get a homogeneous nutrient distribution in laboratory-scale fermenters: 2 L bottles filled with 1.8 L medium and fitted with closures that enabled the carbon dioxide to escape and the samples to be removed. Samples were collected at time 0 (rehydrated yeast) and after 1, 4 and 24 hours. The number of viable cells was monitored by plating in YPD medium.

Protein extraction and two-dimensional electrophoresis

Protein extracts were prepared as described in Blomberg (2002). Briefly, this was as follows: cell suspension was vortexed for 4 x 30 seconds with glass beads containing PMSF as a protease inhibitor (with placement on ice between vortexing) and subsequently boiled for 5 min with SDS/mercaptoethanol buffer. Following nuclease treatment of the cells, protein contents of the extract were estimated using a 2-D quant kit (Amersham Pharmacia Biotech). Soluble proteins were run in the first dimension using a commercial horizontal electrophoresis system (Multiphor II; Amersham Pharmacia Biotech). Forty-five micrograms of protein from whole-cell lysates were mixed with immobilized polyacrilamide gel (IPG) rehydration buffer (8M urea/2% NP-40/10 mM DTT; final volume of 500 µl) and loaded onto polyacrilamide strips. IPG strips with a non-linear pH gradient 3-10 were allowed to rehydrate overnight. Samples were run at 50 µA per strip, in the first step voltage was ramped to 500 V over a period of 5 h, kept at 500 V for 5 h more, ramped again to 3500 V over a period of 9.5 h, and finally kept at 3500 V for 5 h. After the first dimension, IPG strips were then equilibrated twice for 15min in equilibration solution (0.05 M Tris-HCl, pH 8.8, 6 M urea, 30% v/v Glycerol, and 2% w/v SDS), first with 65 mM DTT (reduction step), and finally with 135 mM iodoacetamide (alkylation). The second dimension was carried out in a vertical electrophoresis system (Ettan DALTsix; Amersham Pharmacia Biotech) in a 12.5% (26 cm x 20 cm x 1 mm) polyacrylamide gel where proteins were separated based on molecular size. Electrophoresis conditions were 1 W per gel until the dye front reached the bottom of the gel. Sets of three gels were used for each sampling-time.

Silver staining protocol and image analysis

Staining protocol was done as described by Blomberg (2002). Gels were scanned using an Image Scanner UMAX, Amersham (300 dpi, 12-bit image) that allow us to obtain spot intensities in pixel units. Images were analyzed using PDQuest software (Bio-Rad). Normalization was performed by the mentioned software based in the total in gel density to compensate images differences caused by variations in experimental conditions (e.g. protein loading or staining). Spot

detection was implemented by using PDQuest automated spot detection algorithm. Gel image showing the highest number of spots and the best protein pattern was chosen as a reference template of the image analysis, and spots in the standard gel were then matched across all gels. 2DE gels were separated in 4 different groups corresponding to the already described sampling points: rehydrated yeast and 1, 4 and 24 hours after inoculation in the media. Matching features of the software were used to relate and compare the sets of gels. Finally to achieve maximum reliability and robustness of the results student t test was performed allowing us to identify those sets of proteins that showed a statistically significant difference with a confidence level set at 95%.

In-gel digestion of proteins.

Protein spots were excised manually and then digested automatically using a Proteiner DP protein digestion station (Bruker-Daltonics, Bremen, Germany) according to Schevchenko *et al* (1996). Minor variations of the method were introduced. Gel plugs were reduced with 10 mM dithiothreitol (Amersham Biosciences, Uppsala, Sweden) in 50 mM ammonium bicarbonate (99.5% purity; Sigma Chemical, St. Louis, MO, USA) and alkylated with 55 mM iodoacetamide (Sigma Chemical) in 50 mM ammonium bicarbonate. The gel pieces were then rinsed with 50 mM ammonium bicarbonate and acetonitrile (gradient grade; Merck, Darmstadt, Germany) and dried under a stream of nitrogen. Modified porcine trypsin (sequencing grade; Promega, Madison, WI, USA) at a final concentration of 13 ng μl^{-1} in 50 mM ammonium bicarbonate was added to the dry gel pieces and the digestion proceeded at 37 °C for 6 h. Finally, 0.5% trifluoroacetic acid (99.5% purity; Sigma Chemical) was added for peptide extraction.

MALDI-MS(/MS) and database searching

An aliquot of the above digestion solution was mixed with an aliquot of α -cyano-4-hydroxycinnamic acid (Bruker-Daltonics) in 33% aqueous acetonitrile and 0.1% trifluoroacetic acid. This mixture was deposited onto a 600 μm AnchorChip MALDI probe (Bruker-Daltonics) and allowed to dry at room temperature. MALDI-MS(/MS) data were obtained using an Ultraflex time-of-flight mass spectrometer (Bruker-Daltonics) equipped with a LIFT-MS/MS device (Suckau *et al.*, 2003). Spectra were acquired in the positive-ion mode at 50 Hz laser frequency, and 100 to 1500 individual spectra were averaged. For fragment ion analysis in the tandem time-of-flight (TOF/TOF) mode, precursors were accelerated to 8 kV and selected in a timed ion gate. Ionised fragments generated by laser-induced decomposition of the precursor were further accelerated by 19 kV in the LIFT cell and their masses were analyzed after passing the ion reflector. Measurements were in part performed using post-LIFT metastable suppression, which allowed the removal of the precursor and metastable ion signals produced after extraction from

the second ion source. Peptide mass mapping data was analyzed in detail using flex Analysis software (Bruker-Daltonics). MALDI-TOF mass spectra were calibrated internally using two trypsin autolysis ions with $m/z = 842.510$ and $m/z = 2211.105$; for MALDI-MS/MS, were calibrated with fragment ion spectra obtained for the proton adducts of a peptide mixture covering the 800-3200 m/z region. MALDI-MS and MS/MS data were combined through the MS BioTools program (Bruker-Daltonics) to search the NCBI database using Mascot software (Matrix Science, London, UK) (Perkins *et al.*, 1999).

Results

Experimental design

Commercial ADWY *S. cerevisiae* strain QA23 was used to ferment at low temperature (13°C) for 24 hours. We monitored the cell population during this time (Figure 1). No growth was detected in the two first sampling-times (1 and 4 hours) and the population had barely duplicated after 24 h. Therefore we considered that the cells were found in lag-phase in the 1 h and 4 h samples and in early exponential phase in the 24 h sample. The total cell protein profile was analyzed at these times of fermentation. Rehydrated cells (time 0) were used as the control for this proteomic study.

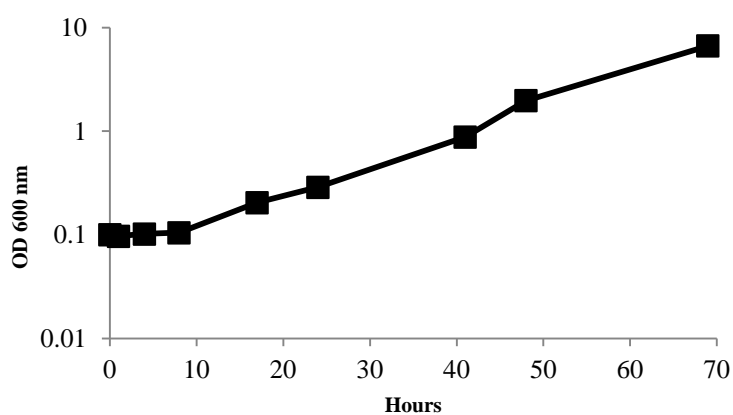


Fig1. Evolution of population (measured as OD 600nm) of QA23 wine yeast strain during the firsts hours of fermentation in synthetic must.

A total of 336 spots were detected on 2-D gel in rehydrated cells (control) (Table 1). After one hour in synthetic must, 61% of detected proteins were matched to proteins on the control gel, whereas for the samples taken at 4h and 24h after inoculation, the total matches were 68% and 52% respectively (Table 1). Qualitative and quantitative differences were detected between matched spots from the corresponding gels of the different collection points. We decided to go into a depth analysis of these proteins that their concentrations were modified at least two-fold (Table 1).

Table 1. Number of modified proteins on 2-D gels during 24 first hours of synthetic must fermentation. Percentage values are given in parentheses. Confidence level was set at 95%.

	Reference gel	Number of Spots		
		Time after inoculation		
		1h	4h	24h
		13°C	13°C	13°C
Detected	336	357	252	380
Matched with reference gel	–	204(61)	228(68)	197(52)
Statistically significant*	–	69(33)	66(29)	77(39)
At least twofold induced	–	1(0.4) α	13(5.9) α	2(0.1) α
At least twofold repressed	–	18(8.8) α	1(0.4) α	11(5.6) α

Percentage values given in parenthesis.

* Number of matched spots that show significative differences with 95% of confidence in t-student test.

Changes in protein profile during the lag phase

The concentration of some yeast proteins statistically increased or decreased two fold during lag phase (1 and 4 h samples) as shown in tables 2 and 3. The concentration of only one protein increased one hour after inoculation while that of 18 proteins decreased (8.8% of matched proteins) in the same period. Four hours after the inoculation, 13 proteins (5.9%) increased at least twofold and just one protein was detected to significantly decrease in comparison to the rehydrated yeast. Most of the proteins that were modified were involved in glycolysis and glucose fermentation. However, the changes were dependent on the time of fermentation. Thus, most of these proteins were present in lower concentrations after 1 h (i.e. Fba1p, Pgc1p, Eno1p, Eno2p, and the key fermentative enzyme Pdc1p) and in higher concentrations 4 h after inoculation than they were in the rehydrated cells. Tdh3p and Adh1p increased at 1 and 4 h respectively. All these proteins, with the exception of Fba1p, represent the enzymes of the lower part of glycolysis and glucose fermentation.

Other enzymes related to the carbohydrate metabolism were the mitochondrial malate dehydrogenase (Mdh1p), whose concentration increased after 4 h, and the D-lactate dehydrogenase (Dld1p), which decreased after 1 h. Mdh1p catalyses interconversion of malate and oxaloacetate in the TCA cycle and Dld1p, which oxidises lactate to pyruvate, is repressed by glucose and is derepressed in ethanol or lactate (Lodi *et al.*, 1999).

Table 2. Proteins whose concentration increased (positive numbers) or decreased (negative numbers) at least two-fold after 1 hour of yeast inoculation.

Gene name	Protein name	Metabolic function	Cellular location	Fold Change
CPR3	Mitochondrial peptidyl-prolyl cis-trans isomerase	Protein folding	cytoplasm, mitochondrion	-2.3
DLD1	D-lactate dehydrogenase	Carbohydrate metabolism	cytoplasm, mitochondrion, membrane, mitochondrial envelope	-3.5
ENO1	Enolase I, Phosphopyruvate hydratase	Glycolysis	cytoplasm, mitochondrion, vacuole	-2.2
ENO2	Enolase II, Phosphopyruvate hydratase	Glycolysis	cytoplasm, mitochondrion, vacuole	-6.3
FBA1	Fructose 1,6-bisphosphate aldolase	Glycolysis	cytoplasm, mitochondrion	-2.7
GDH1	Glutamate dehydrogenase	Amino acid biosynthesis	cytoplasm, nucleus	-2.9
GPD1	Glycerol-3-phosphate dehydrogenase	Response to osmotic stress	cytoplasm, peroxisome	N. D.
HSP12	Heat shock protein 12	Stress response	cytoplasm, membrane, nucleus, plasma membrane	-2.3
HSP60	Heat shock protein 60	Protein refolding after heat shock	cytoplasm, mitochondrion	-3.6
LEU2	Beta-isopropylmalate dehydrogenase	Amino acid biosynthesis	other	-4.0
LYS9	Saccharopine dehydrogenase	Amino acid biosynthesis	cytoplasm	N. D.
MET6	Cobalamin-independent methionine synthase	Amino acid biosynthesis	cytoplasm	-4.8
PDC1	pyruvate decarboxylase	Glycolysis, glucose fermentation	cytoplasm, nucleus	-3.7
PDI1	Protein disulfide isomerase	Protein folding	cytoplasm, endoplasmic reticulum	-2.4
PGK1	3-phosphoglycerate kinase	Glycolysis	cytoplasm, mitochondrion	-2.6
TDH3	Glyceraldehyde-3-phosphate dehydrogenase	Glycolysis	cytoplasm, mitochondrion & cell wall	3.8
TPS1	Trehalose-6-Phosphate Synthase	Stress response	cytoplasm	-5.6
TSA1	Thioredoxin peroxidase	Oxidative stress	cytoplasm	-2.1

N.D.: Not detected at 1h

Table 3. Proteins whose concentration increased (positive numbers) or decreased (negative numbers) at least two-fold after 4 hours of yeast inoculation.

Gene name	Protein name	Metabolic function	Cellular location	Fold Change
ADH1	Alcohol dehydrogenase	Glycolysis, glucose fermentation	cytoplasm	3.4
ATP1	Alpha subunit of mitochondrial ATP synthase	Mitochondrial transport and homeostasis	cytoplasm, mitochondrion, membrane, mitochondrial envelope	2.2
ATP2	Beta subunit of mitochondrial ATP synthase	Mitochondrial transport and homeostasis	cytoplasm, mitochondrion, membrane, mitochondrial envelope	2.2
CYS3	Cystathionine gamma-lyase	Amino acid biosynthesis	cytoplasm	2
ENO1	Enolase I, Phosphopyruvate hydratase	Glycolysis	cytoplasm, mitochondrion, vacuole	2.1
FBA1	Fructose 1,6-bisphosphate aldolase	Glycolysis	cytoplasm, mitochondrion	2.8
GPM1	Phosphoglycerate mutase	Glycolysis	cytoplasm, mitochondrion	2.5
MDH1	Mitochondrial malate dehydrogenase	Tricarboxylic acid (TCA) cycle	cytoplasm, mitochondrion	2
PGK1	3-phosphoglycerate kinase	Glycolysis	cytoplasm, mitochondrion	2.6
POR1	Mitochondrial porin	Mitochondrial homeostasis	cytoplasm, mitochondrion, membrane, mitochondrial envelope, golgi apparatus, endomembrane system	5.2
RPS0B	40S Ribosomal protein	Protein synthesis	cytoplasm, ribosome	5.6
TDH1	Glyceraldehyde-3-phosphate dehydrogenase	Glycolysis	cytoplasm, mitochondrion, cell wall	>10
YHB1	Nitric oxide reductase	Oxidative and nitrosative stress	cytoplasm, mitochondrion	-2.6

Four hours after the inoculation a ribosomal protein (Rps0bp), three mitochondrial proteins (Atp1p, Atp2p and Por1p) and a protein involved in the synthesis of cysteine (Cys3p) were also induced.

Among the proteins whose concentration reduced, we detected an important set related to osmotic (Gpd1p), oxidative (Sod1p, Tsa1p and Yhb1p) and general (Tps1p, Hsp12p and Hsp60p) stresses. The remaining proteins with reduced concentrations are involved in protein folding (Hsp60p, Pdi1p and Cpr3p) and in amino acid biosynthesis (Gdh1p, Leu2p, Lys9p and Met6p). In many cases, the degradation mechanism must be involved in the decrease of these

proteins. We detected fragments of different size belonging to Sod1p in the 1 h sample (data not shown). This degradation was confirmed by a lower concentration of this protein 24 h after inoculation.

Changes in protein profile during the early-exponential phase

Cell population almost duplicated 24 h after inoculation in the synthetic must. Therefore, the cells were at the beginning of the exponential or proliferating phase and the changes in protein concentration should reflect the adaptation of the cellular machinery to biomass production. However, the number of proteins whose amount changed in comparison to those of the rehydrated cells was not very important. Only 2 and 11 proteins were found in higher and lower concentrations respectively. The two increased proteins (Fba1p, Eno1p) (Table 4) are key enzymes of the glycolytic pathway. We also detected a glycolytic protein (Tdh3p) with reduced concentration. Other proteins with decreased concentration were involved in stress response (Hsp12p, Sod1p and Ald3p), amino acid biosynthesis (Gdh1p, Ald3p and Lpd1) and carbohydrate metabolism (Dld1p, Gor1p and Lpd1p). Some proteins can be catalogued in more than one functional category. This is the case with Ald3p which is induced by stress, repressed by glucose and involved in alanine synthesis (Navarro-Avino *et al.*, 1999; White *et al.*, 2003) or Lpd1p whose mutation stops the activity of the glycine decarboxylase, 2-oxoglutarate dehydrogenase, and pyruvate dehydrogenase complexes (Sinclair & Dawes, 1995; Dickinson *et al.*, 1997; Zaman *et al.*, 1999). Gor1p is a glyoxylate reductase which reversibly reduces glyoxylate to glycolate (Rintala *et al.*, 2007). An important trend of most of these proteins with decreased concentration was that 7 out of 11 proteins were mainly located in the mitochondria.

Discussion

The use of ADWY is a widespread practice in wine technology. These ADWY are, after a rehydration process in water a 37°C, inoculated in large fermentation tanks with grape must. This inoculation exposes the yeast cells to strong osmotic, acidic and thermal stresses (especially in refrigerated musts), and the adaptation to the new medium is crucial for a successful fermentation. Our aim in this study was to monitor the proteome changes of *S. cerevisiae* commercial wine yeast during the first hours of fermentation at low temperature. We were interested in both the cellular response to inoculation in a fermentable growth medium and the cold adaptation of the yeast cells. Different DNA microarray analyses have dealt with the evolution of the transcriptome of a wine yeast under these conditions (Rossignol *et al.*, 2006; Novo *et al.*, 2007). The genome-wide transcriptional changes during low-temperature wine fermentations have been also recently analyzed (Beltran *et al.*, 2006). The main relevant differences comparing global transcriptome and proteome analysis is that proteomic changes are

Table 4. Proteins whose concentration was modified at least two-fold during early exponential phase.

Proteins found in higher concentration

Gene name	Protein name	Metabolic function	Cellular location	Fold Change
ENO1	Enolase I, Phosphopyruvate hydratase	Glycolysis	cytoplasm, mitochondrion, vacuole	2.5
FBA1	Fructose 1,6-bisphosphate aldolase	Glycolysis	cytoplasm, mitochondrion	4.8

Proteins found in lower concentration

Gene name	Protein name	Metabolic function	Cellular location	Fold Change
ALD3	Aldehyde dehydrogenase	Amino acid biosynthesis	cytoplasm	N.D.
DLD1	D-lactate dehydrogenase	Carbohydrate metabolism	cytoplasm, mitochondrion, membrane, mitochondrial envelope	4.5
GDH1	Glutamate dehydrogenase	Amino acid biosynthesis	cytoplasm, nucleus	N.D.
HSP12	Heat shock protein 12	Stress response	cytoplasm, membrane, nucleous, plasma membrane	6.7
LPD1	Dihydrolipoamide dehydrogenase	Amino acid biosynthesis	cytoplasm, mitochondrion	3.2
MRP8	Mitochondrial Ribosomal Protein	Translation	cytoplasm, mitochondrion, ribosome	2.7
PIB2	Proteinase B Inhibitor	Proteolysis regulation	cytoplasm, mitochondrion	3
SOD1	Superoxide dismutase	Oxidative stress	cytoplasm, mitochondrion, mitochondrial envelope	2.1
TDH3	Glyceraldehyde-3-phosphate dehydrogenase	Glycolysis	cytoplasm, mitochondrion, cell wall	2.4
GOR1	Putative hydroxyisocaproate dehydrogenase	Oxidoreductase activity	cytoplasm, mitochondrion, nucleus	2.4

N.D. Not detected at 24h

much more limited than transcriptomic ones. Thus, 1874 genes changed their expression more than threefold after inoculation in a synthetic grape must (Rossignol *et al.*, 2006) and 535 ORFs were differentially expressed as a consequence of the temperature of fermentation (Beltran *et al.*, 2006), in comparison with fewer than 20 proteins with modified concentrations detected at each time-point in this study.

Protein changes during the first hour reflected a degradation or modifications in proteins present in the rehydrated yeasts. Our results indicated that the initial stages after rehydration may involve critical changes in yeast metabolism, and one of these may be the active degradation of useless protein and mRNAs (Novo *et al.*, 2007). On the contrary, most proteins increased their concentrations 4 h after inoculation. These protein changes may reflect transcriptional reprogramming after yeast inoculation in a new fermentable medium.

Proteins involved in stress response

An increase in proteins related to stress response may be expected after yeast inoculation. The high sugar concentration, low pH and low temperature may subject the cells to various stressful situations. However our results showed a decrease in proteins related to stress during the time-points studied (especially 1 hour after inoculation). In this case, the decrease in proteins related to stress matched with a down-regulation of most of the genes involved in stress response after yeast inoculation (Rossignol *et al.*, 2006). We should keep in mind that the ADWY have been already subjected to highly stressful conditions during their industrial production (Perez-Torrado *et al.*, 2005). Inoculation in a fresh fermentation medium seems to alleviate the stress exerted during biomass propagation and dehydration.

Schade *et al.* (2004) defined two groups of genes which are transcriptionally activated by cold: the early cold response genes (ECR) (time < 2 h and independent of the transcriptional activator Msn2p/Msn4p) and the late cold response (LCR) genes (time > 12 h and dependent of the transcriptional activator Msn2p/Msn4p). ECR genes were made up of genes implicated in RNA metabolism and lipid metabolism whereas LCR genes included metabolic and stress genes. Contrary to expectations, we did not detect significant differences in proteins encoded by ECR genes and, as mentioned above, stress proteins, included in the LCR genes, decreased their cellular concentration

Tps1p is a typical stress protein which is encoded by the Msn2p/Msn4p regulated-gene *TPS1* and involved in trehalose synthesis. This disaccharide plays an important role as a cell protector and it is found at high levels in ADWY because the industrial conditions of dry cell production cause it to be synthesized. We previously reported the fall in intracellular trehalose in the same wine strain one hour after inoculation (Novo *et al.*, 2003). In this instance, the decrease in this metabolite was matched by a decrease in the enzyme responsible for its synthesis.

It was surprising that Gpd1p, a key enzyme in the osmotic stress response, decreased 1h after inoculation without any further modification being detected. Two issues must be considered. The first one is the high levels of Gpd1p expected in ADWY due to drying conditions (Perez-Torrado *et al.*, 2005). These can mask the response to the high osmotic pressure in the growth media

(Rossignol *et al.*, 2006). The other issue could be again the influence of the rehydration media. Low osmotic pressure supported by the yeast during rehydration process could lead the cell to repress the glycerol catabolic pathway.

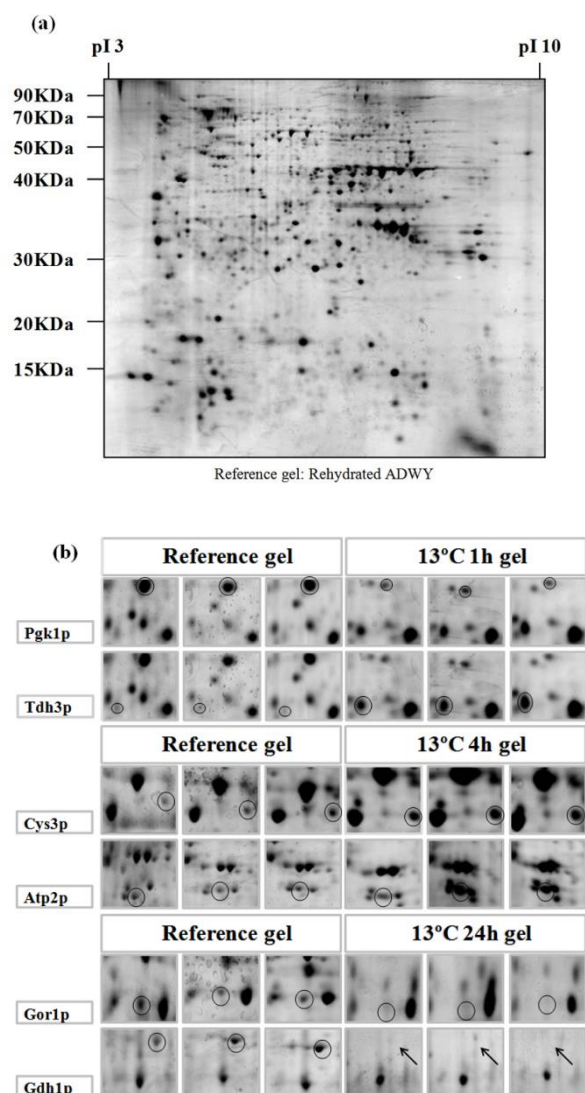


Fig 2. (a) Reference gel corresponding to ADWY after rehydrated on water 30 minutes at 37°C. (b) Evolution of some of the most representative proteins at the different time-points studied.

higher temperature, the increase of Cys3p may fit with this antioxidant response as a consequence of the low growth temperature.

The only protein with higher concentration which could be involved in oxidative stress response is the cystathionase Cys3p. This enzyme is responsible for the novo synthesis of cysteine in yeasts. Matiach & Schröder-Köhne (2001) reported that *cys3* mutant grew perfectly on rich medium at 25°C but was unable to grow on the same medium at 37°C. They provided enough evidence to indicate that this heat-sensitive phenotype was due to oxidative stress rather than simple starvation of cysteine as an amino acid for protein biosynthesis. Cysteine is a constituent of the tripeptide glutathione, the most abundant antioxidative compound in yeast. The *cys3* mutant is depleted significantly in this antioxidant. However, the level of oxidative stress was not increased by high temperature alone. A rapid downshift in the growth temperature of *S. cerevisiae* from 30°C to 10°C resulted in an increase in transcript levels of the antioxidant genes *SOD1*, *CTT1* and *GSH1* (Zhang *et al.*, 2003). Although this hypothesis should be confirmed by trials carried out in the same condition but at

Protein of Glycolysis and carbohydrate metabolism

We did not expect most of the glycolytic proteins to decrease after inoculation in a glucose-rich synthetic must. However, Rossignol *et al.* (2006) had already reported a down-regulation of most of these genes 1 h after inoculation. These authors explained this repression as a general phenomenon during the lag phase in response to new available nutrients. Conversely, most of these proteins increased their concentration 4h and 24 h after inoculation. Most of these enzymes participate in the lower part of the glycolysis, the part of the pathway that leads to ATP generation. Peter-Smits *et al.* (2000) enhanced the glycolytic flux and the fermentative capacity of *S. cerevisiae* through the simultaneous overexpression of these enzymes. The huge amount of sugars in the synthetic must might produce a high ATP demand for sugar phosphorylation (Rizzi *et al.*, 1997). The increase of enzymes from the lower part of glycolysis may balance the drain of ATP. In this strategy to pull the flux through the ATP production stage, a key protein is the glyceraldehyde 3-phosphate dehydrogenase Tdh3p. This enzyme diverts the glycolytic flux to pyruvate production *versus* the dihydroxyacetone and glycerol production. Tdh3p is the first glycolytic protein increasing (after 1 hour) and decreasing (after 24 hours) its concentration. Moreover this increase is matched by a decrease in the glycerol-3-phosphate dehydrogenase Gpd1p, a key enzyme in glycerol synthesis. Therefore, the cellular increase in Tdh3p being concomitant with a decrease in Gpd1p may be considered as a mechanism which activates the glycolytic flux.

Proteins of Nitrogen Metabolism

All the proteins related to nitrogen metabolism were reduced in the time-points studied with the exception of Cys3p. In agreement, Rossignol *et al.* (2006) also detected a repression of the genes involved in nitrogen utilization after inoculation in fermentation medium. Synthetic must is rich in ammonium and amino acids at the beginning of the process. It is known that a rich-nitrogen medium triggers a rapid catabolite repression of the main genes involved in amino acid biosynthesis (Beltran *et al.*, 2004) and that could lead to a reduction in the proteins encoded by these genes.

As mentioned above, the cellular demand of Cys3p may be more closely related to a response to oxidative stress resistance than to protein synthesis. The decrease in Met6p may be also related to the cell's need to synthesize glutathione because both Cys3p and Met6p use homocysteine as substrate for synthesizing cysteine (glutathione) and Methionine respectively.

Mitochondrial proteins

ADWY shifts from a respiratory metabolism during its industrial propagation to a fermentative metabolism during wine fermentation. The inoculation of *S. cerevisiae* cells in a medium with a high sugar concentration triggers the Crabtree effect which means that respiratory metabolism is blocked in favour of the fermentative metabolism. The down-regulation of many genes involved in oxidative metabolism, respiration, TCA cycle, glyoxylate cycle and gluconeogenesis is a typical regulation of the Crabtree effect. However several studies have proved that the Crabtree effect is not immediate and that yeast cells have a period of respirofermentative metabolism. (Postma *et al.*, 1989; Van Urk *et al.*, 1990). In our proteome analysis, we detected several proteins which produce energy through oxidative phosphorylation (Atp1p, Atp2p), mitochondrial homeostasis (Por1p) and TCA cycle (Mdh1p) in higher concentrations after 4 hours. On the other hand, most of the mitochondrial proteins which showed significant differences 24 h after inoculation were present at lower concentrations, suggesting that the change to fermentative metabolism has been completed. A clear indicator of the shift from respiratory to fermentative metabolism is the decrease in the protein Lpd1p. This protein is a component of the pyruvate dehydrogenase complex, the key enzymes in respiratory metabolism.

In conclusion, a set of proteins involved in the adaptive response of a wine *S. cerevisiae* strain to a new fermentative medium has been identified. These proteins mainly included enzymes of the nitrogen and carbon metabolism and proteins related to the cellular stress response. The ADWY inoculation in a synthetic must produced a metabolic change in the cell. However the shift from respiration to fermentation was not immediate in the inoculation because some mitochondrial proteins involved in oxidative metabolism were induced in the 1 h sample. Inoculation in this fresh medium also reduced the cellular concentration of stress proteins produced during industrial production of the ADWY. The only exception was Cys3p which might be involved in glutathione synthesis as a response to oxidative stress. Further studies should confirm the need for glutathione synthesis during the lag phase of the alcoholic fermentation. Contrary to our expectations, we did not detect growth-related proteins during the early exponential phase. A decrease in some enzymes related to amino acid biosynthesis was detected in the three samples analysed. Further transcriptional analysis should confirm the catabolite repression of the genes encoding these proteins and also determine their relation to the protein decrease. A better understanding of the yeast stress response to rehydration and inoculation will lead to improved ADWY handling efficiency in wine making and presumably better control of fermentation start up.

Acknowledgments

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Chapter 4: Functional analysis to identify genes in wine yeast adaptation to low temperature fermentation

ORIGINAL ARTICLE

Functional analysis to identify genes in wine yeast adaptation to low-temperature fermentation

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Keywords

alcoholic fermentation, genetic improvement, *ILV5* gene, proteome, *Saccharomyces cerevisiae*, temperature, yeast.

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Abstract

Aims: To identify genes and proteins involved in adaptation to low-temperature fermentations in a commercial wine yeast.

Methods and Results: Nine proteins were identified as representing the most significant changes in proteomic maps during the first 24 h of fermentation at low (13°C) and standard temperature (25°C). These proteins were mainly involved in stress response and in glucose and nitrogen metabolism. Transcription analysis of the genes encoding most of these proteins within the same time frame of wine fermentation presented a good correlation with proteomic data. Knockout and overexpressing strains of some of these genes were constructed and tested to evaluate their ability to start the fermentation process. The strain overexpressing *ILV5* improved its fermentation activity in the first hours of fermentation. This strain showed a quicker process of mitochondrial degeneration, an altered intracellular amino acid profile and laxer nitrogen catabolite repression regulation.

Conclusions: The proteomic and transcriptomic analysis is useful to detect key molecular adaptation mechanisms of biotechnological interest for industrial processes. *ILV5* gene seems to be important in wine yeast adaptation to low-temperature fermentation.

Significance and Impact of the Study: This study provides information that might help improve the future performance of wine yeast, either by genetic modification or by adaptation during industrial production.

Introduction

Temperature fluctuations are an inevitable aspect of microbial life in exposed natural environments. However, suboptimal temperatures are also common in industrial processes. Low temperatures (10–15°C) are used in wine fermentations to enhance the production and retain flavour volatiles. In this way, white and rosé wines of greater aromatic complexity can be achieved. The improved quality of wines produced at low temperatures can be attributed to greater terpene retention, a reduction in higher alcohols and an increase in the proportion of ethyl and acetate esters in the total volatile compounds (Torija *et al.* 2003; Beltran *et al.* 2008). The optimum fermentation temperature for *Saccharomyces* is between 25 and 28°C. Therefore, among the difficulties inherent to wine

fermentation (high concentration of sugars, low pH, presence of ethanol, nutrient deficiency, etc.), we should add a suboptimal temperature for the primary fermentation agent. Temperature affects both yeast growth and fermentation rate, with lower temperatures giving rise to a very long latency phase of up to 1 week or longer and sluggish fermentations (Meurgues 1996; Bisson 1999), which dramatically increase the duration of alcoholic fermentation, with the consequent energy expenditure.

Little is known about the molecular mechanisms that govern adaptation to cold, whereas the response of *Saccharomyces cerevisiae* to heat-shock stress has been investigated widely (Al-Fageeh and Smales 2006). Low temperature affects a variety of cellular processes in and the characteristics of *S. cerevisiae*. Previous studies have revealed that protein translational rates, cell membrane

Functional analysis to identify genes in wine yeast adaptation to low temperature fermentation

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Running title: Cold adaptation in a wine yeast

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Introduction

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Whereas the response of *S. cerevisiae* to heat-shock stress has been investigated widely, little is known about the molecular mechanisms that govern adaptation to cold (Al-Fageeh and Smales 2006). Low temperature affects a variety of cellular processes and characteristics in *S. cerevisiae*. Previous studies have revealed that protein translational rates, cell membrane fluidity, RNA secondary structure stability, enzymatic activity, protein folding rates and heat shock protein regulation are significantly affected (Schade *et al.* 2004; Aguilera *et al.* 2007; Tai *et al.* 2007; Pizarro *et al.* 2008). The physiological consequences of this altered state are a decreased transport, accumulation of miss-folded proteins and reduced enzyme activities. The cells respond to these modifications in their physiological and biochemical state by rapidly changing processes such as protein phosphorylation and degradation, and longer term effects involving transcriptional changes (Schade *et al.* 2004). To date, most studies have mainly been focused on the genome-wide transcriptional responses to cold-shock (Sahara *et al.* 2002; Homma *et al.* 2003; Schade *et al.* 2004; Murata *et al.* 2005). During wine fermentations, yeasts must adapt to a new medium when they are inoculated in the grape-must (osmotic-shock, pH-shock and temperature-shock). Substantial transcriptional changes have been detected in wine yeast during the lag phase or adaptation period (Rossignol *et al.* 2006; Novo *et al.* 2007). The main responses involved the activation of some genes of the fermentation pathway and of the non-oxidative branch of the pentose pathway, and the induction of a huge cluster of genes related to ribosomal biogenesis and protein synthesis. In addition to these global transcriptional analyses, Salvado *et al.* (2008) analyzed the changes in the protein profile of a wine yeast during the first 24 hours of

fermentation after inoculation in synthetic grape must. Protein changes mainly involved enzymes linked to nitrogen and carbon metabolism, and proteins related to cellular stress response. These changes, which occur during the lag phase, determine proper adaptation to the new medium and affect fermentative capacity and fermentation performance (Quain 1988; Blomberg 1997).

In the present study, we have compared changes in the proteome profile of a commercial wine yeast during the lag phase and initial exponential phase at two fermentation temperatures: optimum temperature (25 °C) and a restrictively low temperature (13 °C). This value of low temperature was used because, in a previous study with fermentations at different temperatures (Llauradó et al., 2005), the best aromatic wine profile was obtained at 13 °C. We identified 9 proteins as representing the most significant changes in proteomic maps during the first 24 hours of fermentation at low and standard temperature (25 °C). A transcription analysis was performed to correlate with the proteomic experiment. Knockout and over-expressing strains were constructed and tested to evaluate their ability to trigger fermentation. This study provides information that might help improve the future performance of wine yeast, either by genetic modification or by adaptation during industrial production.

Materials and methods

Yeast strains and growth conditions

The commercial wine yeast *Saccharomyces cerevisiae* QA23 (Lallemand S.A., Canada) was used in this study because it shows a good fermentative behavior both at low and optimum temperature (Llauradó et al., 2005). This strain was cultivated in a synthetic must (SM) prepared according to Riou *et al.* (1997) but with 200 g l⁻¹ of reduced sugars (100 g l⁻¹ glucose and 100 g l⁻¹ fructose). The Yeast Assimilable Nitrogen (YAN) content was 300 mg N l⁻¹: ammoniacal nitrogen (NH₄Cl) 120 mg N l⁻¹ and amino acids 180 mg N l⁻¹ (expressed as mg N l⁻¹: 4.65 Asp, 11.39 Glu, 10.40 Ser, 47.87 Gln, 3.05 His, 3.40 Gly, 8.87 Thr, 29.60 Arg, 22.90 Ala, 1.51 Tyr, 2.41 Cys, 5.29 Val, 2.93 Met, 11.95 Trp, 3.20 Phe, 3.47 Ile, 5.14 Leu, 1.62 Lys). This active dry wine yeast (ADWY) was rehydrated in water following the manufacturer's recommendations (30 min at 37 °C). After counting under the microscope, the appropriate dilution of the rehydrated QA23 was transferred to SM to achieve an initial cell concentration of 2 x 10⁶ cells ml⁻¹. Cell suspension was incubated at 13 °C and 25 °C with slight shaking to obtain homogeneous nutrient distribution in laboratory-scale fermentations: 2 l bottles filled with 1.8 l medium and fitted with closures that enabled carbon dioxide release and samples to be removed. Samples were collected after 1, 4 and 24 h for the proteomic analysis and at time 20 min, 40 min, 1 h, 2 h, 4 h and 24 h for the transcription analysis. The number of viable cells was monitored by plating on YPD medium.

Construction of deletion mutant and over-expressing strains

In order to simplify the generation of mutant strains, the derivative haploid *boQA23* of the wine strain was constructed by disruption of the *HO* gene and substitution by the KanMX4 cassette (Walker *et al.* 2003). The transformants were sporulated and the spores were selected for their geneticin resistance. The haploid state of spores, which had not re-diploidised due to successful disruption of *HO*, was verified by their failure to sporulate, by PCR determination of their MAT locus constitution (Huxley *et al.* 1990) and by flow cytometry (Bradbury *et al.* 2005). After a screening of twenty of these *HO* disruptants, the haploid strain most similar to the parental wine strain in terms of viability and fermentation capacity was selected to construct the mutants. The KanMX marker of the selected haploid strain *boQA23* was excised using the Cre-*lox* system. This strain was transformed with the plasmid Yep351-Cre-Cyh (Güldener *et al.* 1996), which carries the positive marker CYH^R, conferring resistance to cycloheximide, and the *CRE* gene under the control of the inducible *GAL1* promoter. Expression of the Cre recombinase was induced by shifting cells from YPD to YPGal (galactose) medium.

Knockout strains used in this study were constructed using the KanMX cassette, flanked by 50 nucleotides with homologous sequences within the ORF of each target gene (Güldener *et al.* 1996). Primers used for each gene are shown in Table 1. These primers were used to amplify the KanMX sequence from pUG6 plasmid and obtain the corresponding cassette for each target gene. Derivative haploid *boQA23* transformation was performed using a lithium acetate-based method, as described in Gietz and Woods (2002). After transformation, strain selection was done using geneticin (G418) added to YPD solid media at a concentration of 200 mg l⁻¹. Knockout strains were confirmed by PCR and real-time PCR (rt-qPCR). Over-expressing strains were obtained by using pGREG505 vector within a galactose inducible promoter (pGAL1), as described in Jansen *et al.* (2005). Briefly, the wine yeast *boQA23* was co-transformed with the *SaI* digested pGREG505 plasmid together with the PCR amplified target gene, flanked by recombination sequences homologues to the plasmid ends (primers are shown in Table 1). The lithium acetate transformation method was also used (Gietz and Woods 2002). The cells carrying the multicopy plasmid were selected by growing the transformants in YPD with geneticin (200 mg l⁻¹). Correct plasmid ligation and insertion was confirmed by PCR. During over-expression constructions, *HIS3* fragment, within pGREG505 plasmid, was replaced by target gene.

Table 1 Primers used in this study

Target gene	Primer (FW)	Primer (RV)
<i>GDH</i>^a	GCATCGTTTACGATTGGCTGGATAAGAGGATTT CATAAGGAATATGATG <u>CCGTACGCTGCAGGTCGAC</u> △	ACGAGTAAGGTCATCAATAAGCCTGGTGTC AATCGATGCTTACATACATA <u>ACTAGTGGATCTGATA</u> TC
<i>HSP12</i>^a	ATGTCTGACGCAGGTAGAAAAGGATTCGGTGA AAAAGCTTCTGAAGCTTTC <u>CGTACGCTGCAGGTCGAC</u> △	TTACTTCTGGTTGGGTCTTCTTACCCTGGA CACGACCGGAAACATATT <u>ACTAGTGGATCTGATA</u> TC
<i>HSP26</i>^a	ACAAATTAACATGTCATTTAAACAGTCCATTTTTGAT TTCTTTGACAACACGT <u>TACGCTGCAGGTCGACA</u>	TTCTTGAGAAAGAAACCTCAATCTTCTTACGTTGGT TCTTACCATCCTTCA <u>ACTAGTGGATCTGATATC</u>
<i>PDC1</i>^a	TTTTGGTGGTTCCGGCTTCCTTCCCGATTCCGCC CGCTAA ACGCATATTT <u>CGTACGCTGCAGGTCGACA</u>	GGGTTTTGGAAACCACACTGTTTAAACAGTGT TCCTTAATCAAGGATACC <u>ACTAGTGGATCTGATAT</u> C
<i>TDH1</i>^a	CGCCAGTAGGGTGTGTTGAGCTTAGTAAAAATGT GCGCACCAAGCCTAC <u>CGTACGCTGCAGGTCGAC</u> △	AGGATAATGCGTTCCTTGGCAATAGTCACATA TTGTGGGTATGTGCGTTT <u>ACTAGTGGATCTGATA</u> TC
<i>YHB1</i>^a	CTGGTACATTTTCCCAGAGTACCAGCAAAGGA GGCATCCTTGATGCCTAC <u>GTACGCTGCAGGTCGAC</u> △	TGGACTGGCGTTAACGTGAAGTGAACCTCTGT TTAGCACCTGGCGTTAT <u>ACTAGTGGATCTGATA</u> TC
<i>GDH</i>^b	<u>GAATTCGATATCAAGCTTATCGATACCGTCGAC</u> △ATGTCAGAGCCAGAATTTCAACAAG	<u>GCGTGACATAACTAATTACATGACTCGAGGTC</u> <u>GACTTAAAATACATCACCTTGGTCAAAAC</u>
<i>HSP12</i>^b	<u>GAATTCGATATCAAGCTTATCGATACCGTCGAC</u> △ATGTCTGACGCAGGTAGAAAAGGA	<u>GCGTGACATAACTAATTACATGACTCGAGGTC</u> <u>GACTTACTTCTTGGTGGGTCTTCTTCA</u>
<i>HSP26</i>^b	<u>GAATTCGATATCAAGCTTATCGATACCGTCGAC</u> △ATGTCATTTAACAGTCCATTTT	<u>GCGTGACATAACTAATTACATGACTCGAGGTC</u> <u>GACTTAGTTACCCACGATTCCTG</u>
<i>ILV5</i>^b	<u>GAATTCGATATCAAGCTTATCGATACCGTCGAC</u> △ATGTTGAGAACTCAAGCCG	<u>GCGTGACATAACTAATTACATGACTCGAGGTC</u> <u>GACTTTTATTTGGTTTTCTGGTCTCAAAC</u>
<i>PDC1</i>^b	<u>GAATTCGATATCAAGCTTATCGATACCGTCGAC</u> △ATGTCTGAAATTAATTTGGGTTAA	<u>GCGTGACATAACTAATTACATGACTCGAGGTC</u> <u>GACTTTTATTTGCTTAGCGTTGGTAGC</u>
<i>TDH1</i>^b	<u>GAATTCGATATCAAGCTTATCGATACCGTCGAC</u> △GAATTGCTATTAACGGTTCGGT	<u>GCGTGACATAACTAATTACATGACTCGAGGTC</u> <u>GACTTAAGCCTTGGCAACATATTCG</u>
<i>YHB1</i>^b	<u>GAATTCGATATCAAGCTTATCGATACCGTCGAC</u> △ATGCTAGCCGAAAAAACCCGTT	<u>GCGTGACATAACTAATTACATGACTCGAGGTC</u> <u>GACTTCTCAAACCTGTGAAATCGAAC</u>
<i>ACT1</i>^c	TGGATTCCGGTGATGGTGTT	CGGCCAAATCGATTCTCAA
<i>GDH</i>^c	TGAAACTGGTATCACCTCCGAACA	TCGTTGACGATTTGTTCCAAGGACT
<i>HSP12</i>^c	GGCAGACCAAGCTAGAGATTACATG	AACATATTCGACGGCATCGTT
<i>HSP26</i>^c	CTGGTGAAATCCATCTACCTTGA	TTACCAGAGCTGCTCTCCCTTGAAC
<i>ILV5</i>^c	TGTTCTTGGCTCAATACGACGTC	TCGACGGTTCGTTGAAAAGCT
<i>PDC1</i>^c	CACTTTTCCAGGTGTCCAAA	GGACAGCAAACCTGGCTTGTAA
<i>TDH1</i>^c	AAGAAGGCTGTTAAGGCTGC	CAACGGCATCTTCGGTGTAA
<i>YHB1</i>^c	CAAATGCCTTGGCCACTACT	GACATGGTCCATAAGCACAGAC

^a Primers used for Knockout cassette amplification. Underlining indicates homology to the *loxP-kanMX4-loxP* cassette from plasmid pUG6. The remainder sequences of the primers are homologous to the flanking region of the deleted ORF

^b Primers used for over-expressing strain construction. Underlining indicates the recombination sequences homologues to the plasmid ends

^c Primers used for transcriptomic analysis with rt-qPCR.

For over-expressing strains, the reference or control strain used in the different experiments was the haploid strain *hoQA23*, with the plasmid pGREG505 without the *HIS3* fragment (*hoQA23pGREG*). The overexpression of these genes was tested by rt-qPCR. All the strains (mutant and overexpressing) constructed in this study are shown in Table 2.

Table 2 Original and constructed strains in this study

Strain	Genotype	Definition
QA23	MATa/MAT α	Wine commercial strain
<i>hoQA23</i>	MAT α ; YDL227C::kanMX4	Derivative wine haploid strain
Δ <i>gdh1</i>	<i>hoQA23</i> ; YOR375C::kanMX4	<i>GDH1</i> mutant strain
Δ <i>hsp12</i>	<i>hoQA23</i> ; YFL014W::kanMX4	<i>HSP12</i> mutant strain
Δ <i>hsp26</i>	<i>hoQA23</i> ; YBR072W::kanMX4	<i>HSP26</i> mutant strain
Δ <i>pdcl</i>	<i>hoQA23</i> ; YLR044C::kanMX4	<i>PDC1</i> mutant strain
Δ <i>tdh1</i>	<i>hoQA23</i> ; YJL052W::kanMX4	<i>TDH1</i> mutant strain
Δ <i>yhb1</i>	<i>hoQA23</i> ; YGR234W::kanMX4	<i>YHB1</i> mutant strain
Δ <i>ure2</i>	<i>hoQA23</i> ;YNL229C::kanMX4	<i>URE2</i> mutant strain
<i>hoQA23</i> - pGREG	<i>hoQA23</i> -pGREG505	Haploid strain with empty plasmid
p <i>Gall</i> - <i>GDH1</i>	<i>hoQA23</i> -pGREG <i>GDH1</i>	<i>GDH1</i> overexpressing strain
p <i>Gall</i> - <i>HSP12</i>	<i>hoQA23</i> -pGREG <i>HSP12</i>	<i>HSP12</i> overexpressing strain
p <i>Gall</i> - <i>HSP26</i>	<i>hoQA23</i> -pGREG <i>HSP26</i>	<i>HSP26</i> overexpressing strain
p <i>Gall</i> - <i>ILV5</i>	<i>hoQA23</i> -pGREG <i>ILV5</i>	<i>ILV5</i> overexpressing strain
p <i>Gall</i> - <i>PDC1</i>	<i>hoQA23</i> -pGREG <i>PDC1</i>	<i>PDC1</i> overexpressing strain
p <i>Gall</i> - <i>TDH1</i>	<i>hoQA23</i> -pGREG <i>TDH1</i>	<i>TDH1</i> overexpressing strain
p <i>Gall</i> - <i>YHB1</i>	<i>hoQA23</i> -pGREG <i>YHB1</i>	<i>YHB1</i> overexpressing strain

Protein extraction and two-dimensional electrophoresis

Protein extracts were prepared as described in Blomberg (2002). Briefly, this was as follows: cell suspension was vortexed for 4 x 30 seconds with glass beads containing PMSF as a protease inhibitor (placed on ice between vortexing) and subsequently boiled for 5 min with SDS/mercaptoethanol buffer. Following nuclease treatment of the cells, protein contents of the extract were estimated using a 2-D quant kit (Amersham Pharmacia Biotech, USA). Soluble proteins were run in the first dimension using a commercial horizontal electrophoresis system (Multiphor II; Amersham Pharmacia Biotech, USA). Forty-five micrograms of protein from whole-cell lysates were mixed with immobilized pH gradient (IPG)-rehydration buffer (8 M urea/2% NP-40/10 mM DTT; final volume of 500 μ l) and loaded onto polyacrylamide strips.

The IPG strips with a non-linear pH gradient 3-10 were allowed to rehydrate overnight. Samples were run at 50 μ A per strip, in the first step voltage was ramped to 500 V over a period of 5 h, kept at 500 V for 5 h more, ramped again to 3500 V over a period of 9.5 h, and finally kept at 3500 V for 5 h. After the first dimension, IPG strips were then equilibrated twice for 15 min in equilibration solution (0.05 M Tris-HCl, pH 8.8, 6 M urea, 30% v/v Glycerol, and 2% w/v SDS), first with 65 mM DTT (reduction step), and finally with 135 mM iodoacetamide (alkylation). The second dimension was carried out in a vertical electrophoresis system (Ettan DALTsix; Amersham Pharmacia Biotech, USA) in a 12.5% (26 cm x 20 cm x 1 mm) polyacrylamide gel where proteins were separated based on molecular size. Electrophoresis conditions were 1 W per gel until the dye front reached the bottom of the gel. Sets of three gels were used for each sampling-time.

Silver staining protocol and image analysis

The staining protocol was as described by Blomberg (2002). Gels were scanned using an Image Scanner UMAX, Amersham (300 dpi, 12-bit image) enabling the acquisition of spot intensities in pixel units. Images were analyzed using ImageMaster 2D Platinum (GE Healthcare, USA). Normalization was performed with the aforementioned software based on the total gel density to compensate differences in images caused by variations in experimental conditions (e.g. protein loading or staining). Spots detection was performed with ImageMaster 2D Platinum automated spot detection algorithm. The gel image with the greatest number of spots and the best protein pattern was chosen as a reference template for the image analysis. Spots in the standard gel were then matched across each 2DE gel at a reference temperature (25 °C) for each sampling time (1, 4 and 24 hours after inoculation in the media). Matching features of the software were used to relate and compare the sets of gels. Finally, to achieve maximum reliability and robustness of the results, the Student's t-test was performed allowing us to identify those sets of proteins displaying a statistically significant difference with the confidence level set at 95%.

In-gel protein digestion

Protein spots of interest were excised manually and then digested automatically using a Proteineer DP protein digestion station (Bruker-Daltonics, Germany) according to Schevchenko *et al.* (1996) with minor variations. Gel plugs were reduced with 10 mM dithiothreitol (Amersham Biosciences, Sweden) in 50 mM ammonium bicarbonate (99.5% purity; Sigma Chemical, USA) and alkylated with 55 mM iodoacetamide (Sigma Chemical, USA) in 50 mM ammonium bicarbonate. The gel pieces were then rinsed with 50 mM ammonium bicarbonate and acetonitrile (gradient grade; Merck, Germany) and dried under a stream of nitrogen. Modified porcine trypsin (sequencing grade; Promega, USA) at a final concentration of 13 ng μ l⁻¹ in 50 mM

ammonium bicarbonate was added to the dry gel pieces and digestion proceeded at 37 °C for 6 h. Finally, 0.5% trifluoroacetic acid (99.5% purity; Sigma Chemical, USA) was added for peptide extraction.

MALDI-MS(/MS) and database search

An aliquot of the above digestion solution was mixed with an aliquot of α -cyano-4-hydroxycinnamic acid (Bruker-Daltonics, Germany) in 33% aqueous acetonitrile and 0.1% trifluoroacetic acid. This mixture was deposited onto a 600 μ m AnchorChip MALDI probe (Bruker-Daltonics, Germany) and allowed to dry at room temperature. MALDI-MS(/MS) data were obtained using an Ultraflex time-of-flight mass spectrometer (Bruker-Daltonics, Germany) equipped with a LIFT-MS/MS device (Suckau *et al.* 2003). Spectra were acquired in the positive-ion mode at 50 Hz laser frequency, and 100 to 1500 individual spectra were averaged. For fragment ion analysis in the tandem time-of-flight (TOF/TOF) mode, precursors were accelerated to 8 kV and selected in a timed ion gate. Ionized fragments generated by laser-induced decomposition of the precursor were further accelerated by 19 kV in the LIFT cell and their masses were analyzed after passing the ion reflector. Measurements were in part performed using post-LIFT metastable suppression, which allowed the removal of the precursor and metastable ion signals produced after extraction from the second ion source. Peptide mass mapping data was analyzed in detail using flex Analysis software (Bruker-Daltonics, Germany). MALDI-TOF mass spectra were calibrated internally using two trypsin autolysis ions with $m/z = 842.510$ and $m/z = 2211.105$; for MALDI-MS/MS, spectra were calibrated with fragment ion spectra obtained for the proton adducts of a peptide mixture covering the 800-3200 m/z region. MALDI-MS and MS/MS data were combined through the MS BioTools program (Bruker-Daltonics, Germany) to search the NCBI database using Mascot software (Matrix Science, UK) (Perkins *et al.* 1999).

RNA extraction and cDNA synthesis

Total RNA was isolated from yeast samples as described by Sierkstra *et al.* (1992) and re-suspended in 50 μ l of DEPC-treated water. Total RNA suspensions were purified using the High Pure Isolation kit (Roche Applied Science, Germany) following the protocol provided by the manufacturer. RNA concentrations were determined using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, USA) and the quality of RNA was verified electrophoretically on 0.8% agarose gels. Solutions and equipment were treated so that they were RNase free, as outlined in Amberg *et al.* (2005).

Total RNA was reverse-transcribed with SuperscriptTM II RNase H⁻ Reverse Transcriptase (Invitrogen, USA) in a GenAmp PCR System 2700 (Applied Biosystem, USA). Then, 0.5 μ g of

Oligo (dT)₁₂₋₁₈ Primer (Invitrogen, USA) was used with 0.8 µg of total RNA as template in a reaction volume of 20 µl. Following the protocol provided by the manufacturer, after denaturation at 70 °C for 10 min, cDNA was synthesized at 42 °C for 50 min. Finally, the reaction was inactivated at 70 °C for 15 min.

Real-time quantitative PCR

The PCR primers used in this study are listed in Table 1. The primers were all designed with the available GenBank sequence data and the Primer Express software (Applied Biosystems, USA) in accordance with the Applied Biosystems guidelines for designing PCR primers for quantitative PCR with the exception of the housekeeping gene *ACT1*, previously described by Beltran *et al.* (2004). All amplicons were shorter than 100 bp, which ensured maximal PCR efficiency and, therefore, the most precise quantification.

For each gene, a standard curve was made with yeast genomic DNA. DNA extraction was performed as described by Querol *et al.* (1992), digested by RNase and isolated by two-fold phenol-chloroform extractions and ethanol precipitation. Concentration was determined using a GeneQuant spectrophotometer (Pharmacia, Canada). Serial 10-fold dilutions of DNA were conducted to yield DNA concentrations from 400 to 4×10^{-2} ng µl⁻¹. These dilution series were amplified (in triplicate) by SYBR PCR for each gene to obtain standard curves (see above). The standard curve displays the Ct value *vs.* log 10 of the starting quantity of each standard. The starting quantity of the unknown samples was calculated against the standard curve by interpolation. Gene expression levels are shown as the concentration of the studied gene normalized with the concentration of the housekeeping *ACT1* gene.

The rt-qPCR reaction was performed using SYBR[®] Green I PCR (Applied Biosystems, USA). The 25 µl SYBR PCR reactions contained 300 nmol l⁻¹ of each PCR primer, together with 1 µl cDNA (or 5 µl of each DNA serial dilution for standard tubes) and one time SYBR master mix (Applied Biosystems, USA).

All PCR reactions were mixed in 96-well optical plates (Applied Biosystems, USA) and cycled in a PE Applied Biosystems 5700 thermal cycler under the following conditions: 50 °C for 2 min, 95 °C for 10 min, and 40 cycles at 95 °C for 15 s and at 60 °C for 60 s. All samples were analyzed in triplicate and the expression values were averaged using analysis software (Applied Biosystems, USA).

Determination of fermentation activity

Fermentation activity of deletion mutant and over-expressing strains was analyzed after inoculation in a synthetic grape must (described above) with a population size of 2×10^6 cells ml⁻¹

¹. To obtain this inoculum, the deletion mutant strains were grown in YPD overnight at 28 °C and the over-expressing strains were grown in YPGal with geneticin to induce synthesis of the over-expressed gene at 28 °C. Fermentation volume for all the strains was 400 ml of media in 500 ml bottles. The consumption of the initial 15% of sugars was monitored by measuring the relative density of the media (g l^{-1}) using a Densito 30PX densitometer (Mettler Toledo, Switzerland).

Intracellular amino acid determination

A total of 10^8 cells cultured in YPGal were harvested and washed twice with miliQ water. After that, cells were suspended in 500 μl of miliQ water and incubated at 100 °C for 15 min, centrifuged at 13000 rpm for 10 min at 4 °C. The supernatant was collected and used for analysis.

Amino acids and ammonium ion were determined simultaneously by the method of Gómez-Alonso *et al.* (2007). Briefly, samples (400 μl) were derivatized by 15 μl of diethylethoxymethylenemalonate (Fluka, Germany) in presence of 700 μl of 1M borate buffer (pH 9), 300 μl of methanol and 10 μl of L-2-aminoadipic acid (Internal Standard, 1 g l^{-1}) over 30 min in an ultrasound bath. Then the samples were treated at 80 °C for 2 hours. The analyses were performed on a Agilent 1100 Series HPLC (Agilent Technologies, Germany) comprising a quaternary pump, an autosampler and a multiple wavelength detector at 269, 280 and 300 nm. Nitrogen compound separation of sample (50 μl) was carried out using a 4.6 x 250 mm, 5 μm ACE C18-HL column (Symta, Spain) with a guard column (ACE5 C18-HL) through a binary gradient (Gómez-Alonso *et al.* 2007) at a flow of 0.9 ml min^{-1} . The different nitrogen compounds were identified according to the retention time of corresponding standards and were quantified using the internal standard method.

Mitochondrial staining

DiOC₆ (Invitrogen, USA) was used to stain mitochondria of living cells (Koning *et al.* 1993). Staining protocol and DiOC₆ working concentration were according to manufacturer recommendations. Briefly, $10^6 \text{ cells ml}^{-1}$ were resuspended in 10 mM HEPES buffer, pH 7.4, containing 5% glucose. DiOC₆ was added up to a final concentration of 175 nM. Cells were observed under fluorescence microscopy Leica DM4000 B (Leica Microsystems, Germany) after a room-temperature incubation period of 15 min.

Statistical analysis

Proteomic and transcription data are the result of three independent fermentations at 13 °C and 25 °C. Statistical test for 2DE image analysis was performed using the Student's t-test module of ImageMaster 2D Platinum, with confidence level set at 95%. Significant differences

among strains were checked by means of an analysis of variance, using the one-way ANOVA module of the statistical software package SPSS 13.0 (IBM Corporation. USA). Fermentations with the mutant and over-expressing strains were also repeated at least three times.

The software “MeV 4.8.0 (Multiple Experiment Viewer)” (Madison, USA) has been used to perform a heat-map representing the ratio between gene transcription and protein concentration at both temperatures.

Results

Effect of fermentation temperature on changes in proteome profile

We have compared the changes in proteome profile of the commercial wine strain QA23 after inoculation in a synthetic grape must at two different temperatures, 13 and 25 °C. The differences in protein composition between temperatures were obtained after the comparison between gels from the same time-points after inoculation (1, 4 and 24 h) and by applying a restrictive statistical analysis to obtain a reduced and reliable set of proteins with clear differences between both temperatures (only proteins whose concentration was modified at least twofold) (Fig. 1). There were no significant differences in proteins after 1 h and only Hsp12p presented a higher cellular concentration after 4 h at low temperature (Fig. 1). The remaining proteins with statistically significant differences were observed in the 24-hour sample, including Hsp12p. Most of these proteins can generally be grouped into three main categories: glucose metabolism (Adh1p, Pdc1p and Tdh1p), stress response (Hsp12p, Hsp26p and Yhb1p) and nitrogen metabolism (Gdh1p and Ilv5p). The elongation factor Eft2p, involved in ribosomal translocation during protein synthesis, also showed significant differences between both temperatures.

GENE	FOLD CHANGE	25°C	13°C	PROTEIN NAME	FUNCTION
Glucose metabolism					
ADH1	2.2			Alcohol dehydrogenase	Alcohol dehydrogenase (NAD) activity and methylglyoxal reductase (NADH-dependent) activity
PDC1	0.001			Pyruvate decarboxylase	Pyruvate decarboxylase activity
TDH1	-2.6			Glyceraldehyde-3-phosphate dehydrogenase	Glyceraldehyde-3-phosphate dehydrogenase (phosphorylating) activity
Stress response					
HSP12*	2.2			Heat Shock Protein 12	Molecular function unknown
HSP26	-3.8			Heat Shock Protein 26	Unfolded protein binding
YHB1	4.0			Yeast hemoglobin-like protein	Nitric oxide reductase activity
Nitrogen metabolism					
GDH1	-3.2			Glutamate dehydrogenase	Glutamate dehydrogenase (NADP+) activity
ILV5	2.3			Acetohydroxyacid reductoisomerase	Ketol-acid reductoisomerase activity and mitochondrial DNA stabilization
Elongation factor					
EFT2	4.1			Elongation factor 2	Translation elongation factor activity

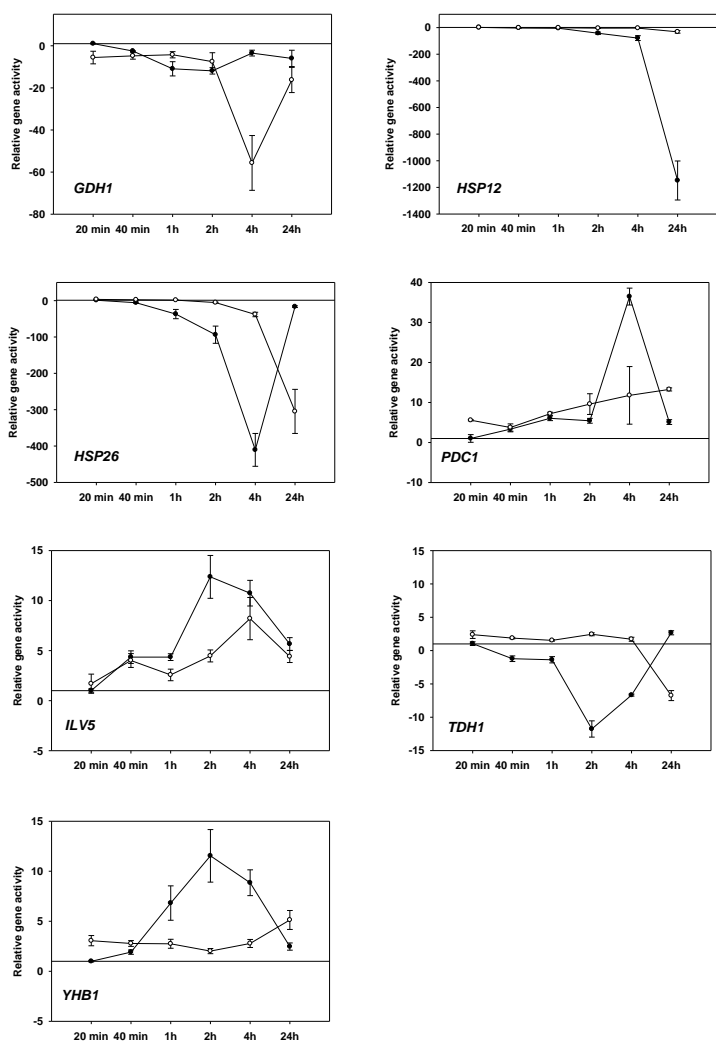
* Protein with different amount in the 4 h sampling point.

Figure 1 Proteins with significant differences of concentration between both temperatures at the same time-points after inoculation (1, 4 and 24 h). Positive and negative values represent higher and lower concentration at 13 °C after 24 h, respectively.

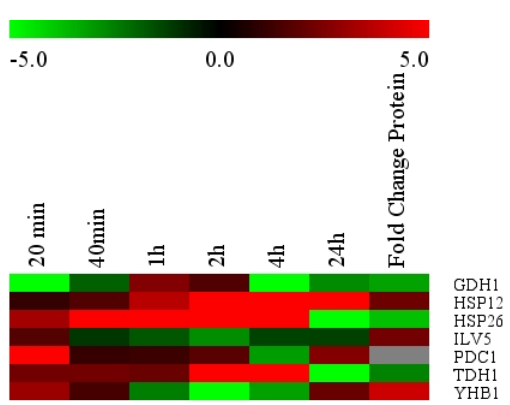
Transcription analysis of selected genes

To check whether differences in protein concentration were also correlated with differences in transcription activity, we also analyzed the relative expression of some selected genes encoding these proteins at both temperatures. Several representatives of each functional category were chosen. The genes analyzed were: *GDH1*, *HSP12*, *HSP26*, *ILV5*, *PDC1*, *TDH1* and *YHB1* (Fig. 2A). We have also represented the ratio 13 °C/25 °C of these relative gene activities in a heat map (Fig. 2B). In order to make easier the comparison between transcripts and protein levels, the last column of this heat map also represents the same ratio of the protein concentration (fold change of Fig. 1).

Generally speaking, we detected a positive correlation between both transcription and translation (Fig. 2B), despite an evident delay in some cases between increased gene activity and protein concentration. The mRNA of the stress proteins, *HSP12* and *HSP26*, decreased at both temperatures from the first sampling time-point until 24h after inoculation. This decrease was faster at 25 °C than at 13 °C, which resulted in increasing values of the ratio 13 °C/25 °C.



A



B

Figure 2 Transcription analyses of selected genes. A) Relative gene expression of the selected genes at different stages of alcoholic fermentation at 25 °C (filled circles) and 13 °C (open circles). Changes in gene activity are shown relative to the expression of the 20-minute sampling time-point at 25 °C (set as value 1). B) Heat map depicts the ratio of the gene expression and protein concentration at 13 °C/25 °C. Positive (red) and negative (green) values represent higher and lower gene expression (protein concentration) at 13 °C, respectively.

Accordingly, Hsp12p was not detected at 25 °C while it was still detectable at 13 °C after 24 hours. At the last sampling time-point (24 h), *HSP26* gene activity increased at 25 °C (ratio 25 °C/13 °C of 18.30). This increase in gene activity also correlated with a protein concentration 3.8 times higher at 25 °C than at 13 °C (Fig. 1). Conversely, the other stress gene *YHB1* showed a stepped increase initially and a decrease after 24 hours at 25 °C. At 13 °C, this increase was smaller initially, although this gene was more active after 24 hours. Again, this higher transcriptional activity correlated with a higher protein concentration at 13 °C after 24 hours.

The *PDC1* gene showed an up-regulation after yeast inoculation at both temperatures. This increase in activity occurred faster at 25 °C, reaching a maximum in the 4h-sample and dropping again in the 24h-sample. Conversely, the maximum activity in cells growing at low temperature was detected in the 24h-sample. In this case, protein differences were observed 24h after inoculation and did not correlate with RNA levels because a higher concentration was detected at 25 °C. Meanwhile, *TDH1* showed a clear correlation between RNA levels and protein concentration after 24 h of inoculation. Tdh1p concentration was 2.6-fold higher at 25 °C while the *TDH1* RNA level was over 18 times higher in the same cells growing at 25 °C. At the other sampling time-points, this gene was down-regulated after inoculation and this decrease was faster at 25 °C.

Transcription levels of nitrogen metabolism-related genes showed different profiles. Maximum *GDH1* activity was detected in the first samples after inoculation. However this gene activity was higher at 25 °C than 13 °C, which correlated with higher protein concentrations at this temperature. *ILV5* showed an up-regulation trend at all sampling time-points, with higher activity at 25 °C. However, protein concentration was higher at low temperature in the 24-hour sample.

Phenotypic evaluation of deletion mutant and over-expressing strains

Once proteins with differential concentration at both temperatures had been identified, the importance of the genes encoding these proteins was determined by deleting or over-expressing these genes in a wine strain, with the exception of $\Delta ilv5$, which was unviable in this genetic background. In order to simplify the generation of mutant strains, we constructed the derivative haploid *boQA23* of the wine strain. This haploid strain did not differ from the wild-type strain QA23 in terms of growth and fermentation rate (data not shown). Transcriptional activity in all mutant strains was determined during the pre-culture in YPD and, as expected, no transcriptional activity was detected. Moreover, the over-expressed genes were controlled by a GAL promoter, which was strongly induced during the pre-culture of the over-expressing strains (pre-culture in YPGal). However, these genes were repressed when strains were inoculated in the synthetic

grape-must, due to the high glucose concentration. As mentioned above, we were interested in the adaptation of the wine yeast to the grape must after inoculation. Thus, with this strategy, the over-expressing strains are enriched in a specific protein during pre-culture, allowing a better adaptation and giving rise to the differential phenotype during the first hours of wine fermentation. Transcriptional activity in all over-expressing strains was also determined during the pre-culture in YPGal and all of them showed an increase in activity ranging from 12-15 times more than the control strain with the empty plasmid pGREG505 (data not shown).

Both mutant and over-expressing strains were phenotypically evaluated according to their fermentation capacity in the first hours of fermentation. To this aim, it was determined the time needed to consume 15% of total sugars in a synthetic grape must during fermentation at 25 °C and 13 °C (Fig. 3). Curiously, all the tested strains showed worse fermentation performance than the reference strains (*hoQA23* for the mutants and *hoQA23pGREG* for the over-expressing strains) at 25 °C. Most of these strains (both mutant and over-expressing) also fermented worse at 13 °C but some of them improved their fermentation capacity at this temperature. The only strain showing significant better fermentation performance was the *pGAL1-ILV5*.

Effect of ILV5 overexpression on nitrogen metabolism and mitochondrial organization

As the *pGAL1-ILV5* strain showed an improved fermentation activity, we decided to perform a more in-depth phenotypic analysis of this strain. *ILV5* is a gene that encodes a mitochondrial protein (Acetohydroxyacid reductoisomerase) involved in branched-chain amino acid (BCA) biosynthesis of leucine, iso-leucine and valine (Zelenaya-Troitskaya *et al.*1995). Thus we analyzed the effect of *ILV5* overexpression on the intracellular amino acid profile. Surprisingly, BCA concentrations were not modified compared to the control strain *hoQA23pGREG*, whereas glutamate, glutamine, arginine and proline were found in higher concentrations in this control strain. On the other hand, alanine and γ -Aminobutyric acid (GABA) were present in higher concentrations in the *pGAL1-ILV5* strain (data not shown).

Intracellular glutamine concentration has been related to the triggering of Nitrogen Catabolite Repression (NCR). NCR involves transcriptional regulation by four members of the GATA family of transcription factors, as well as the regulatory protein Ure2 (Cooper 2002). A glutamine signal activates Ure2, which binds Gln3, retaining it in the cytoplasm and preventing the activation of nitrogen-regulated genes. Conversely, lower levels of cytoplasmic glutamine would induce dissociation of Gln3 from Ure2, dephosphorylation of Gln3, and importation of Gln3 into the nucleus, causing derepression (activation of genes involved in the transport and metabolism of the poorer nitrogen sources) (Magasanik and Kaiser 2002).

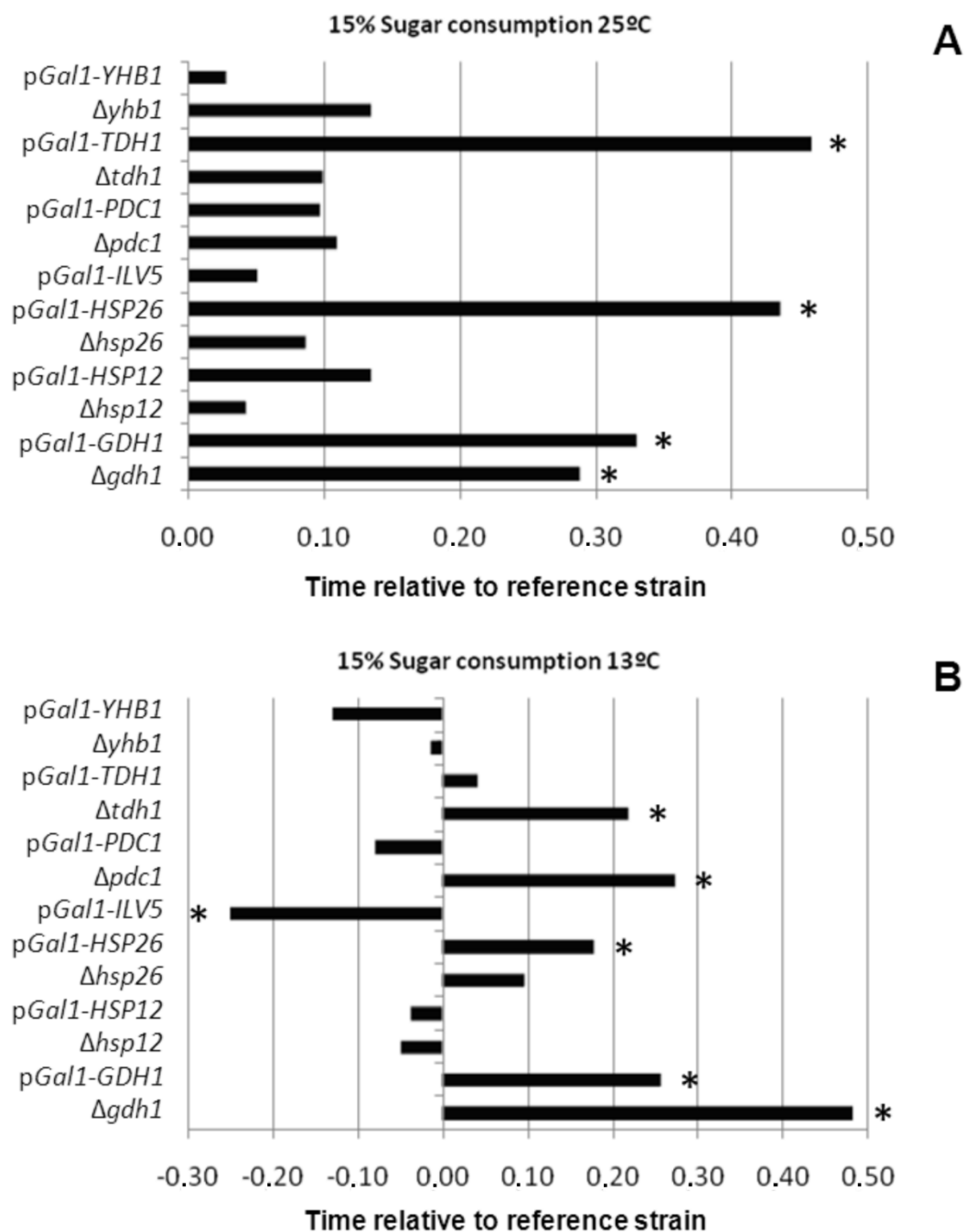


Figure 3 Phenotypic evaluation of deletion mutant and over-expressing strains. Relative values to reach 15% of sugar metabolization by tested strains in synthetic must at 25 (Upper panel, A) and 13 °C (Lower panel, B). Time zero represents the amount of time required by the reference strains (*boQA23* for the mutants and *boQA23pGREG* for the over-expressing strains) to reach 15% of sugar consumption. Negative values represent a shorter time, therefore positive values correspond to longer time. *Significant differences by ANOVA with p-value ≤ 0.05 .

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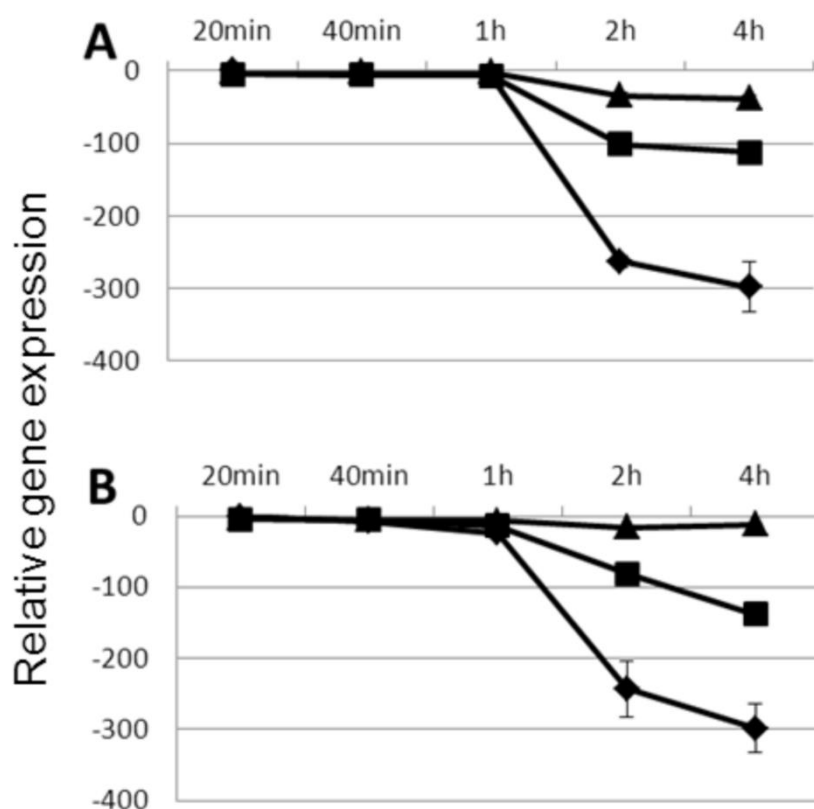


Figure 4 Relative gene expression of the NCR marker genes *GAP1* (Upper panel, A) and *MEP2* (Lower panel, B). Control strain *boQA23pGREG* (◆), *pGAL1-ILV5* (■) and $\Delta ure2$ (▲) strains. These values are relative to gene expression of the sample at time 20 min in the control strain.

Therefore we should expect a more nitrogen-derepressed situation in the *pGAL1-ILV5* strain than in the control strain *boQA23pGREG*. To infer the effect of *ILV5* overexpression on nitrogen regulation we monitored expression levels of two marker genes affected by NCR, *GAP1* and *MEP2*. These genes are strongly repressed in the presence of rich nitrogen sources in the wine yeast used in this study (Beltran *et al.* 2004). We also deleted the *URE2* gene of the *boQA23*

strain ($\Delta ure2$) for use as a NCR derepressed control (Fig. 4). As expected, both genes were repressed one hour after inoculation of the control strain in the fermentation medium. This repression was practically nonexistent in the $\Delta ure2$ strain, while these genes were also repressed in the pGAL1-ILV5 strain, although this repression was much more moderate than in the reference strain. However, Ilv5p is considered a bifunctional protein because it is not only involved in BCA biosynthesis but also plays a role in maintaining mtDNA stabilization (Zelenaya-Troitskaya *et al.* 1995; Bateman *et al.* 2002; Macierzanka *et al.* 2008). We also analyzed mitochondrial organization during the first 24 h of fermentation in the pGAL1-ILV5 and reference strain *hoQA23pGREG* by simple DiOC₆ staining (Fig. 5). In the reference strain, very well-structured mitochondria could be observed in the first two samples (0 and 4 hours after inoculation) whereas these mitochondria developed into unorganized structures in the latter samples (8 and 24 hours). On the other hand, the pGAL1-ILV5 strain showed these unstructured mitochondria from time zero (just before inoculation).

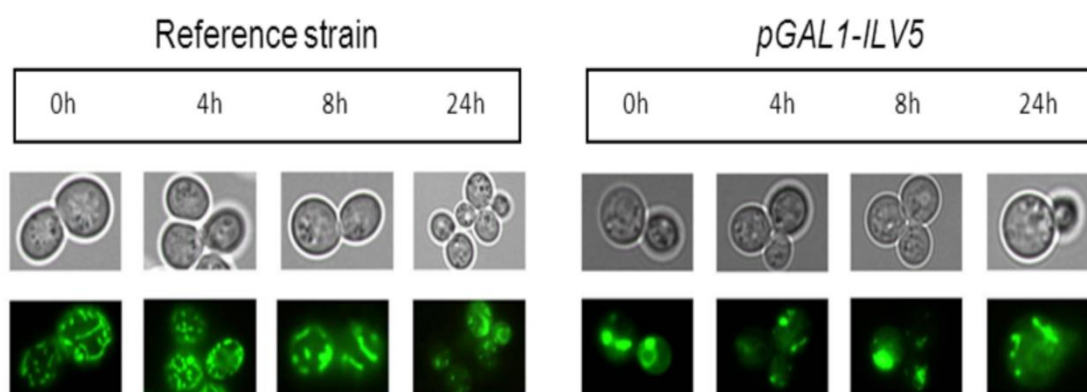


Figure 5 Mitochondrial organization in the strains *hoQA23-pGREG* (reference strain) and pGAL1-ILV5 during the first 24 h of a synthetic grape-must fermentation at 25 °C (DiOC₆ staining).

Discussion

In this study, we were interested in the ability of yeast to adapt to the fermentation medium after inoculation. To this end, first the proteome of a commercial wine yeast strain was compared in the first hours of fermentation at optimum (25 °C) and low temperature (13 °C). The time-frame studied comprised the first 24 hours of fermentation. This period covered the lag and initial log phase of the wine yeast after inoculation. A previous study (Salvadó *et al.* 2008) analyzed the changes occurring in the rehydrated yeast protein profile during the first hours after inoculation under oenological-like conditions at low temperatures. Salvadó *et al.* (2008) detected changes in around 50 proteins, which reflected degradation or modifications in proteins present in the rehydrated yeasts. In the present study, we aimed to identify proteins whose concentration

depended exclusively on the fermentation temperature by comparing the proteome at the same time-points at both temperatures. In this case, we only detected nine proteins with statistically different concentrations, which are mainly involved in stress response and in glucose and nitrogen metabolism. Most of these proteins were also transcriptionally studied during the same time frame of wine fermentation to assess the connection between transcription and translation mechanisms.

In a global transcriptional study of a wine yeast strain during alcoholic fermentation, Rossignol *et al.* (2003) described down-regulation of the stress genes, regulated by the general stress transcription factors Msn2/Msn4, during the exponential (growth) phase and an induction of these genes on entering the stationary phase. The slower growth of cells at low temperature explained the weaker repression of the genes *HSP12* and *HSP26*. Hsp12 was not induced at any of the time-points studied, thus leading to higher protein concentrations 4 and 24 hours after inoculation at 13 °C. Hsp12 is a small heat-shock protein, which is induced under different conditions, such as low and high temperatures, osmotic or oxidative stress and high sugar or ethanol concentrations (Praekelt and Meacock 1990). Recently Pacheco *et al.* (2009) has shown the role played by this protein in freezing-stress tolerance of yeast. Our results also indicate a role in the adaptation to mild cold stress. The other stress protein with high concentrations at low temperatures is Yhb1. This protein plays a role in oxidative and nitrosative stress responses but so far has not been linked to cold adaptation. However, it has previously been reported (Zhang *et al.* 2003) that a rapid downshift in the growth temperature of *S. cerevisiae* from 30 to 10 °C results in an increase in transcript levels of the antioxidant genes *SOD1*, *CTT1* and *GSH1*. In our previous proteomic study (Salvadó *et al.* 2008), we also detected an increase in Cys3, a protein related with antioxidant response, during adaptation to low temperature fermentation. The present study found stronger activity of the *YHB1* gene in the first minutes and 24 hours after inoculation at low temperatures. Thus low temperature might induce stronger oxidative stress in yeast cells.

Salvadó *et al.* (2008) reported an increase in glycolytic enzymes after yeast inoculation. We have detected two of these enzymes, Pdc1 and Tdh1, in a significantly higher concentration at optimum temperature than at low temperature. Conversely to protein concentration, the transcriptional profile of these glycolytic genes showed a higher induction at low temperature in all the samples analyzed, with the exception of *TDH1* after 24 hours at 25 °C. Rossignol *et al.* (2009) also studied the proteome of a wine yeast strain during exponential growth and stationary phase during fermentation. As in our study, glycolysis, amino acid metabolism and stress proteins were the most represented functional categories. They also tried to correlate protein abundance with changes in mRNA by using the data obtained in a previous transcriptome analysis

performed under the same conditions (Rossignol *et al.* 2003). They concluded that proteins and transcripts in the glycolysis category did not show correlation. This lack of correlation can easily be explained by multiple translational and post-translational regulation mechanisms. However other mechanisms such as gene autoregulation could explain why higher protein levels correlate with lower gene activity. This is the case for *PDC1*, which is activated in the absence of Pdc1 (Eberhardt *et al.* 1999).

We constructed mutants and strains over-expressing most of these genes to determine the importance of these genes in yeast growth and fermentation at low temperature. The over-expression of *ILV5* improved the period required to consume the 15% of grape must sugars, which could be related with a better adaptation to the new medium and temperature conditions after yeast inoculation. In the proteome analysis, this protein was in higher concentration at low temperature. It seems logical that the increase in this protein leads to an increase in fermentation capacity at low temperature.

Inoculation of active dry wine yeasts (ADWY) involves a shift from the respiratory metabolism during its industrial propagation to a fermentative metabolism during wine fermentation. In our previous proteome analysis (Salvadó *et al.* 2008), most of the mitochondrial proteins that showed significant differences 24 h after inoculation were present at lower concentrations, suggesting a transition from the respirative to the fermentative metabolism in the first hours of fermentation, which had shifted completely one day after inoculation. This hypothesis is supported by the mitochondrial staining performed in this study (Fig. 6). In the wild strain, the mitochondria were well-structured in the first 4 h after inoculation but these mitochondria developed into unorganized structures after 24 h. However, the overexpression of *ILV5* yielded unorganized mitochondria during the pre-culture phase. These disorganized mitochondria may produce a quick onset of the fermentative metabolism in the *pGAL1-ILV5* strain, which would explain the quicker CO₂ production in the first hours of fermentation. However, the quicker presence of unorganized mitochondria in the *pGAL1-ILV5* strain does not fit with the role previously assigned to this gene in maintaining mtDNA stabilization. Zelanaya-Troitskaya *et al.* (1995) reported that overexpression of *ILV5*, at levels only two- or three-fold, is sufficient to suppress the mtDNA instability phenotype of $\Delta abf2$ (a gene required for the maintenance of the wild-type (ρ^+) mitochondrial DNA).

During this fermentative metabolism, the mitochondrial TCA cycle is not functional, which may alter the intracellular amino acid distribution. TCA compounds, such as oxalacetate and alpha-ketoglutarate, are the precursors of aspartate and glutamate, respectively. The mismatch in the nitrogen metabolism observed in the *ILV5* overexpressing strain yielded a more relaxed NCR

mechanism than the control strain. Intracellular glutamine and ammonium have been reported as the main triggers of this nitrogen repression (Schure *et al.* 2000). The lower concentration of the intracellular glutamine pool (no differences were observed in the ammonium concentration) in the p*GAL1-ILV5* strain can explain this laxer NCR regulation. Owing to the link between nitrogen metabolism and aroma production during wine fermentation (Bell and Henschke, 2005), the influence of this strain to the organoleptic quality of the wines should be analyzed in the future. In a recent study, Chen *et al.* (2011) showed the increase in isobutanol (a fusel alcohol with influence in the aroma of the wine) by simultaneous overexpression of the *ILV2*, *ILV3* and *ILV5* genes.

In conclusion, the use of proteomic and transcriptomic analysis is useful to detect key molecular adaptation mechanisms of biotechnological interest for industrial processes. Although most of these functional analyses were carried out with lab strains, the use of industrial strains and conditions mimicking the industrial environment are recommended. In this study, we have detected genes and proteins with different activity and concentrations at low temperature. Strains that were either mutant for or overexpressed these genes were constructed in a derivative haploid of a commercial strain to determine the importance of these genes in fermentation at low temperature. Most of these strains did not show a differential phenotype to the reference strains in fermentation activity during the first hours of fermentation, with the exception of the strain over-expressing *ILV5*. The mitochondria degeneration process was faster in this p*GAL1-ILV5* strain, which produced an altered intracellular amino acid profile and laxer NCR regulation. It is likely that these physiological differences lead to a quicker onset of the fermentative metabolism, which explains the improved fermentation activity in the early stages. Currently, we are developing a strain over-expressing *ILV5* in the wild commercial strain, using clean and safe integrating methods. Natural grape musts will be fermented at low temperature with this genetically modified strain and an in-depth analysis will be performed of the fitness advantage afforded to this over-expressing strain and of the final quality product.

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General discussion and perspectives

General framework

Yeast is the main microorganism responsible for fermentation process under winemaking, efficiently metabolizing must sugars (glucose and fructose) into ethanol. *S. cerevisiae* has developed mechanisms that allow them to overcome the specific conditions of growth that they must face during alcoholic fermentation (nutrient depletion, increase in ethanol concentration, low pH, temperature changes, etc). New challenges in wine market lead industry to look for new technologies that produce wines with enhanced qualities. Lowering fermentation temperature it's being one of the technological improvements to deal with these challenges. It is noteworthy that temperature has a marked importance on the growth of microorganisms and therefore affects both single species performance and population dynamics during winemaking process. Four different studies were presented in this document contributing with new data to unravel the role of temperature and its influence in yeast evolution, ecology and performance on wine making process.

Temperature on *Saccharomyces* genus ecology and speciation.

Temperature has a marked importance on the growth of microorganisms in industrial environments (Arroyo López et al., 2006; Charoenchai et al., 1998; Serra et al., 2005; Sorensen and Jakobsen, 1997) as well as is a key factor on determining natural distribution of wild species (Lachance et al., 2003; Sampaio and Goncalves, 2008; Sniegowski et al., 2002; Sweeney et al., 2004). There is an increasing interest in determining the influence of temperature in the adaptation of *Saccharomyces* species to both wild and human driven fermentative environments (Goddard, 2008; Sampaio and Goncalves, 2008; Sweeney et al., 2004). Several studies have shown that *S. cerevisiae* is better adapted to grow at higher temperatures than other *Saccharomyces* species (Arroyo-López et al., 2009; Belloch et al., 2008; Kishimoto and Goto, 1995; Naumov, 1996; Serra et al., 2005). In spite of this fact, there was a lack of quantitative data about the whole biological range of temperature in which *Saccharomyces* yeasts are able to grow. In order to fill this gap, we performed a quantitative modeling by using the cardinal temperature model with inflection (CTMI) (Rosso et al., 1993).

From this study we have obtained quantitative data of μ_{max} along the whole biological temperature range as well as data of optimum, maximum and minimum growth temperature of the different species within the *Saccharomyces* genus. Those data has revealed *S. cerevisiae* as the

most thermotolerant species within the genus with the highest optimum (32.3°C) and maximum (45.4°C) growth temperature. Other species of the genus previously reported as more psychrotrophic by literature as *S. bayanus var uvarum* or *S. kudriavzevii* (Belloch et al., 2008; Kishimoto and Goto, 1995; Sampaio and Goncalves, 2008) also show expected profiles in our experiments. In general *Saccharomyces* genus presents species whose optimum growth temperature ranges from 23°C (*S. kudriavzevii*) to 32°C (*S. cerevisiae*). As mentioned above, *S. cerevisiae* is the most competitive species in the genus at high temperature but shows a loss of competitiveness when temperature drops from 20°C.

Our results support the hypothesis that adaptation to grow at different temperatures is an important factor in the ecology of *Saccharomyces* species in wild environments. According to the competitive exclusion principle, niche differentiation is necessary for sympatric coexistence of closely related species. Diverse studies have reported that *S. cerevisiae* and *S. paradoxus* occupy the same ecological niches (oak exudates, oak bark and oak-associated soils) in widely separated woodlands. Those species are typically indistinguishable by the criteria of classical yeast taxonomy, which include phenotypic characters such as cell, spore, and ascus morphology, as well as features of obvious ecological importance such as profiles of assimilation and fermentation of organic compounds (Vaughan-Martini and Martini, 1998). This close phenotypic similarity raises the question of whether sympatric populations of these two species nonetheless differ in some ecologically significant way, as might be predicted by classical ecological theory (Gause, 1936; Hutchinson, 1959). Importance of thermal growth profiles have been reported in both allopatric isolates (Vaughan-Martini and Martini, 1998; Greig et al., 2002) and in populations sampled from a single natural site (Sweeney et al., 2004), indicating that these two species do indeed have different thermal growth profiles in sympatry. In the same fashion, Sampaio and Goncalves (2008) also carried out a study on the influence of temperature on the sympatric association of four *Saccharomyces* species (*S. cerevisiae*, *S. paradoxus*, *S. bayanus* and *S. kudriavzevii*) isolated from oak bark. Their study showed that temperature played a fundamental role in the interactions among the *Saccharomyces* species. They suggested that circadian temperature fluctuations provide a range of temperatures that allows the sympatric association involving species more adapted to grow at high temperatures (*S. cerevisiae* and *S. paradoxus*) and those more adapted to grow at lower temperatures (*S. bayanus* and *S. kudriavzevii*). Therefore growth profiles obtained in our study support with quantitative data the importance of temperature on ecological distribution and sympatric association of the different species of the *Saccharomyces* genus.

Moreover, the analysis of phylogenetic dependence of the CTMI parameters obtained in our experiments showed that there is a significant influence of temperature on *Saccharomyces* genus speciation. Deviation of our data from the null hypothesis of absence of phylogenetic

dependence, showed by Orthonormal approach proposed by Ollier et al. (2006), light up temperature influence on genus speciation. In addition, our results of the R2Max test indicates that a significant part of the variance was explained by a single vector corroborating that a single event, which takes place after the divergence of the *S. arboriculus*, *S. mikatae*, *S. cariocanus*, *S. paradoxus* and the *S. cerevisiae* lineages from the *S. kudriavzevii* and *S. bayanus* lineages, favored the adaptation of the former species to grow at higher temperatures. The analysis also suggests that a second event could now be occurring in the *S. cerevisiae* lineage after its divergence from *S. paradoxus* and *S. cariocanus*, which would explain the higher thermotolerance exhibited by this species. Another interesting point, although it was not revealed by the phylogenetic dependence analysis, is the progressive adaptation of *S. kudriavzevii* to grow at lower temperatures. This was evidenced because *S. bayanus* var. *uvarum*, the first species to diverge within the *Saccharomyces* genus, exhibited higher T_{opt} and T_{max} values than *S. kudriavzevii*. In light of our results, temperature has influenced the evolution of the *Saccharomyces* genus, favoring the adaptation of some species to grow at lower (*S. kudriavzevii*) and higher (especially *S. cerevisiae*) temperatures.

Taking into account our results and previous ecological studies of the *Saccharomyces* genus, circadian temperature oscillations are key driving forces on *Saccharomyces* speciation and also provide conditions in which closely related species within the genus can coexist in sympatry. However more data of thermal profiles from *Saccharomyces* species in sympatric coexistence will confirm the robustness of the hypothesis.

Our studies have pointed that temperature has a clear dependence with *Saccharomyces* phylogeny. This fact correlates with observations on previous studies but is the first time that temperature has been proposed as a key factor on *Saccharomyces* evolution and speciation. Therefore it is necessary to perform new studies using other strains as well as different phylogenetic comparative methodologies to evaluate the phylogenetic dependence, confirm and give more robustness to our conclusions.

Rising temperature to become the Lord of Wineland

Saccharomyces cerevisiae, even when it is scarcely present in the fresh grape juice (Pretorius, 2000; Xufre et al., 2006), becomes the main species during the final stages of wine fermentation. Although the key factor which gives *Saccharomyces* its competitive advantage over remaining wine must microbiota has been studied for several years, it has still not been completely unravel.

Organisms, through their metabolisms, activities and choices, define and partly create their own niches. This process of organism-driven environmental modification is called niche construction. Niche construction regularly modifies both biotic and abiotic sources of natural selection and, in doing so, generate forms of feedback that change the dynamics of the

evolutionary process (Boogert et al., 2006). In wine fermentations, niche construction via ethanol production has been a traditionally accepted hypothesis for explaining how *Saccharomyces* imposes itself over non-*Saccharomyces* species. This hypothesis is supported by the fact that most non-*Saccharomyces* yeasts produce low amounts of ethanol and by the clear correlation between increased ethanol concentrations and reduced non-*Saccharomyces* populations (Goddard, 2008). Recently, Goddard (2008) proposed that temperature could also play an important role in niche construction via metabolic activity of *S. cerevisiae* during wine fermentation. Due to the vigorous fermentation of sugars carried out by this species, a non negligible thermal energy estimated at 104.43 kJ/mol is released during fermentation (Williams, 1982). This energy is responsible for the temperature rise (up to 6°C) that occurs during vinification (Goddard, 2008).

There is a lack of quantitative data supporting or denying any of these two abiotic factors (ethanol production and temperature rise) as being the key driving force in *S. cerevisiae* niche construction strategy that lead it to be the most successful yeast species on winemaking environments. In this study, we have modeled fitness advantage of a commercial strain of *S. cerevisiae* and several non-*Saccharomyces* wine species as a function of temperature and ethanol concentration. Quantitative data obtained from this study has additionally allowed us to predict how temperature and ethanol factors could affect *S. cerevisiae* imposition, by using in-silico hypothetical scenarios. Our results, obtained from assessment of growth profiles and in-silico competitive scenarios, have revealed how ethanol and temperature affect winemaking associated species. Our results show that ethanol rise reduces the competitiveness of *S. cerevisiae*, at least, until the Maximum Inhibitory Concentration (MIC) value of its competitors is reached. Once the ethanol exceeds their MIC value, non-*Saccharomyces* species lose their ability to grow and, consequently, cannot compete with *S. cerevisiae*, which could generally stand higher ethanol levels. These data suggest that *S. cerevisiae* high ethanol tolerance could be one of the key factors to its hegemony at the end of fermentation when ethanol exceeds 9-10%. However, this data cannot explain the progressive imposition of this species during the earlier stages or its dominance over *C. zemplinina*, which was the most ethanol resistant yeast.

Nonetheless, temperature effect, as mentioned above, have been recently proposed by Goddard (2008) as an important factor in the imposition of *S. cerevisiae* over non-*Saccharomyces* yeast during wine fermentations. Goddard's experiment shows that an increase of must temperature over 16 to 23°C, as a consequence of high vigorous respiro-fermentative metabolism exerted by *S. cerevisiae*, favored its rapid cell proliferation and final imposition, regardless of its low initial population frequency. Such data clearly correlate with observations performed in our study in which an increase of temperature confers a clear advantage of *S. cerevisiae* against non-*Saccharomyces* species. Our quantitative models show that *S. cerevisiae* increase its competitive

advantage against Non-*Saccharomyces* species as temperature rise. In addition, when temperature rise above 20°C the growth rate of *S. cerevisiae* increases faster than the non-*Saccharomyces* ones, causing that *S. cerevisiae* theoretical time for imposition drops drastically.

The data in this work were obtained in single culture experiments. This means that other factors such as competition between microorganisms for space and nutrients, oxygen affinity, or the production of toxic compounds (killer factors) were not taken into account (Albergaria et al., 2009; Hanl et al., 2004; Nissen et al., 2004, 2003). Many factors can influence population imposition in mixed cultures. However, we have proved that both factors, temperature and ethanol, can considerably influence the ecology of microorganisms during wine fermentation. Glucose metabolization by yeast under winemaking conditions leads to an increase in both ethanol content and temperature. Our data shows that an increase in ethanol content actually reduces competitive advantage of *S. cerevisiae* against other non-*Saccharomyces* species. These data suggest that *Saccharomyces* niche construction via ethanol production does not provide an ecological advantage, or at least not until ethanol concentrations reach 9-10%. However, temperature rise provides to *S. cerevisiae* a clear competitive advantage against the non-*Saccharomyces* species that usually are found in winemaking fermentations. Therefore, temperature rise produced during wine fermentation has revealed as a convenient environmental modification that confers a competitive advantage to *S. cerevisiae* against other yeast species present in grape must.

It is interesting to point out that these observations make sense in human driven fermentations conditions, where fermentation volume allows biochemical energy released to produce an effective increase of temperature in the fermentation tank. Less clear is how this energy could behave in natural scenarios. Natural niches occupied by *S. cerevisiae* are usually reduced volumes, as small as a drop falling from a damaged grape, oak exudates, surface of flowers, etc. In this kind of environments both thermal exchange with air and ethanol chemical equilibrium will prevent temperature rise as well as high ethanol concentrations. Does niche construction theory via temperature rise or ethanol accumulation make sense out of human driven fermentations? This is an open question that has not been yet addressed. Further studies should be carried out in order to put some light on it.

Yeast journey through modern winemaking at low temperatures

As discussed above, temperature is a key factor that determines fitness advantage among the different yeast species that coexist in human driven fermentations. Temperature has influenced the evolution of the *Saccharomyces* genus and, especially, has favored the adaptation of *S. cerevisiae* to grow at higher temperatures. This natural evolution is confronted nowadays with technological

trends and wine market demands. Wine fermentations carried out at low temperatures are a trendy oenological practice. Low temperature fermentations (10-15 °C) produce wines with an enhanced and more complex flavor and aroma. Low temperatures lead to greater retention of the primary aroma and enhance the production of secondary ones (Beltran et al., 2008, 2006; Torija et al., 2003). There is little information available about microbial dynamics at low temperature fermentations. Few studies suggest that non-*Saccharomyces* species have a higher chance of survival at low temperature fermentations (Fleet, 2003; Heard and Fleet, 1988; Sharf and Margalith, 1983). These findings are supported by our experiments, which also show that low temperature fermentations could reduce the imposition of *Saccharomyces*, thus giving non-*Saccharomyces* species a greater chance to contribute to the final flavor of wine. Our experiments show that when temperature drops from 15°C, time for *S. cerevisiae* imposition increased exponentially. While at temperatures above 25°C time for *S. cerevisiae* imposition remains below 3 days, low temperatures clearly impairs the imposition of *S. cerevisiae* that takes more than 20 days at 10°C. This loss of competitiveness may produce problems in industrial winemaking such as sluggish or stuck fermentations. Higher contribution of wild non-*Saccharomyces* wine microbiota is also favored by low temperature fermentations as shown in our studies. Non-*Saccharomyces* may have a desirable effect in final product in some cases but may also cause off flavors production. Thus, a better management of microbiota become of great importance at low temperature winemaking process.

Nowadays wine industry has, as a widespread practice, inoculation of selected natural wine yeast (mainly *S. cerevisiae*) in order to obtain more reproducible wines by better control of alcoholic fermentation (Ribéreau-Gayon, 2000). Low temperature affects successful proliferation and desired imposition of those inoculated yeast. In addition to the difficulties inherent to wine fermentation, low temperatures affect both yeast growth and fermentation rate. Impair of yeast physiology and metabolism, results in longer lag phases of up to 1 week and sluggish fermentations, which dramatically increase the duration of alcoholic fermentation (Bisson, 1999; Meurgues, 1996). Adaptation to grape-must by yeast has revealed as a key point in successful wine fermentation performed at low temperature.

Several studies have dealt with transcriptional evolution of yeast during wine-like fermentations (Novo et al., 2007; Rossignol et al., 2006). The genome-wide transcriptional changes at low temperature fermentation have been also studied recently by Beltran et al. (2006). This study has shown a response to stress in the first days of fermentation similar to that described as Late Cold Response by Schade et al. (2004), response mediated by transcriptional factors Msn2/Msn4. Moreover over-expression of genes involved in lipid metabolism, nitrogen metabolism and proteins and permeases involved in nutrient transport into the cell were also observed. It was also remarkable the greater activation of genes involved in ribosome synthesis

and transcription (RNA metabolism) observed in those studies. The activation of these genes reflects a compensatory mechanism due to the decrease in the rate of translation because the RNA secondary structures and the increased inactive ribosomes (Al-Fageeh and Smales, 2006). With the same aim of improving understanding of yeast adaptation mechanisms to low temperature, we have studied the proteome evolution of wine yeast in the first 24 hours after inoculation.

Our results show that early protein changes during the first hours after yeast inoculation involve the active degradation or modification of useless proteins, according to mRNA decay observed by Novo et al. (2007). Interestingly, there is a decrease in the amount of stress proteins during the first stages of fermentation, with the only exception of Cys3p, protein involved in glutathione synthesis as a response to oxidative stress. This observation matches with the transcriptional downregulation of most of the genes involved in stress response after yeast inoculation (Rossignol et al., 2006). We should keep in mind that selected yeast strains used in oenology are mostly sold as Active Dry Wine Yeast (ADWY) which must be rehydrated prior to inoculation. ADWY have been already subjected to highly stressful conditions during their industrial production (Pérez-Torrado et al., 2005). Inoculation in a fresh fermentation medium seems to alleviate the stress exerted during biomass propagation and dehydration.

Whereas most proteins increase their concentration 4 hours after inoculation. These protein changes may reflect transcriptional reprogramming after yeast inoculation in a new fermentable medium. We have identified a set of proteins whose amounts increase from 4 hours after inoculation. This set is composed of enzymes (Tdh1p, Tdh3p, Pfk1p, Gmp1p and Eno1p) involved in the lower part of glycolysis, pulling the carbon flux towards the ATP production stage. This scenario is plausible during the first stages of winemaking, where the huge amount of sugars might produce a high ATP demand for sugar phosphorylation. The increase of enzymes of the lower part of glycolysis may balance the drain towards ATP generation. Related to this flux pull towards ATP production, we have detected an increase of mitochondrial proteins involved in oxidative phosphorylation (Atp1p, Atp2p) as well as mitochondrial homeostasis (Por1p) and TCA cycle (Mdh1p). This scenario suggests that during first stages of fermentation respiration is still present in those conditions. Hence our proteomic data correlate with several studies that have proved that Crabtree effect is not immediate and that yeast cells have a period of respiration-fermentative metabolism. Nonetheless most of the mitochondrial proteins that showed significant differences 24h after inoculation were present at lower concentrations, suggesting that the change to fermentative metabolism has been completed.

To date, studies developed at low temperature wine fermentation-like conditions do not show a clear specific low temperature response. In general both transcriptional reprogramming and proteomic changes seem to be more focused on adaptation to grape-must and to alleviate several mismatches produced by low temperature than to develop a specific low temperature adaptation and acclimation process. In general, as showed in our studies, *S. cerevisiae* evolution and speciation have pushed this species to adapt to a better performance at higher fermentation temperature. This fact may be related to its nonspecific response to low temperature showed at both transcriptomic and proteomic level. Comparing proteomic evolution observed at 13°C with that observed at 25°C, little amount of proteins (just 9) show a clear difference between both. Those proteins are mainly involved in stress response and glucose and nitrogen metabolism. When looking at the transcriptomic profile of associated genes, results do not show clear differences in the overall shape of gene expression. Nonetheless we do observe a clear delay in the transcriptomic profile at 13°C compared to 25°C. Therefore, those data suggest a deceleration on the cell reprogramming events required for growth start in new fresh grape-must more than a specific cell roadmap to deal with low temperature.

Low temperature fermentations it's being a challenge to oenological biotechnology. Gene dose modification, deletion and overexpression strains, provide a powerful system for identifying gene roles that may allow developing biotechnological tools to solve low temperature associated challenges. Genetic manipulations produce cell perturbations that allow determine both positive and negative phenotype changes. Actually gene deletion mutant collection have already been used to identify essential genes for growth in cold environments (Abe and Minegishi, 2008). They identify 56 essential genes for growing at low temperature mainly involved in amino acid biosynthesis, microautophagy and sorting of aminoacid permeases, mitochondrial functions, transcriptions and mRNA degradation and ribosome. It is remarkable that mutant collections have been constructed under laboratory background that differ quite a lot from wild/industrial *S. cerevisiae* strains. Therefore observations obtained using those collections may be used as general guides towards the solution but taking into account the limitations of those platforms. In order to get closer to the industrial solution we use gene dose modification under wild/industrial yeast strain derivate. As explained in 4th chapter of this document several mutant strains were tested in order to evaluate phenotype alteration of gene dosage modification. Our results concludes that overexpression of *ILV5* gene confers fermentative advantage during the first stages of grape must fermentation at low temperature. Although the mechanism underlying this phenotypic improvement remains still unclear, we point to two different answers that involve cell amino acid management and mitochondrion organization. It is noteworthy that both issues were also pointed by Abe & Minegishi (2008) in their study with yeast knockout collection. Further investigation

using these functional genomic approaches either by using yeast knockout collections or genetic modified organisms will allow oenological biotechnology to overcome modern winemaking challenges. Our study contributed to focus a bit more those fields within cell functional genomics that may improve in a near future yeast performance low temperature.

In general, studies presented in this thesis, provide oenological biotechnology with a broad range of new and original data that will allow going further in both, developing new biotechnological solutions and performing knowledge-based process management of low temperature fermentations.

Conclusions

Growth profiles obtained in this thesis support with quantitative data the importance of temperature on ecological distribution and sympatric association of the different species of the *Saccharomyces* genus.

Temperature has influenced the evolution of the *Saccharomyces* genus, favoring the adaptation of some species to grow at lower (*S. kudriavzevii*) and higher (especially *S. cerevisiae*) temperatures.

Circadian temperature oscillation is key driving force on *Saccharomyces* speciation and also provides condition in which closely related species within the *Saccharomyces* genus can coexist in sympatry.

According with data obtained in this thesis, temperature has been a key factor on *Saccharomyces* sensu stricto evolution and speciation.

Niche construction theory via temperature rise in winemaking conditions have been supported by quantitative data of main yeast species related with wine fermentation environments.

Growth modeling and in-silico competition scenarios have shown its usefulness to predict yeast competitiveness under wine making fermentation process.

Temperature rise produced during wine fermentation has revealed as a convenient environmental modification that confers a competitive advantage to *S. cerevisiae* against other yeast species present in wine making environments.

Main changes produced in yeast proteome after inoculation of rehydrated ADWY in must at low temperature (13°C) involve proteins related to stress response, carbon metabolism, nitrogen metabolism and mitochondrial enzymes.

Overexpression of *ILV5* gene in wine yeast strain background confer fermentative advantage during the first stages of grape must fermentation at low temperature.

Proteomic and genomic studies performed in this thesis have shown that changes in yeast cells produced by low temperature during wine fermentation correspond more to deceleration on the cell reprogramming events required for growth start in new fresh grape-must than a specific cell roadmap to deal with low temperature.

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