METABOLIC AND MOLECULAR ADAPTATION OF WINE YEASTS AT LOW TEMPERATURE FERMENTATION: STRATEGIES FOR THEIR GENETIC IMPROVEMENT

María López Malo

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María López Malo

Metabolic and molecular adaptation of wine yeasts at low temperature fermentation: strategies for their genetic improvement

Doctoral Thesis

Supervised by: Dr. José Manuel Guillamón Navarro

Departament de Bioquimica i Biotecnologia

Facultat d'Enologia

UNIVERSITAT ROVIRA I VIRGILI

Tarragona 2013

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HAGO CONSTAR que el presente trabajo, con título **"Metabolic and molecular adaptation of wine yeast at low temperature fermentation: strategies for their genetic improvement"**, que presenta la Srta María López Malo, para optar al título de Doctora**,** ha sido realizado bajo mi dirección en el Departamento de Bioquímica y Biotecnología de la Universidad Rovira i Virgili y en el Departamento Biología de Sistemas en Levaduras de Interés Biotecnológico del Instituto de Agroquímica y Tecnología de los Alimentos (IATA) del CSIC en Valencia.

Valencia, 23 de Mayo de 2013

El director de la tesis doctoral

Dr. José Manuel Guillamón Navarro

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AGRADECIMIENTOS

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Nunca pensé que fuera tan difícil escribir estos agradecimientos, pero han pasado tantos años, tantas cosas, tantas experiencias, tantas personas que es difícil resumir en unos párrafos lo agradecida que estoy.

En primer lugar me gustaría agradecer a mi director de tesis, José Manuel Guillamón, todo lo que he aprendido trabajando con él, todo el tiempo que le ha dedicado a esta tesis y sobre todo me gustaría agradecerle el haberme transmitido esa ilusión por la ciencia, esa ilusión que me ha hecho disfrutar tanto de mi trabajo cada día.

Quiero agradecer a Eladio y Amparo que me dieran la oportunidad de conocer a José Manuel. Sin ellos esta tesis no hubiera sido posible.

A todos y cada uno de mis compañeros del IATA, a los que ya no están y a los que están. He pasado momentos maravillosos. Especialmente me gustaría agradecer a mis chicas (y ahora también chicos) que hemos compartido laboratorio. Rosana, muchas gracias por enseñarme tantas cosas, sin ti no podría haber dado mis primeros pasos en el laboratorio!je! Ali me ha encantado haber compartido tantas horas en el laboratorio y fuera de él, tantas horas de xarreta de trabajo y de la vida, gracias por haber estado siempre ahí, gracias por ser tan buena compañera, sin ti esta tesis no hubiera sido igual. Clarita, gracias, gracias! Por ser como eres! Pasamos poco tiempo juntas en el laboratorio, la suerte es que no te fuiste muy lejos y te he podido disfrutar todo este tiempo en el laboratorio de al lado o donde hiciera falta! Fani, pequeño pony! Muchas gracias por apoyarme siempre y confiar tanto en mi! Y por haber sobrevivido tus primeros días en el laboratorio cuando yo te enseñaba!jiji! Me gustaría también agradecer a Víctor y Bruno su apoyo, sobretodo estos últimos meses en los que la locura se apodera de mi.

Muchas gracias también a mis compañeros del 306, con los que compartimos unos añitos laboratorio, Patri, Montse, Jordi, Gloria…gracias por haberme hecho pasar tan buenos momentos! Y como no también me gustaría agradecerles a nuestras compis más recientes del 303, Meri y Bea, gracias por haber sido tan buenas

vecinas de laboratorio y tan buenas amigas en la calle! Y por supuesto a Silvia, gracias por tu ayuda!

También quiero agradecer al resto de compañeros de la tercera planta…Sarita, parece que llegamos a la recta final! Muchos ánimos y muchas gracias por haber compartido tantas cosas conmigo. Amparito, muchas gracias por haber sido mi compi de fiestas, almuerzos, risas…Aunque esos almuerzos de risas nunca podrían haber sido tan graciosos sin Juan! Gracias por haberme alegrado el día durante tanto tiempo! Muchas gracias a los chicos y chicas del 307, 308 y 309! Laura, gracias por cuidar tan bien del HPLC y del gases!jeje!No…gracias por haberme ayudado tanto sobre todo con mis amigas las manoproteinas! Peris, ahora que ya no estás esos cigarritos de mitad mañana me resultan más aburridos, encima Adri ha dejado de fumar, pero con suerte viene a visitarme a menudo! Gracias a los dos! Rosa, Nerea, Anto, David, Eli, Bruno, Jiri, Lupita, Marlen, Ana moltes gràcies. Hay más compañeros que ya se ha ido ya del IATA y que tampoco me puedo quedar si agradecerles esos buenos momentos, Ana, Jordi, Juanan....Me gustaría agradecer especialmente a Aurora y a Isaac que estos últimos meses me ha ayudado mucho con tanta alegría! Gracias por esas noches de jager (jeje), por esas conversaciones de pasillo y por darme tanto apoyo.

Me gustaría agradecer a toda la gente que conocí en Tarragona que me hiciera sentir desde un primer momento como si estuviera en casa. Gràcies a Nicolas per rebrem al teu laboratori. Gracias Maria Jesus por ayudarme con todo el papeleo de la tesis! Anna gràcies per ensenyar-me a treballar amb lípids. Braulio, gràcies per rebrem tan bé! A les meues xiquetes Imma, Marta i Maria vaig gaudir molt de la vostra companyia! Y como no a Angel y Ruben muchas gracias por todo! Zoel, Maite, Gemma moltes gràcies!

En último lugar me gustaría agradecer a mi familia y amigos todo el apoyo que me han ofrecido durante este tiempo. A mis amigos de toda la vida, especialmente a Neus, que me ha regalado una portada tan bonita! A mis amigos de biologia,

gracias! Quiero agradecer a mis padres ese apoyo incondicional y por animarme siempre, millones de gracias por todo. Danielet, muchas gracias por haber estado ahi, a pesar que estes en Brasil siempre has estado! Al final te has librado de mis nervios de última hora!jeje! Guillemet...dir-te gràcies seria poc. Gràcies per animar-me a parlar amb Eladio, ja fa uns anys, gràcies per eixes converses davant d'una birra parlant dels meus resultats, els quals ja te saps tots, gràcies per confiar més en mi que jo mateixa, sense el teu suport tot m'hauria costat més! Moltes gràcies. També vull agrair a la teua família que sempre m'ha recolzat.

MUCHAS GRACIAS!!!

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1. YEAST AND ALCOHOLIC FERMENTATION

It is believed that grapes have been domesticated between the Black Sea and Iran during the 7000 – 4000 BC period. The first evidence of wine making comes from the presence of tartaric acid in an ancient jar dated from 5400 – 5000 BC in the Neolithic site of Tepe in Mesopotamia and from the remains for grape juice extraction from 5000 BC in the Neolithic site of Dikili Tash in Greece (Sicard and Legras, 2011). Colonization by the Romans spread winemaking all around the Mediterranean; by 500 BC wine was being produced in Italy, France, Spain, Portugal and northern Africa. Afterwards was also spread into the Balkan States, Germany and other parts of northern Europe. In 1530 the Spanish introduce the vine into Mexico, Argentina, Peru and Chile. In the same way 1655 Dutch planted vine in South Africa. Planting in California soon thereafter, and in Australia and New Zealand a century later, 1813 (Pretorius, 2000).

However it is only in 1860 when Louis Pasteur discovered that yeast converted sugar into ethanol and carbon dioxide, a process called fermentation. With the knowledge that yeast was responsible of the fermentation, winemakers could control the process from the vineyard to bottling plant. Later in 1890, Müller – Thurgau introduced the concept of inoculating wine fermentations with pure yeast culture (Pretorius, 2000). At the present most of the wine production relies on the use of selected pure yeast culture as an oenological practice to produce wine with desirable characteristics and to guarantee the homogeneity of successive vintages (Schuller, 2010).

1.1 General characteristics of yeast

1.1.1 The *Saccharomyces* genus

Yeast species belonging to the *Saccharomyces* sensu stricto complex contains some of the most important species involved in alcoholic beverages as well as in bread fermentation. These yeasts have a number of unique characters not found in other yeast genera. While a majority of yeast cannot grow in the absence of oxygen, the majority of *Saccharomyces* complex yeast can survive without any oxygen by using the fermentation process (Sicard and Legras, 2011).

Using the biological species definition, yeast of the genus *Saccharomyces* sensu stricto comprises six species and one natural hybrid (Liti *et al*., 2006). Early analysis of *Saccharomyces* strains by interbreeding (Naumov, 1987) and DNA/DNA reassociation (Martini and Martini, 1987; Vaughan-Martini, 1989) were concordant in supporting the existence of three distinct species: *Saccharomyces cerevisiae, S. paradoxus* and *S. bayanus*. These studies also confirmed the identification of one natural hybrid (*S. pastorianus*). Recent studies of compatibility have defined three new species within the complex: *S. cariocanus*, *S. mikatae* and *S. kudriavzevii* (Naumov *et al*., 2000a). Very recently, it was determined that *S. pastorianus* is a hybrid between *S. cerevisiae* and a newly isolated species, *S. eubayanus* (Libkind *et al*. 2011). The nomenclature of *S. bayanus* (*S. bayanus* var. *bayanus*) and *S. uvarum* (*S. bayanus* var. *uvarum*) has been confusing and controversial for decades. However, all known strains of *S. bayanus* appear to be hybrids of *S. eubayanus* and *S. uvarum* that contains contributions from *S. cerevisiae* in at least some cases (Libkind *et al*. 2011).

Some species of the complex have been used by humans for making beverages or bread. Despite *S. cerevisiae* being primarily responsible for alcoholic fermentation, other species of the genus *Saccharomyces,* such as *S. uvarum,* have been isolated during wine and cider fermentation (Masneuf-Pomarède *et al.,* 2010; Naumov *et al*., 2000a) and *S. bayanus* is involved in lager beer fermentation (Sicard and Legras, 2011). Moreover some natural interspecific *Saccharomyces* hybrids were isolated in wine fermentations (González *et al*., 2006). In beer fermentation, the most well-known hybrid is the lager yeast *S. pastorianus* (also known as *S. carlsbergensis*).

1.1.2 Reproduction and cell cycle

Yeast are generally defined and recognized as unicellular fungi of the phyla *Ascomycetes*, *Basidiomycetes* and the imperfect fungi *Deuteromycetes*, characterized by their sexual reproduction mode or lack of it (*Deuteromycetes*). They can multiply asexually through budding, only *Schyzosaccharomyces* genera reproduce by binary fission. The yeast found on the surface of the grape and in wine belongs to *Ascomycetes* and *Deuteromycetes. S. cerevisiae* belongs to *Ascomycetes*, so it can multiply either asexually by vegetative multiplication or sexually by forming ascospores. Under optimal nutritional and cultural conditions *S. cerevisiae* doubles its mass every 90 min. The cell division cycle in vegetative multiplication consists in four phases: G_1 (period preceding DNA synthesis), S (DNA synthesis), G_2 (period preceding the mitosis) and M (mitosis). During asexual reproduction, a bud is forming by the mother cell during S phase. This bud grows into a viable daughter cell throughout the rest of the cell cycle and then separates from the mother at the end of the M phase. Budding is asymmetric in *S. cerevisiae,* in contrast to other eukaryotic cells, and the resulting daughter cell is smaller than its mother cell (Figure 1.1).

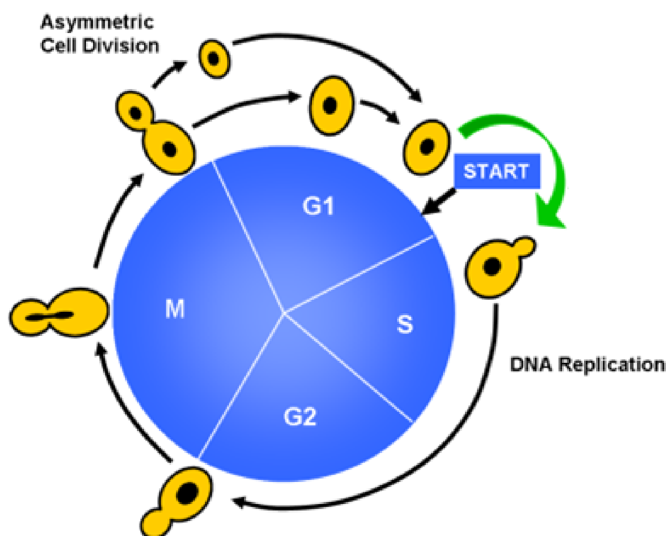

Figure 1.1 A schematic representation of the cell cycle of *S. cerevisiae*.

Sexual reproduction involves the formation of four haploid ascospores (two MATa and two MATα) within an ascus after meiosis and is induced during nutrient starvation, specifically nitrogen and fermentable carbon sources (Taxis *et al*., 2005). MATa spores can only mate with MATα and vice versa, resulting in the fusion of two cells to form a diploid cell (zygote) (Jackson and Hartwell, 1990). Spore released from the ascus can also continue asexual reproduction as haploids for many generations, and are termed heterotallic. Strains in which sex reversals, cell fusion and diploid formation occur are termed homotallic (Figure 1.2).

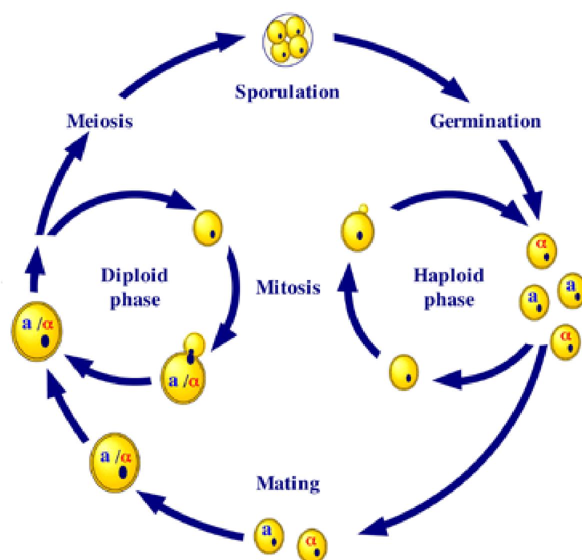

Figure 1.2 A schematic representation of the life cycle of *S. cerevisiae*

1.1.3 Kinetics of yeast growth during wine fermentation

Yeast population growth is the result of cell division and the progression through the cell cycle. Under optimal growth conditions, yeast growth kinetic follows the typical microbial growth curve, comprising three main phases: lag phase, exponential phase and stationary phase (Figure 1.3). The lag phase reflects the time required for yeast cells to adapt to their new environment by synthesizing ribosomes and enzymes needed to establish growth at a higher rate. The duration of this phase depends on firstly the initial population size and secondly environmental conditions. Once the cell starts actively metabolizing, they begin DNA replication and shortly after the cells divide. This begins the second phase of growth called the exponential phase of growth. This is the period in which the cells reproduce at maximum specific growth rate (μ_{max}). The time it takes the culture to double is called generation time. Yeast strain, growth medium, and temperature are important factors in determining the generation time. Industrial fermentations aim to extend this

phase for maximizing the output of biomass and metabolites production (López *et al*., 2004). The third phase in yeast growth is stationary phase, a period of no growth when metabolism slows and cell division is stopped. The factors that cause cells to enter stationary phase are related to change in the environment, such as nutrient deprivation, toxic metabolites and high temperatures. After prolonged periods in stationary phase, cells may die and autolysate.

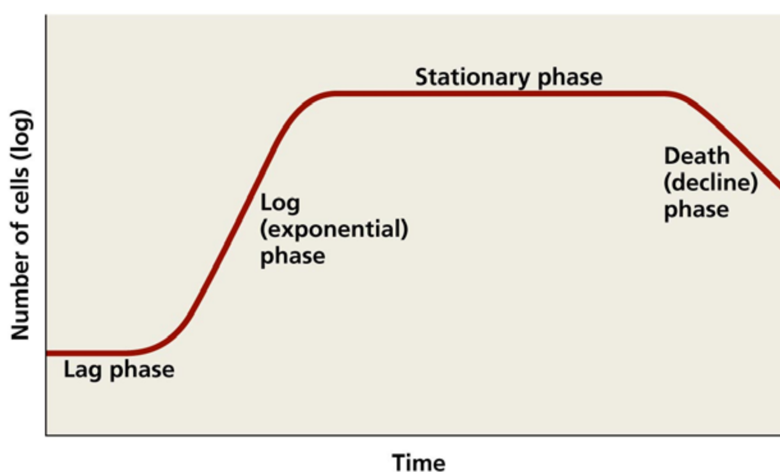

Figure 1.3 Typical yeast growth curve.

1.1.4 Genetic constitution of wine yeast: lab *vs* industrial strains

The majority of laboratory strains of *S. cerevisiae* are either haploid or diploid, whereas industrial wine yeast strains are predominantly diploid or aneuploid, and occasionally polyploidy (Pretorius, 2003). *S. cerevisiae* have a relatively small genome, a large number of chromosomes, little repetitive DNA and few introns (Goffeau *et al.,* 1996). Haploid strains contain 12 to 13 megabases (mb) of nuclear DNA, distributed along 16 linear chromosomes. Each chromosome is a single DNA molecule with a length of about 200 to 2200 kilobases. In addition to the chromosomal DNA exist a non-Mendelian genetics elements

e.g. Ty retrotransposons, 2µm plasmid DNA in the nucleus; 75 kb of mtDNA in the mitocondria; and killer viral-like particles containing dsRNA and prion-like elements in the cytoplasm. The genome of the laboratory strain, S288c, was the first eukaryote for which a fully genome sequence was available. The complete genome sequence defines 5885 open reading frames (ORFs) that are likely to specify protein products in the yeast cell (Goffeau *et al.,* 1996).

Generally, wild type and industrial strains of *S. cerevisiae* are less easily accessible for genetic analysis and manipulation than laboratory strains. Low sporulation frequency and viability of spores, instability of mating types and poor mating efficiencies, and the absence of easily tractable auxotrophic markers are some of the characteristics that compromise user friendliness of industrial yeast species (Winde, 2003). The clear phenotypic differences between industrial and non-industrial strains combined with early indications from single gene studies that suggested substantial novel nucleotide sequences and genomic rearrangements were present across strains of *S. cerevisiae.* Almost a decade passed before genome sequences for additional strains of *S. cerevisiae* became publicly available, RM11-1a and YJM789 (Wei *et al*., 2007). However, in the five years following the sequencing of RM11-1a and YJM789, there has been an exponentially increase in the number of *S. cerevisiae* strains for which whole-genome sequence are available. This was primarily owing to the development of “next generation” DNA sequencing (Borneman *et al*., 2012). The first industrial yeast genome to be assembled primarily from next-generation data was a haploid derivative of a wine yeast strain (Borneman *et al.,* 2008) and there are another five, high quality commercial wine yeast genome assemblies currently available (Borneman *et al.,* 2011; Novo *et al.,* 2009). As more *S. cerevisiae* strains are sequenced, the suitability of S288c as a “reference” strain is becoming less clear, especially as it appears to lack a large number of ORFs found in many others *S. cerevisiae* strains while containing an abnormally high number of Ty transposable

elements. A major finding of *S. cerevisiae* comparative genomics was the discovery of numerous strain-specific ORFs. The genome of the wine yeast EC1118 contains at least two major telomeric insertions relative to the laboratory strain, one on the left arm of chromosome VI and one on the right of chromosome XV. These insertions are shared by subsets of others wine strains such as QA23. The region on chromosome XV has particular interest because has at least three genes that will potentially impact on wine-relevant traits. The first of these is a homolog of the *S. pastorianus* fructose/H^+ symporter (Galeote *et al*., 2010). Two other genes of the genome encode oligopeptide transporters that allow for a grater variety of small peptide to be used as a nitrogen source. One of the most striking aspects of the wine yeast genome is the presence of a cluster of five genes that are postulated to have been horizontally transferred between *S. cerevisiae* and *Zygosaccharomyces spp* (Novo *et al*., 2009). The location, copy-number and exact order of the five genes within the cluster is strain and insertion site dependent. The genesis of these various insertions has been hypothesized to occur via the formation of a circular through an undetermined process that appears to be independent of classical recombinatorial or transposon-based duplication and insertion (Borneman *et al*., 2011).

1.2 Alcoholic fermentation

Alcoholic fermentation is the principal metabolic process in winemaking and is defined as the biotransformation of grape sugars, including glucose and fructose, into ethanol and carbon dioxide (CO_2). The principal responsible for this transformation is the yeast.

Yeasts are strongly inclined to perform alcoholic fermentation under aerobic and anaerobic conditions (Van Dijken *et al.*, 1993). Interestingly the alcoholic fermentation can occur in the presence of oxygen when there is a high concentration of sugars, above 20 g/L, because aerobic respiration is blocked. This phenomenon is named Crabtree effect. So, in *S. cerevisiae*, glucose and fructose are metabolized to pyruvate via glycolitic pathway. Pyruvate is decarboxylated to acetaldehyde, which is then reduced to ethanol. One molecule of glucose or fructose yields two molecules of ethanol and CO_2, theoretically 180 g sugar are converted into 92 g ethanol and 88 g CO_2. However this only could be expected in absence of any yeast growth, production of other metabolites and loss of ethanol as vapor.

In a standard fermentation, about 95% of the sugar is transformed into ethanol and CO_2, 1% into cellular material and 4% into other products such as glycerol. Approximately 60 – 80% of the sugar is fermented during the first half of fermentation then the fermentation rate slows.

1.2.1 Ecology in grape and must fermentation

The grape microbiota vary according to the variety; temperature, rainfall and other climatic influences; soil, fertilization, irrigation and viticultural practices.

Species of the genus *Hanseniaspora spp.* are predominant on the surface of grape berries, accounting for 50 – 75% of the total of the yeast population. Numerically less prevalent than these apiculate yeasts are species of *Candida* (e.g. *C. zemplinina, C. stellata*, *C. pulcherrima*), *Brettanomyces*, *Cryptococcus*, *Kluyveromyces*, *Metschnikowia*, *Pichia* and *Rhodotorula* (Fleet and Heard, 1993; Fleet, 1998). Contrary to popular belief, fermentative species of the genus *Saccharomyces*, predominantly *S. cerevisiae*, occur at extremely low

populations on healthy, undamaged grapes and are rarely isolated from intact berries (< 0.1%) and vineyards soils (Martini *et al.*, 1993), while damaged grapes are believed to be an important source, providing inocula of 10^2 – 10^3 cells/mL (Mortimer and Polsinelli, 1999). In fact the origin of *S. cerevisiae* is quite controversial, one school claims that the primary source of this commercially important yeast is the vineyard, and its presence or absence differ with each plant and grape cluster (Mortimer and Polsinelli, 1999). Others believe the evidence points to a direct association with artificial, man-made environments such as wineries and fermentation plants, and that a natural origin for *S. cerevisiae* should be excluded (Martini, 1993; Vaughan-Martini and Martini, 1995). In fact *S. cerevisiae* is by far the most dominant yeast species colonizing surfaces in wineries, demonstrating the selective effects of grape juice and wine as growth substrates (Pretorius, 2000).

The fermentation of grape must and production of wines is a complex ecological process involving the sequential development of microbial species. The process includes the interaction of fungi, yeast, lactic acid bacteria, acetic acid bacteria, as well as the mycoviruses and bacteriophages affecting these grape-associated microorganism (Fleet and Heard, 1993; Fleet, 1998). Though grape must is relatively complete in nutrient content, it can support the growth of only a limited number of microbial species. The low pH and high sugar content of grape must exert a strong selective pressure on the microorganism, such that only a few yeast and bacterial species can proliferate. Concentration of sulfur dioxide added as an antioxidant and antimicrobial preservative impose additional selection, particularly against undesirable oxidative microbes. The selectivity of fermenting must is further strengthened once anaerobic conditions are established, certain nutrients become depleted and the increasing levels of ethanol start to eliminate alcohol-sensitive microbial species (Henschke, 1997).

1.2.2 Spontaneous *vs* inoculated fermentations

Originally, all wine was made by taking advantage of natural microbiota for spontaneous fermentation. Various yeast found on the surface of grape skins and the indigenous microbiota associated with winery surfaces participate in these spontaneous wine fermentations (Figure 1.4). Some winemakers believe that indigenous yeast present in spontaneous fermentations produce higher concentrations of glycerol and other polyols, yielding wines with a distinct sensorial quality (Fugelsang, 1997).

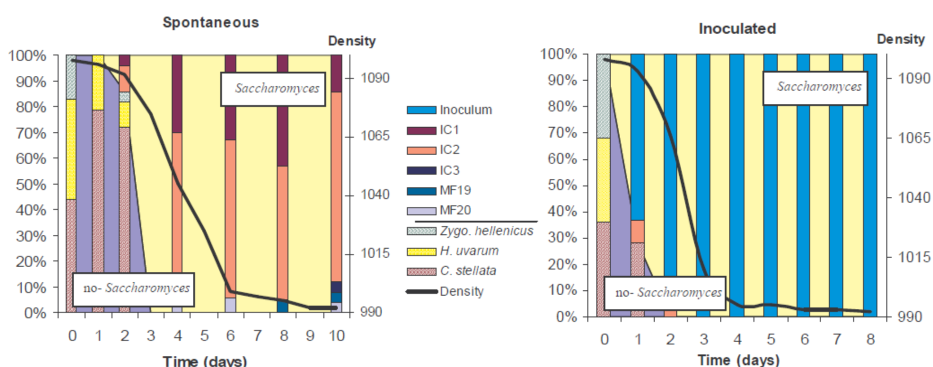

Figure 1.4 Yeast ecology on spontaneous and inoculated Garnatxa grape fermentation (Beltran *et al*., 2002). IC strains are commercial inocula and MF are strains of *Saccharomyces* autochthones.

However, the cast of stylistic characters and the individual and collective contribution of the indigenous yeast to the wine vary. The outcome of spontaneous fermentation depends not only on the number and diversity of yeast present in must but also upon grape chemistry and processing protocol. The combined effect makes the outcome difficult to predict. This lack of predictability/ reproducibility is most troublesome when comparing spontaneous fermentation with that resulting from active dried yeast starters (Pretorius, 2000).

Notwithstanding the fact that spontaneous fermentations usually take longer than most winemakers are willing to accept, and that the outcome is not always what was anticipated, there is no consensus among the world winemakers about using yeast starters (Fugelsang, 1997). At one extreme are those who continue to use solely indigenous yeast, in the belief that the higher yeast diversity, which offers spontaneous fermentations, confers a complexity upon wine. Others prefer to begin with native yeast and later inoculate with a commercial yeast starter. Still others initiate their wine fermentation with starters but a lower than recommended inoculums levels.

In large-scale wine production, however, where fast and reliable fermentations are essential for consistent wine flavor and predictable quality, the use of selected pure yeast inocula of known ability is preferred. These large wineries will be the main beneficiaries of programs aimed at producing new yeast strains with even more reliable performance, reducing processing inputs, and facilitating the production of affordable high-quality wines (Pretorius, 2000).

1.2.3 Causes of stuck and sluggish fermentation

Despite considerable improvements in our ability to monitor and control fermentation, stuck and sluggish fermentations remain major challenges for the wine industry. Bisson (1999) defined incomplete or "stuck" fermentation as those having a higher than desired residual sugar content at the end of alcoholic fermentation (>2-4 g/L), while slow or "sluggish" fermentations are characterized by a low rate of sugar consumption by the yeast (Figure 1.5). The economic and logistic consequence of sluggish and stuck wine fermentations in industrial cellars demand significant investigations into the causes and the determination of methods to avoid this problem. Extensive research has been

conducted since 1977 on elucidating problem fermentations and several causes of sluggish and stuck fermentation have been identified. Factors such as high initial sugar content, nutritional deficiency (nitrogen, vitamin, especially thiamine, oxygen or ergosterol and unsaturated acid); enological practice (excessive must clarification, pH or extreme temperatures); inhibitory substance (high ethanol concentrations, inhibition of yeast cell activity by fermentation by-products, particularly the fatty acids and acetic acid, killer toxins and pesticides) (for review see Malherbe *et al*., 2007).

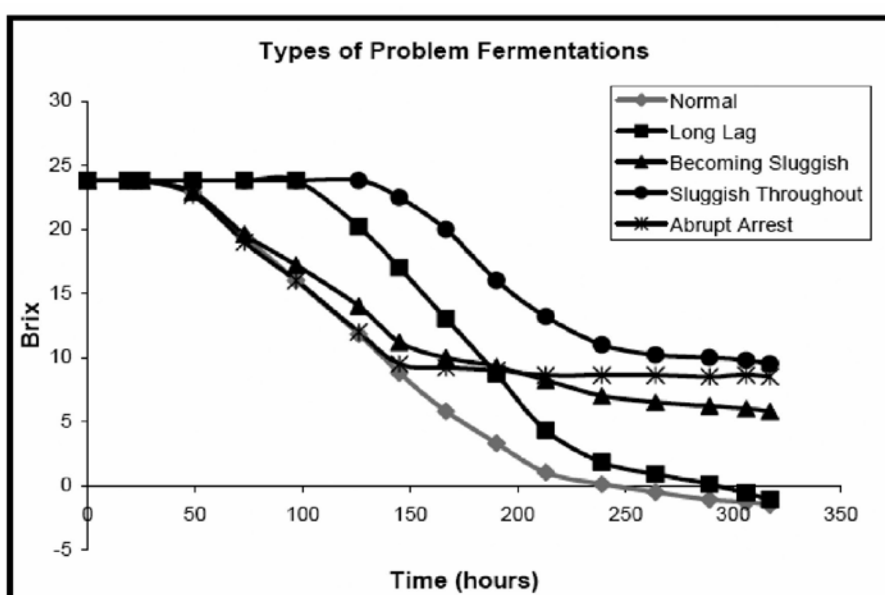

Figure 1.5 Types of problem fermentations (Bisson, 2005)

The effects related to these factors are numerous and include decrease in pH, inhibition of key enzyme activities, and alteration of plasma membrane. These may induce decrease in the metabolism of the yeast cell and consequently decrease in biomass production, cell viability and fermentation rate.

1.3 Yeast and post-genomic era

1.3.1 Genomics, transcriptomics and metabolomics

The combination of large, coordinate research community and small, tractable genome subsequently led to a laboratory strain, *S. cerevisiae* S288c, being the first eukaryote for which a fully characterized genome sequence was available (Goffeau *et al*., 1996). The development of "next generation" DNA sequencing, which collectively describes a group of several new DNA sequencing technologies that have reduced the cost of DNA sequencing by several orders of magnitude, has allowed an exponential increase in the number of *S. cerevisiae* strains for which whole-genome sequences are available. The Genebank DNA repository currently contains 28 assembled genome sequences (mainly in draft format) for strains of *S. cerevisiae*, in addition to another 19 that are available as unassembled sequenced reads. There are also at least 35 sequences available through project-specific websites (Borneman *et al*., 2012).

Functional genomics aims to identify the roles that play in the biology of organism with sequenced genomes. The field encompasses diverse techniques that allow biological study at multiple levels. The transcriptome, analysis of mRNA molecules with the use of full-genome microarrays, is not a direct measure of functionality, but rather a measure of translational potential. The proteome is a "snapshot" of total cellular protein, currently utilizing mainly two-dimensional gel electrophoresis, and the subsequent analysis of gel spots by mass spectrometry techniques. Global protein profile (proteomic) is a true measure of cellular functionality. The metabolome, aiming to analyze the metabolite profile at a given point within cell, is genome independent. Multiple genes may be involved in the synthesis and degradation of a single metabolite and, as such, the exploitation of known genes on metabolic profiles can elucidate functions of unknown genes (Delneri *et al*., 2001). It is the use of

these approaches that will allow the formation of an integrated biology of organism whose genome is fully sequenced (Hoskisson and Hobbs, 2005).

Although most of these functional analyses have been carried out with lab strains, it is important to carry out global functional analysis in industrial strains and in experimental context that approximates industrial conditions. Such studies are not only valuable in terms of basic research but also beneficial for application to the productive sector. This is the case of some studies which dealt with stress conditions frequently found in alcoholic fermentation, such as nutrient limitation (Boer *et al.*, 2003), anaerobic conditions (Kwast *et al.*, 2002), ethanol stress (Alexandre *et al.*, 2001), osmotic stress (Yale and Bohnert, 2001) or cold stress (Beltran *et al.*, 2006; Salvadó *et al.*, 2012). The identification of key genes that are useful for particular aspects of the biotechnological industry might improve the process either through the selection of proper strains or through the genetic modification of current industrial yeasts (Chiva *et al.*, 2012)

1.3.2 Improvement of wine yeast strains

Different wine yeast strains perform differently in fermentations and impart different qualities, such as flavor profiles, to wine and this is genetically determined (Chambers and Pretorius, 2010). These observations provide the basis for strain development programs in which novel yeast strains are generated to, for example, tailor wines to meet demands of specific markets. Indeed there are many attributes of starter culture wine yeast strains that could be improved by introducing novel traits or improving on what is already encoded in the genome (Pretorius, 2000).

Wine yeast can be improved genetically in a number of ways. Some of these methods alter limited regions of the genome, while others are used to recombine or rearrange the entire genome (Pretorius, 2000). Techniques having the greatest potential in genetic programming of wine yeast strains are: clonal selection of variants, mutation and selection, hybridization, rare-mating, spheroplast fusion and gene cloning and transformation.

Selection of variants is a simple and direct method, based on selection of natural genetic variants found within populations of all wine yeast strains due to spontaneous mutations. One kind of selection variants is the genetic drift, which under controlled conditions of growth; a yeast strain reveals slow but distinct changes after many generations. This might be due to a number of different processes, including spontaneous mutation, Ty promoted chromosomal translocations and more frequently, mitotic crossing-over gene conversion. It was shown that successive single-cell cultures of commercial wine yeast strains could result in strains with considerably improved characteristics; this approach is called "evolutionary engineering".

Mutagenesis is the application of mutagens to increase the frequency of mutations in a wine yeast population. The average spontaneous mutation frequency in *S. cerevisiae* at any particular locus is approximately 10^{-6} per generation. The use of mutagens greatly increases the frequency of mutations in a wine yeast population. Mutation and selection appear to be a rational approach to strain development when a large number of performance parameters are to be kept constant while only one is to be changed (Pretorius, 2000). As our ability to analyze whole genome sequences improves via microarray and sequencing-based methods, we can expect more accessible solutions to many biological problems through experimental selection approaches (Dunham, 2010) (Figure 1.6).

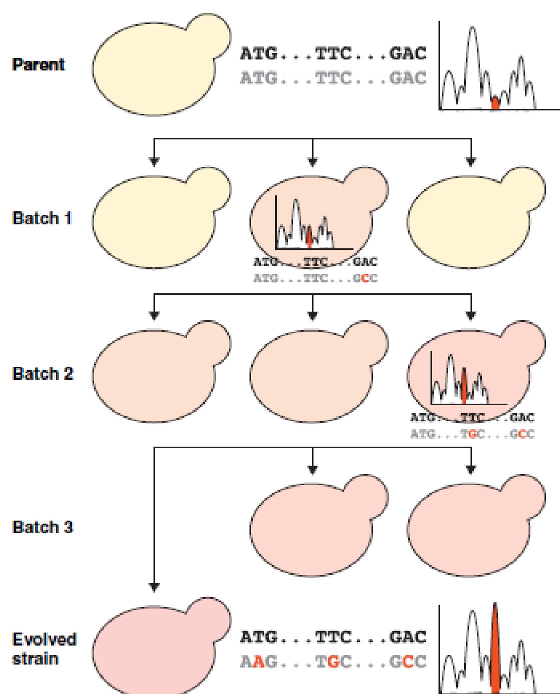

Figure 1.6 Genomic analyses of strains produced by adaptive evolution (Borneman *et al.*, 2012).

Intra-species hybridization entails sporulating diploids, recovering individual haploid ascospores and mating of haploid cells of opposite mating type to produce a new heterozygous diploid. As for all sexual reproduction, the resulting diploid strain may show properties that are different from that of either parental strain. There are several forms of hybridization: spore-cell mating (many wine yeasts are homothallic and require a direct spore cell mating), rare mating (force mating strains that do not express a mating type), cytoinduction (introducing cytoplasmic genetic elements into wine yeast without transfer of nuclear genes) and spheroplast fusion (Pretorius, 2000).

Recent developments of recombinant DNA technologies have enabled manipulation of a specific metabolic pathway by direct approaches, such as

modifying the gene promoter strength or type, performing deletions or introducing new genes into the cell. It has been defined as metabolic engineering, because the improvement is obtained by modification of specific biochemical reactions (Giudici *et al*., 2005). Clonal selection, mutagenesis, hybridization, all have a value in strain development programmes, but these methods lack the specificity required to modified wine yeast in a well-controlled fashion. In contrast, genetic engineering makes it possible to change the characteristics of wine yeast with surgical precision.

Despite very vocal opposition to genetically modified organism (GMOs) and products, in certain countries, the wine industry increasingly is focused on genetic improvement of grapevine and the wine yeast. Significantly, variant mutants, hybrids and cytoinductants are not included in the definition of GMOs. They are therefore not subjected to statutory regulations that pertain to GMOs and are not treated at the same level of public suspicion as are wine yeast that have been transformed with foreign DNA (Pretorius, 2003).

2. LIPID COMPOSITION

S. cerevisiae is a convenient experimental organism for studying the synthesis and intracellular transport of lipids. *S. cerevisiae* can serve as a valuable model for multicellular eukaryotes because of similarity in subcellular structures, and because much is known about the biochemistry and molecular biology of yeast lipids. The ease with which deletion mutants can be made in *S. cerevisiae* has facilitated studies of the function of specific lipids and lipid metabolites. Although most biosynthetic routes of lipids in yeast are similar to those in higher eukaryotes, some specific steps are unique in yeast. Major lipid

components of eukaryotic membranes are phospholipids, sterols, sphingolipids and glycerolipids (Daum *et al*., 1998).

2.1 Phospholipids

2.1.1 Phospholipid structure and function

Phospholipids, which are regarded as a primary structural element of the biological membranes, consist of a glycerol backbone esterified with fatty acids in the *sn*-1 and *sn*-2 positions, and a phosphate group in the *sn*-3 position. One hydroxyl group of the phosphate is linked to a polar head group, which is relevant for classifying the various phospholipids and for the physical properties of these molecules. Fatty acids in the *sn*-1 position are mostly saturated whereas those in the *sn*-2 position are unsaturated (Figure 2.1). The most abundant fatty acids in *S. cerevisiae* are C16:0 (palmitic acid), C16:1 (palmitoleic acid), C18:0 (stearic acid) and C18:1 (oleic acid). Minor amounts of C14:0 (myristic acid) can be detected. Other fatty acids, however, may be present in minor quantities but still be of physiological importance. As an example, phospholipids containing C26 fatty acid appear to be essential for nuclear function (Schneiter *et al*., 1996; Schneiter and Kohlwein, 1997).

Various molecules such as choline (in PC), ethanolamine (in PE), serine (in PS), myoinositol (in PI) and glycerol (in PG) can be linked to the phosphoril group. The distribution of phospholipids is not equal; the inner plasma membrane is enriched in PE, PI and PS, while the external is enriched in PC and sphingolipids.

Although phospholipids are indispensable as bulk components of yeast organelle membranes, it is not clear whether or not all individual classes of

phospholipids are essential. One exception is phosphatidylinositol (PI). Mutation in *PIS1* gene encoding phosphatidylinositol synthase results in lethality (Nikawa *et al.*, 1997). Defects in structural gene encoding enzymes involved in the biosynthesis of phosphatidylserine (PS), phosphatidylethanolamine (PE) and phosphatidylcholine (PC) cause phenotypic changes but are not lethal under laboratory conditions.

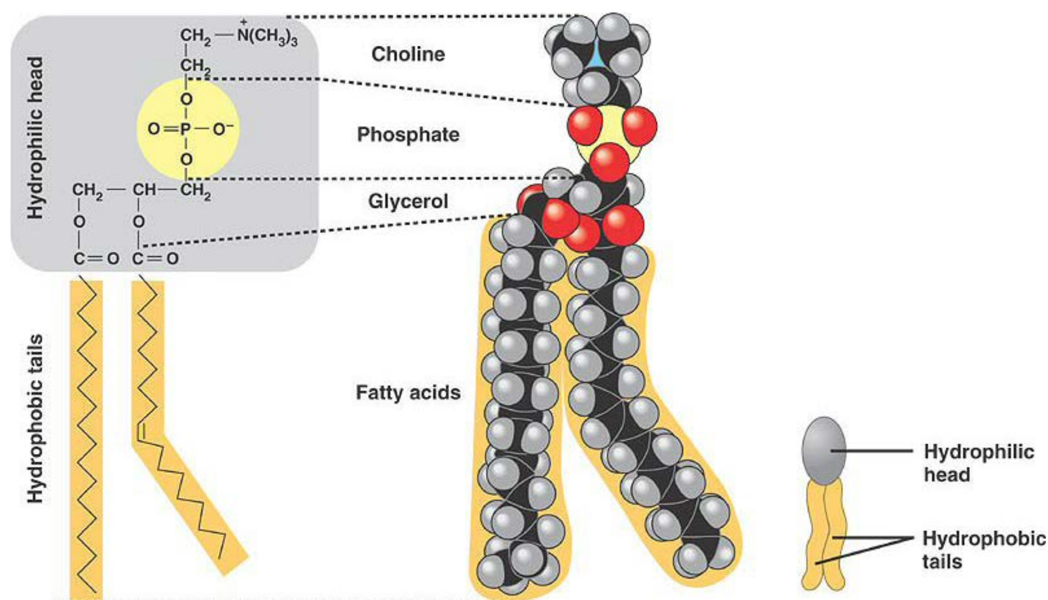

Figure 2.1 Phosphlipid structure

2.1.2 Phospholipid synthesis

It is well accepted that the endoplasmic reticulum and mitochondria are the subcellular compartments that contribute most to yeast phospholipid biosynthesis (for a review see Daum and Vence, 1997). Recently it was recognized that other subcellular compartments, such as the Golgi and lipid

particles contribute to phospholipid synthesis as well (Leber *et al*., 1995; Trotter and Voelker, 1995; Zinser *et al*., 1991).

A general precursor of all glycerolipids is phosphatidic acid (PA). The first step of PA synthesis in yeast is acylation of glycerol-3-phosphate (G-3-P) or dihidroxyacetone phosphate (DHAP), respectively. 1-Acyl-DHAP is then reduced to form 1-acylglycerol-3-phosphate (lysophosphatidic acid; LPA), which is also the direct product of G-3-P acylation. In a second acyltranferase reaction, LPA is converted to PA (Figure 2.2). In *S. cerevisiae*, the highest specific activity of the enzymes(s) that catalyzed the acylation of G-3-P was found in the lipid particle fraction. A central metabolite in phospholipid biosynthesis is CDP-diacylclycerol (CDP-DAG). The yeast gene encoding CDP-DAG synthase is *CDS1*. Cds1p, converts PA to CDP-DAG in a CTP dependent reaction. *CDS1* has a central role in *de novo* glycerolipid synthesis; a high level of CDP-DAG synthase activity favors synthesis of PI over PS. Moreover, CDP-DAG appears to affect both the level of gene expression and enzyme activity.

PI is a phospholipid that is essential for yeast. It is probably not the structural requirement for membrane assembly that makes PI essential but rather its role in cellular signaling as a membrane sensor. PI is synthesized from CDP-DAG and inositol by PI synthase (Pis1p).

PS is only a minor component of total cell phospholipids, but an important intermediate in *de novo* synthesis of the two main yeast phospholipids, PE and PC. Yeast PS synthase is encode by *CHO1* gene and forms PS from CDP-DAG and serine.

The major route of PE synthesis in yeast is the *de novo* pathway through decarboxylation of PS. The majority of PS decarboxylase (PSD) activity has

been localized on the mitochondrial fraction (Kuchler *et al*., 1986). The function of the mitochondrial PSD, Psd1p, is dispensable for the cell, even in the absence of ethanolamine, which is substrate for PE biosynthesis through the Kennedy pathway. Residual PSD activity in Δ*psd1* strains was attributed to Psd2p, which accounts for 5 – 10 % of the cellular PSD activity *in vitro* (Trotter and Voelker, 1995). The intracellular location of Psd2p is not completely clear; a Golgi/vacuole compartment has been suggested to harbor Psd2p.

In absence of exogenous choline, PC is synthesized primarily a three-step methylation of PE. These reactions are catalyzed by two independent methyltransferases, PE methyltransferase (Pem1p/Cho2p) and phospholipid methyltransferase (Pem2p/Opi3p), which are localized in the endoplasmic reticulum.

An alternative route for the synthesis of PE and PC in yeast is the Kennedy pathway originally describe by Kennedy and Weis, (1956). This route is a salvage pathway for yeast cells which are either unable to synthesize PS or lack of PSD activity, provided that sufficient ethanolamine/choline is present in the growth medium. Alternatively, ethanolamine can be provided by the action of phospholipases type D or by degradation of long-chain sphingoid base phosphate. Some of the genes involved in the ethanolamine branch of the Kennedy pathway also catalyze reactions involved in the utilization of choline through the choline branch of this pathway. The first step of the CDP ethanolamine pathway, phosphorilation of the ethanolamine by choline kinase (Cki1p), is catalyzed by such an enzyme with dual substrate specificity. Ethanolaminephosphate is then converted to CDP-ethanolamine by ethanolaminephosphate cytidyltransferase (Ect1p/Muq1p).

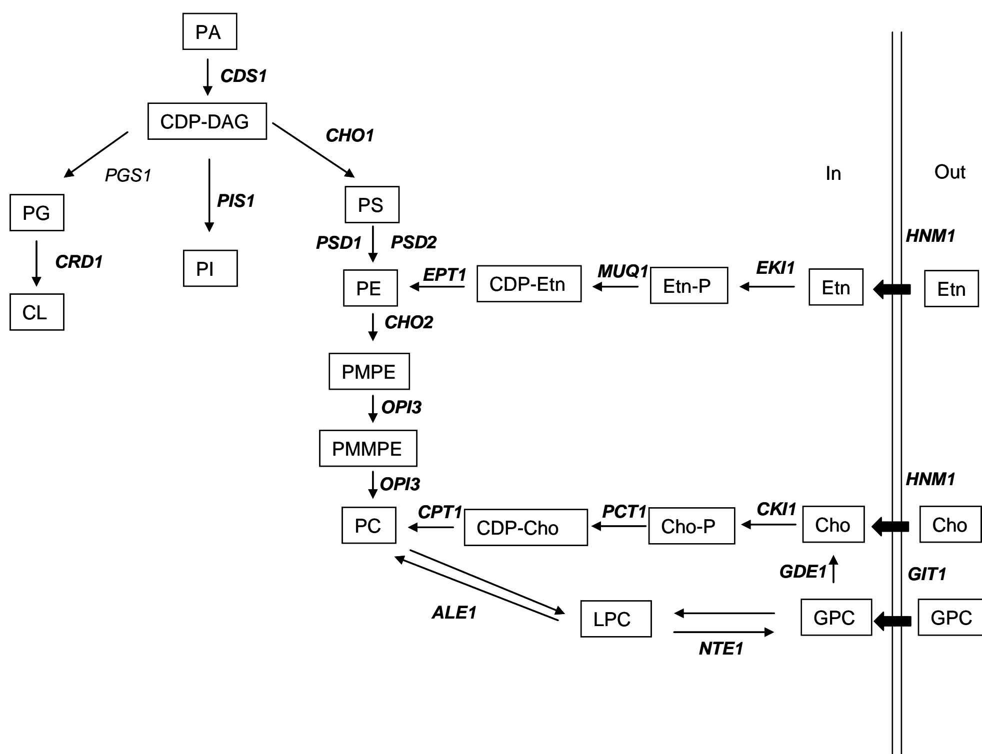

Figure 2.2 Phospholipid biosynthetic pathways in *S. cerevisiae*.

Finally PE is formed in the endoplasmic reticulum by CDP-ethanolamine:1,2-diacylglycerol ethanolaminephosphotransferase (Ept1p). Similar to the utilization of free ethanolamine, yeast can incorporate choline into PC through the Kennedy pathway. This pathway is not only active when exogenous choline is present but also functions continuously to recycle degradation products of PC. The enzymes involved in the choline branch are choline kinase (Cki1p), cholinephosphate cytidyltransferase (Pct1p/Cct1p) and CDP-choline:1,2-diacylglycerol cholinephosphotransferase (Cpt1p).

Phosphatidylglycerol (PG) is a minor phospholipid component of yeast subcellular membranes. Cardiolipin (CL) comprises 10 – 15% of mitochondrial phospholipids, especially when mitochondria are fully developed in the presence of non-fermentable carbon sources. PG and CL are closely related

biosynthetically. First, phosphatidylglycerophosphate (PG-P) is synthesized from CDP-DAG and glycerol-3-phosphate by the action of phosphatidylglycerophosphate synthase, Pgs1p. The next reaction in CL synthesis is dephosphorylation of PG-P yielding PG. As the ultimate step, CL is formed by reaction PG with a second molecule of CDP-DAG through catalysis performed by CL synthase, encoded by *CRD1*.

2.2 Sterols

2.2.1 Sterol structure and function

Sterols are essential lipid components of eukaryotic membranes and have been shown to be responsible for a number of important physical characteristics of membranes. Sterols are important regulators of membrane permeability and fluidity. Although other membrane lipids play a role in defining these properties, eukaryotic cells are unable to maintain viability without sterol.

The fungal sterol, ergosterol, differs from the animal sterol, cholesterol, by the presence of unsaturations at C7, 8 in the ring structure and at C22 in the side chain and by the presence of a methyl group at C24 on the side chain. Common to all sterols is the saturation at C5,6 and the presence of the hydroxyl group at C3 (Figure 2.3). The latter provides the only hydrophilic component of the molecule and allows for the proper orientation of the sterol molecule in the membrane.

Ergosterol is not present in equal amounts in all membranes. Ergosterol is found in highest concentrations in the plasma membrane and in the secretory vesicles (Zinser and Daum, 1995). In contrast, the membrane ergosterol content in the microsomes, mitochondria and other intracellular membranes is

significantly lower and the relative concentration of sterol intermediates is increased. Yeast differs from most eukaryotes in that the mitochondria ergosterol is concentrated in the inner membrane rather that in the outer membrane. The highest concentration of sterol found in yeast is in lipid particles which are primarily comprised of triacylglycerol and steryl esters.

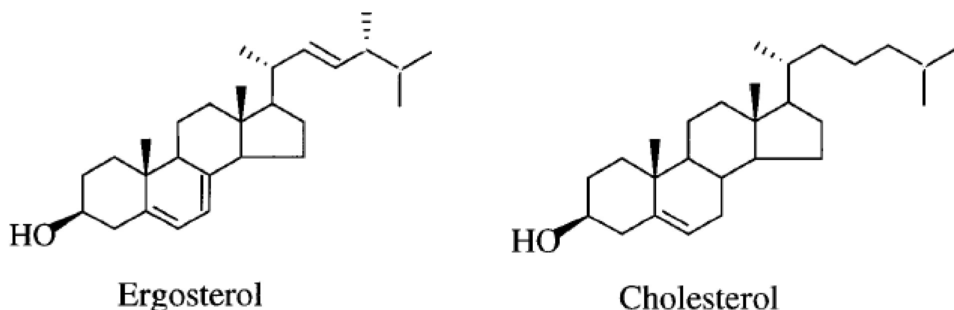

Figure 2.3 Ergosterol and cholesterol structure (Daum *et al*., 1998).

2.2.2 Sterol biosynthesis

Sterol biosynthesis is a major metabolic commitment on the part of the cell and involves over 20 distinct reactions.

The first portion of the pathway is initiated with acetyl-CoA and ends with the formation of farnesyl pyrophosphate, a pivotal intermediate which is the starting point of several essential pathways. The synthesis of heme, quinones and dolichols involve the participation of farnesyl components derived from this pathway. In addition, the farnesyl units and the related geranyl and geranylgeranyl species are important for the posttranslational modification of proteins that require hydrophobic membrane anchors for proper placement and

function. For this reason, mutations in this portion of the pathway are lethal, since multiple essential metabolic products cannot be synthesized.

The first step in the pathway is the synthesis of acetoacetyl-CoA from two molecules of acetyl-CoA, a reaction catalyzed by acetoacetyl-CoA thiolase, encoded by the *ERG10* gene (Figure 2.4). The second reaction is the conversion of acetoacetyl-CoA to hydroxymethylglutaryl-CoA (HMGCoA) by the *ERG13* gene product, HMGCoA synthase. The third enzyme in the pathway is HMGCoA reductase and catalizes the mevalonic acid formation. Yeast has two copies of the gene encoding HMGCoA reductase, *HMG1* and *HMG2*. Step 4 is catalyzed by the mevalonate kinase, which is encoded by the *ERG12* gene. Step 5 also involves a phosphorylation catalyzes by phosphomevalonate kinase, which is encoded by *ERG8*. The *ERG19* gene encodes the mevalonate pyrophosphate decarboxylase, which converts mevalonate pyrophosphate to isopentenyl pyrophosphate (IPP). The subsequent step converts IPP to dimethylallyl pyrophosphate (DMAPP) through the action of isopentenyl diphosphate isomerase, a product of *IDI1* gene. The final step in the early portion of the pathway is the conversion of DMAPP to farnesyl and geranyl pyrophosphate. This step is accomplished by the action of farnesyl (geranyl) pyrophosphate synthase, a product of the *ERG20* gene. The enzyme combines DMAPP and IPP to form geranyl pyrophosphate (GPP). The same enzyme is then able to extend GPP by combining it with a second IPP to form farnesylpyrophosphate (FPP).

The conversion of FPP to the end-product sterol represents an 11-step pathway dedicated to ergosterol biosynthesis (Figure 2.5). Since yeast requires sterol, one would predict that the first three steps (formation of squalene to lanosterol) would be essential, since no sterol molecule is synthesized to this point. Squalene synthase, a product of the *ERG9* gene, combines two FPP molecules

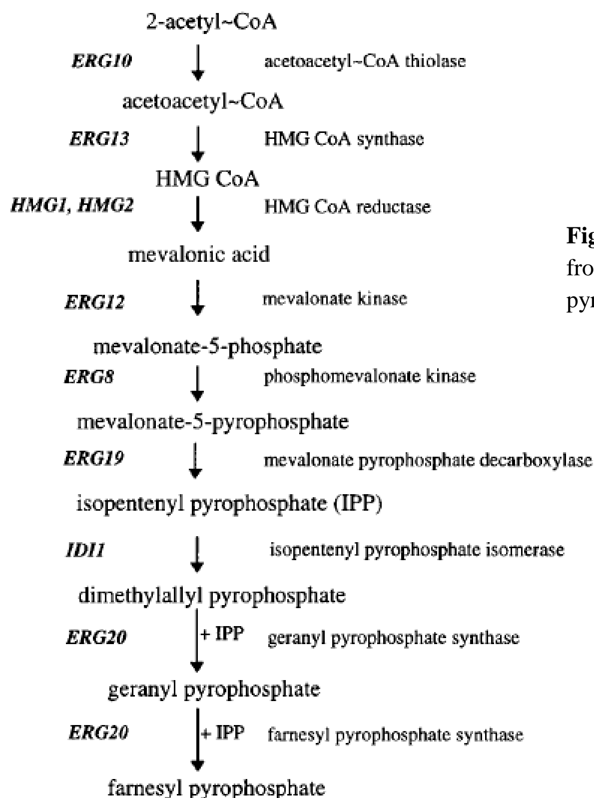

Figure 2.4 The mevalonate payhway from acetyl-CoA to farnesyl pyrophosphate (Daum *et al*., 1998).

to form squalene. This step is an ideal place for regulation since the enzyme competes with other enzymes for the FPP substrate. The *ERG1* gene encodes squalene epoxidase, which converts squalene to 2,3-oxidosqualene. This oxygen-requiring step precludes sterol synthesis in anaerobic conditions, thus requiring the addition of exogenous sterol for growth. The third step represent the conversion of 2,3-oxidosqualene to lanosterol, the first sterol molecule of the pathway. Lanosterol synthase, the product of the *ERG7* gene, performs several ring closures and bond cleavages in this very complex reaction. Lanosterol C-14 demethylase, the product of the *ERG11* gene is an essential cytochrome P-450 enzyme, and converts lanosterol to 4,4-dimethylcholesta-8,14,24-trienol. Proteins levels of the enzymes have been shown to respond to oxygen concentration, carbon source and other growth conditions. The *ERG24*

gene encodes the sterol C-14 reductase which converts 4,4-dimethylcholesta-8,14,24-trienol to 4,4-dimethylcholesta-8, 24-dienol. The removal of the two methyl groups from the C-4 position remains the final step in yeast sterol biosynthesis; this process of demethylation is very complex. There are three genes evolved this process, *ERG25*, *ERG26* and *ERG27* which encoded the C-4 methyloxidase, C-3 sterol dehydrogenase and C-3 keto reductase. The first enzyme needs oxygen for the reaction. Zymosterol, the product of the C-4 demethylation step, serves as the substrate for the sterol C-24 methyltransferase, which is encoded by *ERG6*.This step has been of particular interest, since it represents a step not found in cholesterol biosynthesis. *ERG2* encode C-8 isomerase converts zymosterol to episterol. The next step in sterol biosynthesis is a desaturation which adds the C-5,6 double bounds by the sterol C5 desaturase, encoded by *ERG3*. This process also needs oxygen. The final two steps of the pathway involve the introduction of the double bond at C-22 and the reduction of the double bond at C-24. The reduction of C-22 is accomplished by the sterol C-22 desaturase, a product of the *ERG5* gene. Finally, the production of ergosterol results from the action of sterol C-24 reductase, an enzyme encode by *ERG4* gene.

2.3 Sphingolipids

2.3.1 Sphingolipid structure and function

Sphingolipids are abundant components of the *S. cerevisiae* plasma membrane, with smaller amounts found in other cellular membranes. Complex yeast sphingolipids comprises about 30% of the phosphorylated membrane lipids and nearly 7% of the mass of the plasma membrane (Patton and Lester, 1991). Like phospholipids and sterols, sphingolipids play structural roles in membranes, and like other membrane lipids they play additional roles as second messengers

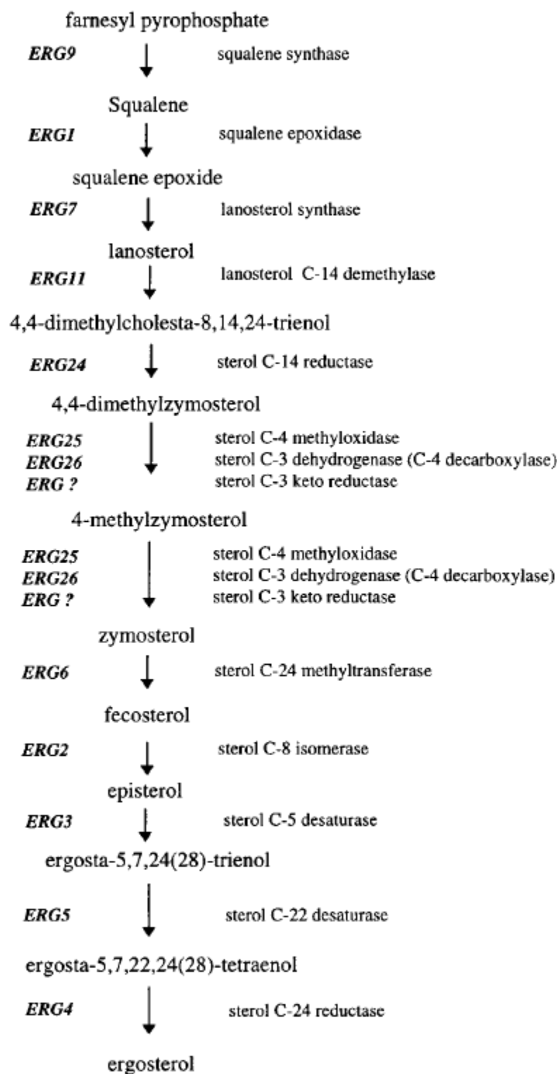

Figure 2.5 The ergosterol biosynthetic pathway from FPP to ergosterol (Daum *et al*., 1998).

for regulating signal transduction pathways. Some of these functions as signaling molecules are to regulate growth, response to heat stress, cell wall synthesis and repair, endocytosis and dynamics of the actin cytoskeleton in response to stresses. Others functions include roles in protein trafficking/exocytosis, lipid raft or microdomains, calcium homeostasis,

longevity and cellular aging, nutrient uptake, cross-talk with sterols and the action of some antifungal agents (Dickson *et al*., 2006).

Sphingolipids are defined by so-called long-chain bases, which in *S. cerevisiae* are usually 18 carbon linear alkanes containing hydroxyls on C-1 and C-3 and an amino group on C-2. The two long-chain bases in *S. cerevisiae* are dihidrosphingosine or sphinganine (DHS) and phytosphingosine (PHS), having hydroxyls on both C-3 and C-4 (Figure 2.6). The amino group is linked to a fatty acid to form a ceramide. Ceramides are modified on the C-1 hydroxyl to give complex sphingolipids. Fungal sphingolipids have inositolphosphate attached to C-1.

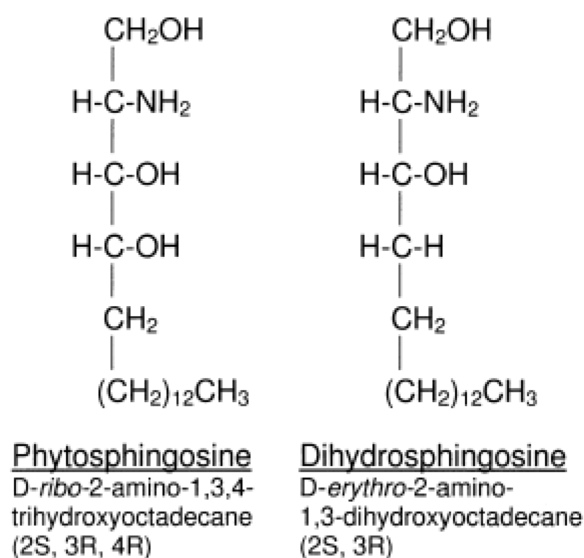

Figure 2.6 Diagram of sphingolipids (Dickson and Lester, 1999)

2.3.2 Sphingolipid biosynthesis

Sphingolipid synthesis begins with the condensation of serine and palmitoyl-CoA to yield the long-chain base 3-ketodihydrosphingosine (Figure 2.7). This reaction is catalyzed by serine palmitoyltransferase (SPT). Two essential

genes, *LCB1* and *LCB2*, are necessary for SPT activity and are thought to encode subunits of the enzyme (Hanada *et al*., 1997; Nagiec *et al*., 1994). In the second step in sphingolipid synthesis 3-ketodihydrosphingosine is reduced in a reaction utilizing NADPH to produce the long-chain base DHS. The 3-ketosphinganine reductase is encoded by *TSC10*. DHS is then attached to a C_{26} fatty acid via an amide linkage by either two ceramide synthases, Lag1p and Lac1p, to yield dihidroceramide which is then hydroxylated at C-4 by Sur2p/Syr2p to give a phytoceramide. Alternatively, DHS can be hydroxilated by Sur2p to form PHS which is then amide linked to C_{26} fatty acid to yield phytoceramide. Ceramide synthases in yeast, but not in mammals, contains another subunit, Lip1p.

Once made, ceramides must be transported from the ER to the Golgi apparatus where the polar head groups are added. Ceramide is probably delivered to the outer leaflet of the Golgi apparatus bilayer. Upon reaching the Golgi membrane, ceramide incorporates into the outer leaflet and then either flips spontaneously or is flipped enzymatically (it is not clear which mechanism is more important) to the inner leaflet so that it is accessible to the enzymes that attach polar head groups, which are in the lumen. It must then be flipped to the inner leaflet in order for inositol phosphate to be transferred from PI onto the C_1OH of ceramide from the first complex sphingolipid, inositol phosphoceramide (IPC). This transfer is catalyzed by inositol phosphorylceramide synthase (IPC synthase), encode by the *AUR1* gene (Nagiec *et al*., 1997). The second complex sphingolipid is mannose-phosphoceramide (MIPC), formed by transfer of mannose from GDP-mannose onto inositol 2-OH moiety of IPC. This reaction requires three proteins Csg1p (Sur1p), Csg2p and Csh1p. The third and most complex sphingolipid in yeast, mannose-(inositol-P)$_2$-ceramide (M(IP)$_2$C) is made by transfer of a second inositol phosphate from PI to MIPC, a reaction that requires the *IPT1*. Also two ceramidases, Ydc1p and Ypc1, have been identified in *S. cerevisiae*.

PHS and DHS derived from turnover of ceramide as well as that derived from the *novo* synthesis can be phosphorilated by two LCB kinases, Lcb4p and Lcb5p to yield PHS-1-P and DHS-1-P. Lcb5p is a small fraction of a total LCB kinase acitivity. PHS-1-P and DHS-1-P have two fates in yeast. They can be dephosphorylated by either two phosphatases Lcb3p (Ysr2p) or Ysr3p (Lbp2p) (Mao *et al*., 1997; Qie *et al*., 1997,) or they can be cleaved by a LCB phosphatase lyase Dpl1p (Saba *et al*., 1997), to yield ethanolamine phosphate and a C_{16} aldehyde.

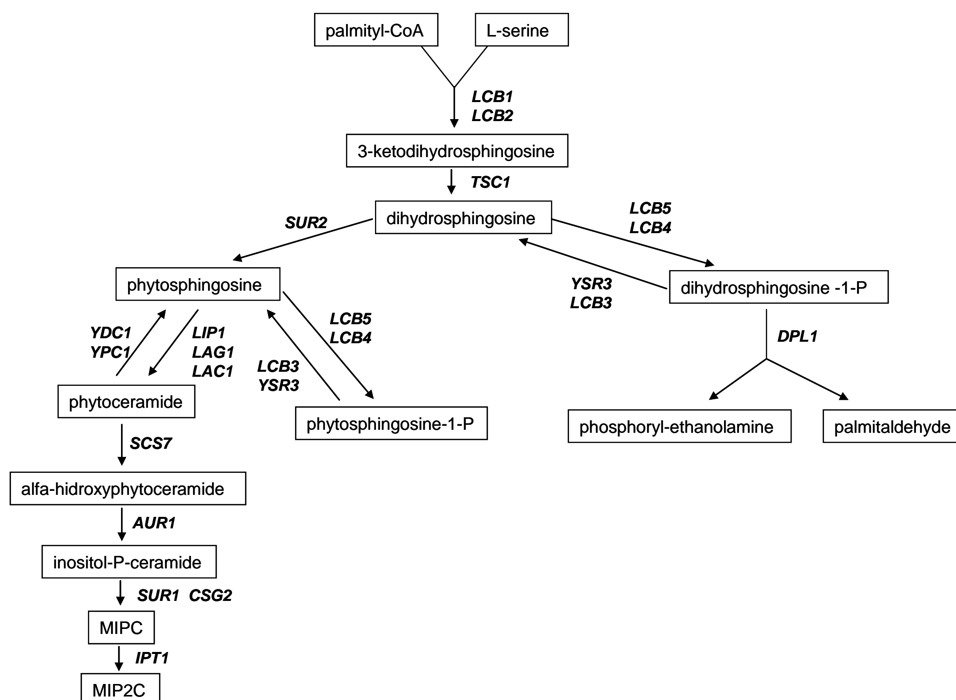

Figure 2.7 Phospholipid biosynthetic pathways in *S. cerevisiae*.

2.4 Fatty acids

The fatty acid composition can greatly influence the physical state of phospholipids and thereby affect membrane fluidity and permeability. The major species are C16 and C18 fatty acids with or without double bond.

Genes involved in the formation of fatty acids in yeast are *ACC1*, encoding acetyl-CoA carboxylase, and *FAS1* and *FAS2*, encoding the β and α subunits of the fatty acid synthase complex. An acyl-CoA binding protein, Acb1p, is thought to bind the acyl chain and hand it over to cellular processes that consume acyl-CoA.

Pre-existing fatty acid yeast can be modified by desaturation, elongation and hydroxylation. Mono-unsaturation of fatty acids is catalyzed by a single essential desaturase, Ole1p (Stukey *et al*., 1989). Elongation of fatty acids is accomplished by introducing malonyl-CoA as a C2 unit into pre-existing fatty acids. This reaction is catalyzed by the product of the *ELO1* gene (Dittrich *et al*., 1998; Toke and Martin, 1996). Subsequently, two genes structurally related to *ELO1*, namely *ELO2* and *ELO3*, were identified which encode components of the very long chain (C24 and C26) fatty acid elongation system (Oh *et al*., 1997).

3. LOW TEMPERATURE FERMENTATIONS

Many factors such as must composition, juice clarification, the temperature of fermentation or the yeast strain inoculated strongly affect alcoholic fermentation and aromatic profile of wine (Ribéreau-Gayon *et al*., 2000). With the effective control of fermentation temperature by the wine industry, low temperature fermentation (10 – 15 ºC) are becoming more frequent due to the

aim of producing white and "rosé" wines with more pronounce aromatic profile (Beltran *et al*., 2006; Llauradó *et al*., 2005; Molina *et al*., 2007; Torija *et al*., 2003a). This practice is limited to fermentation of white and "rosé" wine since in red winemaking higher temperatures (22 – 28 ºC) are needed for extracting phenolic compounds from the skin of the grape. Low temperatures increase not only the retention but also the production of some volatiles compounds (Killian and Ough, 1979). In these conditions greater concentration of aroma compounds are produced, such as esters that impart sweet and fruity aromas and lesser amounts of unpleasant compounds are produced, such as certain higher alcohols and acetic acid (Beltran *et al*., 2006). Another interesting aspect is that low temperatures notably reduce the growth of acetic and lactic acid bacteria, facilitating the control of alcoholic fermentation (Ribéreau-Gayon *et al*., 2000).

Despite fermentations at low temperature have interesting improvements; this practise also has some disadvantages. The optimal growth temperature of the wine yeast *S. cerevisiae* is around 32 ºC (Salvadó *et al*., 2011). Restrictive low temperature increases the lag phase and reduces the growth rate, producing sluggish and stuck fermentations (Bisson, 1999).

3.1 Effect of temperature on yeast ecology and growth

Temperature of fermentation directly affects the microbial ecology of the grape must and the biochemical reactions of the yeast (Fleet and Heard, 1993). Several authors have suggested that some species of non-*Saccharomyces* have a better chance of growing at low temperature than *Saccharomyces* (Heard and Fleet, 1988; Sharf and Margalith, 1983,) because they can increase their ethanol tolerance (Gao and Fleet, 1988). Also the number of different species,

as well as their endurance during alcoholic fermentation is conditioned by both the temperature of the must and the temperature during fermentation. These changes determine the chemical and organoleptic qualities of the wine (Fleet and Heard, 1993).

On the other hand, while the toxic effects of ethanol serve to poison other competing yeast in the community, it also appears that *S. cerevisiae* is better adapted to other stresses imposed by the juice environment at higher temperatures. An increase of temperature from 16 to 23 ºC, as a consequence of the highly vigorous fermentative consumption of sugars, favoured the rapid growth of *S. cerevisiae* cells and final imposition (Goddard, 2008). Similar results showed Salvadó *et al*. (2011) that found higher increases of the μ_{max} of the *S. cerevisiae* wine strains than the μ_{max} of non-*Saccharomyces* at temperatures above 20 ºC.

Temperature affects also to yeast viability, which decreases as the temperature increases (Torija *et al*., 2003b). At low temperatures, maximal population remained constant throughout the alcoholic fermentation.

3.2 Cryotolerant yeast

The selection of yeast able to ferment at low temperature is of great interest for winemaking industry (Castellari *et al*., 1994; Giudici *et al*., 1998, Massoutier *et al*., 1998). *S. cerevisiae* is the main responsible for the alcoholic fermentation, however other species of the genus *Saccharomyces* such as *Saccharomyces bayanus* var. *uvarum* have been isolated in wine and cider fermentation (Masneuf-Pomarède *et al*., 2010; Naumov *et al*., 2000b). It has been also isolated hybrid strains of *S. cerevisiae* x *Saccharomyces kudriavzevii* from Austrian vineyards (Gangl *et al*., 2009). Nevertheless, *S. kudriavzevii* has only

been isolated from natural environments, firstly in Japan (Naumov *et al.*, 2000a) and recently in Europe (Lopes *et al.*, 2010; Sampaio *et al.*, 2008). These two species, *S. bayanus var. uvarum* and *S. kudriavzevii* are considered the most psychrotrophic species of *Saccharomyces* genus (Figure 3.1) (Belloch *et al.*, 2008; Salvadó *et al.*, 2011; Serra *et al.*, 2005). Furthermore these two cryotolerant species posses other advantages compared to *S. cerevisiae* concerning valuable organoleptical properties such as higher production of glycerol and succinic acid and lower of ethanol (Arroyo-López *et al.*, 2010; Masneuf-Pomarède *et al.*, 2010; Serra *et al.*, 2005). However, both *S. bayanus var. uvarum* and *S. kudriavzevii* has the disadvantage, from the oenological point of view, that are less tolerant to ethanol than *S. cerevisiae*. Therefore *S. cerevisiae* ended up imposing in competence with these species during wine fermentation conditions (Arroyo-López *et al.*, 2010). Thus hybridization is a good option since hybrid yeast acquires physiological properties from both parents, e.g. alcohol and glucose tolerance of *S. cerevisiae* and the low temperature tolerance of *S. bayanus var. uvarum* and *S. kudriavzevii*.

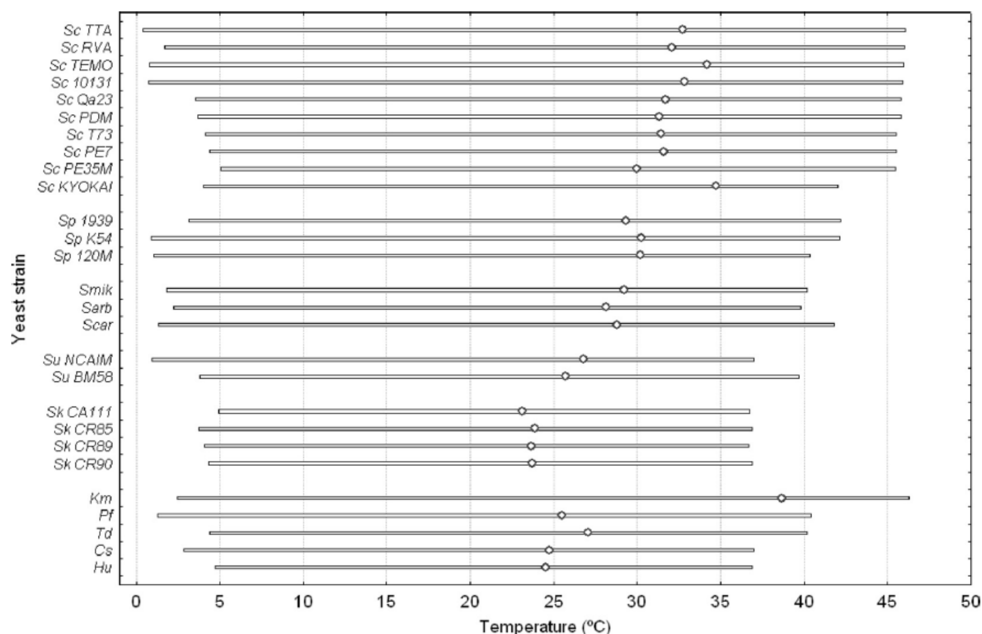

Figure 3.1 Temperature ranges in which 27 yeast strains were able to grow. Optimum temperature of each strain is marked by the circles on bars. *Sc*, *S. cerevisiae*; *Sp*, *S. paradoxus*; *Smik*, *S. mikatae*; *Sarb*, *S. arboriculus*; *Scar*, *S. cariocanus*; *Su*, *S. bayanus* var. *uvarum*; *Sk*, *S. kudriavzevii*; *Km*, *Kluyveromyces marxianus*; *Pf*, *Pichia fermentans*; *Td*, *Torulaspora delbrueckii*; *Cs*, *Candida stellata*; *Hu*, *Hanseniaspora uvarum*. (Salvadó *et al*., 2011).

3.3 Effect of temperature on fermentation kinetic

Temperature has an impact on yeast development and fermentation kinetics (Fleet and Heard, 1993; Ribéreau-Gayon *et al*., 2000). The effect of temperature (ranging from 10-15 ºC) on fermentation efficiency varies markedly for different *S. cerevisiae* strains and the ability of a strain to ferment well at cold temperatures depends on its cold tolerance and the rate of adaptation (Torija *et al*., 2003a). Cold fermentation temperatures significantly decrease the speed of fermentation and the maximal fermentation rate, whilst increasing the lag period and the overall duration increases between twice and three times more (Beltran *et al*., 2008) (Figure 3.2). Sugar uptake and

consumption is also decreased at low temperature and cells consume less nitrogen. These factors greatly increase the chances of stuck and sluggish fermentations.

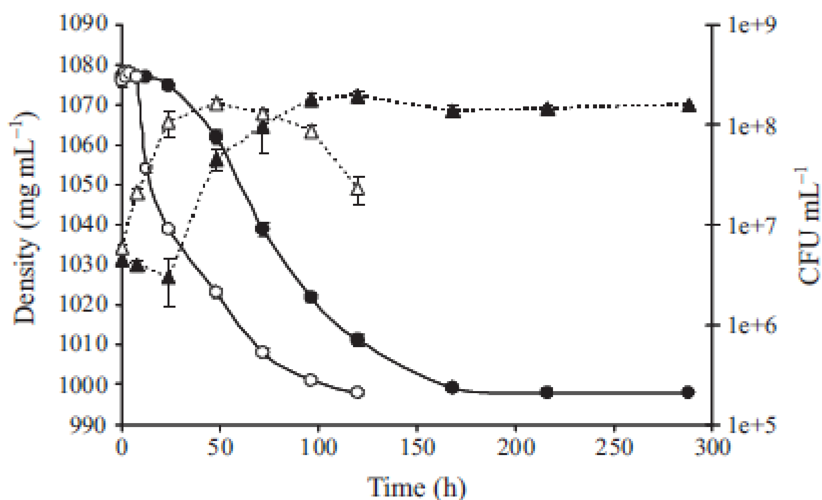

Figure 3.2 Fermentation kinetics (density reduction) and growth (CFU mL^{-1}). Filled symbols indicated fermentation at 13 ºC, and open symbols indicated fermentation at 25 ºC (Chiva *et al*., 2012)

3.4 Effect of temperature on yeast metabolism

Furthermore temperature affects biochemical activities of yeast, and as a result, the modified production of secondary metabolites such as glycerol, acetic acid, succinic acid, or aromatic compounds and ethanol. These changes determined the organoleptical qualities of the final product (Fleet and Heard, 1993).

3.4.1 Effect of temperature on alcohol and by-products synthesis

The final concentration of alcohol decrease as the temperature increased, which has been related to a drop in the ethanol yield and a reduce use of substrate (Casey and Ingledew, 1986). Torija *et al*. (2003b) reported that in their results the substrate was completely used, and although losses due to evaporation of high temperatures are expected, the main cause of the reduction is probably the increase of products of other metabolic pathways such as glycerol, acetic acid, etc. Thus, the diversion of carbon into various sinks is altered at low temperatures, resulting in higher ethanol yield and modified production of secondary metabolites. The decrease in production of acetic acid and possibly acetaldehyde allows more carbon to be diverted towards the formation of ethanol (Llauradó *et al*., 2005). However the decrease in production of glycerol suggested by Torija *et al*, (2003b) at low temperature is unlikely since glycerol accumulation and synthesis is widely reported to be greater at low temperature (Aguilera *et al*., 2007; Llauradó *et al*., 2005, Panadero *et al*., 2006).

3.4.2 Influence of temperature on volatile compounds

The aroma of wines is a unique mixture of volatile compounds originated from grapes (varietal aromas), secondary products formed during wine fermentation (fermentative aromas) (Figure 3.3) and ageing (post-fermentative aromas) (Lambrechts and Pretorius, 2000; Swiegers and Pretorius, 2005). The aroma complexity dramatically increases during alcoholic fermentation as a result of the synthesis of important volatile compounds by the wine yeast and the release of some varietal aroma precursors (Swiegers *et al*., 2005). The volatile compounds synthesised by wine yeast include higher alcohol (fusel, marzipan and floral aromas), medium- and long-chain volatile acids (fatty, cheesy and

sweaty aromas), acetate esters and ethyl esters (fruity and floral aromas) and aldehydes (buttery, fruity and nutty aromas), among others (Lambrechts and Pretorius, 2000, Delfini *et al*., 2001). Fermentations at low temperature increased the concentration of ethyl and acetate esters and overall medium-chain fatty acids (MCFA), although this increase is strain dependent (Torija *et al*., 2003a, Molina *et al*., 2007).

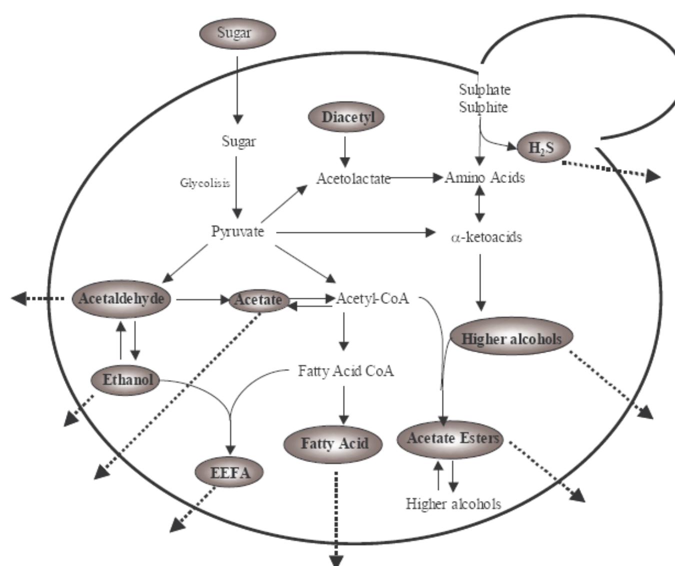

Figure 3.3 A schematic representation of the formation of aroma compounds by yeast (adapted from Lambrechts and Pretorius, 2000)

Moreover, Beltran *et al.* (2008) observed that low temperature fermentation produced wine with increasing floral (fatty acid ethyl ester), fruity (fusel alcohol acetates) yeast aromas and maintained a high levels of varietal aromas (terpens), in addition they observed a decreased in volatile acidity and fusel alcohol concentrations.

3.5 Effect of temperature on biological membranes

Biological membranes are the first barrier between the cell interior and its environment and a primary target for damage during cold stress. Incubation at low temperature increases the molecular order of membrane lipids by rigidification (Russell, 1990). Thus, a decrease in temperature leads to a decrease in the membrane fluidity, and the membrane could change from the fluid crystalline phase to the gel phase (Figure 3.4). The temperature in which the membrane changes of phase is known as transition temperature (T_m) (Beney and Gervais, 2001; Thieringer *et al.*, 1998). Several factors affect the T_m of the phospholipids, but the most important is the nature of the fatty acid present in the molecule. The T_m increases with the length of the chain of fatty acid while both the degree of unsaturation and the position of the double bound, mainly located in the centre of the chain, decrease the T_m. Also the nature, size and charge of polar head group of phospholipids have an effect on T_m and the physical state of the membrane. Lipids such as PC, PS, PI and sphingolipids, which have head group and acyl chain with comparable cross-sectional area, are cylindrical and organize easily in bilayers. However, lipids which have smaller head groups than acyl chains, such PE, CL and sterols, are cone shaped, and at a high concentration in the membrane may locally induce a high membrane curvature and membrane-packing defect (van der Rest *et al.*, 1995). These changes in membrane fluidity alter various functions of membrane-bound proteins, such as import and export of metabolites and proteins across plasma membrane-bound proteins. Thus, yeasts have developed several mechanisms to maintain appropriate fluidity of its membranes regardless of the ambient temperature. The most described mechanism is the increase of the unsaturation degree of fatty acids, mainly palmitoleic (C16:1) and oleic (C18:1). Another way to increase membrane fluidity is to decrease the chain length (ChL) of fatty acids by increasing the synthesis of medium

chain fatty acids (MCFA; C6 to C14) (Beltran *et al*., 2008; Torija *et al*., 2003a). In a recent work, Redón *et al*. (2011) also reported new common changes in the lipid composition of different industrial species and strains of *Saccharomyces* after low temperature growth. Despite specific/species dependent responses, the results showed that, at low temperature, the MCFA

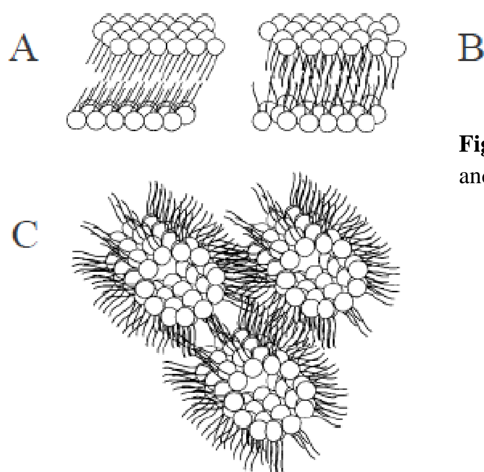

Figure 3.4 Organization of phospholipids (Beney and Gervais, 2001)

A) Bilayer in the gel phase

B) Bilayer in the liquid crystalline phase

C) Hexagonal (non-bilayer)

and triacylgliceride (TG) content increase, whereas the phosphatidic acid content (PA) and the PC/PE ratio decreased. Moreover, in a similar study, Tronchoni *et al.* (2012) compared the lipid composition of different *S. kudriavzevii* strains, and hybrid strains between *S. cerevisiae* and *S. kudriavzevii*. In these strains also the PC/PE ratio decreased and a common response to low temperature was an increase in TG and sterol esters (SE), the main storage lipids.

The lipid composition of a cell can also be influenced by the environment since yeast has the capacity to include fatty acids from the medium in its own phospholipids (Benchekroun and Bonaly, 1992; Rosi and Bertuccioli, 1992; Thurston *et al*., 1981). In grapes, UFA represents the major component of total lipids. The most abundant is linoleic acid (C18:2), followed by oleic (C18:1), linolenic (C18:3) and palmitoleic acid (C16:1) (Castela *et al*., 1985). Of the

SFA, palmitic acid (C16) is the most abundant (Bertrand and Miele, 1984, Castela *et al*., 1985). Yeast can include these lipids in its own membranes, modifying its structure

3.6 Effect of temperature on nitrogen transport

Yeast metabolism mainly depends on the uptake of nutrient driven by permeases. A decrease in membrane fluidity results in slower lateral diffusion of membrane proteins, decreased activity of membrane-associated enzymes, and a major reduction in membrane transport (Vigh *et al*., 1998). Membrane permeases are highly temperature dependent, because changes in temperature can cause conformational changes to their structure (Entian and Barnett, 1992).

The nitrogen fraction in grape must consist of organic (amino acids) and inorganic (ammonium) nitrogen. At optimum temperature, ammonium is the preferred nitrogen source for biomass production while amino acids are preferentially used during the stationary phase (Beltran *et al*., 2005). However, at low temperature, amino acids were preferentially consumed during exponential phase (Beltran *et al*., 2007). One explanation for this different pattern of nitrogen consumption might be the slower flux of sugars into glycolytic pathway (slow fermentation rate) at low temperature, which determines a lower availability of carbon skeletons for amino acid biosynthesis (Beltran *et al*., 2007).

There are also differences in the pattern of amino acid consumption between optimal and low temperature. Glutamine was consumed much more at 25 ºC than at 13 ºC whereas tryptophan was consumed more at low temperature (Table 3.1) (Beltran *et al*., 2007). The uptake of tryptophan as a rate-limiting step in growth at low temperature has already been reported (Tokai *et al*., 2000). The overexpression of the gene encoding the high-affinity tryptophan

permease Tat2p gave the yeast cell good capacity to grow at low temperature (Abe and Horikoshi, 2000, Nagayama *et al.*, 2004). Other amino acids whose uptake increased at low temperature were arginine and glutamic acid.

Table 3.1 Final consumption of amino acids (mg L^{-1}) by yeast cells fermenting at 25 ºC and 13 ºC (Beltran *et al.*, 2007).

	25°C	13°C	Ratio
His	41.4	24.2	1.7
Ser	21.0	12.8	1.6
Gln	266.3	175.3	1.5
Thr	79.8	51.6	1.5
Val	25.4	17.4	1.5
Tyr	5.1	3.8	1.3
Phe	28.7	23.1	1.2
Ile	29.6	26.9	1.1
Lys	11.9	10.9	1.1
Leu	39.4	42.7	0.9
Met	19.2	20.7	0.9
Trp	96.7	144.9	0.7
Glu	4.8	11.1	0.4
Arg	7.8	28.5	0.3
Gly	0.9	0.0	
Ala	0.0	3.0	
Asp	0.0	4.1	
Suma	668.4	593.8	1.1

3.7 Role of reserve carbohydrates

Glycogen and trehalose are the main reserve carbohydrates in yeast cells (for a review see François and Parrou, 2001). During cold stress yeast cells synthesize large amounts of protective compounds, such as trehalose and glycerol, to preserve and defend internal cellular components. Glycogen provides a readily mobilizable carbon and energy source while the yeast adapts to new growth medium (Pretorius, 2000). Besides glycogen breakdown is accompanied by sterol formation, which is essential for yeast vitality and successful fermentation (François *et al.*, 1997). Trehalose protects cells by

preserving the integrity of biological membranes and stabilizing proteins in their native state (Lucero *et al*., 2000). Trehalose synthesis is stimulated by heat shock and osmotic stress (De Virgilio *et al*., 1994; Gounalaki and Thireos, 1994; Ribeiro *et al*., 1994) and its accumulation correlates with thermotolerance of yeast cells (Piper, 1998, Singer and Lindquist, 1998a, Singer and Lindquist, 1998b).

It has been reported that trehalose is essential for the viability of the *E. coli* cells at low temperature (Kandror *et al*., 2002). Sahara *et al*. (2002) and Schade *et al*. (2004) observed that the accumulation of trehalose and glycogen is induced during cold response. The production of glycogen is also induced after cold shock; however its role in the cold response has not yet been elucidated (Aguilera *et al*., 2007). Exogenous trehalose restored the viability of yeast cells during freezing by possible protection of the cellular membrane (Diniz-Mendes *et al*., 1999). The disaccharide trehalose could act as a chemical chaperone for membrane and protein stabilization (Sahara *et al*., 2002).

In fermentation conditions yeast cells began to accumulate storage carbohydrates from growth arrest (Novo *et al*., 2003; Rossignol *et al*., 2003). Novo *et al*. (2003) observed a correlation with the initial degradation of trehalose and glycogen by yeast and the timing of lag phase. On the other hand, similar accumulation profile of both reserve carbohydrates was found along the fermentation at 13 ºC and 25 ºC.

3.8 Stress and low temperature

In the past years many attempts have been made to elucidate the cold response in *S. cerevisiae* using the DNA microarray technology. Low temperature has several effects on biochemical and physiological properties in yeast cell and therefore modifying a large set of genes.

There are several aspects that need to be taken into account in order to interpret the effects of low temperature. Primarily, the response to low temperature can be categorized into two different groups based on the severity of the temperature. Cold shock is defined as the response of cells when exposed to temperatures of 10 – 15 ºC, whereas freeze shock occurs at temperatures of <10 ºC (Al-Fageeh and Smales, 2006).

Examination of different microarray-based studies in *S. cerevisiae* after a decrease in temperature reveals that part of the genetic response to low temperature seems to be time dependent. Sahara *et al*. (2002) found three different phases according to the expression profile: (i) in the early phase genes involved in RNA polymerase I and rRNA processing are up-regulating; (ii) in the middle phase genes involved in cytosolic ribosomal proteins are up-regulated; (iii) in the late phase genes involved in general stress response are up-regulated. On the other hand similar results were shown by Schade *et al.* (2004) who identified two distinct responses during cold adaptation: early cold response (ECR) during ≤ 2 h and are induced genes implicated in RNA metabolism and lipid metabolism; and late cold response (LCR) during ≥ 12 h and are induced genes which encode proteins involved in protecting the cell against variety of stresses. Thus, in both studies the late response is overlapped with the environmental stress response (ESR). Genes induced during ESR are involved in a variety of cellular functions such as protein folding and degradation, transport and carbohydrate metabolism. Repressed ESR genes

generally are involved in cell growth-related process, including RNA metabolism, nucleotide biosynthesis, secretion and ribosomal performance. The regulation of ESR is determined by the function of two transcription factors, Msn2p and Msn4p that bind to stress response elements (STREs) in the promoters on their target genes (Görner *et al*., 1998; Martinez-Pastor *et al*., 1996; Schmitt and McEntee, 1996). In contrast, the transcriptional response of the ECR genes was Msn2p/Msn4p independent and seems to be cold specific response.

From several studies on genome wide expression analysis, at low temperature, using DNA microarray in *S. cerevisie* (Homma et al., 2003; Murata *et al*., 2006; Sahara *et al*., 2002; Schade *et al*., 2004) we could know many genes commonly induced in all these works. Some genes involved in lipid metabolism are induced during ECR, in order to control the membrane fluidity. The fatty acid desaturase gene *OLE1* is induced to counteract the decrease in membrane fluidity (Nakagawa *et al*., 2002). Furthermore other lipid genes are induced at low temperature such as *INO1* and *OPI3* (Murata *et al*., 2006). Tai *et al*. (2007) also showed that lipid metabolism genes were the only ones whose activity was clearly regulated by low temperature in batch and chemostat cultures.

Other cold-induced genes during ECR were those encoding RNA helicase, RNA-binding proteins and RNA-processing proteins, e.g. *NSR1*, required for normal pre-rRNA processing (Kondo and Inouye, 1992) or *DBP2*, a RNA helicase (Barta and Iggo, 1995).

One of the major ECR genes groups includes the family of temperature inducible proteins (*TIP*). The *TIP* family has been renamed the *DAN/TIR* family to incorporate the *TIP*-related (*TIR*) genes and Delayed Anaerobic (*DAN*) genes (Abramova *et al*., 2001). The nine members of *DAN/TIR* family

are serine- and alanine-rich cell wall mannoproteins which function as "low temperature growth genes" by maintaining cell wall integrity and aiding the adaptation to extreme temperature shifts (Abramova *et al*., 2001; Homma *et al*., 2003; Kondo and Inouye, 1991; Schade *et al*., 2004). It has been shown that the *DAN/TIR* genes are induced under cold temperature stress particularly when there is a lack of oxygen (Abramova *et al*., 2001). Seripauperin (*PAU*) family genes (*PAU1*, *PAU2*, *PAU3*, *PAU4*, *PAU5*, *PAU6* and *PAU 7*) were also induced at low temperature, which have been shown to display phospholipid interacting activity (Zhu *et al*., 2001).

During LCR a variety of *HPS* genes (*HSP12*, *HSP26*, *HSP42*, *HSP104*, *YRO2* and *SSE2*) were also found induced, suggesting a requirement for nuclear chaperones for protein folding and maintaining protein conformation in the cold (Schade *et al*., 2004). In addition genes belonging to the glutathione/glutaredoxine system (*GTT2*, *HYR*, *GPX1*, *TTR1* and *PRX1*) were induced in the LCR (Murata *et al*., 2006; Schade *et al*., 2004). These genes were previously shown to be induced by oxidative stress and they were also implicated in detoxification processes (Gasch *et al*., 2000).

Despite the plethora of low-temperature transcriptome datasets, major questions still have to be addressed. There are major discrepancies in the low-temperature transcriptome data already published (Homma *et al.,* 2003; Murata *et al.,* 2006; Sahara *et al.,* 2002; Schade *et al.,* 2004). For instance, the observed expression of ribosomal protein (RP) genes has some inconsistencies. Although Sahara *et al*. (2002) reported an increased of many RP genes during a temperature downshift to 10 ºC, a similar temperature downshift resulted in a totally different transcriptional response in the study by Schade *et al*. (2004). Second, although the induction of genes involved in reserve carbohydrates seems to be a consistent feature of cold shock, trehalose is only indispensable for survival in near-freezing conditions (Kandror *et al*., 2004). Furthermore,

above 10 ºC Δ*tps1*Δ*tps2* double mutant showed no growth defects or viability loss (Panadero *et al.*, 2006).

Most of these studies have mainly been focused on the genome-wide transcriptional responses to cold-shock (Homma *et al.,* 2003; Murata *et al.,* 2006; Sahara *et al.,* 2002; Schade *et al.,* 2004). In interpreting effects of low temperature and other environmental parameters on microbial physiology, the time scale of exposure is essential. Sudden exposure to environmental changes (e.g. cold shock) is likely to trigger rapid, highly dynamic stress response phenomena (adaptation). While acclimation is a prolonged exposure of an organism to nonlethal stimuli in which its regulatory mechanisms have resulted in full adaptation of genome expression to the environmental conditions. On an even longer time scale, mutational changes lead to evolutionary adaptation of the genome itself (Brown *et al.*, 1998).

In batch cultures the specific growth rate (μ) is strongly affected by temperature, which makes it impossible to dissect temperature effects on transcription from effects of specific growth rate. This is relevant because specific growth rate as such has a strong impact on genome-wide transcript profiles (Castrillo *et al.,* 2007; Regenberg *et al.,* 2006). Furthermore all culture variables (e.g. intra- and extracellular metabolites) evolve in time and result in complex data patterns and make the identification of temperature-specific responses very difficult (Tai *et al.*, 2007). In contrast to batch cultures, chemostat cultures enable accurate control of specific growth rate, independent of other culture conditions. In chemostat cultures, the dilution rate (D) is defined as the ratio of the flow rate of the ingoing medium (f, $L \cdot h^{-1}$) and the culture volume (V, L). When the culture volume is kept constant by continuous removal of culture broth, a steady-state will be reached in which the specific growth rate (μ, h^{-1}) is equal to the dilution rate (D). In steady-state chemostat culture the concentrations of all metabolites and substrates are constant in time.

In a decisive study, Tai *et al.* (2007) compared their transcriptomic results obtained in a steady-state chemostat culture with other previous genome-wide transcriptional studies of batch cultures at low temperature. This comparison revealed large differences between transcriptional response during long-term low temperature acclimation and the transcriptional response to rapid transition to low temperature. In contrast to observations in cold-shock and batch culture studies, transcript levels of environmental stress response genes and trehalose biosynthesis genes were reduced at 12 ºC. Interestingly, lipid metabolism genes were the only ones whose activity was clearly regulated by low temperature.

Although cold stress has been widely studied, very little is understood with regards to how cells adapt to cold temperatures during processes such as winemaking, where multiple stresses are present. Beltran *et al.* (2006) compared the expression patterns of the wine yeast QA23 during the industrial fermentation carried out at 13 ºC and 25 ºC. From a total of 535 ORFs that were significantly differentially expressed between 13 ºC and 25 ºC fermentations, two significant programs were identified. A cold-stress response was expressed at the initial stage of the fermentation and this was followed by a transcription pattern of up-regulated genes concerned with the cell cycle, growth control and maintenance in the middle and late stages of the process at 13 ºC with respect to 25 ºC. These expression patterns were correlated with higher cell viability at low temperature. The other relevant difference was that several genes implicated in cytosolic fatty acid synthesis were down-regulated whereas that involved in mitochondrial short-chain fatty acid synthesis were up-regulated in fermentation process conducted at 13 ºC compared to that at 25 ºC. These transcriptional changes were qualitatively correlated with improved resistance to ethanol and increased production of short-chain (C_4 – C_8) fatty acids and their corresponding esters at 13 ºC as compared to 25 ºC.

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STRATEGIES FOR THEIR GENETIC IMPROVEMENT
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BACKGROUND AND OBJECTIVES

The selection of suitable microorganisms for use in industrial processes is a key issue in food technology. Due to the demanding nature of the modern winemaking practice and sophisticated wine markets, there is an ever-growing quest for specialized wine yeast strains that possess a wide range of optimized, improved or novel oenological properties.

This PhD has been carried out in the Oenological Biotechnology group at the Department of Biochemistry and Biotechnology (Faculty of Oenology, Rovira i Virgili University, Tarragona, Spain) and in the System biology in biotechnological yeasts group located at the Institute of Agrochemistry and Food Technology (IATA) of the Spanish Scientific Research Council (CSIC), Valencia (Spain). When I joined the research group, one of the research objectives was to focus on studying wine yeast metabolism at low temperature fermentation. Low temperature fermentations are an interesting application in the wine industry, especially in the production of wines with more pronounced aromatic profiles. This work has been supported by grants from the Spanish government (AGL2007-65498-C02-02 and AGL2010-22001-C02-01). The first project focused on studying the importance of lipid metabolism at low temperature fermentation, whereas the second project centred on determining the molecular and physiological mechanisms that determine greater tolerance at low temperature.

As fermentation temperature is effectively controlled by winemakers, low temperature fermentations (10–15ºC) are becoming more frequent. White and “rosé” wines produced at low temperature are often considered to have improved sensory qualities. However, there are certain drawbacks to low temperature fermentations, such as reduced growth rate, a long lag phase, and sluggish or stuck fermentations. Thus, very few commercial wine strains available on the market are well-adapted to ferment at low temperature. Our research group has long since been studying the mechanism of adaptation of wine yeast *Saccharomyces*

cerevisiae to low temperature fermentation. The results obtained in previous studies done by our group demonstrate that low temperature does not only prevent loss of primary (varietal) aromas by evaporation, but also increases the synthesis of secondary aromas (Llauradó *et al*., 2002; Torija *et al*., 2003). Other studies have analyzed the transcriptional and proteomic differences between low and optimal temperature fermentations (Beltran *et al*., 2006; Salvadó *et al*., 2012). Lipid composition of cellular membranes has been directly related with the yeast adaptive response at different environmental temperatures in many studies (Beltran *et al*., 2006; Beltran *et al.,* 2008; Redón *et al.,* 2009; Redon *et al*., 2011; Redon *et al*., 2012; Torija *et al.,* 2003). The effect of low temperature fermentation on nitrogen metabolism has also been studied (Beltran *et al*., 2007). This information enabled us to improve haploid derivative wine yeast by genetic engineering by overexpressing several genes that previously related with low temperature adaptation (Chiva *et al*., 2012). Moreover, some studies have focused on studying the cryotolerant *Saccharomyces* species, *Saccharomyces bayanus* var. *uvarum* and *Saccharomyces kudriavzevii,* by mainly analyzing the lipid composition of their membranes (Torija *et al*., 2003; Redón *et al*., 2011; Tronchoni *et al*., 2012).

In this context, the main objectives of this thesis work were to study the molecular and metabolic mechanisms of adaptation of wine yeast to low temperatures and to develop well-adapted wine yeast strains to low temperature fermentation. To do this, we analyzed the metabolome of commercial *S. cerevisiae* wine strain QA23, which has been our model strain in most of the studies mentioned above. The metabolome of this strain, grown at optimal and low temperatures, was compared with the metabolome of two strains belonging to the cryotolerant species: *Saccharomyces bayanus* var. *uvarum* and *Saccharomyces kudriavzevii*. We also did an in depth analysis of the genes involved in the lipid metabolism of *S. cerevisiae,* and the selected genes were deleted or overexpressed in a derivative haploid of this commercial wine strain. The growth and fermentation performance of the

constructed mutants and overexpressing strains were analyzed at optimum and low temperature. However, another important goal of this thesis consisted in obtaining a genetically improved strain in its fermentation capacity at low temperature. Using a batch serial dilution, and by mimicking the wine fermentation conditions at 12 ºC, we selected and developed well-adapted wine yeast strains to ferment at low temperature, and we attempted to decipher the process underlying the phenotypes obtained.

These general objectives have been dealt in the following partial objectives:

Metabolomic comparison of *Saccharomyces cerevisiae* and cryotolerant species *S. bayanus* var. *uvarum* and *S. kudriavzevii* during wine fermentation at low temperature. We aim to determine the metabolic differences in commercial wine yeast growing at 12 ºC and 28 ºC in a synthetic must. In an attempt to also detect interspecific metabolic differences, we characterized the metabolome of *Saccharomyces bayanus* var. *uvarum* and *Saccharomyces kudriavzevii* growing at 12 ºC, and we compared it with the metabolome of *S. cerevisiae* at the same temperature. These results are reported in Chapter 1.

Lipid metabolism of *Saccharomyces cerevisiae*: its importance in growth and wine fermentation at low temperature. Many studies have reported the relevance of lipid composition in the yeast adaptive response to low temperature. To achieve this objective, we analyzed the growth of knockouts in phospholipids, sterols and sphingolipids, from the EUROSCARF collection of the *Saccharomyces cerevisiae* BY4742 strain at low and optimal temperatures. These results enabled us to identify the genes involved, which were also deleted or overexpressed in a derivative haploid of the commercial wine strain. The phenotypic differences in the mutant and overexpressing strains correlated with changes in their lipid composition. These results are reported in Chapter 2**.** Furthermore, we aimed to determine the influence of these genes on growth and fermentation performance

during wine fermentations at low and optimal temperatures. These results are reported in Chapter 3.

Wine yeast improved in low temperature fermentation by evolutionary engineering: deciphering the molecular basis. There are very few commercial wine strains available on the market which are well-adapted to ferment at low temperature (10-15 ºC). For this reason, the selection and development of new *S. cerevisiae* strains with improved fermentation abilities at low temperature is of much interest for future biotechnological applications. The aims of this study were to select and develop wine well-adapted yeast strains to ferment at low temperature, and to decipher the process underlying the phenotypes obtained. To achieve this objective, a collection of commercial wine strains was inoculated in a synthetic must at 12 ºC and was grown in a batch serial dilution for 200 generations, with and without an initial mutagenesis. Afterward, the growth and fermentation performance of the evolved strain were characterized. Finally, to gain a better understanding of the low temperature adaptation of the evolved strain, we sequenced the whole genome of the parental and evolved strains to identify the mutations which contributed to the improved phenotype at low temperature. Moreover we also analyzed the gene expression of the improved and parental strains. The global genome and transcriptome of these strains evidenced the molecular basis of the low temperature adaptation.

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Chapter 1

Metabolomic comparison of *Saccharomyces cerevisiae* and the cryotolerant species *S. bayanus* var. *uvarum* and *S. kudriavzevii* during wine fermentation at low temperature

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PlosOne 8(3): e60135.

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ABSTRACT

Temperature is one of the most important parameters affecting the length and rate of alcoholic fermentation and final wine quality. Wine produced at low temperature is often considered to have improved sensory qualities. However, there are certain drawbacks to low temperature fermentations such as reduced growth rate, long lag phase, and sluggish or stuck fermentations. To investigate the effects of temperature on commercial wine yeast, we compared its metabolome growing at 12 ºC and 28 ºC in a synthetic must. Some species of the *Saccharomyces* genus have shown better adaptation at low temperature than *Saccharomyces cerevisiae*. That is the case of the cryotolerant yeasts *Saccharomyces bayanus* var. *uvarum* and *Saccharomyces kudriavzevii*. In an attempt to detect inter-specific metabolic differences, we characterized the metabolome of these species growing at 12 ºC which we compared with the metabolome of *S. cerevisiae* (not well adapted at low temperature) at the same temperature. Our results show that the main differences between the metabolic profiling of *S. cerevisiae* growing at 12 ºC and 28 ºC were observed in lipid metabolism and redox homeostasis. Moreover, the global metabolic comparison among the three species revealed that the main differences between the two cryotolerant species and *S. cerevisiae* were in carbohydrate metabolism, mainly fructose metabolism. However, these two species have developed different strategies for cold resistance. *S. bayanus* var. *uvarum* presented elevated shikimate pathway activity, while *S. kudriavzevii* displayed increased NAD^+ synthesis.

Keywords: metabolome, cold, yeast, wine, fermentation.

INTRODUCTION

Temperature is one of the main relevant environmental variables that microorganisms have to cope with. The natural environment for the majority of microorganisms, including yeast species, exhibits temporal fluctuations in temperature on scales ranging from daily to seasonal. In addition, temperature is a key factor in some industrial processes involving microorganisms. Low temperatures (10-15 ºC) are used in wine fermentations to enhance production and to retain flavor volatiles. In this way, white and "rosé" wines of greater aromatic complexity can be achieved (Beltran *et al.,* 2008; Torija *et al.,* 2003). However the optimal growth temperature of the wine yeast *Saccharomyces cerevisiae* is around 32 ºC (Salvadó *et al.,* 2011). Thus low-temperature fermentation has its disadvantages such an increased lag phase and a reduced growth rate, producing stuck and sluggish fermentations (Bisson, 1999). Therefore the quality of wines produced at low temperature depends on the ability of yeast to adapt to cold.

Despite *S. cerevisiae* being primarily responsible for alcoholic fermentation, other species of the genus *Saccharomyces,* such as *S. bayanus* var. *uvarum,* have been isolated during wine and cider fermentation (Masneuf-Pomarède *et al.,* 2010; Naumov *et al*., 2000a). Moreover natural interspecific *Saccharomyces* hybrids have been isolated in wine fermentations (González *et al*., 2006). These authors identified and characterized new hybrids between *S. cerevisiae* and *S. kudriavzevii*, between *S. cerevisiae* and *S. bayanus*, as well as a triple hybrid *S. bayanus* x *S. cerevisiae* x *S. kudriavzevii*. However *S. kudriavzevii* has been isolated only from natural environments and was formally described from decaying leaves in Japan (Naumov *et al*., 2000b). Recently, Sampaio and Gonçalves (2008) and Lopes *et al.* (2010) also isolated new strains in Portugal and Spain, respectively. These two species, *S. bayanus* var. *uvarum* and *S. kudriavzevii* are considered the most psychrotrophic species of the *Saccharomyces* genus (Belloch *et al*., 2008; Salvadó *et al.,* 2011; Serra *et al*., 2005). Moreover, these two cryotolerant species possess

other advantages as compared to *S. cerevisiae* in terms of valuable organoleptic properties, such as greater glycerol production and lower ethanol production (Arroyo-López *et al*., 2010; Serra *et al*., 2005).

In past years, some attempts have been made to elucidate the cold response in *S. cerevisiae* using a variety of high-throughput methodologies. Some studies have analyzed the genome-wide transcriptional response of *S. cerevisiae* to low temperatures. These studies have mainly focused on cold shock (Homma *et al*., 2003; Murata *et al*., 2006; Sahara *et al*., 2002; Schade *et al*., 2004). Schade *et al*. (2004) identified two distinct phases during the cold shock response: 1) an early cold response (ECR) occurring within the first 12 h after exposure to low temperature and 2) a late cold response occurring beyond 12 h after exposure to low temperature. ECR-induced genes are implicated in RNA and lipid metabolism, whereas the genes induced during LCR mainly encode the proteins involved in protecting the cell against a variety of stresses. In fact, the LCR response is very similar to the general stress response mediated by transcription factors Msn2p/Msn4p. However, the response type depends on the length of exposure to stressful conditions. Sudden exposure to environmental changes (e.g., cold shock) is likely to trigger a rapid, highly dynamic stress-response (adaptation). Prolonged exposure to non lethal stimuli leads to acclimation; i.e., establishment of a physiological state in which regulatory mechanisms, like gene expression, fully adapt to suboptimal environmental conditions (Tai *et al.,* 2007). Tai *et al.* (2007) compared their transcriptomic results obtained during cold acclimation in a steady-state chemostat culture with other previous genome-wide transcriptional studies of batch cultures at low temperature, and they found major discrepancies among the low-temperature transcriptome datasets. The authors partially explained these major differences by the cultivation method used in different transcriptome experiments. Although batch cultures are well-suited to study low temperature adaptation dynamics, they are poorly adapted to study prolonged exposure to low temperature. In such cultures, the specific growth rate (μ) is strongly affected by

temperature, which makes it impossible to dissect temperature effects on transcription from specific growth rate effects. Two recent chemostat studies (Castrillo *et al.*, 2007; Regenberg *et al.*, 2006) also found that the growth rate itself has a strong effect on transcriptional activity. Furthermore, chemostat cultures help to accurately control the specific growth rate, so the concentration of all the metabolites is constant over time, thus providing a good platform to study microbial physiology and gene expression. These genome-wide studies have also tackled the transcriptional response of *S. cerevisiae* (Beltran *et al.*, 2006), *S. kudriavzevii* and their natural hybrids (Combina *et al.*, 2012) in enological conditions.

In addition to transcriptomic, there are the other so-called "omics" currently available, such proteomics and metabolomics. In our group, we previously studied the proteome changes of a commercial wine yeast strain during the first hours of fermentation at low temperature (Salvadó *et al.,* 2008). However, there is a limited body of knowledge on the application of metabolomics to the winemaking process, and it has never been applied to understand yeast cold adaptation under fermentation conditions. The metabolome comprises the complete set of metabolites, these being the non genetically encoded substrates, intermediates and products of the metabolic pathways associated with a cell. By representing integrative information across multiple functional levels and by linking DNA encoded processes with the environment, the metabolome offers a window to the map core attributes responsible for different phenotypes (Nielsen and Jewett, 2007). Few studies have applied metabolomics to the winemaking process. Skogerson *et al.* (2009) determined the metabolite profiles of white wines belonging to different grape varieties. As far as we know, comprehensible metabolic profiles have not yet been determined in yeast growing under conditions that mimic industrial fermentations.

The aim of this study is to improve the feasibility of low-temperature wine fermentation by identifying biomarkers and metabolic adaptations at low

temperature in the industrial wine yeast strain QA23, and to detect differential metabolites that distinguish two cryotolerants *S. bayanus* var. *uvarum* and *S. kudriavzevii* species from *S. cerevisiae,* whose growth potential is limited at this temperature. To achieve these objectives, we analyzed the global metabolic profiling of *S. cerevisiae* growing in a steady-state chemostat at 12 ºC and 28 ºC at the same growth rate. Thus, for a better understanding of the low-temperature adaptation of *S. bayanus* var. *uvarum* and *S. kudriavzevii*, we characterized the metabolome of these species which we compared with the metabolome of commercial wine yeast QA23 at 12 ºC.

MATERIAL AND METHODS

Yeast strains and culture conditions

A commercial *S. cerevisiae* (*Sc*) wine strain (QA23, Lallemand S.A., Canada), a *S. bayanus* var. *uvarum* (*Su*) strain (CECT12600) and a *S. kudriavzevii* strain (*Sk*) (CR85) (Lopes *et al*., 2010) were used in this work. The *Sc* strain was grown at a dilution rate (D) of 0.04 h^{-1} at 12 ºC and 28 ºC in 2 L chemostat (Biostat ® B, Braun Biotech International, Sartorius Group, Germany) with a working volume of 0.75 L. The *Su* and *Sk* strains were grown under the same conditions, but only at 12 ºC. A temperature probe connected to a cryostat controlled the cultures grown at 12 ºC.

Cultures were grown in the synthetic grape must (SM) derived from that described by Bely *et al.* (1990). The medium composition included 200 g/L of sugars (100 g/L glucose + 100 g/L fructose), 6 g/L malic acid, 6 g/L citric acid, 1.7 g/L YNB without ammonium and amino acids, anaerobic factors (15 mg/L ergosterol, 5 mg/L sodium oleate and 0.5 mL/L tween 80) and 60 mg/L potassium disulfite. The assimilable nitrogen source used was 300 mg N/L (120 mg N/L as ammonium and 180 mg N/L in an amino acid form). pH was measured online and kept constant at 3.6 by the automatic addition of 2M NaOH and 1M HCl. The stirrer was set at 100

rpm. Biomass and extracellular metabolites were constant for at least five volume changes before sampling. Sampling of cells from continuous cultures was carried out following the "general sample preparation recommendations for metabolon studies" of Metabolon, Inc. (Durham, NC, USA). When the steady-state was reached, a volume of approximately 30 units of OD_{600} was centrifuged at 1000 g for 3 min at 4 ºC. After supernatant removal, cell suspension was washed with PBS, transferred to a 1.5 to 2 mL microcentrifuge tube and centrifuged again in the same conditions. The pellet was flash-frozen with liquid nitrogen and stored at -80 ºC.

HPLC analysis

Glucose, fructose, glycerol and ethanol were analyzed in all the supernatant samples. Analytical HPLC was carried out in a Surveyor Plus Chromatograph (Thermo Fisher Scientific, Waltham, MA) equipped with a refraction index detector, autosampler and a UV-Visible detector. Prior to injection, samples were centrifuged at 13300 rpm for 5 min, and samples were diluted 10-fold and filtered through 0.22-µm pore size nylon filters (Micron Analitica, Spain). A total volume of 25 µL was injected into a HyperREZ XP Carbohydrate H+8 µm column (Thermo Fisher Scientific) assembled to its correspondent guard. The mobile phase used was 1.5 mM H_2SO_4 with a flux of 0.6 mL/min and a column temperature of 50 ºC. The concentration of each was calculated using external standards. Each sample was analyzed in duplicate.

Nitrogen content analysis

Ammonia concentration was measured with a kit using an enzymatic method (Roche Applied Science, Germany). The concentration of free amino acid nitrogen was determined using the σ-phthaldehyde/N-acetyl-L-cysteine spectrophotometric assay (NOPA) procedure (Dukes and Butzke, 1998). The results were expressed as mg nitrogen/mL.

Metabolic Profiling

Frozen samples of cells, OD of approximately 30 units, were submitted to Metabolon, Inc. (Durham, NC, USA) for sample extraction and analysis. The sample preparation process was carried out using the automated MicroLab STAR® system from the Hamilton Company. Sample preparation was conducted using a proprietary series of organic and aqueous extractions. The resulting extract was divided into two fractions; one for analysis by liquid chromatography / mass spectrometry (LC / MS) and one for analysis by gas chromatography / mass spectrometry (GC / MS). Samples were placed briefly in a TurboVap® (Zymark) to remove the organic solvent. The LC / MS extracts reconstituted under acidic conditions were gradient-eluted using water and methanol, both containing 0.1% formic acid, while basic extracts, which also used water / methanol, contained 6.5 mM ammonium bicarbonate. The samples destined for the GC / MS analysis were re-dried under vacuum desiccation for a minimum of 24 h prior to being derivatized under dried nitrogen using bistrimethyl-silyl-triflouroacetamide (BSTFA).

The LC / MS portion of the platform was based on a Waters ACQUITY UPLC and a Thermo-Finnigan LTQ mass spectrometer, which consisted of electrospray ionization (ISI) and a linear ion-trap (LIT) mass analyzer. The derivatized samples for GC / MS were analyzed in a Thermo-Finnigan Trace DSQ fast-scanning single-quadripole mass spectrometer using electron impact ionization. The GC column was 5% phenyl and the temperature ramp went from 40°C to 300°C in a 16-minute period. Accurate mass determination and MS / MS fragmentation (LC / MS), (LC / MS / MS) were carried out in a Thermo-Finnigan LTQ-FT mass spectrometer, which had an LIT front end and a fourier transform ion cyclotron resonance (FT-ICR) mass spectrometer backend.

Compounds were identified by comparison to Metabolon's library entries of purified standards or recurrent unknown entities. Data were normalized to the correct variation resulting from instrument inter-day tuning differences. Raw area

counts for each compound were corrected in run-day blocks by registering the medians to equal one and by normalizing each data point proportionately.

Statistical data processing

The metabolic data are the result of five replicates for each fermentation (temperature and strains). Significant differences between the supernatants of *Sk*, *Su* and *Sc* at both temperatures and the other two species were determined by *t*-tests. The statistical level of significance was set at $P \leq 0.05$.

For the metabolic profiling data, two types of statistical analysis were performed. Following log transformation and imputation with minimum observed values for each compound, Welch's two sample t-tests were used to identify the metabolites that differed significantly between the experimental groups. An estimate of the false discovery rate (q-value) was calculated to take into account the multiple comparisons that normally occur in metabolomic-based studies. A low q-value ($q<0.10$) indicates high confidence in a result. For classification purposes, we mainly used random forest analyses. Random forests create a set of classification trees based on the continual sampling of experimental units and compounds. Statistical analyses were performed with the R program.

Principal component analysis (PCA) was done using the *vegan* package (the *rda* function) of the R v.2.15 statistical software (R Development Core Team, 2010).

RESULTS

Global biochemical profiles comparison among the three species

This comparative biochemical study aimed to characterize the metabolic adaptations defining *S. cerevisiae* grown at a near-optimal temperature (i.e., 28 °C) versus a non optimal temperature (i.e., 12 °C), and to compare and contrast its profile to *Saccharomyces* genus members (*S. bayanus* var. *uvarum* and *S. kudriavzevii*) with greater tolerance at low temperature.

Our experimental design was based on continuous-culture fermentations. This system offers a stable and controlled environment for cells by maintaining constant biomass and concentrations of nutrients and products (Clement *et al.*, 2011), thus making the comparison between fermentation conditions and strains more feasible. All the cultures were grown at the same dilution rate (D), which corresponded to the maximum D of the control condition (*Sc* at 12 ºC). When the steady-state was reached (after five volume changes), the sampling of supernatants and cells was done. Table 1 shows the physiological data of the three *Saccharomyces* strains and the concentration of the main compounds of oenological interest in the supernatant during the steady-state. As seen, few sugars were consumed from the SM fed to the continuous cultures, and *Sc* at 28 ºC was the strain which more consumed sugars. Intriguingly, cryotolerant strains *Su* and *Sk* consumed less than 5% of the initial sugar content. These parameters clearly indicate that the metabolic study fitted with the first wine fermentation stages (initial exponential phase). In accordance with this fermentation stage, residual nitrogen (both ammonium and amino acids) has hardly been consumed, showing high concentrations in the supernatant. The high concentrations of sugars and nitrogen indicated that these chemostat cultures were not carbon-limited or nitrogen-limited. As the steady-state was reached in all the cultures, another nutrient, or group of nutrients, must be limiting growth. The physiological data showed remarkable differences between *Sc* and the cryotolerant species *Su* and *Sk*. Regardless the growth temperature, *Sc* showed higher sugar consumption rate and glycerol and ethanol production rate than *Su* and *Sk*. Conversely, *Su* and *Sk* presented higher biomass yields than *Sc*. Likewise, the cultures of *Sc* did not show significant differences in biomass yields, in glucose and ammonium consumption rate and ethanol production rate between both temperatures. Conversely, *Sc* at 12 ºC showed greater amino acid consumption and lower glycerol production rates than *Sc* at 28 ºC.

Table 1. Physiological characteristics of *Saccharomyces* strains and extracellular metabolites during the stedady-state of continuous cultures

	Sc 28 ºC	*Sc* 12 ºC	*Su*	*Sk*
Extracellular metabolites				
Glucose ($g\ L^{-1}$)	72.31 ± 1.73*	78.89 ± 1.02	89.09 ± 1.14*	92.02 ± 1.01*
Fructose ($g\ L^{-1}$)	83.88 ± 2.00	83.53 ± 1.40	93.18 ± 1.06*	95.08 ± 1.33*
Amino acids ($mg\ NL^{-1}$)	145.76 ± 8.55*	80.19 ± 4.00	150.73 ± 2.91*	148.55 ± 4.07*
Ammonium ($mg\ L^{-1}$)	90.71 ± 5.95*	55.18 ± 2.93	105.44 ± 6.03*	121.94 ± 3.29
Glycerol ($g\ L^{-1}$)	4.62 ± 0.17*	1.60 ± 0.09	1.17± 0.09*	0.91 ± 0.04*
Ethanol ($g\ L^{-1}$)	19.97 ± 0.45*	13.98 ± 0.78	6.38 ± 0.54*	4.76 ± 0.12*
Physiological data				
Biomass ($g\ DW\ L^{-1}$)	1.55 ± 0.07*	1.15 ± 0.07	0.93 ± 0.11	0.77 ± 0.06*
$Y_{hexoses\text{-}X}$ ($g_{DW} \cdot g_{hexoses}^{-1}$)	0.0 4 ± 0.00	0.03 ± 0.00	0.06 ± 0.0*	0.06 ± 0.01*
$q_{glucose}$ ($g_{glucose} \cdot g\ DW^{-1} .\ h^{-1}$)	-0.71 ± 0.05	-0.73 ± 0.04	-0.44 ± 0.02*	-0.42 ± 0.06*
$q_{fructose}$ ($g_{fructose} \cdot g\ DW^{-1} .\ h^{-1}$)	-0.42 ± 0.05*	-0.57 ± 0.05	-0.26 ± 0.02*	-0.26 ± 0.06*
q_{aa} ($mg_{Naa} \cdot g\ DW^{-1} .\ h^{-1}$)	-0.88 ± 0.22*	-3.47 ± 0.14	-1.3 ± 0.02*	-1.68 ± 0.22*
q_{NH4} ($mg_{NH4} \cdot g\ DW^{-1} .\ h^{-1}$)	-1.67 ± 0.08	-1.16 ± 0.37	-0.62 ± 0.32	0.00± 0.00*
$q_{glycerol}$ ($g_{glycerol} \cdot g\ DW^{-1} .\ h^{-1}$)	0.12 ± 0.00*	0.06 ± 0.00	0.05 ± 0.00*	0.05 ± 0.00*
$q_{ethanol}$ ($g_{ethanol} \cdot g\ DW^{-1} .\ h^{-1}$)	0.52 ± 0.01	0.49 ± 0.03	0.28 ± 0.03*	0.25 ± 0.00*

*Significant differences (p value≤ 0.05) compared to the control condition (*Sc* 12 ºC).

The comparison of the global metabolic profiles of the control condition *Sc*-12 ºC versus the other continuous-cultures (*Sc*-28 ºC, *Su*-12 ºC and *Sk*-12 ºC) resulted in 295 molecules identified from a total of 1700 molecules contained within the Metabolon library (Table S1). These compounds included a wide variety of metabolic classes: amino acids, peptides, carbohydrates, energy metabolisms, lipids, nucleotides, cofactors, vitamins and xenobiotics.

The differences in metabolite concentration were used to perform a hierarchical clustering (Fig. 1A), grouping to *Sc*-12 ºC and *Sc*-28 ºC in a sub-cluster and *Su* and *Sk* in another one. In fact, cryotolerant strains showed a very similar metabolic profile, with increases and decreases in the same metabolic compounds. Although clear differences can be observed in the metabolic profile of *Sc* at both temperatures, differences between this species and the two cryotolerant species were greater. In order to detect the metabolites which better defined the metabolic profiles of these three species, we used a principal component analysis (PCA) (Fig. 1B). The two first components were retained, explaining 96.1% of total variance. Te first component (PC1) explained 70.3% of variation and was marked by high positive component loadings for fructose (+0.88), glucose-6-P (+0.31) and mannose-6-P (+0.309). The second component (PC2) explained 25.8% of variation and was marked by high positive component loadings for the isobaric compounds fructose 1,6-diphosphate, glucose 1,6-diphosphate and myo-inositol 1,4 or 1,3 diphosphate (+0.938) and for the nitrogen compound gamma-aminobutyrate (GABA) (+0.25). No highly negative component loadings were observed for either of both axes. These results indicate that the three species were separated by their differences in carbohydrate metabolism, mainly glucose and fructose metabolism. *Sk* samples were distributed in the lower right quadrant and were characterized by higher concentration of fructose, glucose 6-P and manose 6-P whereas the *Su* samples were situated in the upper quadrant and showed higher concentration of the isobaric compounds (Fig. 1B).

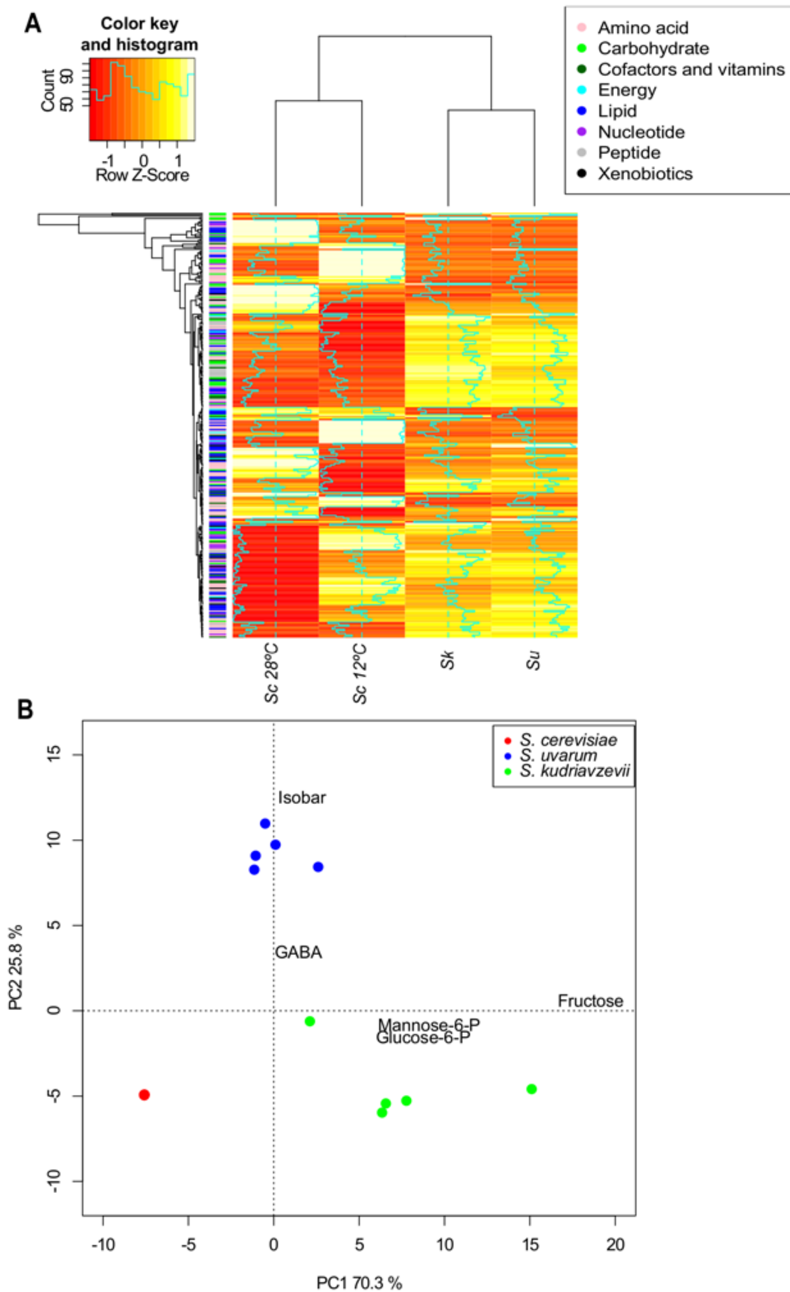

Figure 1. Global biochemical profiles comparison made of the three species. **A)** Hierarchical clustering of all the species is computed after standardizing metabolites to the Z-score. **B)** Biplot of the first two components of the PCA according to the metabolic composition. Isobar = isobaric compounds fructose 1,6-diphosphate, glucose 1,6-diphosphate and myo-inositol 1,4 or 1,3 diphosphate.

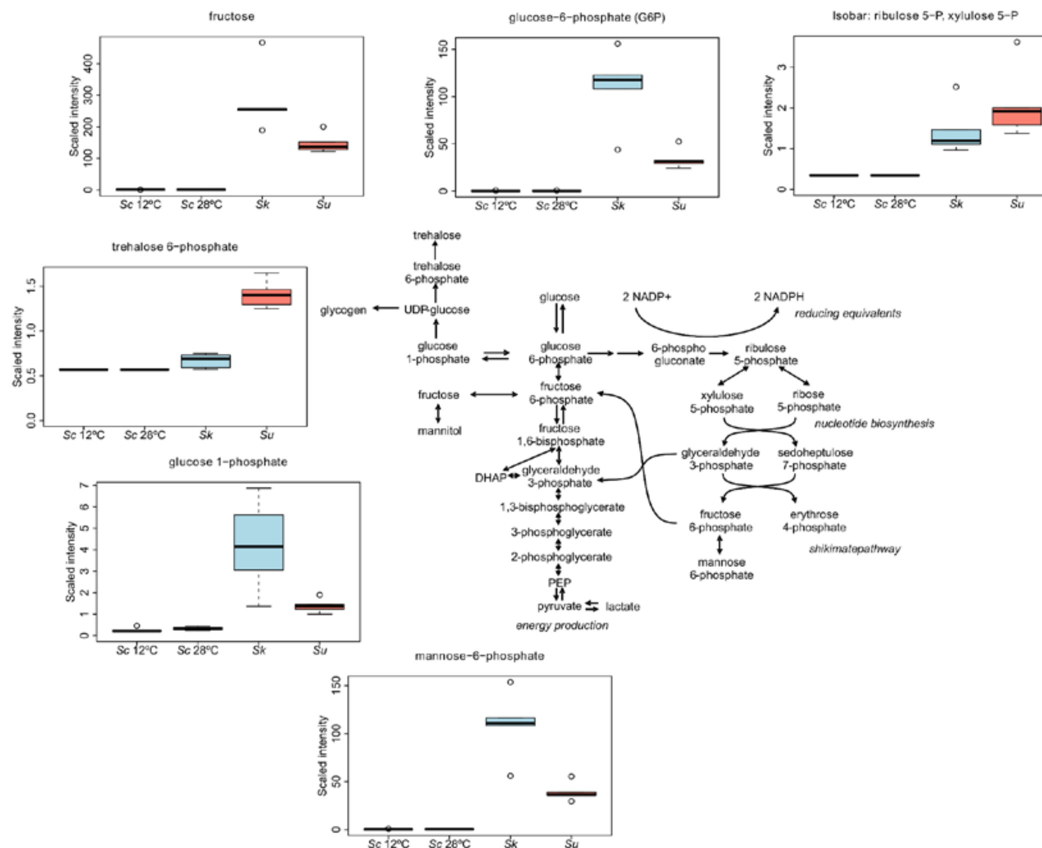

Figure 2. Carbohydrate metabolism: glycolytic pathway, trehalose synthesis, intermediates of the pentose phosphate pathway and protein mannosylation. Differentially produced metabolites within *Su* and *Sk* compared to *Sc*. Box legend: bar inside the box represents the median value, upper bar represents maximum of distribution, lower bar represents minimum of distribution, and the circle represents extreme data points.

The increased levels of these and other compounds of the carbohydrate metabolism are also shown in Fig. 2. Trehalose, but mainly its intermediates glucose-1-phosphate and trehalose-6-phoshate, significantly increased in the cryotolerant species at low temperature. Likewise, intermediates of the pentose phosphate pathway (i.e., isobaric compounds ribulose-5-phosphate and xylulose-5-phosphate), glycolysis (glucose-6-phosphate) and protein mannosylation (mannose-6- phosphate) pathways were all elevated in both *Su* and *Sk* (Fig. 2).

Comparison of the metabolic profile of the *Sc* growing at 12 ºC and 28 ºC

When the metabolic profiles of *Sc* growing at 12 ºC and 28 ºC were compared, the statistical t-test showed that more than half the 295 detected biochemicals had significantly changed. These compounds included a wide variety of classes. The majority of differences were observed in the amino acid and lipid classes (Fig. 3A). Another statistical method, known as Random Forest classification, was used to narrow down this large number of changes to a list of biochemicals, which was predicted to have the greatest influence on distinguishing between the *Sc* grown at both temperatures. The Random Forest classification identified a set of metabolites that could be used to separate the two growing conditions with 100% predictive accuracy (Fig. 3B). Among the top 30 metabolites contributing to the classification result, many were connected to membrane lipid metabolism and redox homeostasis.

Preservation of membrane fluidity is a key cold adaptive response and can be accomplished by increasing the degree of phospholipid acyl-chain unsaturation and by shortening the chain length of these fatty acids. However, only a few unsaturated fatty acids decreased at 28 ºC (i.e., myristoleate C14:1 and palmitoleate C16:1), and several more increased, such as linoleate (C18:2) and linolenate (C18:3) (Table S1). We also observed elevated lanosterol levels, intermediates of sterol biosynthesis, in the *Sc* cells grown at 28 ºC. In addition, the *Sc* grown at optimal temperatures displayed high levels of glycerophosphorylcoline (GPC), 2-

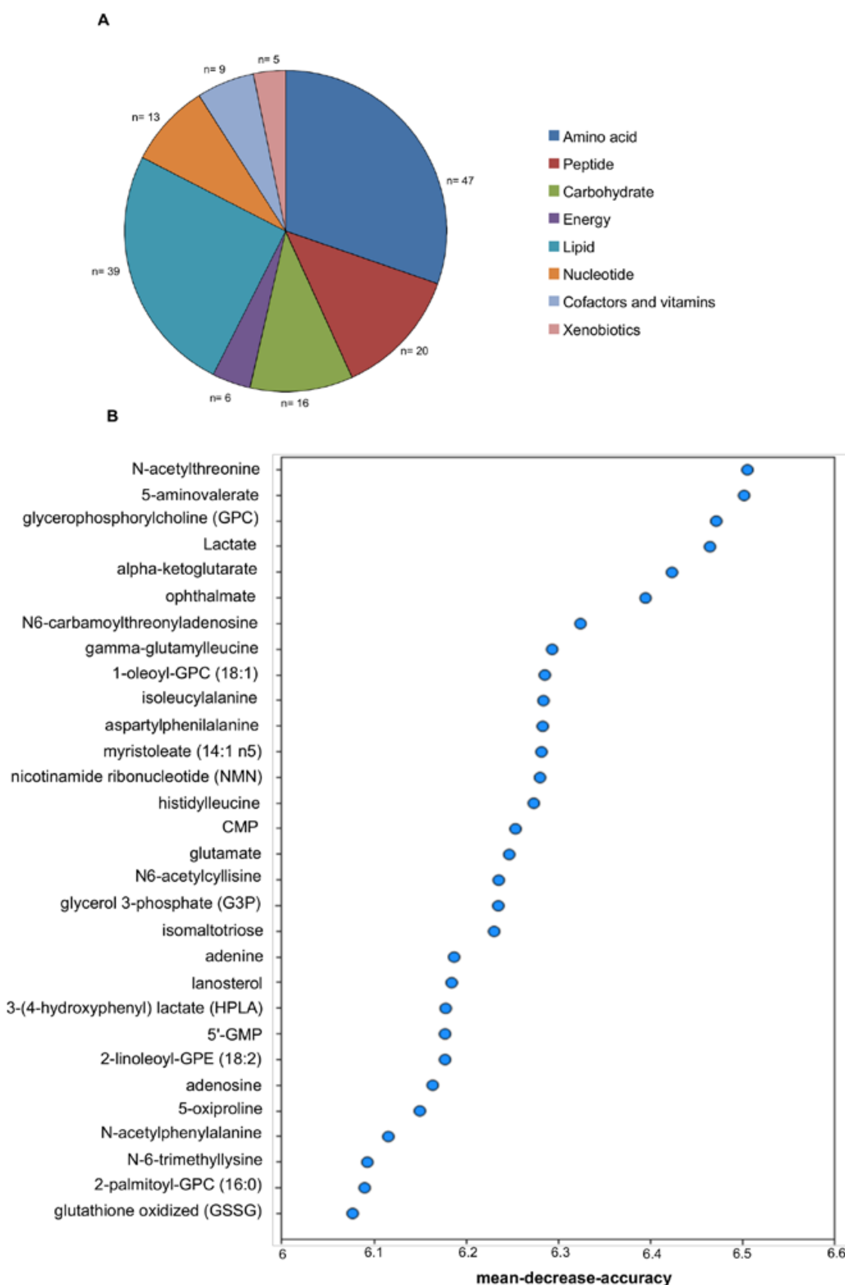

Figure 3. Principal metabolic differences between *Sc* growing at 12 ºC and at 28 ºC. A) Class distribution of the different identified metabolites. n = number of metabolites in each class. B) The Random Forest statistical analysis of the metabolomic data was used to identify the top 30 biochemicals with the greatest influence in distinguishing different groups. Metabolites are listed on the y-axis in order of importance, which importance decreasing from top to bottom. The mean decrease in accuracy for each metabolite is plotted on the x-axis.

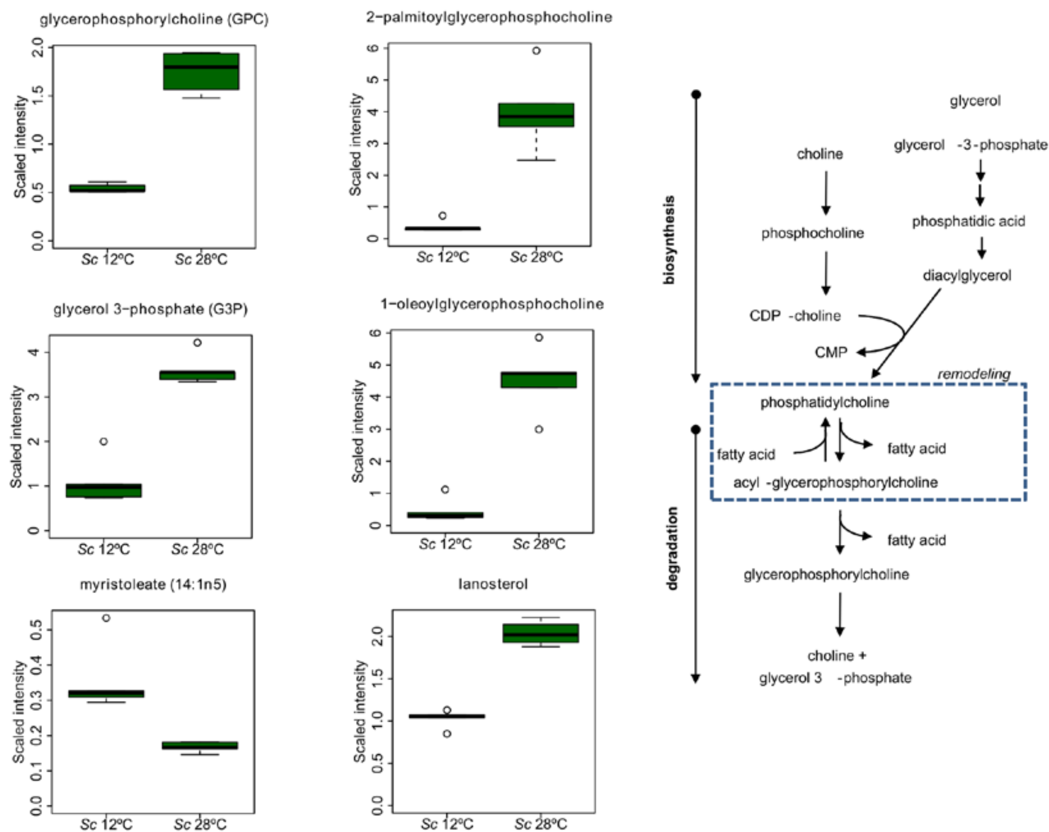

Figure 4. Phosphatidylcholine biosynthesis and degradation in *S. cerevisiae***.** Metabolic differences in the *Sc* growing at 12 ºC and 28 ºC. Box legend: bar inside the box represents the median value, upper bar represents maximum of distribution, lower bar represents minimum of distribution and the circle represents extreme data points.

palmitoylglycerophosphocholine, glycerol-3-phosphate and 1 oleoyglycerophosphocholine (Fig. 4).

Furthermore, we observed relative elevations in 5-oxoproline, reduced glutathione, gamma-glutamylcysteine and ophthalmate, as well as a relative reduction of oxidized glutathione in the *Sc* cells grown at 28 ºC, indicating that the cells grown at the higher temperature are better poised to deal with oxidative stress or that the *Sc* growing at 12 ºC undergo more oxidative stress (Fig. S1).

Comparison of the metabolic profile of *Sc* and *Su* grown at 12 ºC

In the same way as in the comparison of the metabolic profile of *Sc* at both temperatures, the statistical t-test revealed that more than half the 295 detected biochemicals had significantly changed. In this case, major differences were observed for amino acids, lipids and carbohydrates (Fig. 5A). Moreover, the Random Forest classification identified multiple metabolites connected to the fructose metabolism as discriminating factors to separate *Su* from *Sc* growing at 12 ºC (Fig. 5B). We also observed that shikimate, its metabolic intermediates, glucose-6-phospate and phosphoenolpyruvate, and a terminal product, tryptophan, were identified by the Random Forest analysis (Fig. 5B), all of them significantly increasing in *Su* (Fig. 6).

In addition, lysine and multiple precursors, including 2-aminoadipate, alpha-ketoglutarate and glutamate, were identified by the Random Forest analysis as compounds that separate these two species (Fig. 5B). Except for alpha-ketoglutarate, the initial substrate of the pathway, lysine and its pathway intermediates, such a 2-aminodipate, saccharopine and homocitrate, were significantly reduced in *Su* (Fig. S2).

Comparison of the metabolic profile of the *Sc* and *Sk* grown at 12 ºC

In the case of *Sk,* the statistical t-test showed that 179 of the 295 detected biochemicals had significantly changed as compared with *Sc*. Major differences

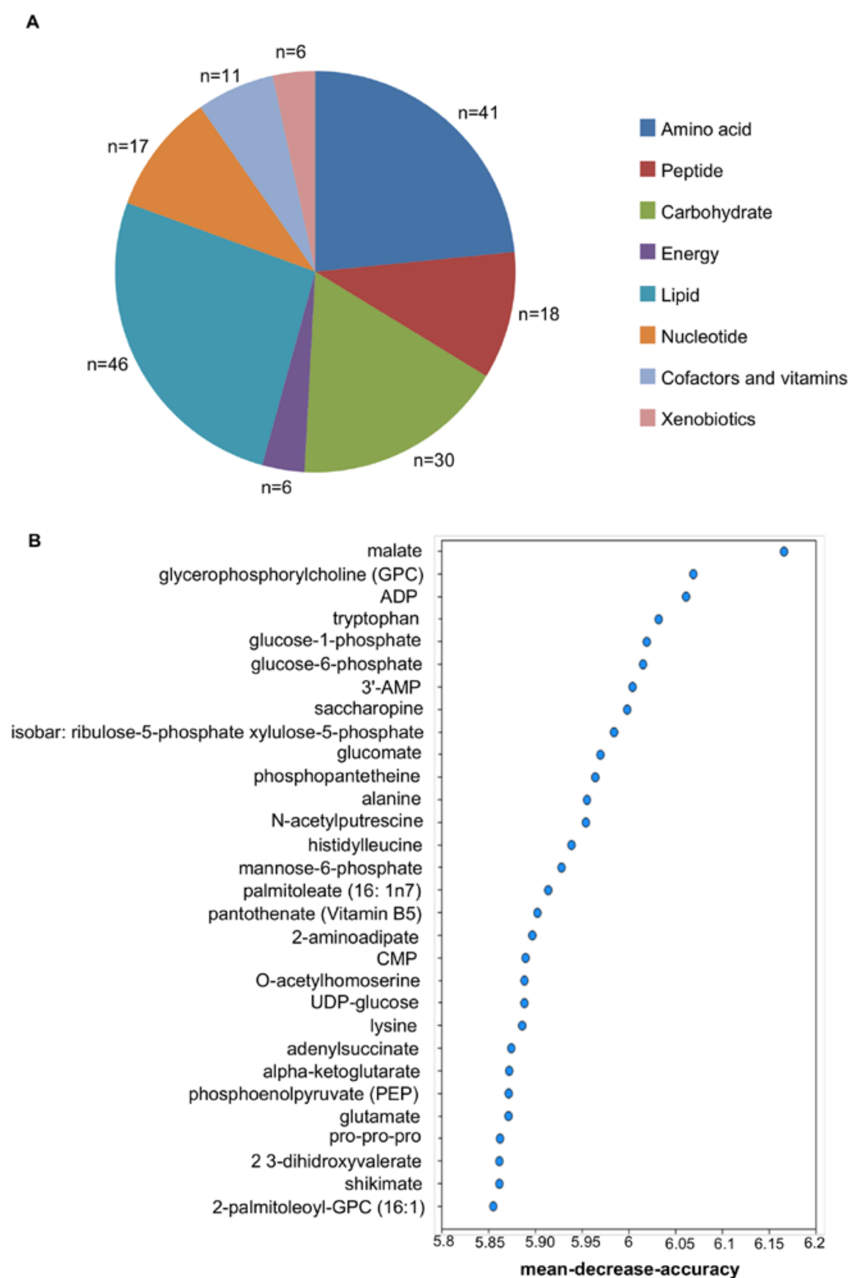

Figure 5. Principal metabolic differences between *Sc* and *Su* at 12 ºC. A) Class distribution of the different identified metabolites. n = number of metabolites in each class. B) The Random Forest statistical analysis of metabolomic data was used to identify the top 30 biochemicals with the greatest influence in distinguishing the different groups. Metabolites are listed on the y-axis in order of importance, with importance decreasing from top to bottom. The mean decrease in accuracy for each metabolite is plotted on the x-axis.

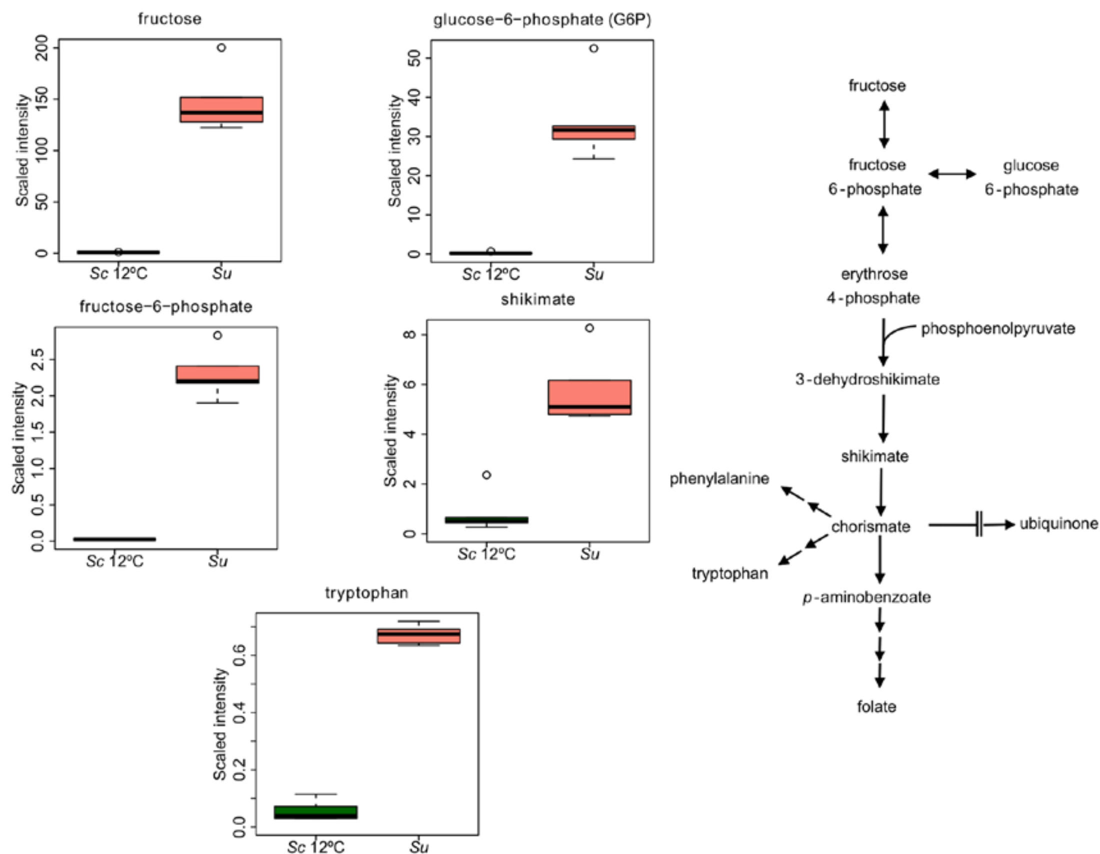

Figure 6. The Shikimate pathway. Differentially produced metabolites within *Su* and *Sc*. Box legend: bar inside the box represents the median value, upper bar represents maximum of distribution, lower bar represents minimum of distribution and the circle represents extreme data points.

were detected for amino acids, carbohydrates and lipids (Fig. 7A). Moreover, the Random Forest classification identified multiple metabolites connected to the NAD^+ and chitin biosynthesis as discriminating factors to separate both species (Fig. 7B).

NAD^+ can be synthesized by *de novo* synthesis originating with tryptophan or by the conversion of vitamins nicotinate and nicotinamide. Multiple compounds representing each of these arms of NAD^+ production showed different concentration in *Sk* (Fig. 8). Elevated tryptophan and the corresponding decreases in kynurenine and quinolinate suggest that the *de novo* synthesis of NAD^+ has a blockage in the first steps of the route in *Sk*. On the other hand, the increases in nicotinate, nicotinate ribonucleoside and nicotinamide ribonucleotide point to the salvage pathway as responsible for the elevations of NAD^+ and NADH observed (Fig. 8).

Multiple compounds, including glucosamine, N-acetylglucosamine and erythronate, related to the synthesis of cell wall polysaccharide chitin were identified by the Random Forest classification as separating factors for *S. kudriavzevii* (Fig. 7B and Fig. S3).

DISCUSSION

Temperature is one of the most important parameters affecting the length and rate of alcoholic fermentation and final wine quality. Many winemakers prefer low-temperature fermentation (10–15 ºC) for the production of white and “rosé” wines because it improves the taste and aroma characteristics (Beltran *et al.,* 2008; Torija *et al.,* 2003). Although *S. cerevisiae* is always predominant in wine fermentations, a drop in temperature affects its competitiveness. It is obvious that the direct effect of lowering temperature is to slow down the metabolic activity of the yeast cell, which accounts for reduced growth and longer fermentation process at 12 ºC. Thus, we characterized the metabolome of a commercial wine strain *Sc* growing at 12 ºC and 28 ºC. A metabolome analysis is a powerful tool to understand metabolic

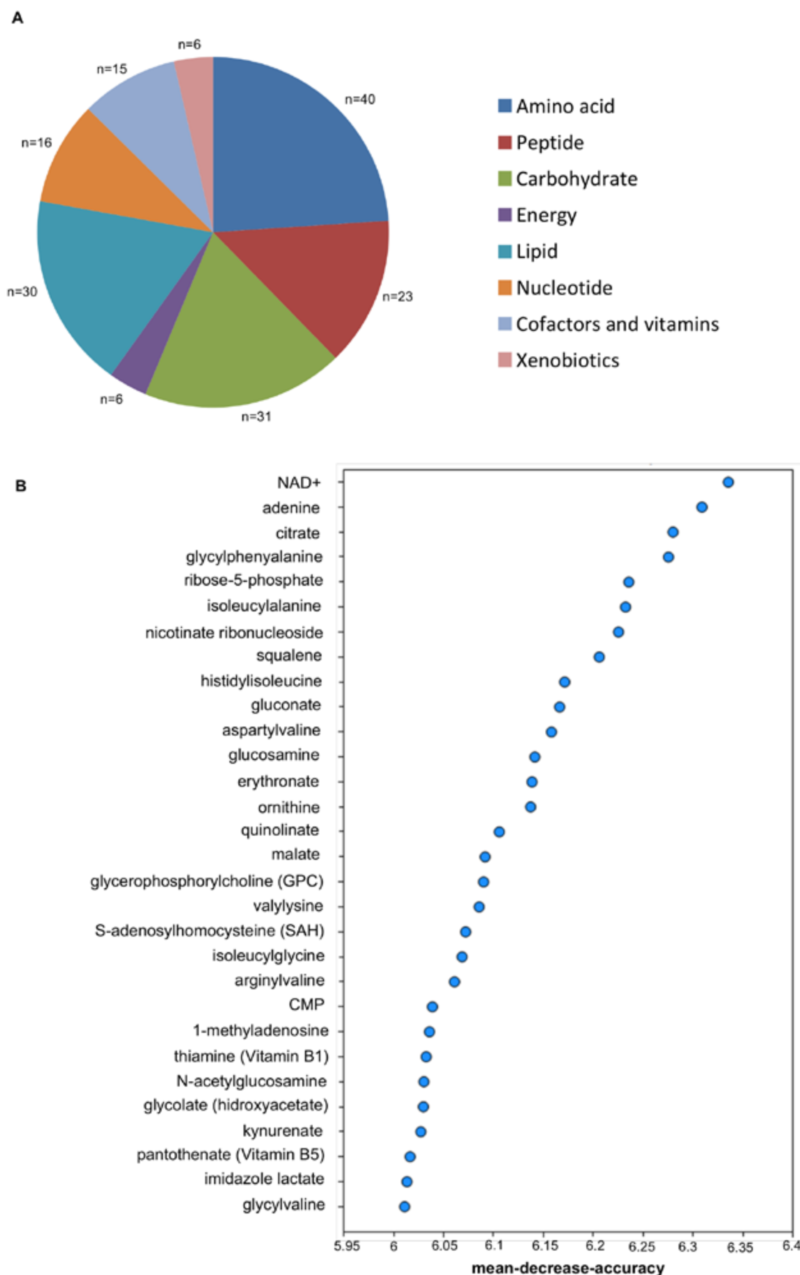

Figure 7. Principal metabolic differences between *S. cerevisiae* and *S. kudriavzevii* growing at 12 ºC. A) Class distribution of the different identified metabolites. n = number of metabolites in each class. B) The Random Forest statistical analysis of metabolomic data was used to identify the top 30 biochemicals with the greatest influence in distinguishing the different groups. Metabolites are listed on the y-axis in order of importance, with importance decreasing from top to bottom. The mean decrease in accuracy for each metabolite is plotted on the x-axis.

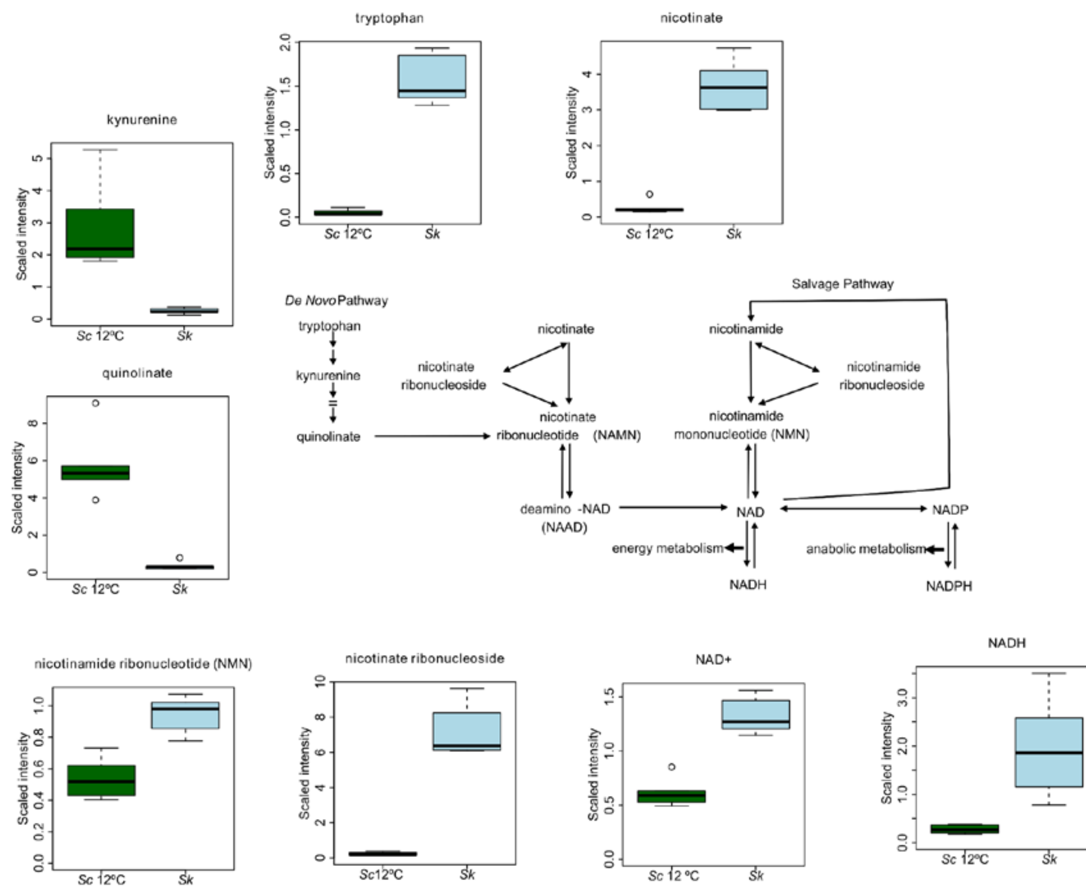

Figure 8. The NAD$^+$ biosynthesis pathway. Differentially produced metabolites within *Sk* and *Sc*. Box legend: bar inside the box represents the median value, upper bar represents maximum of distribution, lower bar represents minimum of distribution and the circle represents extreme data points.

changes in response to different environmental conditions, such as cold temperatures. In past years, some attempts have been made to elucidate the cold response of the same wine yeast *Sc* using a variety of high-throughput methodologies, such as transcriptomics (Beltran *et al.,* 2006) or proteomics (Salvadó *et al.,* 2012). These metabolic changes could be due to temperature-mediated transcriptional and/or to post-transcriptional effects on yeast. However, multiple genes may be involved in the synthesis and degradation of a single metabolite. This makes the metabolome an appropriate level for studying phenotypic responses.

Notwithstanding, the fact that *S. cerevisiae* is the predominant species responsible for alcoholic fermentation, other species of the genus *Saccharomyces*, such as *S. bayanus* var. *uvarum* (Masneuf-Pomarède *et al*., 2010) or the hybrid strains of *S. cerevisiae* x *S. kudriavzevii* (González *et al*., 2006), have shown to be better adapted at low temperatures during winemaking. Thus, these two species are often referred to as being cryotolerant (Salvadó *et al.,* 2011; Sampaio and Gonçalves, 2008) and are good models to study adaptation at low temperature. To this end, we also characterized the metabolome of *Su* and *Sk* growing at 12 ºC which we compared with the metabolome of *Sc* (not well-adapted at low temperature) at 12 ºC. This comparison revealed to us that inter-specific differences were even greater than temperature-dependent metabolic changes. As we did not analyze the metabolic profiling of *Su* and *Sk* at 28 ºC, we cannot ascertain that the changes observed in the metabolism of these species were due to low temperature. In a further study, the metabolic adaptation of these cryotolerant species at low temperature should be dealt with. The main differences between the metabolic profiling of *Sc* growing at 12 ºC and 28 ºC were observed in amino acid and lipid classes. These differences in the amino acid metabolism could be connected with the different consumption rate of the organic nitrogen at both temperatures (Table 1). Several studies have reported the importance of lipid composition in the yeast adaptive response at low temperature (Beltran *et al.,* 2008; Henschke and Rose,

1991; Redón *et al*., 2011; Torija *et al.,* 2003). Furthermore, Tai *et al.* (2007) observed that the only clear group of genes that were commonly regulated in a low-temperature chemostat and batch-culture studies was involved in lipid metabolism. Incubation at low temperature increases the molecular order of membrane lipids by rigidification (Russell, 1990). Yeasts are known to have developed several strategies to maintain appropriate membrane fluidity. The most commonly studied involves increased unsaturation and reduced average chain length (ChL) of fatty acids (FA) (Beltran *et al*., 2008; Torija *et al*., 2003). Our data reveal that the cells growing at low temperature increase the unsaturated fatty acids (UFA) of C14 and C16 (myristoleate and palmitoleate), but decrease other unsaturated fatty acids (linoleate C18:2 and linolenate C18:3). The result is a global reduction in the chain length of FA as a main strategy to enhance membrane fluidity. The increase in the medium chain FA as a caprylate (C8:0) in the cells cultured at 12 ºC also supports the predominance of this strategy.

Our results also show lower levels of the metabolites connected with phosphatidilcholine (PC), such as glycerophosphorylcoline (GPC), 2-palmitoylglycerophosphocholine and 1-oleoyglycerophosphocholine, in the *Sc* grown at 12 ºC. Redon *et al*. (2011) and Tronchoni *et al*. (2012) also reported a lowered ratio of phosphatidilcholine (PC)/phosphatidylethanolamine (PE) when this species was cultured at low temperature. Regarding the sterol pathway, the strategy for counteracting the membrane rigidity in the cells grown at 12 ºC increased in ergosterol, but decreased in their intermediates squalene and lanosterol.

Another set of metabolites that distinguished the *Sc* grown at 12 ºC and 28 ºC were the metabolites belonging to the glutathione/glutaredoxin system, biochemicals that help cells detoxify reactive oxygen species (ROS). Cross-talk between signal transduction in response to the temperature downshift and oxidative stress has been previously reported by Zhang *et al*. (2003), who observed that the mRNA levels of *SOD1*, *CTT1* and *GSH1* significantly rose by a temperature downshift from 30 ºC

to 10 ºC. Moreover, and consistent with this idea, Schade *et al*. (2004) showed that the transcriptional profile of the glutathione/glutaredoxin system genes suggests activation during the LCR (Late Cold Response). In a proteomic study of the response of the same wine yeast at low temperature, Salvadó *et al*. (2012) also detected a higher concentration of Yhb1 (Yeast hemoglobin-like protein), with functions in oxidative stress response.

The global metabolic comparison made of the three species revealed that the main differences between the two cryophilic species and *S. cerevisiae* were in the carbohydrate metabolism. The *Sk* and *Su* strains had significantly higher levels of glucose and fructose, and most of the intermediates were of the higher part of glycolysis (C6 sugars), the pentose phosphate pathway and the trehalose metabolism. The easiest explanation is that these strains present a higher sugar uptake at low temperature. Yet when considering the residual sugars in the supernatant of steady-state cultures (Table 1), *Sk* and *Su* had consumed less sugars than *Sc* at both temperatures. It is well-known that *Sc* is the species with the greatest fermentative competitiveness. This fitness advantage has been related with quicker sugar uptake and speedier flux by the glycolysis pathway than its competitors (Piškur *et al*., 2006), thus enabling better ethanol yields, which allow niche construction via ethanol production (Arroyo-López *et al*., 2010). The comparison of sugar consumption and ethanol production rates clearly confirmed this higher fermentative performance of *Sc* in comparison with *Su* and *Sk* (Table 1). Thus, the higher concentration of glucose, fructose and the intermediates of other biochemical pathways in the *Sk* and *Su* strains may be related with a slower glycolytic flux, mainly at the level of conversion of hexoses into trioses (higher concentration in *Sc*).

Despite the main differences among the three species lying in the carbohydrate metabolism, other important differences have been be detected. Shikimate, its metabolic intermediates, glucose-6-phosphate and phosphoenolpyruvate, which are shared with the glycolysis pathway, and a terminal product, tryptophan, were

identified by the Random Forest analysis as factors separating *Su* from *Sc*. Shikimate is an important precursor for aromatic amino acids tyrosine, phenylalanine and tryptophan. It is well-documented that tryptophan uptake is a limiting factor for yeast cell growth at low temperature (Abe and Horikoshi, 2000). These authors postulated that the increased rigidity of the plasma membrane at low temperature especially impairs tryptophan transport, and they also proved that the overexpression of high-affinity tryptophan transporter Tat2p improves cell growth at low temperature (Abe and Horikoshi, 2000). Thus, the increased tryptophan biosynthesis in *Su* can counteract the transport problem and improve its growth at low temperature.

Another amino acid biosynthesis route also showed differences in its intermediates and the final product separating the *Su* and *Sc* strains. Apart from alpha-ketoglutarate, the initial substrate of the pathway, lysine and its pathway intermediates (e.g., 2-aminoadipate, saccharopine, and homocitrate) significantly reduced in *S. bayanus* var. *uvarum*. Homocitrate synthase (HCS) catalyzes the conversion of alpha-ketoglutarate into homocitrate, and represents the rate-limiting step of lysine synthesis. The observed pattern of changes strongly suggests that HCS, a highly regulated enzyme, activity is inhibited in *S. bayanus* var. *uvarum.* Yet no obvious relationship to its ability to grow at 12 °C has been identified in the scientific literature.

Regarding *Sk*, it is characterized by increased NAD^+ synthesis and cell wall synthesis. NAD^+ is a primary cofactor for the reductive reactions of energy metabolism and can serve as a marker of cellular energy status. Additionally, it is a co-substrate for certain NAD^+-dependent histone deacetylases of the sirtuin family, which play a role in gene silencing and in regulating cellular metabolism (Lu and Lin, 2010.). The relative elevation of NAD^+ and NADH suggest that *Sk* has considerable nucleotide cofactor capacity to catalyze the reactions related to energy metabolism, as well as those reactions controlled by NAD^+-dependent sirtuins. Furthermore, *Sk* has elevated several compounds relating to the synthesis of the

cell wall polysaccharide chitin. It is known that many cold shock proteins in *S. cerevisiae* are serine/threonine-rich mannoproteins localized in the cell wall (Abe, 2007; Abramova *et al*., 2001; Murata *et al*., 2006). Thus, cell wall composition appears to be important during cold adaptation.

In summary, with this global metabolic profiling study, we have detected the main metabolites involved in low temperature response in three different yeast species from the genus *Saccharomyces.* We have confirmed the importance of lipid metabolism in the cold adaptive response in *S. cerevisiae*, especially the lesser amount of phosphatidylcholine and its derivatives. We have also observed that two cryotolerant species, *S. bayanus* var. *uvarum* and *S. kudriavzevii*, differ from *S. cerevisiae* in terms of their capacity to use fructose at 12 ºC. Nevertheless, these two species have developed different strategies for cold resistance. *S. bayanus* var. *uvarum* showed great shikimate pathway activity, while *S. kudriavzevii* presented increased NAD^+ synthesis. The differences in these metabolites of cold tolerance and the winemaking process suggest that it is possible to segregate the metabolism of *S. cerevisiae* from that of *S. uvarum* and *S. kudriavzevi*, and that it may provide a basis for understanding why *S. cerevisiae* performs better at 28 °C, while its *Saccharomyces* counterparts do better at 12°C. This study advances our understanding of the cold response within the *Saccharomyces* genus at the metabolic level and can be used as a basis for future biotechnological applications for low-temperature wine fermentations.

ACKNOWLEDGMENTS

This work has been financially supported by the grants AGL2010-22001-C02-01 from the Spanish government and PROMETEO/2009/019 from Generalitat Valenciana, awarded to JMG and AQ, respectively. MLM also wishes to thank the Spanish government for her FPI grant. We also want to thank Guillem Salazar for his help in the statistical analysis.

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Chapter2

Phenotypic analysis of mutant and overexpressing strains of lipid metabolism genes in *Saccharomyces cerevisiae*: implication in growth at low temperatures

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International Journal of Food Microbiology 162, 26 – 36.

UNIVERSITAT ROVIRA I VIRGILI
METABOLIC AND MOLECULAR ADAPTATION OF WINE YEASTS AT LOW TEMPERATURE FERMENTATION:
STRATEGIES FOR THEIR GENETIC IMPROVEMENT
María López Malo
DL: T. 1275-2013

ABSTRACT

The growing demand for wines with a more pronounced aromatic profile calls for low temperature alcoholic fermentations (10 – 15 ºC). However, there are certain drawbacks to low temperature fermentations such as reduced growth rate, long lag phase and sluggish or stuck fermentations. The lipid metabolism of *Saccharomyces cerevisiae* plays a central role in low temperature adaptation. The aim of this study was to detect lipid metabolism genes involved in cold adaptation. To do so, we analyzed the growth of knockouts in phospholipids, sterols and sphingolipids, from the EUROSCARF collection *Saccharomyces cerevisiae* BY4742 strain at low and optimal temperatures. Growth rate of these knockouts, compared with the control, enabled us to identify the genes involved, which were also deleted or overexpressed in a derivative haploid of a commercial wine strain. We identified genes involved in the phospholipid (*PSD1* and *OPI3*), sterol (*ERG3* and *IDI1*) and sphingolipid (*LCB3*) pathways, whose deletion strongly impaired growth at low temperature and whose overexpression reduced generation or division time by almost half. Our study also reveals many phenotypic differences between the laboratory strain and commercial wine yeast strains, showing the importance of constructing mutant and overexpressing strains in both genetic backgrounds. The phenotypic differences in the mutant and overexpressing strains were correlated with changes in their lipid composition.

Keywords: lipids, mutant, overexpressing strains, cold, yeast

Abbreviations: FA: Fatty Acids; MCFA: Medium Chain Fatty Acids; UFA: Unsaturated Fatty Acids; SFA: Saturated Fatty Acids; ChL: Chain Lengths; TG: Triacylglyceride; DG: Diacylglyceride; PL: Phospholipid; PI: Phosphatidylinositol; PS: Phosphatidylserine; PE: Phosphatidylethanolamine; PC: Phosphatidylcholine; PG: Phosphatidylglycerol; CL: Cardiolipin; PA: Phosphatidic Acid; NL: Neutral Lipid; SE: Sterol Esters

INTRODUCTION

Temperature fluctuations are an inevitable part of microbial life in exposed natural environments; however, sub-optimal temperatures are also common in industrial processes. Low temperatures (10-15 ºC) are used in wine fermentations to enhance production and retain flavor volatiles. In this way, white and "rosé" wines of greater aromatic complexity can be achieved (Beltran *et al.,* 2008; Torija *et al.,* 2003). The optimum fermentation temperature for *Saccharomyces* is between 25 and 28 ºC. Therefore, among the difficulties inherent to wine fermentation (high concentration of sugars, low pH, presence of ethanol, nutrient deficiency, etc.), we should add a sub-optimal 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 one week or longer and sluggish fermentations (Bisson, 1999; Meurgues, 1996), dramatically lengthening alcoholic fermentation with the consequent energy expenditure.

Low temperature has several effects on biochemical and physiological properties in yeast cells: low efficiency of protein translation, low fluidity membrane, change in lipid composition, slow protein folding, stabilization of mRNA secondary structures and decrease in enzymatic activities (Aguilera *et al.*, 2007; Hunter and Rose, 1972; Sahara *et al.,* 2002; Schade *et al.,* 2004). To date, most studies have mainly focused on the genome-wide transcriptional responses to cold-shock (Beltran *et al.*, 2006; Homma *et al.,* 2003; Murata *et al.,* 2006; Sahara *et al.,* 2002; Schade *et al.,* 2004). In a decisive study, Tai *et al.* (2007) compared their transcriptomic results obtained in a steady-state chemostat culture with other previous genome-wide transcriptional studies of batch cultures at low temperature. Interestingly, lipid metabolism genes were the only ones whose activity was clearly regulated by low temperature. This is consistent with the notion that after a temperature downshift, homeoviscous adaptation of the membrane composition is

essential for growth (Beltran *et al.*, 2006; Beltran *et al.*, 2007; Hunter and Rose, 1972; Torija *et al.*, 2003).

Biological membranes are the first barrier between the cell interior and its environment and a primary target for damage during cold stress. Major lipid components of eukaryotic membranes are phospholipids, sterols, sphingolipids and glycerolipids. The main species of fatty acids of *Saccharomyces cerevisiae* are C16 and C18, with or without a double bond. The composition of these lipid components is important for the physical properties of the membrane, such as fluidity. Incubation at low temperature increases the molecular order of membrane lipids by rigidification (Russell *et al.*, 1990). Yeasts are known to have developed several strategies to maintain appropriate membrane fluidity. The most commonly studied involves the increase in unsaturation (mainly palmitoleic C16:1 and oleic C18:1 acids). Phospholipids with unsaturated fatty acids (UFA) have a lower melting point and greater flexibility than phospholipids with saturated acyl chains. Another way of increasing membrane lipid fluidity is to decrease the chain length (ChL) of these FA by increasing the synthesis of medium chain fatty acids (MCFA; C6 to C14) (Beltran *et al.,* 2008; Torija *et al.,* 2003). Recently, Redón *et al.* (2011) also reported new common changes in the lipid composition of different industrial species and strains of *Saccharomyces* after low temperature growth. Despite specific strains/species dependent responses, the results showed that at low temperatures the MCFA and triacylglyceride (TG) content increased, whereas the phosphatidic acid content (PA) and the phosphatidylcholine/ phosphatidylethanolamine (PC/PE) ratio decreased. Reshaping the plasma membrane composition might be a good strategy for adapting yeast cells to low temperatures, reducing the lag-phase and speeding up fermentation onset. In this respect, knock-out or overexpression of genes related with lipid metabolism can modify the architecture of this plasma membrane. In a preliminary study (Redón *et al.*, 2012), we tested various phospholipid mutants from the EUROSCARF collection of *S. cerevisiae* BY4742 to ascertain whether the suppression of some

genes could improve the fermentation vitality of the cells at low temperature. The aim of this study was to detect key genes in the lipid metabolism pathways which play an important role in the adaptation of *S. cerevisiae* to low temperature. To achieve this objective, we analyzed the growth of several knockouts of phospholipid, sterol and sphingolipid pathways at 12 ºC and 28 ºC and compared them to the wild type BY4742. This first screening of the laboratory strain enabled us to select genes for deletion and overexpression in the genetic background of a derivative haploid of the commercial wine strain, QA23. The phenotypic differences in the mutant and overexpressing strains were correlated with the changes in their lipid composition.

MATERIAL AND METHODS

Strains and growth media

S. cerevisiae strains used in this study were: a total of 34 phospholipids, sterols and sphingolipids mutants of the laboratory strain BY4742 (MATα, his3*Δ* 1; leu2*Δ* 0; lys2*Δ* 0; ura3*Δ* 0), from the EUROSCARF collection (Frankfurt, Germany) and the derivative haploid of the commercial wine strain QA23 (Lallemand S.A., Canada), *ho*QA23 (Salvado *et al*., 2012).

These strains were cultured on SC (6.7 g/L Difco Yeast Nitrogen Base (w/o amino acids), 20 g/L glucose, 0.83 g/L synthetic complete drop-out mix (2 g Histidine, 4 g Leucine, 2 g Lysine 1.2 g Uracil)). They were grown in Erlenmeyer flasks (250 mL) filled with 50 mL of medium, fitted with cotton and shaken at 200 rpm at 30 ºC for 48 h. The population inoculated in every flask was 2 x 10^6 cells/mL from an overnight culture in YPD at 30 ºC.

Construction of mutant and overexpressing strains

Mutated genes which showed growth insufficiency in the background of the laboratory strain BY4742 were deleted on the derivative haploid of a commercial

wine strain, *ho*QA23. Genes were deleted using the short flanking homology (SFH) method based on the KanMX4 deletion cassette (Güldener *et al.*, 1996). The primers used for amplification of the *loxP-KanMX4-loxP* cassette from the plasmid pUG6 have 50-nucleotide extensions corresponding to regions upstream of the target gene start codon (forward primer) and downstream of the stop codon (reverse primer). The PCR fragments were used to transform the haploid *ho*QA23 strain using the lithium acetate procedure (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. Total DNA from transformants resistant to G418 Geneticin was analyzed by PCR using primers upstream and downstream of the deleted region combined with primers of the *KanMX* gene.

The genes, whose deletion significantly impaired growth in the *ho*QA23, were overexpressed by cloning into the centromeric plasmid pGREG505, as described in Jansen *et al.* (2005). All genes were amplified from approximately 600 nucleotides upstream of the start codon to 400 nucleotides downstream of the stop codon to ensure that the promoter and terminator regions were included. The PCR protocol involved an initial denaturation at 94 ºC (2 min), followed by 30 cycles of 10 s at 94 ºC, 30 s at 49-50 ºC (depending on the different primers) and 3-4 min at 72 ºC (depending on the different PCR product lengths). The last cycle was followed by a final extension step of 10 min at 72 ºC. PCR fragments were generated with oligonucleotides that contained the short sequences rec5 (forward) and rec2 (reverse), which are homologous to the sequences in the plasmid (about 35 bp). The plasmid was linearized by *Sal*I digestion and digested with *Asl*I to avoid sticky ends and to make the recombination process easier (Jansen *et al.*, 2005). The wine yeast *ho*QA23 was co-transformed with the digested pGREG505 plasmid together with the PCR amplified target gene, flanked by recombination sequence homologues to the plasmid ends. This co-transformation promotes an *in vivo* homologous recombination between both fragments. This recombination process also deleted the *GAL1* promoter of the plasmid (the genes were cloned with their

own promoters). The transformants were selected by Geneticin resistance, which is encoded by the *KanMX* gene in the plasmid. Correct yeast transformations were verified by plasmid DNA isolation using a modification of the protocol described by Robzyk and Kassir (1992) and subsequently amplification with the Illustra TempliPhi Amplification Kit (GE Healthcare, UK). Then, to verify the correct integration of the gene into the vector, plasmids were checked by PCR using primers specified for sequences rec5 and rec2. All the strains (mutants and overexpressing) constructed in this study are shown in Table 1.

Table 1. Strains constructed in this study

Strain	**Genotype**	**Definition**
*ho*QA23	MATα; YDL227C::kanMX4	Derivative wine haploid strain
Δpsd1	MATα; YNL169c::kanMX4	*PSD1* mutant strain
Δopi3	*ho*QA23; YJR073C::kanMX4	*OPI3* mutant strain
Δerg3	*ho*QA23; YLR056W::kanMX4	*ERG3* mutant strain
Δerg6	*ho*QA23; YML008C::kanMX4	*ERG6* mutant strain
Δlcb3	*ho*QA23; YJL134W::kanMX4	*LCB3* mutant strain
Δlcb4	*ho*QA23; YOR171C::kanMX4	*LCB4* mutant strain
Δdpl1	*ho*QA23; YDR294C::kanMX4	*DPL1* mutant strain
*ho*QA23 pGREG	*ho*QA23-pGREG505	Haploid strain with empty plasmid
pGREG *PSD1*	*ho*QA23-pGREG *PSD1*	*PSD1* overexpressing strain
pGREG *CHO2*	*ho*QA23-pGREG *CHO2*	*CHO2* overexpressing strain
pGREG *OPI3*	*ho*QA23-pGREG *OPI3*	*OPI3* overexpressing strain
pGREG *ERG3*	*ho*QA23-pGREG *ERG3*	*ERG3* overexpressing strain
pGREG *ERG6*	*ho*QA23-pGREG *ERG6*	*ERG6* overexpressing strain
pGREG *IDI1*	*ho*QA23-pGREG *IDI1*	*IDI1* overexpressing strain
pGREG *OLE1*	*ho*QA23-pGREG *OLE1*	*OLE1* overexpressing strain
pGREG *LCB3*	*ho*QA23-pGREG *LCB3*	*LCB3* overexpressing strain
pGREG *LCB4*	*ho*QA23-pGREG *LCB4*	*LCB4* overexpressing strain
pGREG *DPL1*	*ho*QA23-pGREG *DPL1*	*DPL1* overexpressing strain

Generation time (GT)

Growth was monitored at 600 nm in a SPECTROstar Omega instrument (BMG Labtech, Offenburg, Germany) at 12 ºC and 28 ºC. Measurements were taken, after pre-shaking the microplate for 20 s, every half hour over 3 days. However at 12 ºC the microplate had to be incubated outside the SPECTROstar spectrophotometer and then transferred inside to take measurements every 8 h during the lag phase and every 3 h during the exponential phase. The microplate wells were filled with 0.25 mL of SC medium, reaching an initial OD of approximately 0.2 (inoculum level of 2 x 10^6 CFU/mL). Uninoculated wells for each experimental series were also included in the microplate to determine, and consequently subtract, the noise signal. All experiments were carried out in triplicate.

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

$$y=D*\exp\{-\exp[((\mu_{max}*e)/D)*(\lambda-t))+1]\}$$

where $y=\ln(OD_t/OD_0)$, OD_0 is the initial OD and OD_t is the OD at time t; $D=\ln(OD_t/OD_0)$ is the asymptotic maximum, μ_{max} is the maximum specific growth rate (h^{-1}), and λ the lag phase period (h) (Salvadó *et al.,* 2011). Generation time (GT) was calculated using the equation $GT=\ln 2/\mu_{max}$. To normalize, this value was divided by the GT of *S. cerevisiae* BY4742 and hoQA23 (control strains).

Spot test

To analyze growth phenotypes of mutant strains, cells grown on SC to stationary phase (OD600 ~ 4) were harvested by centrifugation, washed with sterile water, resuspended in sterile water to an OD (600 nm) value of 0.5, followed by serial dilution of 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4}. From each dilution, 3.5 μL was spotted onto SC agar plates. The plates were incubated at 28 ºC and 12 ºC for 2-9 days.

Lipid extraction

Mutant and overexpressing strains were grown in SC for 48 hours at 28 ºC. Geneticin was also added (200 mg/L) to the SC medium of the overexpressing strains to stabilize the plasmid and promote overexpression of the genes. Cells were frozen until the different lipid analyses. Prior to lipid extraction, a solution of 100 µL of cold methanol and 20 µL of EDTA 0.1 mM was added to the yeast cells (5-10 mg dry mass) with 1 g glass beads (0.5 mm, Biospec Products) in Eppendorf, and then mixed for 5 minutes in a mini-bead-beater-8 (Biospec Products, Qiagen). Lipid extraction was performed in two steps with 1 mL chloroform/methanol (2:1, v/v, for 1 hour), one step with 1 mL chloroform/methanol (1:1, v/v, for 1 hour) and one step with 1 mL chloroform/methanol (1:2, v/v, for 1 hour). All the organic phases were transferred to a 15 mL glass screw tube and cleaned twice by adding KCl 0.88% (1/4 of a total volume of the extract). After vortexing and cooling at 4 ºC for 10 minutes, the samples were centrifuged at 3000 rpm for 5 minutes. The inferior organic phase was collected and finally concentrated to dryness under nitrogen stream. The residue was dissolve in 100 µL of chloroform/methanol (2:1, v/v).

Separation and quantification of the yeast phospholipids (PLs) by HPTLC

The yeast extract phospholipids were separated by one-dimensional HPTLC on silica gel $60F_{254}$ plates (10 x 20cm, 200 µm) with chloroform: acetone: methanol: glacial acetic acid: water (65:15:10:10:5, v/v/v/v/v). After charring the plate with 10% $CuSO_4$ in 8% H_3PO_4 and heating to 160 ºC for 4 min, the PLs were identified by known standards purchased from Sigma: phosphatidylinositol (PI), phosphatidylserine (PS), phosphatidylcholine (PC), phosphatidylglycerol (PG), phosphatidylethanolamine (PE), cardiolipin (CL) and phosphatidic acid (PA). The plates were scanned and each spot of the image was quantified in terms of integrated optical densities (IOD) with ImageJ software (a public domain, Java-based image processing program developed at the National Institute of Health).

Calibration curves were constructed by applying standards to each plate in the range of 1-4 µg/µL to quantify the PLs. These values were related to the dry weight of cells and expressed as a percentage of the total PLs extracted.

Yeast neutral lipid (NL) composition by thin-layer chromatography (TLC)

NL composition of yeast was separated by one-dimensional TLC on silica gel $60F_{254}$ (10 x 20 cm, 250 µm) (Merck, Germany). The plate was developed in three steps: the first step with hexane, tert-Butyl methyl ether (MTBE) and glacial acetic acid (50:50:2) to 35mm, the second step with hexane, tert-Butyl methyl ether (MTBE) and glacial acetic acid (80:20:1) to 60mm and the last step with hexane to 85mm. The standard lipids lanosterol, ergosterol, squalene, cholesteryl oleate, ethyl oleate, diolein, triolein were purchased from Sigma and were applied to every plate in the range of 1-4 µg/µL. After TLC, lipids were charred with 10% $CuSO_4$ in 8% H_3PO_4 and heated to 160 ºC for 4 min on a TLC Plate Heater (CAMAG). Plates were scanned and each spot of the image was quantified as integrated optical densities (IOS) with ImageJ software (a public domain, Java-based image processing program developed at the National Institute of Health). Calibration curves were constructed by applying standards to each plate in the range of 1-4 µg/µL to quantify the NLs. These values were related to the cell dry weight and expressed as a percentage of total NLs extracted.

Determination of total yeast fatty acids

Yeast cells (5-10 mg dry mass) were placed in sealed tubes with a Teflon-lined screw cap and saponified using a 1 mL of 5% NaOH in 50% methanol/water (Rozès *et al.*, 1992). The tubes were placed in a dry bath (100 ºC) for 5 min. Samples were vortexed and then the tubes were placed in a dry bath (100 ºC) for another 25 min. Then the saponified material was cooled to room temperature and 2 mL HCl 6M was added. Free fatty acids were extracted by adding 500 µL hexane: tert-Butyl methyl ether (MTBE) (1:1, v/v). Each tube was vortexed twice

for 30 seconds. The organic phase was collected after centrifugation at 3000 rpm for 3 min.

Analytical gas chromatography was performed on a Hewlett-Packard 6850 (Agilent Technologies). 1µL of cellular extract was injected (splitless, 1 min) into a FFAP-HP column (30m x 0,25mm x 0.25µm from Agilent) with an HP 6850 automatic injector. The initial temperature was set at 140 ºC and increased by 4 ºC/min up to 240 ºC. Injector and detector temperatures were 250 ºC and 280 ºC, respectively. The carrier gas (helium) was at a flow rate of 1.4 mL/min. Heptanoic and heptadecanoic acids (10 and 40 mg/mL, respectively) were added as internal standards before cell saponification. Relative amounts of fatty acids were calculated from their respective chromatographic peaks. These values were related to the dry weight of cells and expressed as a percentage of the total fatty acid extracted (Redón *et al*., 2009).

Statistical data processing

All experiments were repeated at least three times, and data are reported as the mean value ± SD. Significant differences between the control strain, the mutant and the overexpressing strains were determined by *t*-tests (SPSS 13 software package). The statistical level of significance was set at $P \leq 0.05$. Principal component analysis (PCA) was done using *vegan* package (*rda* function) from the statistical software R v.2.15 (R Development Core Team, 2010).

RESULTS

Generation time (GT)

Determination of generation time and spot test in BY4742 lipid mutants

In order to determine the importance of lipid metabolism genes on growth at low temperature, we determined GT and carried out spot test for the screening of the

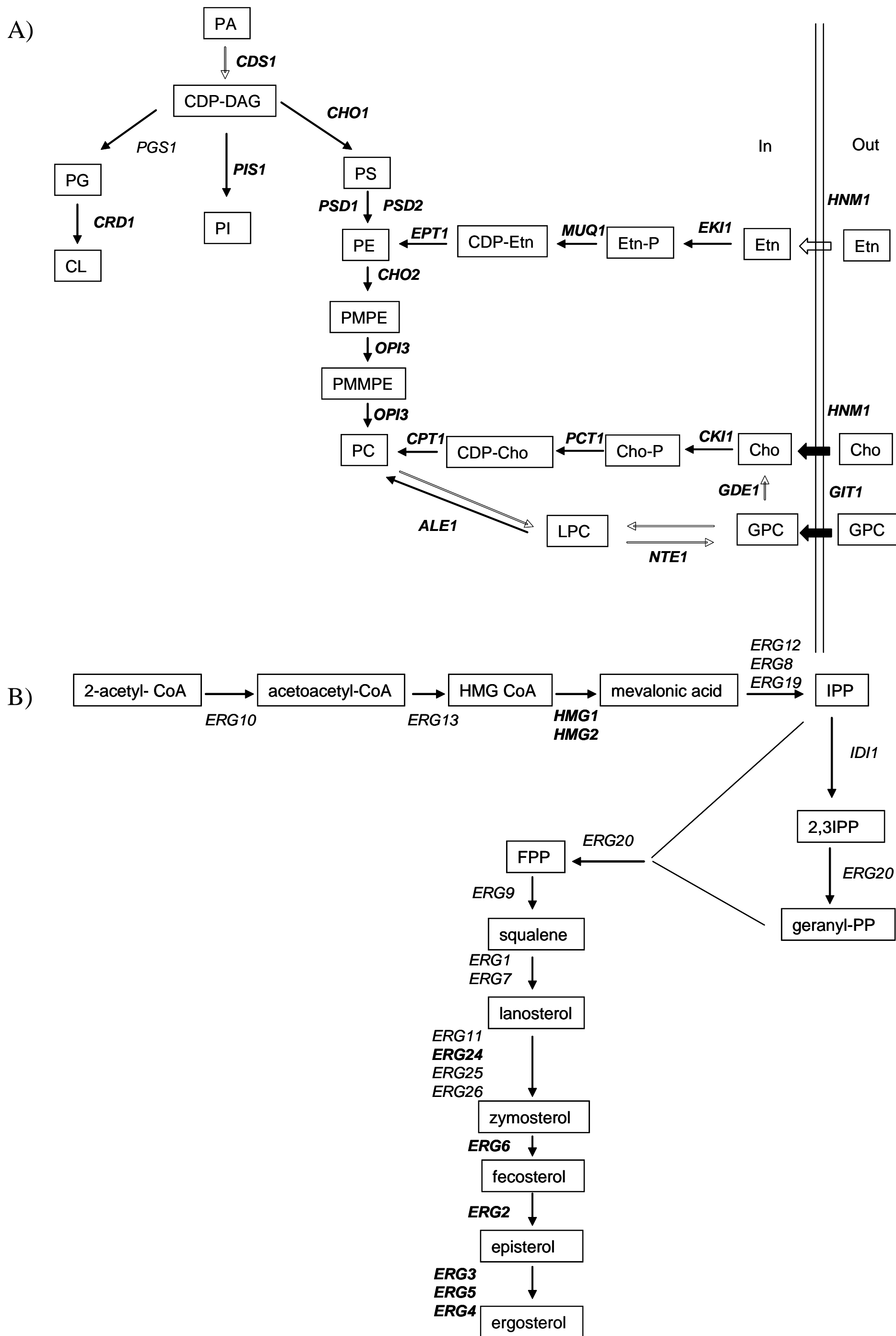


Figure 1. Diagrams of the major pathways for lipid biosynthesis in *S. cerevisiae*: **A)** Phospholipid pathway **B)** Sterol pathway **C)** Sphingolipid pathway. Genes in bold indicate viable mutants.

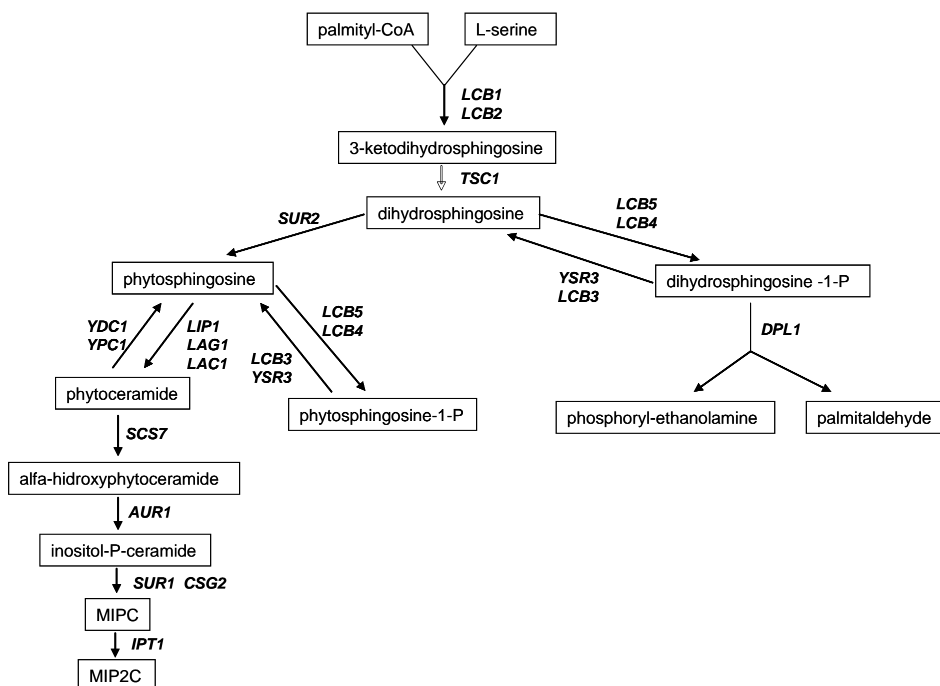


Figure 1. Continued

BY4742 lipid mutants at 12 ºC and at 28 ºC. Most of the deleted genes analyzed (some deletions produce unviable phenotypes) are shown graphically in their respective pathways (Fig. 1). The GT of these mutant strains is also grouped on the basis of the biosynthesis pathway to which they belong (phospholipids, sterols and sphingolipids) (Fig. 2).

The strains with deletions in the genes *OPI3*, *CHO2* and *PSD1*, encoding enzymes of the phospholipid pathway; *ERG24*, *ERG6* and *ERG3*, from the sterol biosynthesis pathway; and *DPL1*, involved in sphingolipid pathway significantly increased their GT at 12 ºC compared to the control strain BY4742. Some of these mutant strains also showed significant differences at 28 ºC, but, in any case, these were not as extreme as at 12 ºC (Fig. 2).

Conversely, some mutant strains improved their relative growth. A remarkable decrease in GT was detected for the deletion of *ERG2*, involved in the synthesis of a precursor of ergosterol (episterol). Likewise, several mutant strains of the

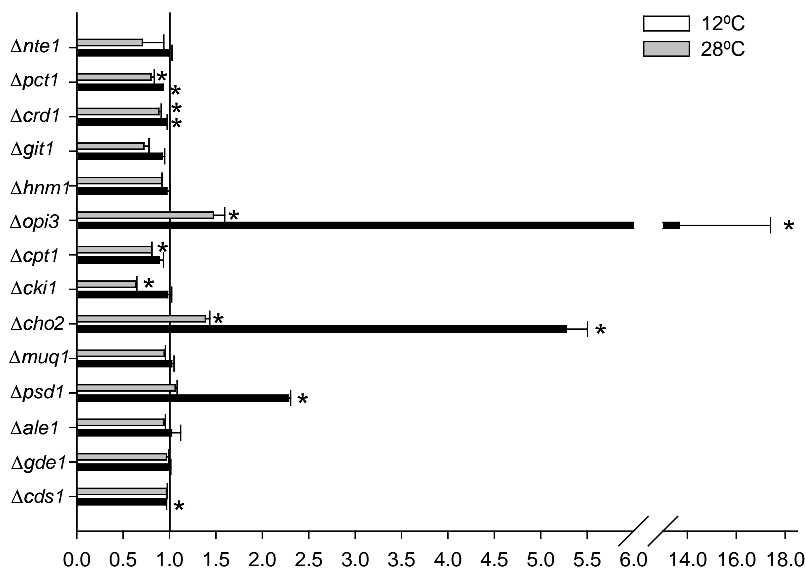
sphingolipid synthesis, such as *Δysr3, Δcgs2, Δipt1, Δsur2, Δydc1, Δlcb4* and *Δlcb3*, also decreased their GT significantly compared to control strain BY4742.

This data on growth in liquid SC were corroborated by a drop test on a SC agar plate at 12 ºC and 28 ºC. Generally, the same mutant strains also showed an impaired growth at low temperature whereas they were hardly affected at 28 ºC. As an example, the drop test for the *ERG* genes (ergosterol pathway) is shown in Figure 2D. Only the deletion of cardiolipin synthesis (*CRD1*) led to worse growth on solid medium than in liquid medium at 12 ºC (data no shown).

Determination of generation time in lipid mutants and overexpressing strains of hoQA23

A total of 15 genes, whose deletion showed significant differences in GT in BY4742, were also deleted in the haploid wine strain *ho*QA23. The first remarkable result was the difference in growth behavior observed depending on the genetic background of the strains in which the genes were deleted. In contrast to the laboratory strain, no deletion yielded better growth than the parental wine strain *ho*QA23 (Fig. 3A). Most of the deleted genes from the sphingolipid pathway with a lower GT in BY4742 mutants did not show differences or displayed slow growth (i.e. *Δlcb3*) in the *ho*QA23. Other remarkable differences between both strains were the phenotypes observed for the mutant strains of genes *CHO2* and *CRD1* in the wine strain *ho*QA23. The *Δcho2* strain was unable to grow in SC medium (only grew in YPD) but growth was recovered when SC medium was supplemented with choline. Thus the mutation of this gene caused auxotrophy for choline in the wine strain *ho*QA23. Regarding *CRD1*, we were unable to delete this gene in *ho*QA23 because the *CRD1* knock-out made this strain unviable. We confirmed that *CRD1* is required for viability of this wine strain by further deleting one of the copies of the diploid commercial strain QA23. This heterozygous mutant strain (*CRD1*/*Δcrd1*) was sporulated but only the spores of the wild copy (non Geneticin

A)



B)

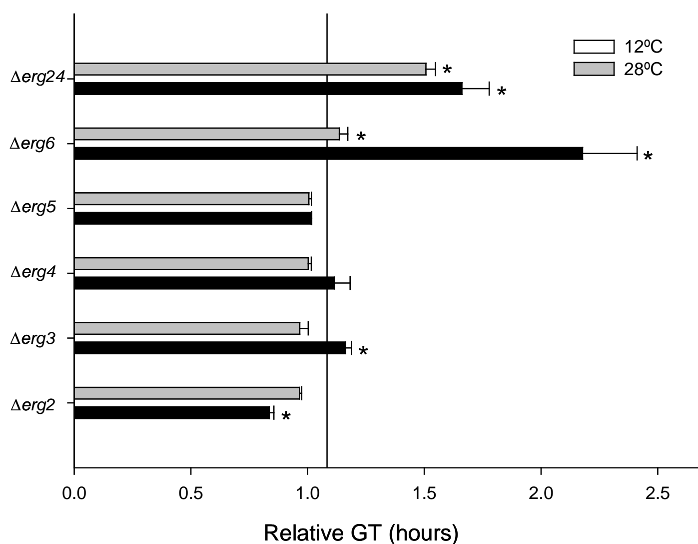
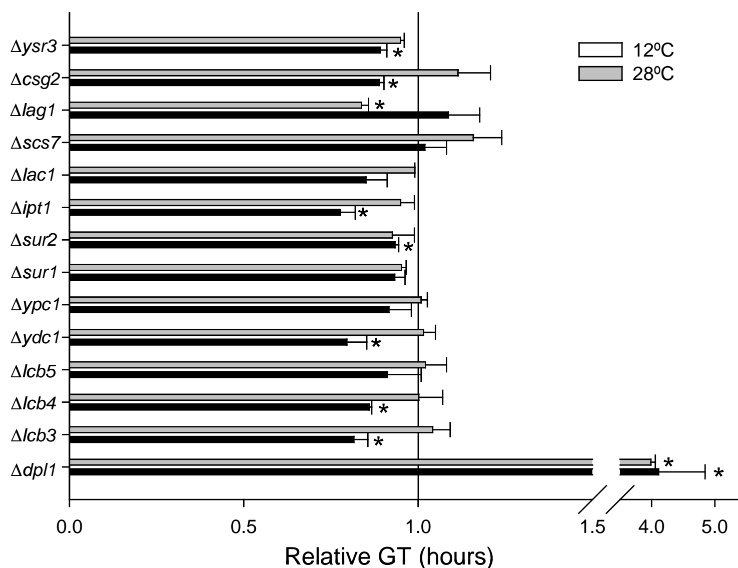


Figure 2. Growth of lipid mutant strains compared with control strain BY4742. Generation time of **A)** phospholipid, **B)** sterol, and **C)** sphingolipid mutants grown at 12 ºC (black bars) and at 28 ºC (gray bars). The GT of the mutant strains was compared to GT of control strain BY4742 (normalized as value 1). The duplication time for this control strain was 20.24 h at 12 ºC and 3.09 h at 28 ºC. **D)** Results of sterol mutant spot test at 12º C and 28 ºC. *Significant differences compared with the wild type at the same temperature.

C)



D)

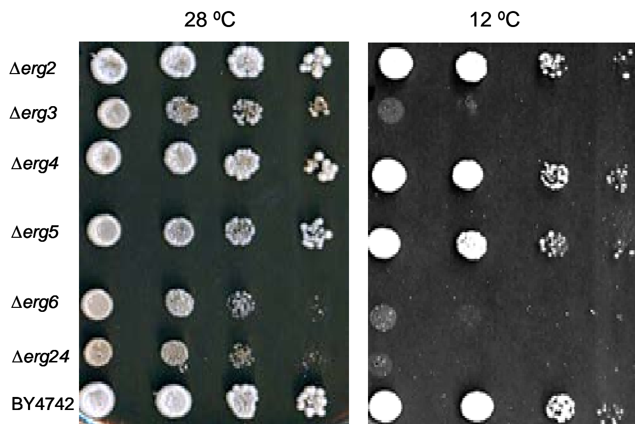


Figure 2. Continued.

resistant) were recovered in the YPD medium. The heterozygous mutant strain (*CRD1*/*Δcrd1*) did not show any differences in terms of GT with the parental strain QA23 (data not shown). Thus this mutation produces unviability but not haploinsufficiency.

The mutant strains with significant differences in GT are shown in Figure 3A. *Δerg3, Δpsd1* and *Δopi3* showed the most important increases in GT (more than 15 hours). These two latter phospholipid mutants also presented impaired growth at 28

ºC, but, as in the case of BY4742, these increases in GT were much more moderate than at low temperature.

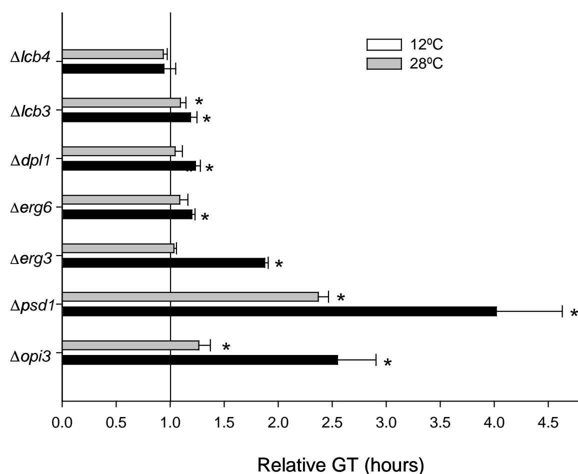
The six genes whose deletion produced slowest growth were also overexpressed in the wine strain *ho*QA23. Although *Δlcb4* did not show significant differences in GT, we decided to overexpress this gene because it encodes the enzyme Lcb4, a sphingoid long-chain base kinase, which catalyzes the reversible step of Lcb3, and has been related with heat shock adaptation (Dickson *et al.,* 2006). We also constructed strains overexpressing genes *IDI1* and *OLE1*, whose deletion produced an unviable phenotype (Giaever *et al.,* 2002) and *CHO2*, whose knock-out also yielded an auxotrophic phenotype for choline. *IDI1* is involved in the ergosterol biosynthesis pathway (Fig. 1) and *OLE1* encodes the only desaturase in *S. cerevisiae*, required for monounsaturated fatty acid synthesis (Mitchell and Martin, 1995). The overexpression of the selected genes decreased GT at low temperature, although only the strains pGREG *LCB3*, pGREG *IDI1*, pGREG *ERG3*, pGREG *OPI3* and pGREG *PSD1* showed significant decreases in GT (more than five hours) (Fig. 3B).

Lipid composition

The lipid composition (fatty acids, phospholipids and neutral lipids) of the mutant and overexpressing strains selected in the previous section was compared with the control strains (*ho*QA23 and *ho*QA23 pGREG). It is worth mentioning that TLC enables us to detect only the main metabolites of the phospholipid and ergosterol biosynthesis pathways. Unfortunately we were not able to analyze sphingolipids with the methodology available in our laboratory. The percentages of the different lipids in the constructed strains are shown in Table S1. The impact of deleting or overexpressing a gene on the compounds of its respective pathway is graphically shown in Figure 4.

As expected, the most important modification in phospholipid composition was observed in the mutant strains of phospholipid pathway *Δpsd1* and *Δopi3*, which

A)



B)

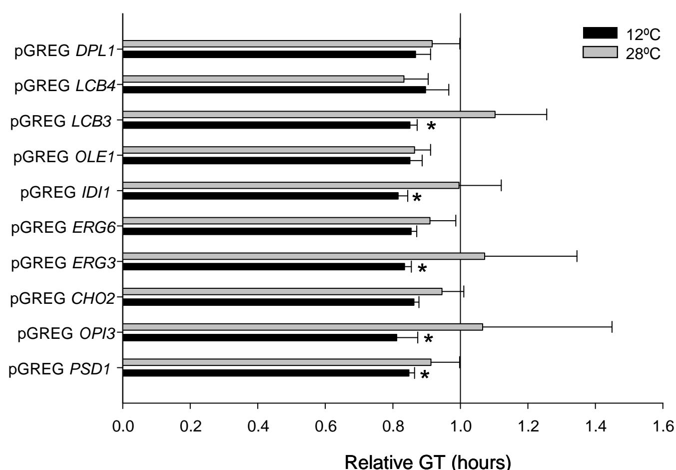


Figure 3. Growth of lipid mutant and overexpressing strains compared with their control strains. Generation time of **A)** mutant and **B)** overexpressing strains grown at 12 ºC (black bars) and at 28 ºC (gray bars). The GT of the mutant and overexpressing strains compared to GT of their control strains *ho*QA23 and *ho*QA23 pGREG (normalized as value 1). The GT for control strains was the following: 15.94 h and 2.58 h for *ho*QA23 and 12.18 h and 3.13 h for *ho*QA23 pGREG at 12 ºC and 28 ºC, respectively. *Significant differences compared with the control strains at the same temperature.

showed a significant increase in PI and important reduction of PS, PC and PE (Fig. 4 A.1). In fact, PE and PC were not detected in *Δpsd1* and *Δopi3* respectively. Moreover, the blockage in PC synthesis in *Δopi3* yielded a new band on HPLTC

plates, which may suggest the detection of PMPE and PMMPE intermediates (Fig. 1A). For Δ*psd1*, the strong PE reduction seemed to be compensated by a significant increase in CL (Fig. 4 A.1). It should be kept in mind that we cannot analyze PL composition of *Δcho2* because this mutant was unable to grow in SC medium.

It should be highlighted that the parental *ho*QA23 (control strain of the mutants; panel A) and the same strain transformed with the empty vector pGREG (control strain of the overexpressing strains; panel B) differed in the composition of some PLs. These differences may be explained by the presence of Geneticin in the growth medium of the overexpressing strains and resistance to this antibiotic encoded in the plasmid. For all overexpressing strains, the most important changes in their PL composition were observed in pGREG *CHO2* (Fig. 4 B.1). Contrary to the expected result, overexpression of *CHO2* induced a significant increase in PE and CL percentage, but a decrease in PC. In fact, most of the overexpressing strains showing significant differences seemed to follow the same trend: to decrease their PC content and increase in PE and CL percentage, except pGREG *OLE1* and pGREG *DPL1* which had less PE (Table S1).

In contrast to PL composition, the mutant and overexpressing strains involved in sterol synthesis did not show important changes in sterol composition (Fig. 4 A.2 and B.2). The most remarkable trend is that mutant strains decreased TG whereas the overexpressing strains increased the sterol esters.

As expected, pGREG *OLE1* significantly increased UFA (mainly in palmitoleic acid C16:1) and decreased saturated fatty acids (SFA) (mainly in palmitic acid C16) (Fig. 5). However most of the overexpressing strains which showed significant differences in their GT (pGREG *OPI3*, pGREG *IDI1* and pGREG *LCB3*) also significantly increased the UFA/SFA ratio (Table S1). In the case of the mutant strains, the general trend was an increase in C16 and C16:1 and a decrease in C18 and C18:1. This increase in shorter-chain fatty acids resulted in a decrease in the average fatty acid chain length of most of the mutant strains. As a

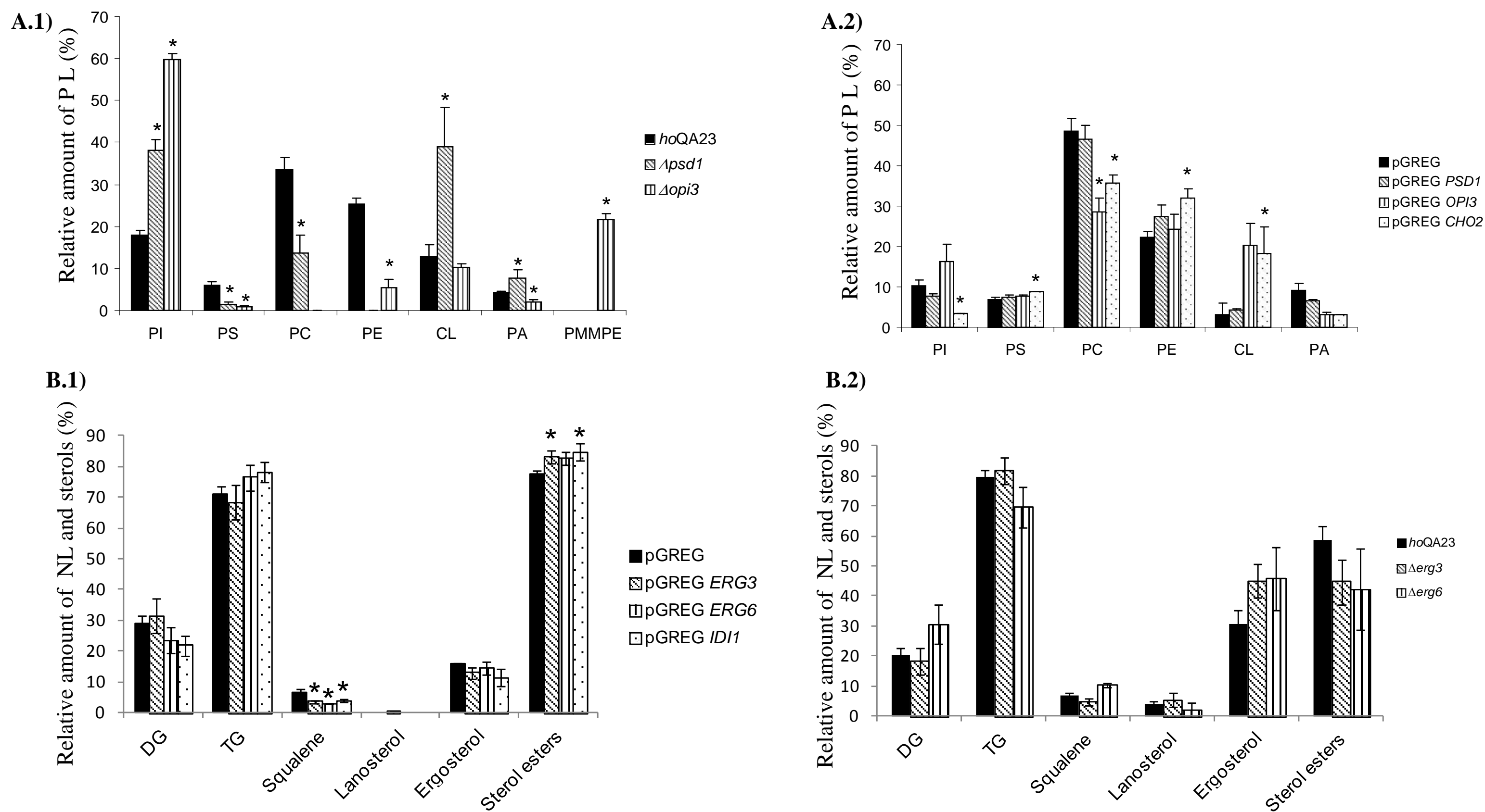


Figure 4. Percentages of phospholipids (A), neutral lipids and sterols (B) for the mutant (1) and overexpressing (2) strains of these biosynthetic pathways. *Significant differences compared with their respective control strains.

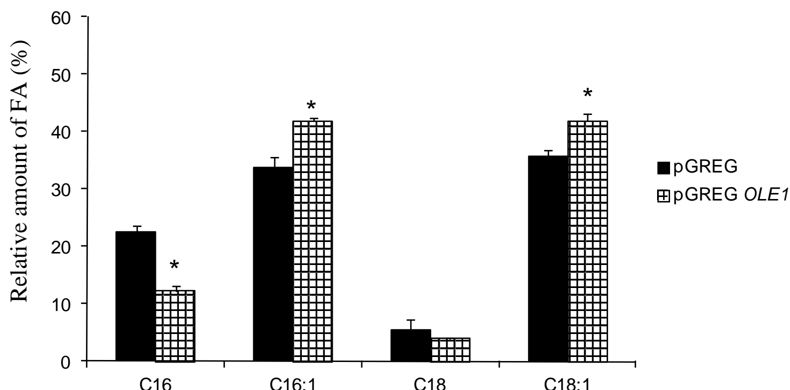


Figure 5. Percentages of fatty acids of pGREG *OLE1* strain and their control strain, *ho*QA23 pGREG. *Significant differences compared with their respective control strains.

paradigm of this trend, we were able to detect the myristoleic acid (C14:1) in *Δerg*6 whereas C18 was undetectable in *Δlcb3* (Table S1).

Principal component analysis (PCA)

In order to explore the effect of the deletion and overexpression of the target genes in lipid composition, a PCA was performed on the 19 strains using the untransformed relative concentration of the 18 compounds measured in all strains (Fig. 6). The two first components were retained explaining 80.8% of the total variance. The first component explained 66.2% of the variation and was marked by high positive component loadings for sterol esters (+0.605) and PC (+0.430) and high negative loadings for PI (-0.429) and FA (-0.406). The second component explained 14.6% of the variation and was marked by high positive component loadings for sterol esters (+0.620) and PI (+0.511) and high negative loadings for PE (-0.395) and TG (-0.320).

The general pattern provided by the PCA is the formation of two groups: deletion and overexpressing strains associated with low and high amounts of sterol esters,

respectively. Moreover deletion strains were grouped by PI content and overexpressing strains by PE content.

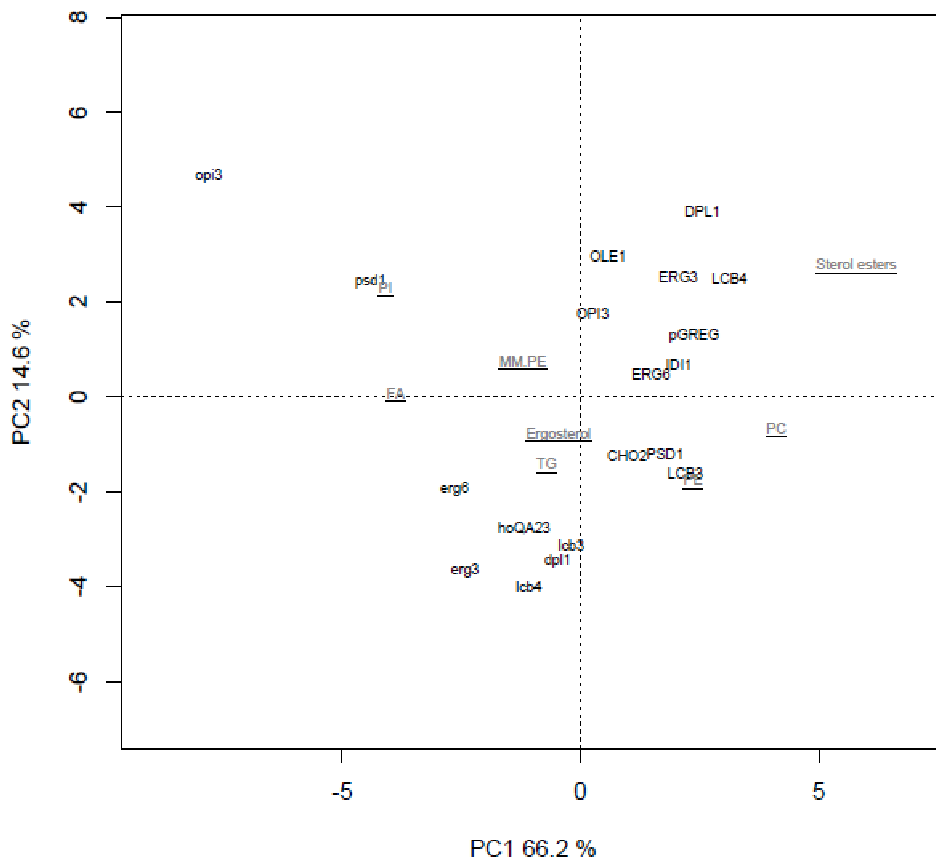


Figure 6. Biplot of the first two components of the PCA according to the lipid composition. Variables are represented by gray underlining and samples are represented by black underlining: deletion strains (lower-case letters) and overexpressing strains (capital letters).

DISCUSSION

Yeast adaptation at low temperature is an interesting feature from an industrial viewpoint, especially in the wine industry, where low temperatures are used to enhance production and retain flavor volatiles. Lipid composition of the cellular

membranes has been directly related with yeast adaptive response at different environmental temperatures in many studies (Beltran *et al.,* 2008; Henschke and Rose, 1991; Redón *et al.,* 2011; Torija *et al.,* 2003). A possible adaptation might be the reshaping of the plasma membrane composition, which would reduce the lag phase, increase growth and speedup fermentation onset. In a recent study, we modified lipid composition by incubating yeast cells in the presence of different lipid compounds, improving growth and fermentation activity at low temperature (Redón *et al.,* 2009). Another strategy to redesign the cellular lipid composition is to alter transcriptional activity by deleting or overexpressing key genes of lipid metabolism. This latter strategy has previously been and successfully assayed, though not as comprehensively as in this study. Some authors have overexpressed the gene encoding the *S. cerevisiae* desaturase *OLE1* (Kajiwara *et al.*, 2000) or other heterologous desaturases (Rodríguez-Vargas *et al*., 2006) in order to increase the degree of unsaturation and membrane fluidity, while improving the cold resistance of these engineered strains. In a recent work (Redón *et al*., 2012), we have also detected an improved or impaired fermentation vitality in some mutants of the phospholipid biosynthesis. In the present study, we have screened most of the mutants of the laboratory strain BY4742 encoding enzymes of the phospholipid, sterol and sphingolipid pathways in terms of their growth capacity at low temperature. The GT of these mutant strains was used to select genes which were further deleted in a derivative industrial strain. Again, the deleted genes showing impaired growth at low temperature were overexpressed in the genetic background of this industrial strain. The main objective of this study was to identify lipid-metabolism genes that play a key role in the adaptive response of wine yeast to low temperature and to verify the correlation between growth at low temperature and lipid composition.

Phenotypic differences between strains mutated in the same gene constructed in the laboratory and wine yeast showed the importance of the genetic background (Pizarro *et al.,* 2008; Redón *et al.,* 2011). In the wine strain *ho*QA23, the deletion

of *CRD1* led to unviability whereas BY4742 was hardly affected by the deletion of this gene. *CRD1* encodes cardiolipin synthase which catalyzes the last step in CL synthesis, but it is not essential for growth (Breslow *et al.,* 2008). Thus, other complementary mutations confer synthetic lethality in the haploid wine strain *ho*QA23. *ERG24* provides another example of the genetic background effect. This gene encodes a C-14 sterol reductase and the mutants accumulate the abnormal sterol ignosterol (ergosta-8,14 dienol), and are viable under anaerobic growth conditions but unviable on rich medium under aerobic conditions (Marcireau *et al.,* 1992). We detected important impaired growth in the BY4742 *Δerg24*, although this mutation hardly affected growth fitness in *ho*QA23. However, in spite of these differences, we detected gene deletions which significantly affected growth fitness at low temperature in both studied strains.

The mutants in the PL synthesis pathway *Δpsd1* and *Δopi3* (and *Δcho2* in the BY4742) showed the greatest increases in terms of GT in comparison with the parental strains. These genes encode the enzymes involved in synthesis of the most important plasma membrane phospholipids, PE and PC, by the *de novo* pathway (Daum *et al*., 1998). As expected, these mutant strains were characterized by a strong reduction in the proportion of PE and PC. *S. cerevisiae* had two PS-decarboxylases, one located in the mitochondrial inner membrane (encoded by *PSD1*) and another located in the Golgi and vacuolar membranes (encoded by *PSD2*). Daum *et al.* (1998) reported *PSD1* had no effect on cell viability because *Δpsd1* had residual PSD activity attributed to Psd2p. However the presence of the isoenzyme Psd2p was not enough to counterbalance the lack of Psd1p growing at low temperature. The decrease in PE and PC in the *Δpsd1* and *Δopi3* strains was counterbalanced by the increase in PI and CL. All these PLs have the same precursor CDP-DAG and, the blockage in the PE and PC biosynthetic branch increased the flux in the other two branches, leading to PI and CL increases (Fig. 1A). Contrary to PL mutant strains, the overexpression of genes *OPI3* and *PSD1* produced a very significant reduction in GT in the wine strain at low temperatures.

Enhanced growth in the PL overexpressing strains could be correlated with changes in lipid composition; however, these overexpressing strains did not significantly increase PE and PC. Even the overexpression of *OPI3*, which catalyzes the last two steps in PC biosynthesis, decreased the proportion of this PL. This metabolic route must be fine-tuned to avoid imbalances in PL proportion as a consequence of increasing the gene-dosage of some enzymes in the pathway.

The deletion and overexpression of *ERG3* also produced a phenotype with worse and better growth, respectively, in comparison with the parental strain. This gene encodes a sterol desaturase, which catalyzes the insertion of a double bond into episterol, a precursor in ergosterol biosynthesis. The deletion of this gene has previously been related with cold sensitivity (Hemmi *et al.*, 1995). These authors correlated the growth defect at low temperature with a defect in tryptophan uptake in the Δ*erg3* mutants. Another overexpressed gene in the ergosterol pathway that significantly reduced its duplication time was *IDI1* (mutant strain is unviable). We selected this gene because Beltran *et al.* (2006) previously reported a strong up-regulation of this gene at low temperature fermentation in a global transcriptomic analysis of the same industrial wine yeast. *IDI1* encodes the isopentenyl-diphosphate delta-isomerase which catalyzes the isomerization between isopentenyl pyrophosphate and dimethylallyl pyrophosphate (Fig. 2A). In terms of neutral lipid composition, the overexpression of this gene did not change the proportion of the main sterols substantially. As in the PL overexpressing strains, it is difficult to correlate improved growth with changes in the composition of the main metabolites of the pathways involving these genes. It must be borne in mind that our methodology was unable to detect ergosterol precursors. Thus, the possibility that deletion or overexpression may produce changes in the concentration of these precursors cannot be ruled out.

Finally, only the mutation and overexpression of the sphingolipid gene *LCB3* yielded a significant increase and decrease, respectively, in GT. This gene encodes a phosphatase with specificity for dihydrosphingosine-1-phosphate, regulating

ceramide and long-chain base phosphate levels and involves in incorporation of exogenous long-chain bases in sphingolipids (Mao *et al.*, 1997; Mandala *et al.*, 1998; Qie *et al.*, 1997). Intermediates in sphingolipid biosynthesis, such as sphingolipid long-chain bases (LCBs), dihydrosphingosine (DHS) and phytosphingosine (PHS) (see Figure 1C), have been identified as secondary messengers in signaling pathways that regulate the heat stress response (Ferguson-Yankey *et al.*, 2002; Jenkins *et al.*, 1997). Thus, it cannot be ruled out that these sphingolipid intermediates may also contribute to the cold stress response. Unfortunately we were not able to determine how the deletion or overexpression of this gene affected the content of these intermediates.

In an attempt to correlate growth of the different constructed strains at low temperature and modification in their lipid composition, we performed a PCA. This is a useful tool for identifying similarity and difference patterns among strains for which many data are analyzed. The PCA data clearly separated the mutant strains (left panel) from the overexpressing strains (right panel). Although genes involved in different lipid pathways were deleted or overexpressed, a general modification of the lipid profile can be ascribed to both groups of strains. The mutant strains tended to increase PI and FA, whereas the overexpressing strains increased Sterol Esters (SE) and the phospholipids PC and PE. Both lipid compounds have been linked to low temperature growth or fermentation activity in previous works by our research team. Redon *et al.* (2011) compared the lipid composition of strains, belonging to different *Saccharomyces* species and isolated from different fermentative processes (wine, beer, bread), after growing at optimum (25 ºC) and low temperatures (13 ºC). A common change in all the strains under study was the increase in PE and reduction in the PC/PE ratio. In a similar study, Tronchoni *et al.* (2012) also compared the lipid composition of different *S. kudriavzevii* strains (a more psychrophilic species than *S. cerevisiae*) and hybrid strains between *S. cerevisiae* and *S. kudriavzevii*. In these strains, in terms of neutral lipids, a common response to low temperature was an increase in TG and SE, the main storage lipids.

These storage lipids are mainly synthesized during the stationary phase, when the growth is arrested, and there is an excess of intermediates of the biosynthetic pathways (Czabany *et al.,* 2007), in a similar manner to the accumulation of carbohydrates such as glycogen and trehalose. Thus, the excess of intermediates of the sterol pathway in the overexpressing strains can produce an increase in the synthesis of sterol esters.

In conclusion, here we report a study aiming to detect the role of key lipid metabolism genes in promoting better growth at low temperature. The study has identified genes involved in the phospholipid (*PSD1* and *OPI3*), sterol (*ERG3* and *IDI1*) and sphingolipid (*LCB3*) pathways whose deletion strongly impaired growth at low temperature, whereas their overexpression reduced generation or division time by almost half. The study also reveals the importance of constructing mutant and overexpressing strains in the genetic background of commercial wine yeast, given the many phenotypic differences observed between these and the laboratory strain. As the impact of all these genes can be modulated by the genetic background, new strains should be tested in future studies to ensure the universality of these mechanisms of adaptation at low temperature. Moreover, further research will test these strains with improved growth during grape must fermentations and analyze their growth behavior and fermentation performance. This information may help to improve the future performance of wine yeast at low temperature, either by genetic modification or by the selection of strains with a better genetic makeup in terms of low temperature adaptation.

ACKNOWLEDGMENTS

This work was financially supported by the grants from the Spanish government (projects AGL2010-22001-C02-02 and AGL2010-22001-C02-01, awarded to NR and JMG, respectively). MLM also wants to thank to Spanish government for her FPI grant.

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DL: T. 1275-2013

Chapter 3

Functional analysis of lipid metabolism genes in wine yeasts during alcoholic fermentation at low temperature

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International Journal of Food Microbiology (Submitted)

UNIVERSITAT ROVIRA I VIRGILI
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ABSTRACT

Wine produced by low temperature fermentation is mostly considered to have improved sensory qualities. However few commercial wine strains available in the market are well adapted to ferment at low temperature (10–15 ºC). The lipid metabolism of *Saccharomyces cerevisiae* plays a central role in low temperature adaptation. One strategy to modify lipid composition is altering transcriptional activity by deleting or overexpressing key genes of lipid metabolism. In a previous study, we identified genes of the phospholipid, sterol and sphingolipid pathways which impacted on growth capacity at low temperature. In the present study, we aimed to determine the influence of these genes in fermentation performance and growth during wine fermentations at low temperature. To this aim, we constructed mutant and overexpressing strains in the background of a derivative commercial wine strain. The increase in gene-dosage of some of these lipid genes, such as *PSD1*, *LCB3* and *OLE1,* improved both growth and fermentation activity during fermentations at low temperature, thus confirming their positive role during wine yeast adaptation at low temperature. In terms of lipid composition, the improved phenotypes correlated with PE and UFA increases whereas the chain length of the FA decreased (conversion of C18 into C16).

Keywords: wine, yeast, cold response, lipids, mutant, overexpressing strains

Abbreviations: FA: Fatty Acids; MCFA: Medium Chain Fatty Acids; UFA: Unsaturated Fatty Acids; SFA: Saturated Fatty Acids; ChL: Chain Length; TG: Triacylglyceride; DG: Diacylglyceride; PL: Phospholipid; PI: Phosphatidylinositol; PS: Phosphatidylserine; PE: Phosphatidylethanolamine; PC: Phosphatidylcholine; PG: Phosphatidylglycerol; CL: Cardiolipin; PA: Phosphatidic Acid; NL: Neutral Lipid; SE: Sterol Ester.

INTRODUCTION

Temperature is one of the most important parameters affecting the length and rate of alcoholic fermentation and the final quality of wine. Many winemakers prefer low temperature fermentation (10–15 ºC) for the production of white and "rosé" wine because improved the characteristics of taste and aroma. This improved quality can be attributed not only at the prevention of volatilization of primary aromas, also to the increase of synthesis of secondary aromas. Thus the final wine has a greater retention of terpenes, a reduction in higher alcohols and an increase in the proportion of ethyl and acetate esters in the total volatile compounds (Beltran *et al*., 2008; Llauradó *et al*., 2002; Torija *et al*., 2003). Another positive aspect is that low temperatures reduce the growth of acetic and lactic bacteria, making easier to control alcoholic fermentation.

Despite fermentations at low temperature have interesting improvements, this practise also has some disadvantages. The optimal growth and fermentation temperature for *Saccharomyces cerevisiae* is 25–28 ºC. Restrictive low temperature increases the lag phase and reduces the growth rate, producing sluggish and stuck fermentations (Bisson, 1999). Therefore the quality of wines produced at low temperature depends on the ability of the yeast to adapt to cold. Biological membranes are the first barrier between the interior of the cell and their environment, and a primary target for damage during cold stress. The main lipid components of eukaryotic membranes are phospholipids, sterols, sphingolipids and glycerolipids. The major species of fatty acids of *S. cerevisiae* are C16 and C18 without or with one double bond. A balance mixture of lipid components is important for the physical properties of the membrane, such as fluidity. Incubation at low temperature increases the molecular order of membrane lipids by rigidification (Rusell, 1990). The membrane is essentially modified from liquid crystalline form to a gel state (Thieringer *et al*., 1998). These changes in the membrane fluidity alter various functions of membrane-bound proteins, such as

import and export of metabolites and proteins across plasma membrane. Yeasts have developed several mechanisms to maintain appropriate fluidity of its membranes regardless of the ambient temperature. The most described mechanism is the increase of the unsaturation degree of fatty acids (mainly palmitoleic (C16:1) and oleic (C18:1) acids). Phospholipids with unsaturated fatty acids (UFA) have a lower melting point and more flexibility than phospholipids with saturated acyl chains. Another way of increasing membrane lipid fluidity is to decrease the chain length (ChL) of these FA by increasing the synthesis of medium chain fatty acids (MCFA; C6 to C14) in hypoxic conditions (Beltran *et al.,* 2008; Torija *et al.,* 2003). Recently, Redón *et al.* (2011) also reported new common changes in the lipid composition of different industrial species and strains of *Saccharomyces* after low temperature growth. Despite specific strains/species dependent responses, the results showed that at low temperatures the MCFA and triacylglyceride (TG) content increased, whereas the phosphatidic acid content (PA) and the phosphatidylcholine/phosphatidylethanolamine (PC/PE) ratio decreased.

In *S. cerevisiae*, these metabolic changes are primarily governed by regulation of the transcriptional activity of the genes involved in the lipid biosynthesis pathway. Tai *et al.* (2007) compared different genome-wide transcriptional studies of *S. cerevisiae* growing at low temperature and concluded that lipid metabolism genes were the only ones whose activity was clearly regulated by low temperature. In previous studies of our group, we screened the importance of most of the genes belonging to the phospholipid, sterol and sphingolipid pathways in the adaptation at low temperature by analyzing the effect on the growth and on the vitality of the deletion and overexpression of these genes in a laboratory and industrial strain (López-Malo *et al*., 2013; Redón *et al*., 2012). From these previous studies, the genes whose deletion and overexpression showed a greatest effect on growth were *PSD1* and *OPI3,* of the phospholipid metabolism; *ERG3, ERG6* and *IDI1,* of the ergosterol pathway; *LCB3, LCB4* and *DPL1,* belonging to the sphingolipid pathway and *OLE1*, the only desaturase of *S. cerevisiae*. The aim of the present

study was to study these selected genes in depth in a context which mimicked the wine fermentation conditions. Firstly, we analysed gene activity of these selected genes throughout several fermentations of synthetic grape must at low temperature in the wild, mutant and overexpressing strains. Afterwards we characterized the effect of the mutations and overexpressions in a wine yeast on the growth and fermentation activity during wine fermentations at low and optimum temperature. The increase in gene-dosage of some of these lipid genes improved both growth and fermentation activity during fermentations at low temperature, thus confirming their positive role during wine yeast adaptation at low temperature.

MATERIAL AND METHODS

Strains and growth media

The mutant and overexpressing strains used in this study were constructed in our previous work (Lopez-Malo *et al*., 2013) in the background of a derivative haploid of the commercial wine strain QA23 (*ho*QA23) (Lallemand S.A., Canada) (Salvadó *et al*., 2012). The genes which were deleted and overexpressed are listed in Table 1. The genes *IDI1* and *OLE1* were only overexpressed because their deletion produced an unviable phenotype (Giaever *et al*., 2002). The haploid QA23 strain transformed with the empty plasmid pGREG505 (*ho*QA23-pGREG) was used as control of the overexpressing strains.

These strains were cultured in the synthetic grape-must (SM) (pH 3.3) described by Riou *et al*. (1997) but with 200 g/L of reducing sugars (100 g/L glucose + 100 g/L fructose) and without anaerobic factors (Beltran *et al*., 2004). The following organic acids were used: malic acid 5 g/L, citric acid 0.5 g/L and tartaric acid 3 g/L. The following mineral salts were used: KH_2PO_4 750 mg/L, K_2SO_4 500 mg/L, $MgSO_4$ 250 mg/L, $CaCl_2$ 155 mg/L, NaCl 200 mg/L, $MnSO_4$ 4 mg/L, $ZnSO_4$ 4 mg/L, $CuSO_4$ 1 mg/L, KI 1 mg/L, $CoCl_2$ 0.4 mg/L, H_3BO_3 1 mg/L and $(NH4)_6Mo_7O_{24}$ 1 mg/L. The following vitamins were used: myo-inositol 20 mg/L,

calcium panthothenate 1.5 mg/L, nicotinic acid 2 mg/L, chlorohydrate thiamine 0.25 mg/L, chlorohydrate pyridoxine 0.25 mg/L and biotine 0.003 mg/L. The assimilable nitrogen source used was 300 mg N/L (120 mg N/L as ammonium and 180 mg N/L in amino acid form).

The population inoculated in the synthetic grape-must came from an overnight culture in YPD at 30 ºC. After counting under the microscope, the appropriate dilution of the overnight culture was transferred to SM to achieve an initial cell concentration of 2 x 10^6 cells/mL.

Gene expression analysis by real-time quantitative PCR

Total RNA of 10^8 cell/mL was isolated as described by Sierkstra *et al*. (1992) and re-suspended in 50 µL of DEPC-treated water. Total RNA suspensions were purified using the High Pure Isolation kit (Roche Applied Science, Germany) according to the manufacturer's instructions. RNA concentrations were determined using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, USA), and the quality of the RNA was verified electrophoretically on a 0.8 % agarose gel. Solutions and equipment were treated so that they were RNase free as outlined in Sambrook *et al*. (1989).

Total RNA was reverse-transcribed with Superscript™ II RNase H^- Reverse Transcriptase (Invitrogen, USA) in a GenAmp PCR System 2700 (Applied Biosystems, USA). The reaction contained 0.5 µg of Oligo $(dT)_{12-18}$ Primer (Invitrogen, USA) and 0.8 µg of total RNA as template in a total reaction volume of 20 µL. As directed by the manufacturer, after denaturation at 70 ºC for 10 min, cDNA was synthesized at 42 ºC for 50 min, and then the reaction was inactivated at 70 ºC for 15 min.

The primers were designed with the *Saccharomyces* Genome Database (SGD) with the exception of the housekeeping gene *ACT1*, which was previously described by Beltran *et al*. (2004). All amplicons were shorter than 100 bp, which ensured maximal PCR efficiency and the most precise quantification. Real-Time

Table 1: List of lipid genes used in this study

Standard Name	Systematic name	Molecular funtion	Substract	Product
PSD1	YNL169c	Phosphatidylserine decarboxilase I	PS	PE
CHO2	YGR157W	Phosphatidylethanolamine N-Metyltransferase	PE	M-PE/ MM-PE
OPI3	YJR073C	Phospholipid methyltranferase	MM-PE	PC
ERG3	YLR056W	C-5 sterol desaturase	Episterol	5,7,24(28)-ergostatrienol
ERG6	YML008C	Sterol 24-C methyltransferase	Zymosterol	Fecosterol
IDI1	YPL117C	Isopentenyl-diphosphate delta-isomerase	Delta3-isopentenyl-PP	Dimethylallil-pyrophosphate
LCB3	YJL134W	Sphingosine-1-phosphate phosphatase	DHS-P PHS-P	DHS PHS
LCB4	YOR171C	D-erythro-sphingosine kinase	DHS PHS	DHS-P PHS-P
DPL1	YDR294C	Sphinganine-1-phosphate aldolase	DHS-P	Palmitaldehide Phosphoryl-ethanolamine
OLE1	YGL055W	Stearoyl-CoA-desaturase	Saturated fatty acids	Unsaturated fatty acids

Quantitative PCR was performed using LightCycler® 480 SYBR Green I Master (Roche, Germany). The SYBR PCR reactions contained 2.5µM of each PCR primer, 5 µl cDNA and 10 µL of SYBR Green I Master (Roche, Germany) in a 20 µL reaction.

All PCR reactions were mixed in LightCycler® 480 Multiwell Plate 96 (Roche, Germany) and cycled in a LightCycler® 480 Instrument II, 96 well thermal cycler (Roche, Germany) using the following conditions: 95 ºC for 5 min, and 45 cycles at 95 ºC for 10 sec, at 55 ºC for 10 sec and 72 ºC 10 sec. Each sample had two controls which were run in the same quantitative PCR: NAC (No Amplification Control; sample without reverse transcriptase reaction) to avoid the interference by contaminant genomic DNA and NTC (No Template Control; sample without RNA template) to avoid interference by primer-dimer formation. All samples were analyzed in triplicate with LightCycler® 480 Software, version 1.5 (Roche, Germany) and the expression values were averaged. Gene expression levels are shown as the concentration of the studied gene normalized to the concentration of the housekeeping gene, *ACT1* and referenced to the control (*ho*QA23-pGREG). Values higher than one indicates higher gene expression than the control whereas values lower than one indicates lower gene expression in comparison to the control.

Generation time

Growth was monitored at 600 nm in a SPECTROstar Omega instrument (BMG Labtech, Offenburg, Germany) at 12 ºC and 28 ºC. Measurements were made every half hour during 3 days after a pre-shaking of 20 s for 28 ºC microplate. However at 12 ºC the microplate had to be incubated outside the SPECTROstar spectrophotometer and then transferred into it to take the measurements every 8 h during lag phase and every 3 h during exponential phase. The wells of the microplate were filled with 0.25 mL of SM, reaching an initial OD of approximately 0.2 (inoculum level of ~ 2 x 10^6 cells/mL). Uninoculated wells for

each experimental series were also included in the microplate to determine, and consequently subtract, the noise signal. All experiments were carried out in triplicate.

Growth parameters were calculated from each treatment by directly fitting OD measurements versus time to the reparametized Gompertz equation proposed by (Zwietering *et al*., 1990):

$$y=D*\exp\{-\exp[((\mu_{max}*e)/D)*(\lambda-t))+1]\}$$

where $y=\ln(OD_t/OD_0)$, OD_0 is the initial OD and OD_t is the OD at time t; $D=\ln(OD_t/OD_0)$ is the asymptotic maximum, μ_{max} is the maximum specific growth rate (h^{-1}), and λ is the lag phase period (h) (Salvadó *et al*., 2011). Generation time was calculated using the equation $t_d=\ln2/\mu$.We normalised this value by dividing with its control, the generation time of the *S. cerevisiae ho*QA23 or *ho*QA23-pGREG.Values lower than 1 indicated lower generation time, whereas values greater than 1 indicated greater generation time compared to the control.

Fermentations

Fermentations were performed at 28 ºC and 12 ºC, with continuous orbital shaking at 100 rpm. Fermentations were done in laboratory-scale fermenters using 100 mL bottles filled with 60 mL of SM and fitted with closures that enabled the carbon dioxide to escape and samples to be removed. The population inoculated in every flask was 2 x 10^6 cells/mL from an overnight culture in YPD. Fermentation was monitored by measuring the density of the media (g/L) using a Densito 30 PX densitometer (Mettler Toledo, Switzerland). Fermentation was considered to be completed when density was below 998 g/L. Also yeast cell growth was determined by absorbance at 600 nm. Geneticin was also added (200 mg /L) to the SM of the overexpressing strains to ensure plasmid stability.

Determination of total yeast fatty acids

Yeast cells (5-10 mg dry mass), collected at mid-fermentation (T50) at 12 ºC, were placed in sealed tubes with a Teflon-lined screw cap and saponified using a 1 mL of 5% NaOH in 50% methanol/water (Rozès *et al*. 1992). The tubes were placed in a dry bath (100 ºC) during 5 min. After vortexing samples and then the tubes were placed in a dry bath (100 ºC) 25 min more. Then the saponified material was cooled a room temperature and 2 mL HCl 6M was added. Free fatty acids were extracted by adding 500 µL hexane: tert-butylmethyl ether (MTBE) (1:1, v/v). Each tube was vortexed twice for 30 sec. The organic phase was collected after centrifugation at 3000rpm for 3min.

Analytical gas chromatography was performed on Hewlett-Packard 6850 (Agilent Technologies, USA). 1µL of cellular extract was injected (splitless, 1 min) into a FFAP-HP column (30m x 0.25mm x 0.25µm from Agilent) with an HP 6850 automatic injector. The initial temperature was set at 140 ºC and increased by 4 ºC/min up to 240 ºC. Injector and detector temperatures were 250 ºC and 280 ºC, respectively. The carrier gas (helium) at a flow rate 1.7 mL/min. Heptanoic and heptadecanoic acids (10 and 40 mg/mL respectively) were added as internal standards before the saponification of cells. Relative amounts of fatty acids were calculated from their respective chromatographic peaks areas. These values were related to the dry weight of cells and expressed as a percentage of the total fatty acid extracted, (Redón *et al*., 2009).

Lipid extraction

Yeast cells (5-10 mg dry mass) were collected at mid-fermentation. Prior to lipid extraction, a solution of 100 µL of cold methanol and 20 µL of EDTA 0.1 mM was added to the yeast cells with 1 g glass beads (0.5 mm, Biospec Products, USA) in Eppendorf, and then mixed for 5 min in a mini-bead-beater-8 (Biospec Products, Qiagen, USA). Lipid extraction was performed in two steps with 1mL chloroform/methanol (2:1, v/v, for 1 h), one step with 1mL chloroform/methanol

(1:1, v/v, for 1 h) and one step with 1mL chloroform/methanol (1:2, v/v, for 1 h). All the organic phases were transferred in a 15 mL glass screw tube and cleaned twice by adding KCl 0.88% (1/4 of a total volume of the extract). After vortexing and cooling at 4 ºC for 10 min, the samples were centrifuged at 3000 rpm for 5 min. The inferior organic phase was collected and finally concentred to dryness under nitrogen stream. The residue was dissolve in 100 µL of chloroform/methanol (2:1, v/v).

Separation and quantification of the yeast phospholipids (PLs) by HPTLC

The yeast extract phospholipids were separated by one-dimensional HPTLC on silica gel $60F_{254}$ plates (10 x 20 cm, 200 µm) with chloroform: acetone: methanol: glacial acetic acid: water (65:15:10:10:5, v/v/v/v/v). After charring the plate with 10% $CuSO_4$ in 8% H_3PO_4 and heating at 160 ºC for 4 min, the PLs were identified by known standards purchased from Sigma: phosphatidylinositol (PI), phosphatidylserine (PS), phosphatidylcholine (PC), phosphatidylglycerol (PG), phosphatidylethanolamine (PE), cardiolipin (CL) and phosphatidic acid (PA). The plates were scanned and each spot of the image was quantified in terms of integrated optical densities (IOD) with ImageJ software (a public domain, Java-based image processing program developed at the National Institute of Health, USA). Calibration curves were constructed by applying standards to each plate in the range of 0.5-4 µg/µL to quantify the PLs. These values were related to the dry weight of cells and expressed as a percentage of the total PLs extracted.

Yeast neutral lipid (NL) composition by thin-layer chromatography (TLC)

NL composition of yeast was separated by one-dimensional TLC on silica gel $60F_{254}$ (10 x 20 cm, 250 µm) (Merck, Germany). The plate was developed in three steps. First step with hexane, tert-butylmethyl ether (MTBE) and glacial acetic acid (50:50:2) to 35mm. Second step with hexane, tert-butylmethyl ether (MTBE) and glacial acetic acid (80:20:1) to 60mm. last step with hexane to 85mm. The standard

lipids lanosterol, ergosterol, squalene, cholesteryl oleate, ethyl oleate, diolein, triolein were purchased from Sigma (Germany) were applied to every plate in the range of 0.5-4 µg/µL. After TLC, lipids were charred with 10% $CuSO_4$ in 8% H_3PO_4 and heated at 160 ºC for on a TLC Plate Heater (CAMAG). Plates were scanned and each spot of the image was quantified as integrated optical densities (IOS) with ImageJ software (a public domain, Java-based image processing program developed at the National Institute of Health, USA). Calibration curves were constructed by applying standards to each plate in the range of 0.5-4 µg/µL to quantify the NLs. These values were related to the dry weight of cells and expressed as a percentage of the total NLs extracted.

Statistical data processing

All experiments were repeated at least three times, and data is reported as the mean value ± SD. Significant differences between the control strain, the mutant and the overexpressing strains were determined by *t*-tests (SPSS 13 software package, USA). The statistical level of significance was set at $P \leq 0.05$.

RESULTS

Gene activity and overexpression

Gene activity of the selected genes at 12 ºC vs 28 ºC

In order to analyze the changes in gene activity at low temperature of the selected genes (*PSD1*, *CHO2*, *OPI3, ERG3*, *ERG6, IDI1*, *LCB3*, *LCB4*, *DPL1* and *OLE1)* we analyzed their expression in the control *ho*QA23 strain at 12 ºC and 28 ºC. Samples were taken at the lag (3 h at 28 ºC and 8 h at 12 ºC) and exponential phase (24 h at 28 ºC and 48 h at 12 ºC) during fermentation. Relative values of gene expression (GE) of the ratio GE12ºC/GE28ºC are shown in Fig. 1. Higher and lower values than one indicate higher and lower gene expression at 12 ºC in comparison with 28 ºC. With few exceptions, these lipid genes showed higher

activity during the lag or adaptation period at low temperature and, conversely, they were more active during the exponential phase at 28 ºC.

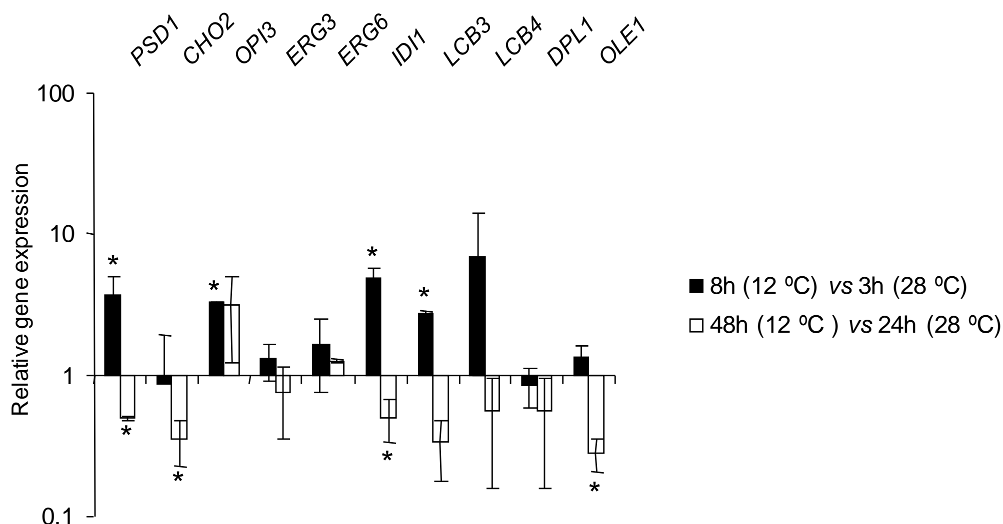


Figure 1: Relative expression of the selected genes in the haploid strain hoQA23 at different stages of alcoholic fermentation at 12 ºC and 28 ºC. Values represent the gene expression ratio 12 ºC/28 ºC of the same fermentation phase (lag and exponential phases).

Verification of overexpression

Once it was determined gene activity at both temperatures in key phases of wine fermentation, we aimed to validate and quantify the overexpression of the constructed strains. Samples were taken before inoculation (time 0) and at the same time-points (8 and 48 h) at low temperature. Relative gene expression values of the overexpressing strains, normalized with the values of the control haploid strain (*ho*QA23-pGREG), are shown in Fig. 2. All the constructed strains showed an increase in the overexpressed gene activity which ranged from 3.5 to 68 fold more than the control strain in most of the time-points analyzed. Thus we can verify that the constructed strains were overexpressing the gene of interest.

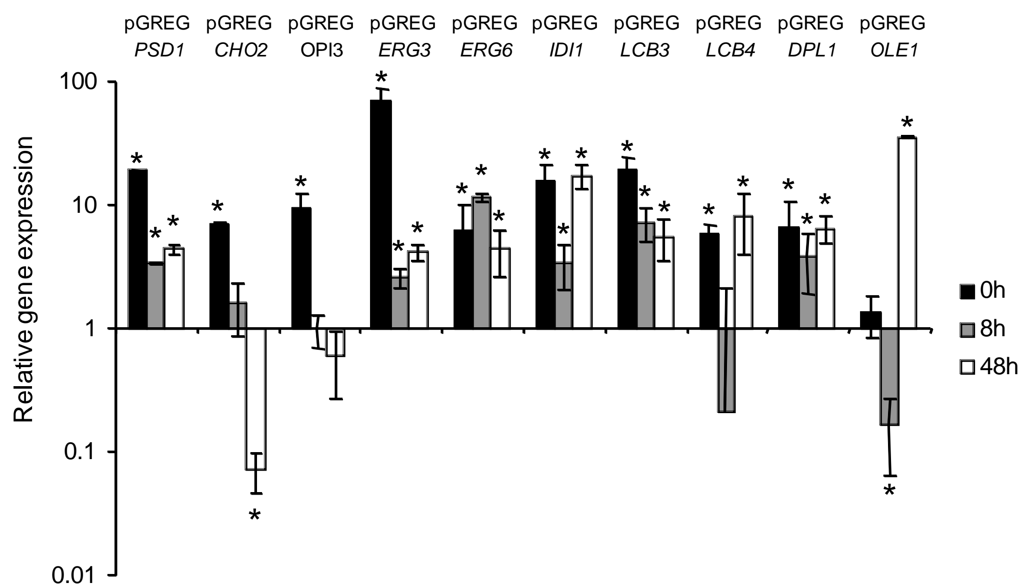


Figure 2: Relative expression of the overexpressed genes. Changes in gene activity in the overexpressing strains are shown relative to the control *ho*QA23-pGREG (set as value 1).

Phenotype effect of mutant and overexpressing strains

Determination of generation time (GT)

In order to determine the importance of the deletion or overexpression of the selected genes on growth at low temperature in wine fermentation, we calculated the GT of the mutant and overexpressing strains at 12 ºC and 28 ºC in a synthetic grape-must (SM) (Fig. 3). All the phospholipid and sterol mutants showed worse growth than the control strain at 12 ºC, whereas the sphingolipid mutants did not present significant differences at this temperature. Several mutants also increased their GT at 28 ºC compared with *ho*QA23, however, the differences were much higher at 12 ºC than at 28 ºC in *Δpsd1*and *Δerg3*.

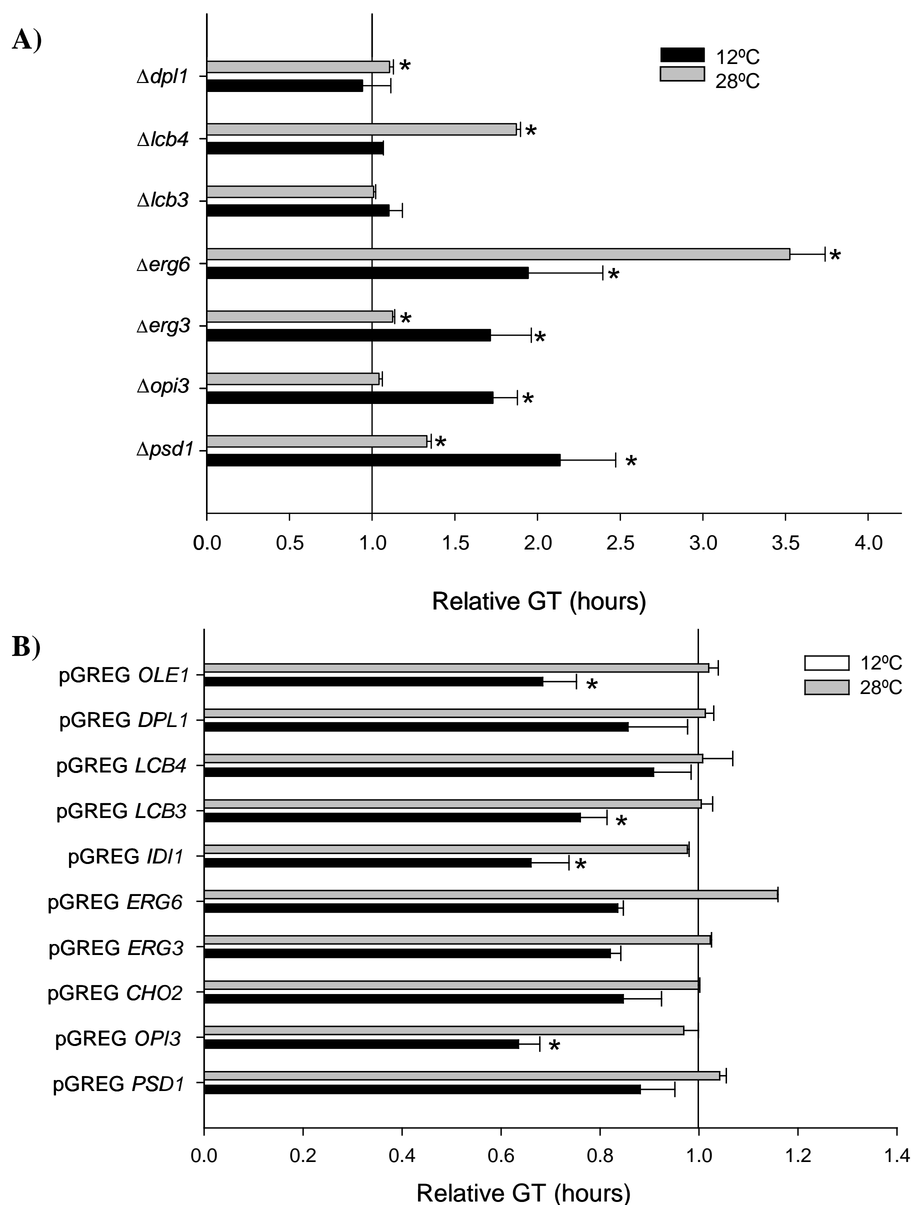


Figure 3: Generation time (GT) of **A)** mutant and **B)** overexpressing strains grown at 12 ºC (black bars) and at 28 ºC (grey bars) normalized with the GT of their control strains *ho*QA23 and *ho*QA23 pGREG (normalized as value 1). The GT for control strains was the following: 11.59 h ± 3.12 h and 3.48 h ± 0.06 h for *ho*QA23 and 13.83 h ± 0.05 h and 3.63 h ± 0.05 h for *ho*QA23-pGREG at 12 ºC and 28 ºC, respectively. *Statistically significant differences (P-value ≤ 0.05) compared with the control strain at the same temperature.

Likewise the GT of the overexpressing strains was also determined (Fig. 3). Most of the overexpressing strains showed a strong reduction in GT at low temperature. Nevertheless only pGREG *OPI3*, pGREG *IDI1*, pGREG *LCB3* and pGREG *OLE1* had significant differences.

Fermentation activity of mutant and overexpressing strains

The fermentation kinetics of the mutant and overexpressing strains were estimated by calculating the time needed to ferment 5% (T5), 50% (T50) and 100% (T100) of the sugars in the SM (Fig. 4). T5, T50 and T100 approximately match with the beginning (lag phase), middle (end of exponential phase) and end of fermentation, respectively. It should be highlighted that the parental *ho*QA23 (control strain) and the same strain transformed with the empty vector pGREG (control strain of the overexpressing strains) had differences in the T5, T50 and T100. These differences may be explained by the presence of Geneticin in the fermentations of the overexpressing strains and their resistance to this antibiotic encoded in the plasmid.
The deletion of some genes impaired the fermentation performance at low temperature of the wine strain. This was especially remarkable for *Δpsd1* and *Δerg3,* which underwent a significant delay during the beginning of the process (T5) (more than 30 and 60 hours respectively). The *Δpsd1*, *Δopi3*, *Δerg3* and *Δerg6* mutant strains also needed more time to ferment 50% of the sugars (T50) and did not finish the fermentation process at low temperature. Although not as strong, a similar delay in fermentation was also observed at 28 ºC for the *Δopi3, Δerg3* and *Δerg6* strains, but not for the *Δpsd1*. This strain was very affected at low temperature but not at all at 28 ºC. Other genes whose deletion affected to the fermentation capacity both at low and optimum temperature were *Δlcb4* and *Δlcb3.* This latter gene deletion produced a stuck fermentation at 28 ºC.
Conversely several overexpressing strains showed a quicker fermentation activity at low temperatures. Overexpression of *OLE1*, *DPL1* and *LCB3* resulted in a shorter T5, T50 and T100. Although the pGREG *PSD1* did not start the

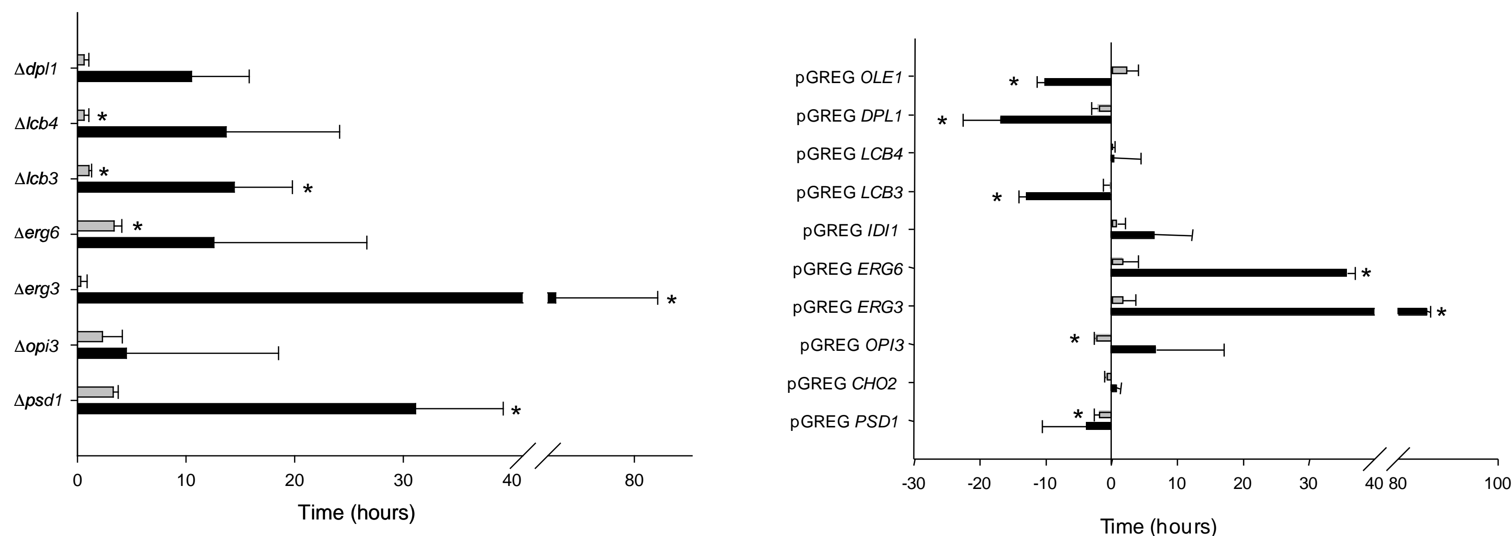
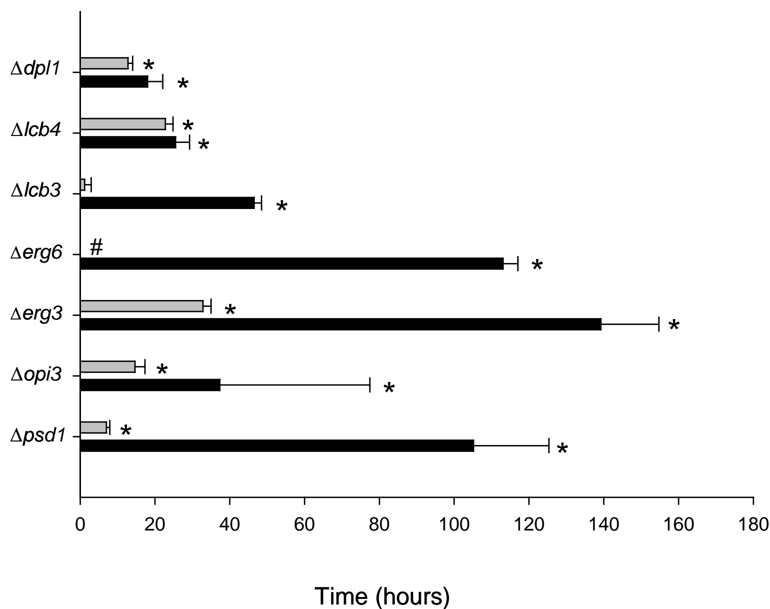


Figure 4: Determination of the time required by the mutant and overexpressing strains to ferment 5% (T5), 50% (T50) and 100% (T100) of the initial sugar content in a synthetic must at 12 ºC (black bars) and 28 ºC (grey bars). Positive and negative values represent the increases and decreases in time (hours) of the mutant and overexpressing strains compared to control strains (normalized as value 0). The fermentation time of the control strains are: *ho*QA23 at 12 ºC T5 = 27 h ± 3.18 h, T50 = 96.19 h ± 3.97 h T100 = 251.44 h ± 10.34 h; at 28 ºC T5 = 6.23 h ± 0.93 h, T50 = 44.95 h ± 0.93 h, T100 = 131.14 h ± 2.32 h and *ho*QA23-pGREG at 12 ºC T5 = 41.63 h ± 7.16 h, T50 = 119.81 h ± 11.93 h T100 = 271.69 h ± 21.48 h; at 28 ºC T5 = 8.49 h ± 0.64 h, T50 = 38.40 h ± 1.56 h, T100 = 121.73 h ± 3.36 h. # Indicates stuck fermentation before T50 or T100. * Indicates statistically significant differences (P-value ≤ 0.05).

T50



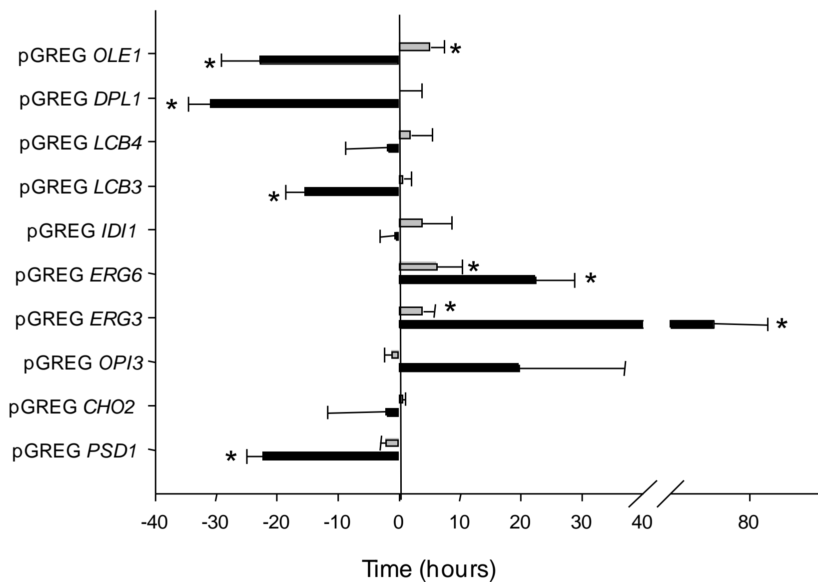
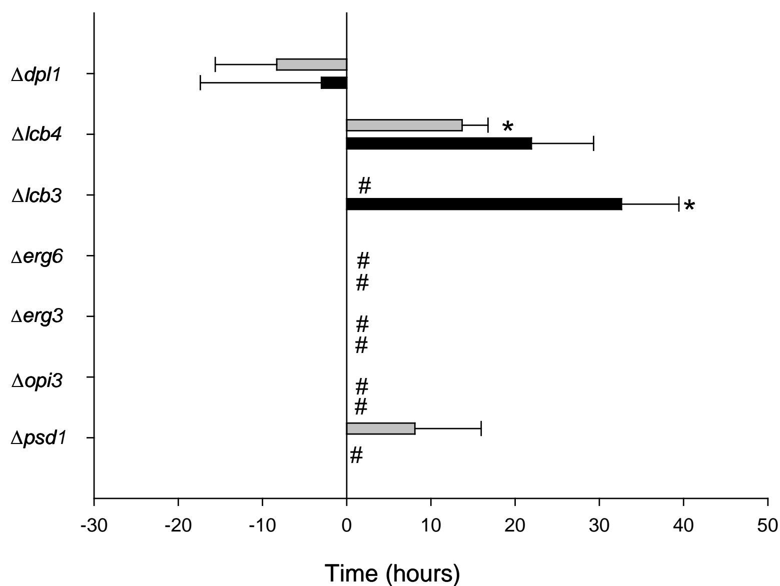


Figure 4. Continued.

T100



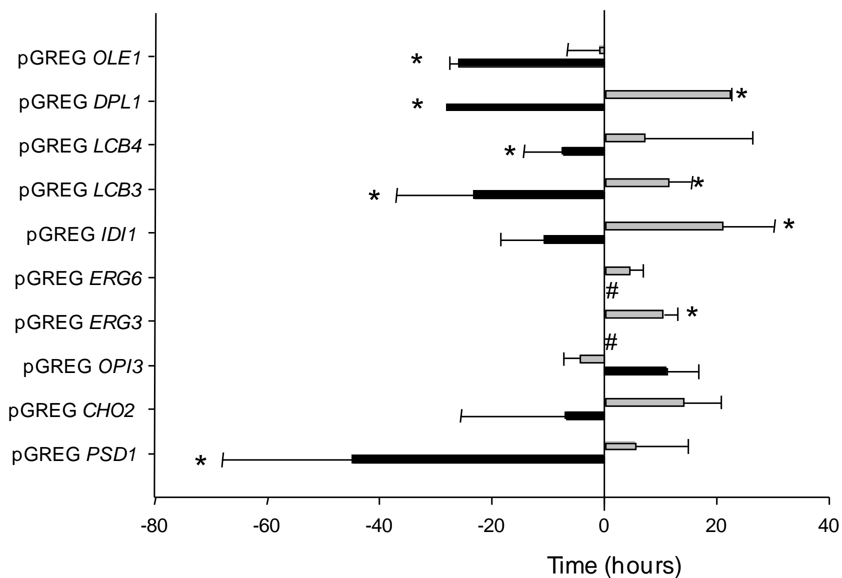


Figure 4. Continued

fermentation before than the control, this strain had a higher fermentation activity at T50 and finished almost two days before the fermentation compared with the control *ho*QA23-pGREG strain. On the other hand, overexpression of *ERG3* and *ERG6* resulted in a strong delay throughout the fermentation process at 12 ºC. pGREG *ERG3* and pGREG *ERG6* had a longer T5 and T50 and were not be able to finish the fermentation. Analyzing the fermentation rate at 28 ºC, interestingly the overexpression of *PSD1* and *OLE1* did not have any effect in fermentation length at 28 ºC. However pGREG *LCB3* and pGREG *DPL1* had longer length fermentation than the control strain at optimal growth temperature. Moreover the overexpressing *IDI1* and *ERG3* also delayed the fermentation end at 28 ºC.

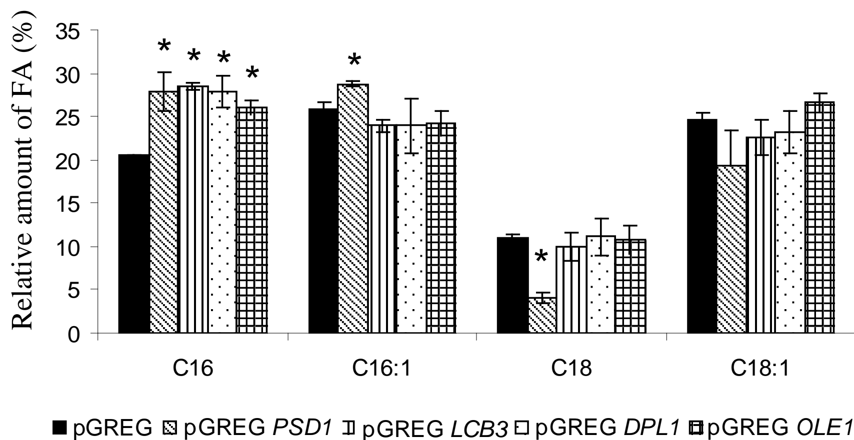
Lipid composition of improved overexpressing strain in fermentation at low temperature

In an attempt to correlate the improved fermentation activity of the overexpressing strains with the lipid composition, we analyzed the fatty acids, phospholipids and neutral lipids (DG, TG and sterols) of the four strains which showed a short fermentation length at low temperature. Unfortunately we were not able to analyze sphingolipids with the available methodology in our laboratory.

Modification of fatty acids composition

All improved overexpressing strains, pGREG *PSD1*, pGREG *LCB3*, pGREG *DPL1* and pGREG *OLE1,* showed a significant increase in palmitic acid (C16) compared with the control *ho*QA23-pGREG strain (Fig. 5A) The strain with deeper modifications in its fatty acid profile was pGREG *PSD1*, the one showing better fermentation performance at low temperature. This strain also increased palmitoleic acid (C16:1) and reduced the content in stearic acid (C18). This increase in both saturated and unsaturated C16 fatty acids fatty acids together with the decrease in C18 led to a significant decrease in the average of the total fatty acid chain lengths (data not shown).

A)



B)

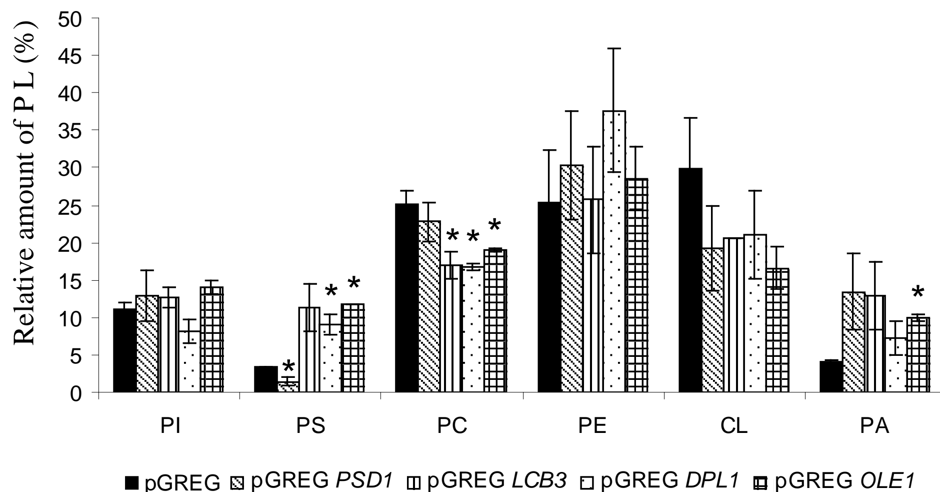


Figure 5: Percentages of **A)** fatty acids and **B)** phospholipids of the overexpressing strains with improved phenotype at 12 ºC and the control *ho*QA23-pGREG. *statistically significant differences compared with the control strain (P-value ≤ 0.05).

Modification of phospholipid and neutral lipid composition

Only tree phospholipids had significant variations respect to the control: PS, PC and PA (Fig. 5B). pGREG *PSD1* decreased its PS percentage whereas pGREG *DPL1* and pGREG *OLE1* showed an increase in this PL. PC percentage was decreased in strains which overexpressed *LCB3*, *DPL1* and *OLE1*. pGREG *OLE1* also significantly increased its PA percentage. Finally, although non-significantly, *PSD1* and *DPL1* overexpressing strains increased the PE percentage.

The analysis of neutral lipids did not show any significant differences among the overexpressing strain and the control strain *ho*QA23-pGREG (data not shown).

DISCUSSION

Low temperature fermentations are becoming more frequent in wine industry to enhance production and retain flavor volatiles, obtaining wines with more pronounced aromatic profiles. However this practice has some disadvantages, like an increase in the risk of stuck or sluggish fermentation. Changes in plasma membrane composition have been directly related with the yeast adaptive response at different environmental temperatures in many studies (Beltran *et al.,* 2008; Henschke & Rose, 1991; Redón *et al.,* 2011; Torija *et al.,* 2003). A possible adaptation might be a reshaping of the plasma membrane composition which would reduce the lag phase, increase growth and speedup the beginning of fermentation. One strategy to redesign the cellular lipid composition is altering transcriptional activity by deleting or overexpressing key genes of lipid metabolism. In previous studies (Lopez-Malo *et al*., 2013; Redón *et al*., 2012), we screened most of the mutants of the laboratory strain BY4742 encoding enzymes of the phospholipid, sterol and sphingolipid pathways in their growth capacity at 12 ºC and vitality of phospholipid mutants at low temperature. From this data, we selected 10 genes whose deletion, overexpression or both showed a differential phenotype at low temperature. In the present study, we aimed to determine the

influence of these genes in fermentation performance and growth during wine fermentations at low temperature. To this objective, we firstly analysed the transcriptional activity of these genes in the lag and exponential phases of a derivative wine yeast after inoculation in a synthetic grape must (SM) fermentation.

Most of the selected genes showed an up-regulation at low temperature during lag phase. This result evidences the importance of these lipid genes in the adaptation of the wine yeast after the inoculation to the grape must at low temperature. Previous functional genomic analysis of yeast response during low temperature exposure showed that some genes involved in phospholipid synthesis were induced under these conditions (Murata *et al.*, 2006). Our results showed that *PSD1* and *OPI3* increased their gene expression during the lag phase when yeasts were growing at 12 ºC compared with growth at 28 ºC. The up-regulation by cold fitted with a strong growth and fermentation impairments of the corresponding mutant strains Δ*psd1* and Δ*opi3* and an improved phenotype of the overexpressing strains pGREG *PSD1* and pGREG *OPI3*. However, the gene which showed a more specific response to low temperature was *PSD1* because its deletion or overexpression hardly affected to growth or fermentation performance at optimum temperature. *PSD1* encodes a phosphatidylserine decarboxilase (Psd1p) of the mitochondrial membrane which converts PS into PE. Recent works related increases of PE or decreases in the ratio of PC/PE as a general response to low temperature in different strains and species of *Saccharomyces* (Redón *et al.*, 2011; Tronchoni *et al.*, 2012). The *PSD1* overexpressing strain increased PE and decrease PC, although none of these changes were significant. Other reported mechanisms of cold adaptation are the increase in the degree of unsaturation of fatty acids (FA) (Sakamoto and Murata, 2002) and the decrease in the average chain length of these FA (Torija *et al.*, 2003). The pGREG *PSD1* strain adapted its FA content to both mechanisms by increasing the C16 and C 16:1 and decreasing the C18.

Beltran *et al.* (2006) previously reported that several genes of sterol metabolism were differentially expressed in winemaking fermentations at low temperature. However, we did not observe transcriptional differences between *ERG3* and *ERG6* at 12 ºC and 28 ºC in the two fermentation stages studied. These genes are involved in the last steps of ergosterol biosynthesis and their function must be crucial for growth and fermentation activity because the respective Δ*erg3* and Δ*erg6* mutant strains were strongly affected in both activities, with little influence of the fermentation temperature. The deletion of *ERG3* has previously related with cold sensitivity as a consequence of a decrease in the tryptophan uptake (Hemmi *et al.*, 1995). Conversely to the expected, the strains overexpressing these two genes also showed an impaired phenotype in growth and fermentation. Although we did not detect significant changes in the concentration of the main sterols, the increase in the gene-dosage of some enzymes of the pathway can be counterproductive for the cell for unbalancing the proportion of some sterol intermediates.

On the other hand, we did observe an increase of the *IDI1* transcriptional activity at lag phase. In the abovementioned work of Beltran *et al.* (2006) this gene showed the strongest up-regulation at the middle to late phases of the low temperature fermentation. *IDI1* encodes the isopentenyl-diphosphate delta-isomerase which catalyzes the isomerization between isopentenyl pyrophosphate and dimethylallyl pyrophosphate (DMPP), an intermediate in the ergosterol synthesis. These authors correlated the upregulation of this gene more with a better cell growth than with the ergosterol synthesis because DMPP, the product of *IDI1*, is also implicated in tRNA synthesis. Our results confirmed that the overexpression of this gene led to a significant lower generation-time (GT) at low temperature. However this better growth of the pGREG *IDI1* strain did not correlate with and improved fermentation activity at low temperature. We could not analyse the phenotype of the Δ*idi1* because this mutant strain is unviable.

There is little information about the relation of sphingolipids with cold stress. The most remarkable connection is that *YPC1*, which encodes an alkaline ceramidase

involved in the synthesis of phytoceramides, showed a consistent transcriptional up-regulation at low temperature (Tai *et al*., 2007). In our study, *LCB3* was also up-regulated during the lag phase of the low temperature fermentation and its overexpression improved both growth and fermentation activity at low temperature. The other sphingolipid gene whose overexpression improved fermentation activity (not growth) was *DPL1*. *LCB3* encodes a phosphatase capable of dephosphorylating long-chain bases, DHS-1-P and PHS-1-P and *DPL1* encodes the lyase that cleaves the same long base phosphates (Dickson and Lester, 1999). Sphingolipids are bio-effectors molecules and second messengers in important cellular events, such as cell growth, cell senescence, apoptosis and stress. Levels of ceramide, DHS and phosphorylated sphingoid bases may be critical to determining whether the yeast cells proliferate or undergo growth arrest (Mao *et al*., 1999). Mandala *et al*. (1998) demonstrated that Δ*lcb3* and Δ*dpl1* had a dramatically enhanced survival upon severe heat shock. Conversely, according to our data, the overexpression of these genes improved growth and fermentation performance at low temperature. Unfortunately we have not been able to determine the modification in the main sphingolipid compounds as consequence of overexpression of these genes.

Finally another gene whose overexpression (the mutant is unviable) showed a differential phenotype at low temperature was *OLE1*. This gene encodes the only desaturase of *S. cerevisiae*, converting saturated FA acyl-CoA substrates to mono-UFA via oxygen-dependent mechanism (Stukey *et al*., 1989). In spite of the previously reported overexpression of this gene at low temperature (Beltran *et al*., 2006; Schade *et al*., 2004), we did not observe overexpression or higher UFA content in the fermentation points analyzed. Nevertheless, the pGREG *OLE1* showed improved fermentation performance throughout the process and shorter generation time than the control at low temperature. Remarkably, the overexpression did not affect either fermentation rate or generation time at 28 ºC.

An improved phenotype in growth and fermentation activity at low temperature of an *OLE1* overexpressing strain was previously reported by Kajiwara *et al*. (1999). In our previous study, we detected key lipid metabolism genes in promoting better growth at low temperature (Lopez-Malo *et al*., 2013). This previous study was carried out in minimal medium (SC) to avoid the interferences of other stresses exerted during wine fermentation (osmotic, pH, ethanol, etc.). However we already stated that these mutant and overexpressing strains with differential phenotypes at low temperature should be tested in an environment mimicking grape must fermentation. The aim of this study has been to confirm the importance of these genes in growth and fermentation activity at low temperature by using a synthetic grape must. Most of the results have supported the screening carried out in the previous study because the mutants constructed showed an impaired growth and fermentation activity whereas the overexpressing strains of these genes reduced the generation time and the fermentation length. In terms of lipid composition, the improved phenotypes correlated with PE and UFA increases whereas the chain length of the FA decreased (conversion of C18 into C16). However, the deletion or overexpression of a gene did not always correlate with the accumulation or shortage of the lipid compound expected, either by fine-tuning regulation of the metabolic pathways or by limitation of the analytical technique used. In summary, genes as *PSD1*, *LCB3* and *OLE1* have shown a specific and crucial role in cold adaptation, and the genetic manipulation of these genes may improve the performance of wine yeasts under low temperature fermentations. However, as wine industry is very reluctant to use genetically modified strains (GMO), this information can be also useful for the selection of future strains with higher transcriptional or metabolic activity in these genes.

ACKNOWLEDGMENTS

This work was financially supported by the grants from the Spanish government (projects AGL2010-22001-C02-02 and AGL2010-22001-C02-01, awarded to NR

and JMG, respectively). MLM also wants to thank to Spanish government for her FPI grant.

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UNIVERSITAT ROVIRA I VIRGILI
METABOLIC AND MOLECULAR ADAPTATION OF WINE YEASTS AT LOW TEMPERATURE FERMENTATION:
STRATEGIES FOR THEIR GENETIC IMPROVEMENT
María López Malo
DL: T. 1275-2013

Chapter 4

Genetic improvement of a wine yeast in its fermentation performance at low temperature by evolutionary engineering: deciphering the molecular basis

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In preparation

ABSTRACT

Wine produced at low temperature is often considered to improve sensory qualities. However, there are certain drawbacks to low temperature fermentations such as reduced growth rate, long lag phase, and sluggish or stuck fermentations. Selection and development of new *Saccharomyces cerevisiae* strains well adapted at low temperature has interest for future biotechnological applications. The objectives of this study were to select and to develop wine yeast strains well adapted to ferment at low temperature, and decipher the process underlying the phenotypes obtained. To do so, we used a batch serial dilution, mimicking the wine fermentation conditions at 12 ºC. The strain P5 showed to be the most competitive among the 27 commercial strains because it was able to impose in the process after a few generations. After 200 generations under selective pressure growth (12 ºC), with or without mutagen, we isolated evolved strains. Growth and fermentation kinetics of evolved strain were compared to ancestral strain. The evolved strains (P5-M, P5-C, P17-M and P17-C) showed an improved growth and fermentation performance at low temperature. In an attempt to decipher the molecular mechanisms underlying the new phenotype the genome of the evolved strains P5-C and P5-M were sequenced. We also analyzed the global gene expression of the evolved strain P5-M and compared with the transcriptome of the parental strain P5-O. DNA microarray results showed that many genes of metabolism, specifically carbohydrate metabolism, were up-regulated in the evolved P5-M strain, whereas many genes involved in transcription were down-regulated. The most remarkable result was that 4 genes belonged to *DAN*/*TIR* family (*DAN1, TIR1, TIR4* and *TIR3*) showed the highest up-regulation in P5-M compare to P5-O. These cell wall mannoproteins genes have been widely linked to low temperature response.

Keywords: wine, yeast, experimental evolution, cold, fermentation

INTRODUCTION

Many factors such as grape must composition, juice clarification, fermentation temperature or the yeast strain inoculated strongly affect alcoholic fermentation and aromatic profile of wine (Ribéreau-Gayon *et al*., 2000). With the effective control of fermentation temperature by the winemakers, low temperature fermentations (10–15 ºC) are becoming more frequent. Low temperatures are used in wine fermentations to prevent the loss of primary (varietal) aromas by evaporation, and to also increase the synthesis of secondary aromas, mainly ethyl and acetate esters (Beltran *et al*., 2006; Torija *et al*., 2003). In this way, white and "rosé" wines of greater aromatic complexity can be achieved. The optimum fermentation temperature for *Saccharomyces cerevisiae* is between 25 and 28 ºC. Thus low-temperature fermentation has some disadvantages such an increased lag phase and a reduced growth rate, producing stuck and sluggish fermentations (Bisson, 1999). Therefore the quality of wines produced at low temperature depends on the ability of yeast to adapt to cold.

Nowadays there are more than 200 commercial wine yeasts in the market (Richter *et al.,* 2013). Although some of these commercial strains are described as psychrotrophic, few of them indeed perform well during wine fermentations at low temperature (Llauradó *et al*., 2005). Thus, the selection of yeast able to ferment at low temperature is of great interest for winemaking industry yet (Catellari *et al*., 1994; Giudici *et al*., 1998; Massoutier *et al*., 1998). However the natural phenotypic diversity regarding to low temperature fermentation could be very limited in *S. cerevisiae* strains, the less psychrotrophic species of the *Saccharomyces* genus. An appealing alternative is the development of genetically improved new strains of *S. cerevisiae* better adapted to grow at low temperature. The wine yeast can be improved genetically in different ways. In the last decades many efforts have been made to engineering wine yeast strain with improved

characteristics (Dequin, 2001; Donalies *et al*., 2008; Husnik *et al*., 2006). However metabolic engineering has some limitations: 1) requirement of extensive biochemical and genetic information of the metabolism of interest; 2) the complexity of cellular physiological response, such as activation of an alternative metabolic pathway; 3) difficulties of cloning in industrial strain mainly resulting from their genetic complexity and 4) regulatory issues such the use of genetically modified organism (GMO) in food industry (Çakar *et al*., 2012). On the other hand, as a non-recombinant approaches to obtain a desired phenotype could be used the called "evolutionary engineering". Strategies based on evolutionary engineering are attractive because they may generate improved strains that will not be considered as GMOs, and will most likely have higher public acceptance. Evolutionary engineering were used for the generation of new industrial strain (Sonderegger and Sauer, 2003; Guimarães *et al*., 2008; Teunissen *et al*., 2002). The major area where this approach was used with yeast is bioethanol production, however, only a few studies reported the development of improved wine yeast strains by evolutionary engineering (Cadière *et al*., 2011; McBryde *et al*., 2006).
Experiments for many generations under conditions to which yeast are not optimally adapted selects for fitter genetic variants. Culturing populations of *S. cerevisiae* under long-term pressures undergoes a series of adaptive shifts, these shifts have been observed to occur on the order of once every 50 generations (Ferea *et al*., 1999). On the other hand, the initial mutagenesis (physical or chemical) of the culture to be evolved provides an increase of genetic diversity (Çakar *et al*., 2005). This sort of experiments also shed light on larger question about the molecular basis underlying the improved phenotype. The evolutionary engineering provides the opportunity to study evolutionary adaptation, analyzing changes in gene expression patterns following adaptive evolution in yeast or analyzing the genome structure and organization or the whole genome sequence of evolved strains (Araya *et al*., 2010; Dunham *et al*., 2002; Ferea *et al*., 1999).

The first aim of this study was to assess the most competitive strains growing in wine fermentation conditions at low temperature. To this end, we performed a growth competition assay among 27 commercial wine strains inoculated at the same size in a synthetic grape must. In spite of the economical and industrial importance of these strains, their phenotypic variation in the main oenological traits has been poorly investigated, and particularly those related to the optimum growth temperature (Salvadó *et al.*, 2011) and their ability to adapt to low temperature fermentation. The second goal was to obtain an improved strain growing and fermenting at low temperature by engineering evolution. To this aim, we maintained the growth competition in synthetic grape must during 200 generations to force mutations which produce phenotypes with improved growth in this medium. One of these evolved cultures was previously mutagenized to increase the mutation rate. Finally we aimed to decipher the molecular basis underlying this improvement by analyzing the genomic and transcriptional differences between the parental strain and the evolved strain at low temperature.

MATERIAL AND METHODS

Strains and mutagenesis

A pool of 27 commercial wine yeast strains was used in this study. These strains were typing by their interdelta sequences (Legras and Karst, 2003), so these strains were called according to their delta pattern (from P1 to P27). Before to chemical mutagenesis, yeast cells of each strain were overnight grown in 3 mL of YPD at 30 ºC and 200 rpm. A mixed culture with the same proportion of each strain was prepared to a final cell concentration of 2 x10^8 cell/mL. This mixed culture was divided into two lots. One lot was mutagenized with ethyl methanesulfonate (EMS), following the protocol described by Winston (2008), and the another one was used as non-mutagenized control. Both mutagenized and non-mutagenized

cultures were further used as inoculums of the competition and evolution experiment.

Competition experiments and experimental evolution

After mutagenesis procedures, cells were transferred to synthetic grape must (SM), derived from that described by Bely *et al.* The SM composition included 200 g L^{-1} of sugars (100 g/L glucose + 100 g/L fructose), 6 g/L malic acid, 6 g/L citric acid, 1.7 g/L YNB without ammonium and amino acids, anaerobic factors (15 mg/L ergosterol, 5 mg/L sodium oleate and 0.5 mL/ L tween 80) and 60 mg /L potassium disulfite. The assimilable nitrogen source used was 300 mg N/L (120 mg N/L as ammonium and 180 mg N/L in an amino acid form).

The competition experiments and experimental evolution was based on batch serial dilution. Batch cultures were done in laboratory-scale fermenters using 100 mL bottled filled with 60 mL of SM and fitted with closures that enabled the carbon dioxide to escape and samples to be removed. The population inoculated in every flask was an OD approximately of 0.2. Batch selection was performed at 12 ºC, with a continuous orbital shaking at 100 rpm for 200 generations.

Cultures were allowed to grow through a normal growth curve, with a weekly transfer of a small volume (the volume necessary to inoculate an OD of 0.2) of the expanded culture into 60 mL fresh medium. Batch cultures were plating on solid YPD at initial point (0), at 10, 50, 100, 150 and 200 generations and 50 colonies of each sampling point were randomly selected and were kept at -80 ºC in 35% (v/v) glycerol for further genotyping analysis.

Growth of cultures was monitored by measuring the absorbance at 600nm every 48 h. The numbers of generations were calculated using the equation: $n = (\log N_t - \log N_0)/\log 2$, where n is the number of generations, N_0 is the initial OD and N_t is the OD at time t. Thus, generation time (GT) was calculated using the equation $GT = t/n$.

Fermentations

Fermentations were preformed in laboratory-scale fermenters using 100 mL bottles filled with 60 mL of SM and fitted with closures that enabled the carbon dioxide to escape and samples to be removed. Fermentations were run at 28 ºC and 12 ºC, with continuous orbital shaking at 100 rpm. The population inoculated in every flask was 2 x 10^6 cells/mL from an overnight culture in YPD. Fermentation was monitored by measuring the density of the media (g/L) using a Densito 30 PX densitometer (Mettler Toledo, Switzerland). Fermentation was considered to be completed when density was below 998 g/L. Cell growth was determined by absorbance at 600 nm (OD_{600}).

Interdelta sequences typing

Yeast typing was performed by delta elements amplification from genomic DNA. PCR amplifications were carried out in a 50 µL reaction, containing 5 µL (0.1 – 100 ng) of DNA, 1 µL 200 µM dNTPs, 1 µL 10 µM primers, 5 µL 10 x PCR buffer, 2.5 µL 50 mM $MgCl_2$, 1 µL BSA 200 µg/mL, 0.2 µL of Taq polymerase, and 33.3 µL water to complete the 50 µL. The conditions for delta sequences amplification were those described by Legras and Karst (2003). Amplification products were separated by electrophoresis on 1.5% (w/v) agarose gels.

Transcriptome analysis

Yeast cells (10^8 cell/mL) were collected at exponential growth phase during fermentation at 12 ºC from three independently cultured replicates. RNA was isolated as described by Sierkstra *et al*. (1992) and was re-suspended in 50 µL of DEPC-treated water. Total RNA suspensions were purified using the High Pure Isolation kit (Roche Applied Science, Germany) according to the manufacturer's instructions. Solutions and equipment were treated so that they were RNase free as outlined in Sambrook *et al*. (1989).

Microarray services were provided by the IRB Functional Genomics Core Facility, including quality control tests of total RNA by Agilent Bioanalyzer and Nanodrop spectrophotometry. RNA expression profiling was performed following the Pico Profiling method (Gonzalez-Roca *et al*, 2010). Briefly, cDNA library preparation and amplification were performed from 25ng total RNA using WTA2 (Sigma-Aldrich) with 17cycles of amplification. 8µg cDNA were subsequently fragmented by DNaseI and biotinylated by terminal transferase obtained from GeneChip Mapping 250K Nsp Assay Kit (Affymetrix). Hybridization mixture was prepared according to Affymetrix protocol. Each sample was hybridized to a GeneChip Yeast Genome 2.0 Array (Affymetrix). Arrays were washed and stained in a Fluidics Station 450 and scanned in a GeneChip Scanner 3000 (both Affymetrix) according to manufacturer's recommendations. CEL files were generated from DAT files using GCOS software (Affymetrix). To generate the log2 expression estimates, overall array intensity was normalized between arrays and the probe intensity of all probes in a probe set summarized to a single value using RMA (Robust Multichip Average) algorithm (Irizarry *et al*, 2003) in Genomics Suite 6.6 (Partek). Log2 ratios were used to calculate the differential expression between strains. We tested genes with at least two fold differences in transcript levels (log2 fold were $\leq$-1 or $\geq$ 1) between strains. Genes were considered to have significant differential expression if the p-values of the Student's t-test, after applying the Benajamini and Hochberg (BH) method to adjust for false discovery rate (FDR) (Benajamini and Hochberg, 1995) were $\leq$0.05. To group genes into functional categories was used the GO term Finder, in MIPS Functional Catalogue (http://mips.helmholtz-muenchen.de/proj/funcatDB/).

Illumina sequencing library prep

Illumina sequencing libraries were constructed from YMD1803:P5-O, YMD1805:P5-M, YMD1807:P5-C. Genomic DNA was extracted using the

Hoffman-Winston DNA prep. Bar-coded DNA fragment libraries were prepared using a Nextera DNA sample preparation kit (Epicentre Biotechnologies, Madison, WI) following standard procedures and published recommendations (Adey *et al.*, 2010).

Briefly, 50ng of yeast genomic DNA from each strain was tagmented (tagged and fragmented) by the Nextera transposome. The tagmented DNA was purified following AMPure (Agencourt) purification protocol. Purified tagmented DNA libraries were PCR amplified using the Nextera PCR Master Mix. PCR amplified libraries were cleaned following AMPure (Agencourt) purification procedures, and submitted for sequencing.

Genome mapping and variant calling

We collected 591,334 paired-end, 100bp, quality-filtered reads from P5-O, 1,793,478 from P5-M, and 2,370,026 from P5-C using the Illumina Hiseq 2000 platform. These strains were sequenced a second time with a final yield of 2,138,346 paired-end reads from P5-O, 6,459,516 from P5-M, and 9,441,932 from P5-C. Reads were aligned to the sacCer3 reference sequence using BWA (Li and Durbin, 2009) using default parameters for paired-end reads. Mapped reads were converted to a SAM file format for each strain. A file containing uniquely mapped reads was generated from the original SAM file and obtained a final percent coverage of 89.5%, 93% and 93.2% coverage for P5-O, P5-M and P5-C respectively. A final filtered mpileup file was generated for each strain using samtools (Li *et al.*, 2009) with a -C50 filter as recommended by BWA.

For SNP calling, a filtered VCF file was generated using bcftools, generated from the filtered mpileup file with duplicate reads removed. The filtered VCF file contained 4,278 variants from P5-O, 8,212 from P5-M and 14,584 variants from P5-C. Additional filtering using in-house python scripts separated variants that were only identified in the evolved strain, removed variants that were called in both

the evolved and ancestral strain and annotated the final variant call list (Pashkova *et al.*, 2013). We manually examined 96 variants from YMD1805 and 65 variants from YMD1807 using Integrative Genome Viewer (James et al., 2011) (IGV) for further prioritization.

Determination of mannoprotein content

Total mannoproteins released during fermentation were quantified at the end of the fermentative process. The relative mannoprotein content of the yeast cell wall was also determined. Yeast cells were collected at exponential growth phase during fermentation at 28 ºC and 12 ºC, 24 h and 96 h respectively, and at the end of fermentation, from three independently cultured replicates.

We used the method for mannoprotein quantification described by Quirós *et al* (2011). 3 mL of supernatant were gel filtered through 30 x 10 mm Econo-Pac®10 DG disposable chromatography columns (Bio-Rad Laboratories, Hercules, CA) and eluted with 4 ml distilled water. Then, 3 ml of the eluted fraction were filtered again using the same type of columns and eluted with 4 ml of distilled water. Two aliquots of 2 ml were concentrated in 2 ml screw-capped microtubes (QSP, USA) using Concentrator Plus (Eppendorf, Germany) at 60 ºC until complete evaporation. To determine the relative mannoprotein content of the yeast cell wall, 2 mL of each fermentation were centrifuged and cells washed with 1 mL of sterile distilled water. Resulting pellets were carefully resuspended in 100 µl of 1M H_2SO_4. Tubes were tightly capped and placed in a bath at 100 ºC for 5.5 h to undergo acid hydrolysis. After this treatment, tubes were briefly spun down, 10-fold diluted using 900 µl of miliQ water.

The samples were filtered through 0.22-µm pore size nylon filters (Micron Analitica, Spain) and were subjected to HPLC analysis for quantification of the glucose and mannose released during hydrolysis. Analytical HPLC was carried out in a Surveyor Plus Chromatograph (Thermo Fisher Scientific, Waltham, MA)

equipped with a refraction index detector. A total volume of 25 µL was injected into a HyperREZ XP Carbohydrate H+8 µm column (Thermo Fisher Scientific) assembled to its correspondent guard. The mobile phase used was 1.5 mM H_2SO_4 with a flux of 0.6 mL/min and a column temperature of 50 ºC. For the preparation of a standard curve, serial aqueous dilutions of commercial mannan (Sigma–Aldrich: Fluka) containing 10 different concentrations, ranging from 400 to 1 mg/L, were prepared and subjected to the hydrolysis described above.

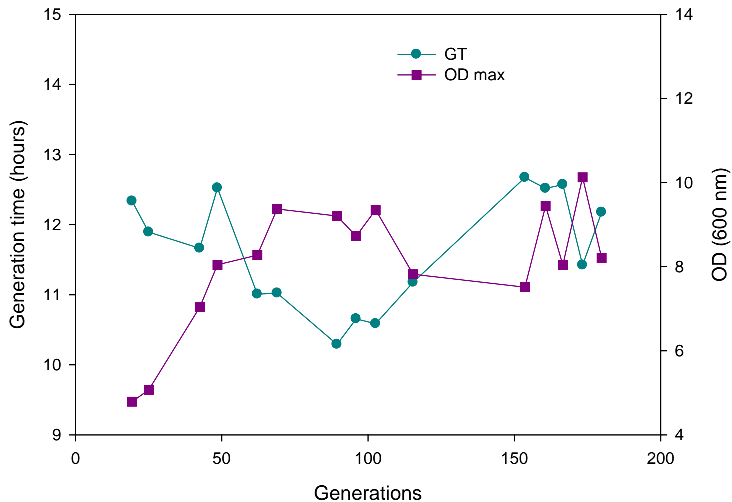
RESULTS

Evolution of growth at batch selection cultures

Growth of batch cultures was monitored during whole selection process (Fig. 1). The most remarkable result was the increase of maximum OD at the end of the experiment, more than twice, at both cultures. The maximum OD at the beginning (first generations) was around 4.5, reaching values around 8 and 10 at the end of the experiment (generation 200) for the non-mutagenized and mutagenized cultures respectively. In both cultures, the most striking improvement was occurred in the first 50 generations. However the highest values were observed at generation 173 at the non-mutagenized culture (10.13) and at generation 92 at the mutagenized culture (12.53).

Despite the lower GT observed between the generation 50 and 150 at non-EMS treated culture, we did not observe differences between the initial and final GT (around 12 h). Conversely we observed clear differences between the initial (14.29 h) and the final (10.25 h) GT at EMS treated culture. It is important to notice that the initial GT at the mutagenized culture was higher than at the non mutagenized culture, maybe as consequence of the EMS effect on the survival cells.

A)



B)

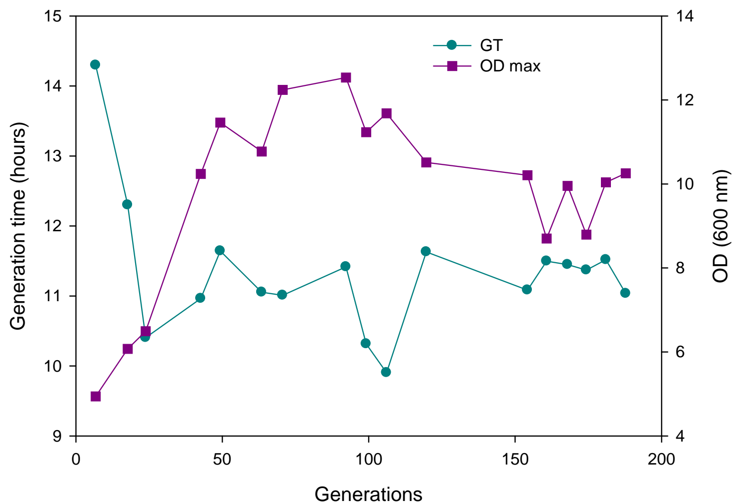


Figure 1: Evolution of growth (generation time and maximum OD_{600}) at batch selection cultures in a synthetic must at 12 ºC, without mutagenesis treatment (A) and with EMS mutagenesis treatment (B).

Competition experiments

During competition experiments, we analyzed the evolution of the 27 commercial strains, which made up the mixed inoculums, by delta sequence typing, at generations 10, 50, 100, 150 and 200 at both batch selection cultures (with and without EMS). The competence results showed similar patterns between cultures

treated and non-treated with mutagen (Fig. 2). At generation 10, only 7 strains out of the 27 making up the initial inoculum were present at the non-EMS treated culture. The number of strains was also strongly diminished at generation 10 in the EMS mutagenized culture; only 9 strains (33.3% of the total) were present.

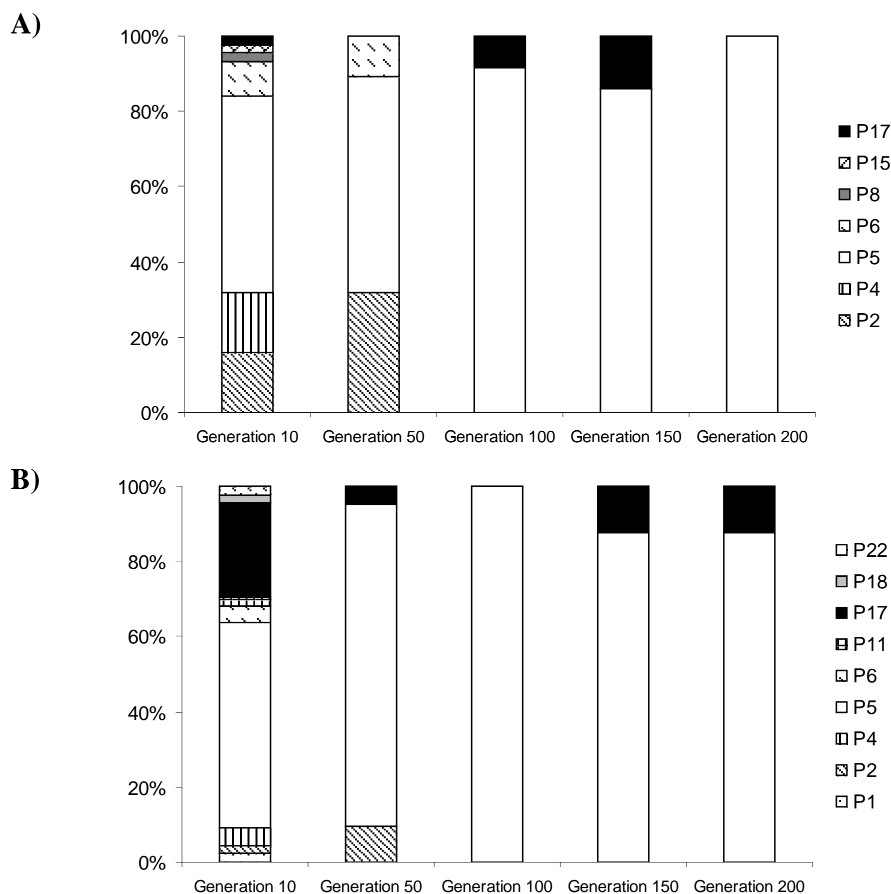


Figure 2: Percentages of *S. cerevisiae* strains during batch selection cultures at generations 10, 50, 100, 150 and 200, without mutagenesis treatment (A) and with EMS mutagenesis treatment (B).

Furthermore, the strain P5 already represented more than the 50% in both cultures. Interestingly other 3 strains (P2, P6 and P17) were detected in both cultures at generation 10. At generation 50 only 3 strains were present at both cultures. The

strain P5 again was the most abundant, representing the 57% and the 85% at non-mutagenized and mutagenized culture respectively. At this generation, the P2 strain, with a lower percentage than the major P5 strain, was also identified in both cultures. From the generation 100 to 200, 1or 2 strains (P5 and P17) were found in both cultures. The strain P5 always represented around the 90% of the analyzed colonies. At the end of the cultures, only the strain P5 was present at the non-mutagenized culture whereas a small percentage of the P17 (12.5%) strain was also identified in the EMS mutagenized culture.

Fermentation performance of evolved strains

Experiments of longer duration than 20-50 generations, under conditions in which yeast are not optimally adapted, select fitter genetic variants (Ferea *et al*., 1999). Thus, we analyzed the fermentation performance of the two strains isolated at the end of the serial batch cultures (P5 and P17). To distinguish between the strains isolated from the non-mutagenized or the mutagenized culture, the strain code was complemented with a C (control) or a M (mutagenized) respectively. The fermentation kinetics of these evolved strains were compared with those of the original commercial strains (identified with the letter O).

The fermentation kinetic of the original and evolved strains was estimated by calculating the time needed to ferment 5% (T5), 50% (T50) and 100% (T100) of the sugars in the SM (Table 1) and is graphically plotted in figure 3. T5, T50 and T100 approximately match with the beginning (lag phase), middle (end of exponential phase) and end of fermentation, respectively.

All the evolved strains showed a better fermentation performance that the original strains at 12 ºC (Fig. 3 and Table 1).The most remarkable improvement in the time to complete the fermentation at low temperature was observed in the P5-M. This strain took around 350 hours to finish the fermentation whereas the parental strain (P5-O) was unable to consume all the sugars after 30 days (720 hours) of

Table 1: Determination of the time required by the evolved and original strains to ferment 5% (T5), 50% (T50) and 100% (T100) of the initial sugar content in a synthetic must at 12 ºC and 28 ºC.

Strain	P5-O	P5-C	P5-M	P17-O	P17-C	P17-M
28 ºC						
T5	6.3 ± 0.74	5.98 ± 0.81	10.04 ± 0.81*	7.71 ± 0.90	9.96 ±1.83	9.28 ± 0.34*
T50	38.54 ± 0.74	38.54 ± 2.25	37.7 ± 0.98	50.39 ± 2.05	48.93 ±1.55	48.14 ± 0.90
T100	127.38 ± 3.43	126.31 ± 5.64	89.47 ± 11.84*	147.27 ± 3.98	125.29 ± 21.15	149.7 ± 0.00
12 ºC						
T5	29.625 ± 3.25	20.81 ± 0.80*	22.88 ± 2.60*	65.74 ± 5.21	64.61 ± 4.27	54.09 ± 9.63
T50	211.88 ± 48.94	172.88 ± 47.43	104.63 ± 8.11*	335.83 ± 14.39	203.98 ± 9.82*	210.36 ± 9.04*
T100	-	505.69 ± 97.85*	349.13 ± 68.85*	-	539.43 ± 5.21*	570.1 ± 8.46*

* Statistically significant differences (P-value ≤ 0.05) compared with their control strain at the same temperature

- Unfinished fermentation

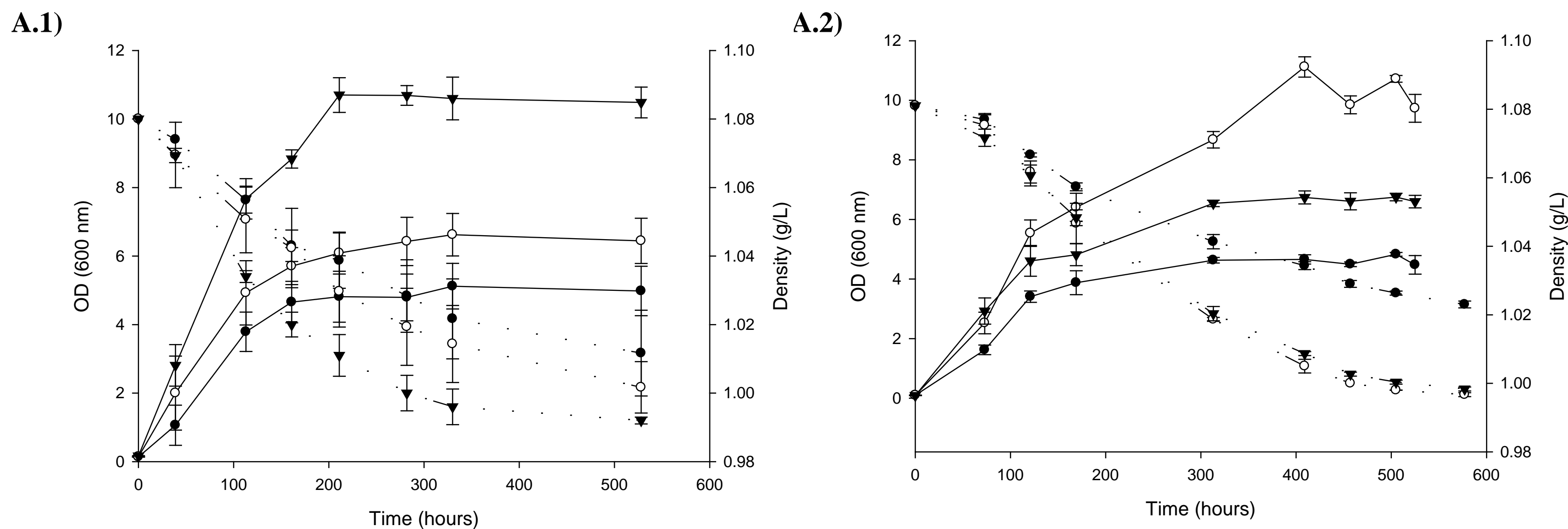


Figure 3: Fermentation kinetics (measured as density reduction; dashed lines) and growth (measured as OD_{600}; solid lines) of evolved and parental strains: P5 strains at 12 ºC (A.1) and 28 ºC (B.1) and P17 strains at 12 ºC (A.2) and 28 ºC (B.2). Original strains are represented as filled circles; strains from non-EMS treated cultures are represented as open circles; strains from EMS treated cultures are represented as filled triangles.

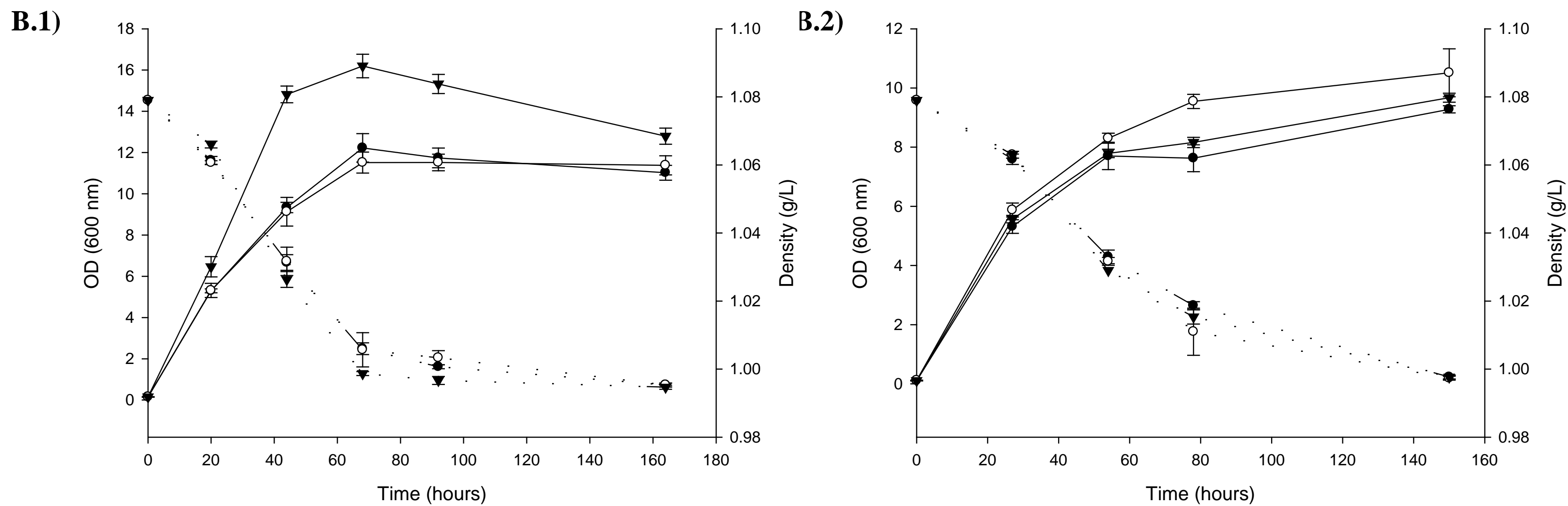


Figure 3: Continued

fermentation. The strain P5-M also reached a maximum OD two fold higher than the original strain P5-O (Fig. 3). The other evolved strains, P5-C, P17-C and P17-M, showed a similar fermentative behavior, finishing the fermentations in more than 500 hours. In any case, these three evolved strains also improved fermentation performance and biomass production (higher OD yield) than the parental strains at low temperature (The P17-O was also unable to consume all the sugars after 30 days).The P17-O reached similar OD values to the P5-M.

To correlate the improvement in fermentation kinetics with adaptation at low temperature, we also analyzed the fermentation behavior of evolved strains at 28 ºC (Table 1). Interestingly the P5-C and P17-C did not show differences in fermentation rate and biomass production in comparison with their parental strains. A little delay was observed for P5-M and P17-M at the beginning of the process, with a bigger T5 than the parental strains. In spite of this longer lag phase, the P5-M also finished the fermentation before than the parental strain at optimum temperature. However, in general for all the strains, the differences in fermentation rate and growth at 28 ºC were not as extreme as those observed at low temperature.

Changes in the genome of the evolved strains P5-C and P5-M in comparison with its parental strain P5-O

The whole genomes of the evolved mutants, P5-C and P5-M were sequenced and compared with that of the parent strain, P5-O. We identified 72 and 10 single nucleotide polymorphism (SNPs), in P5-M and P5-C genomes respectively. A large percentage of the identified mutations were non-synonymous in coding regions, 55% in P5-M strain and 40% in P5-C. Only about 4.1% in P5-M strain and 1% of P5-C were changes in regulatory sequences 5’ upstream of the ORF (Table S1 and S2).

MIPS categories constitute an interesting tool to find significant overrepresented functional groups in a gene set. This functional analysis was done with the non-

synonymous SNPs in P5-M (Table 2). The mutated genes were classified into 11 functional categories. The most representative functional categories were "Protein fate (folding, modification, destination)" (35.5%), "transport routes" (25%), "general transcription activities" and "translational control" (12.5%).

The three genes 5' upstream mutated were *MNS1, ODC2* and YPR174C. *MNS1* encodes an alpha-1,2-mannosidase that catalyzes the removal of one mannose residue from a glycosylated protein. *ODC2* encodes a mitochondrial inner membrane transporter, exports 2-oxoadipate and 2-oxoglutarate from the mitochondrial matrix to the cytosol for use in lysine and glutamate biosynthesis and in lysine catabolism. YPR174C encodes a protein of unknown function, binds phosphatidylinositol and phosphatidylethanolamine.

Table 2: Significant MIPS categories of mutated genes in P5-M compared with P5-O. Numbers of genes are between brackets.

FUNCTIONAL CATEGORY
01.01.06.05.01.01 biosynthesis of homocysteine (1)
10.03.05.03 cell cycle dependent actin filament reorganization (1)
11.02.03.01 general transcription activities (5)
12.07 translational control (5)
14 PROTEIN FATE (folding, modification, destination) (15)
14.07 protein modification (10)
14.07.03 modification by phosphorylation, dephosphorylation, autophosphorylation (5)
20.03.01.05 nuclear pore forming protein (1)
20.09 transport routes (10)
20.09.01 nuclear transport (4)
20.09.01 nuclear transport (4)

Changes in the gene expression of the evolved strain P5-M in comparison with its parental strain P5-O

The global gene expression of the best strain performing fermentation at low temperature P5-M was analyzed during SM fermentation at 12 ºC and compared with its parental strain P5-O. The physiological condition chosen for analyzing the

gene expression was at mid-exponential growth phase (96 h) (Fig. 3). The comparative results of transcriptional profiles revealed that only 2.6% (161/6124) of yeast genes showed at least 2-fold significant difference in transcript levels (Table S3). Of these, 107 genes showed a decreased expression in the evolved strain compared to the parental, whereas only 54 genes had an increased expression in P5-M.

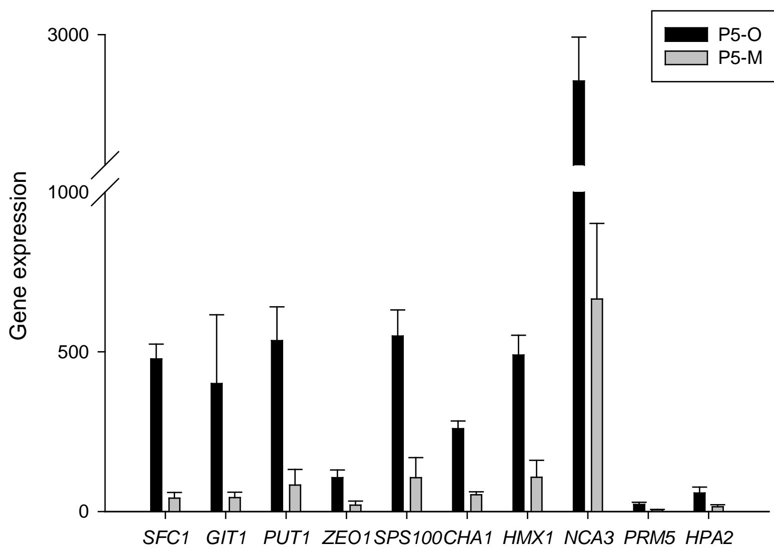
MIPS categories analysis was done with up- and down-regulated genes, with at least two fold differences in transcript levels, in P5-M compared with P5-O (Table 3). The up-regulated genes were classified into 25 functional categories. However, a large percentage of P5-M up- regulated genes (40%) belonged to the functional category "metabolism", these genes were mainly classified into the sub-category "C-compound and carbohydrate metabolism". The other two most representative functional categories of up-regulated genes were "cell rescue, defense and virulence" (16%) and "interaction with environment" (14.8%). On the other hand, down-regulated genes were classified into 24 functional categories, those with large percentage of genes were: "transcription" (29.90%), "protein with binding function or cofactor requirement" (24.29%) and "mitotic cell cycle and cell cycle control" (12.5%).

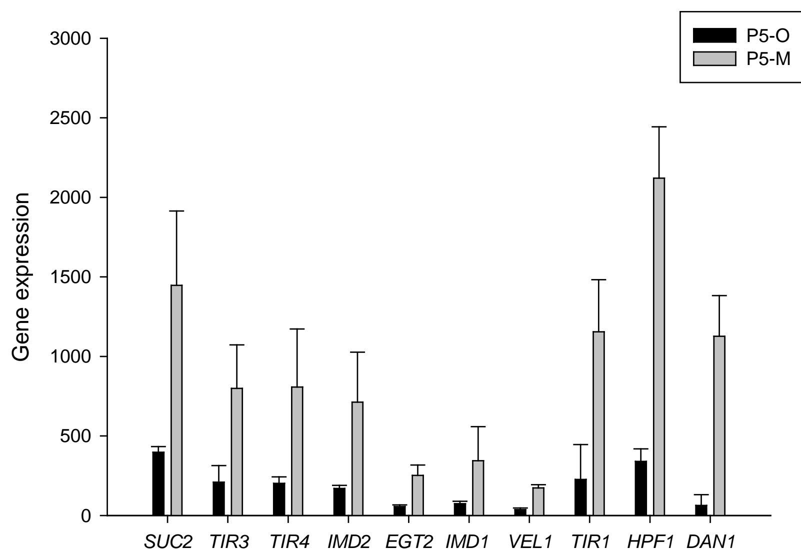
The 10 strongest down- and up-regulated genes in P5-M compared to P5-O are shown in figure 4. The three genes that showed the highest down-regulation were: *SCF1* (encodes a mitochondrial succinate-fumarate transporter), *GIT1* (encodes a plasma membrane which mediates the uptake of glycerophosphoinositol and glycerophosphatidylcholine, Git1p) and *PUT1* (encodes the proline oxidase, Put1p). The most remarkable result related to the 10 highest up-regulated genes in P5-M was that 4 of these genes were belonged to *DAN*/*TIR* family (*DAN1, TIR1, TIR4* and *TIR3*). These cell wall mannoproteins genes have been widely linked to low temperature response. Interestingly, we also observed other mannoprotein gene

Table 3: Significant up- and down regulated GO terms in P5-M at low temperature fermentation compared with P5-O. Numbers of genes are between brackets.

	FUNCTIONAL CATEGORY
	01 METABOLISM (22)
	01.03.01.03 purine nucleotide/nucleoside/nucleobase anabolism (2)
	01.05 C-compound and carbohydrate metabolism (12)
	01.05.02 sugar, glucoside, polyol and carboxylate metabolism (3)
	01.05.02.07 sugar, glucoside, polyol and carboxylate catabolism (3)
	01.05.03 polysaccharide metabolism (3)
	01.07.04 utilization of vitamins, cofactors, and prosthetic groups (1)
	02.13.01 anaerobic respiration (1)
	10.03.03 cytokinesis (cell division) /septum formation and hydrolysis (6)
UP	16.13 C-compound binding (3)
	16.13.01 sugar binding (3)
	18.01 regulation by (2)
	20.01.13 lipid/fatty acid transport (3)
	20.01.25 vitamine/cofactor transport (2)
	20.03 transport facilities (5)
	20.03.02 carrier (electrochemical potential-driven transport) (2)
	20.03.02.03 antiporter (2)
	20.03.25 ABC transporters (2)
	30.01.05.01.03 MAPKKK cascade (2)
	32 CELL RESCUE, DEFENSE AND VIRULENCE (9)
	32.01 stress response (8)
	34 INTERACTION WITH THE ENVIRONMENT (8)
	34.07 cell adhesión (4)

	34.07.01 cell-cell adhesión (4)
	42.01 cell wall (5)
DOWN	01.01.09.02.02 degradation of serine (1) 01.05.06 C-2 compound and organic acid metabolism (2) 01.05.06.07 C-2 compound and organic acid catabolism (2) 02.16 fermentation (3) 02.16.01 alcohol fermentation (2) 02.16.13 acetate fermentation (1) 02.45 energy conversion and regeneration (3) 10.03.01 mitotic cell cycle and cell cycle control (13) 10.03.02 meiosis (7) 10.03.04 nuclear and chromosomal cycle (7) 10.03.04.03 chromosome condensation (3) 11 TRANSCRIPTION (32) 11.04 RNA processing (19) 11.04.01 rRNA processing (17) 12.01 ribosome biogenesis (13) 16 PROTEIN WITH BINDING FUNCTION OR COFACTOR REQUIREMENT (structural or catalytic) (26) 16.03 nucleic acid binding (13) 16.03.03 RNA binding (8) 16.21.01 heme binding (1) 20.01.03.03 C4-dicarboxylate transport (e.g. malate, succinate, fumarate) (2) 41 DEVELOPMENT (Systemic) (4) 41.01 fungal/microorganismic development (4) 41.01.01 mating (fertilization) (4) 42.01 cell wall (8)



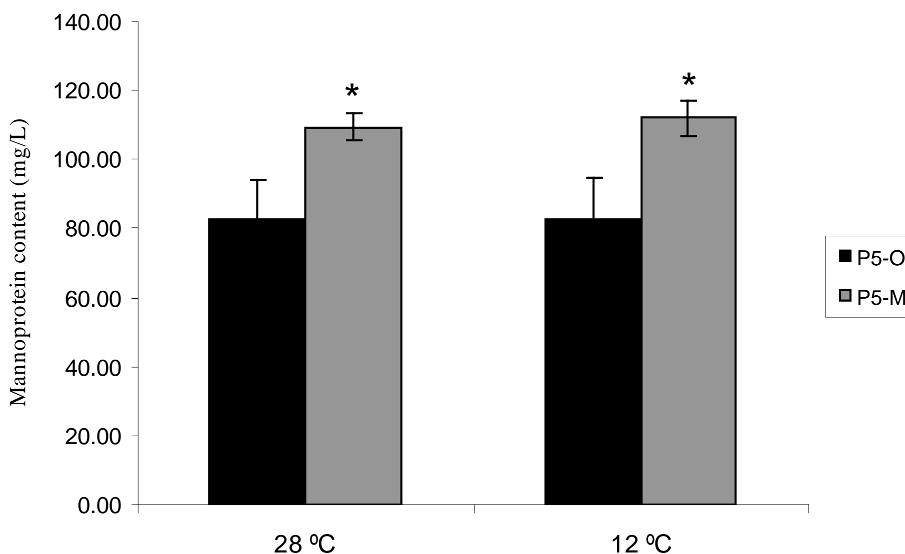


Figures 4: The 10 most down-regulated (A) and up-regulated (B) genes in P5-M (black bars) compared to P5-O (grey bars) during fermentation at 12 ºC.

overexpressed, *HPF1*. This gene encodes a haze-protective mannoprotein that reduces the particle size of aggregated protein in white wines.

Mannoprotein content in wine and the cell wall yeast

The amounts of mannoproteins released by P5-M and P5-O strains during fermentation at 28 ºC and 12 ºC were measured (Figure 5). We observed that mannoprotein content was higher at final fermentation in fermentations carried out by P5-M strain, regardless the temperature. On the other hand we only observed significant differences in cell wall mannoproteins at mid-exponential growth phase (96 h) at low temperature fermentation (Table 4).



Figures 5: Final concentrations of the mannoprotein released by P5-O and P5-M during fermentation at 28 ºC and 12 ºC

* Statistically significant differences (P-value ≤ 0.05) compared with their control strain at the same temperature

Table 4: Mannoprotein content in cell wall yeast (mg mannoprotein/mg dry weight (DW)) at mid-exponential growth phase (24 h at 28 ºC and 96 h at 12 ºC) during fermentation and at final fermentation (FF).

Strain	P5-O	P5-M
28 ºC		
24 h	96.34 ± 12.58	110.54 ± 10.84
FF	101.90 ± 7.74	96.21 ± 8.21
12 ºC		
96 h	99.82 ± 4.38	132.44 ± 18.32*
FF	122.94 ± 10.79	125.61 ± 5.00

* Statistically significant differences (P-value ≤ 0.05) compared with their control strain at the same growth phase and temperature.

DISCUSSION

White and "rosé" wine produced by low temperature fermentation is mostly considered to have improved sensory qualities (Beltrán *et al*., 2008; Llauradó *et al*., 2002; Torija *et al*., 2003). However few commercial wine strains available in the market are well adapted to ferment at low temperature (10–15 ºC). For this reason, the selection and development of new *S. cerevisiae* strains with improved fermentation abilities at low temperature has interest for future biotechnological applications. Evolutionary engineering is a powerful approach with widespread use in improving industrially important and genetically complex properties of *S. cerevisiae* and other microorganism (Çakar *et al*., 2012). Moreover, strategies based on evolutionary engineering are attractive, because they generate non-GMO improved strains that can be quickly transferred and used in industry.

In this study we used a batch serial dilution, in a context which mimicked the wine fermentation conditions at 12 ºC, to perform a competition experiment and experimental evolution. Twenty-seven commercial strains were used to inoculate the cultures. In addition, to increase the genetic variation, the same amount of cells was mutagenized to inoculate the EMS treated culture. During experimental evolution we observed a huge decrease of GT in mutagenized culture within the first 25 generations, reaching similar values to initial GT in non-EMS treated culture. This rapid growth improvement in the culture should be determined by the elimination of the worst cold adapted mutants. When we compared the evolution of EMS and non-EMS treated cultures we observed that higher values of OD were reached between the generation 50 and 100 in both cultures. This fact indicates that the ability to grow well at low temperatures might have been acquired in the range of these generations. On the basis of this result, experimental evolution for improving low temperature growth should not be longer of 100 generations. It is also important to note that the mutagenized culture had higher values of OD and shorter GT than non-EMS culture. This result also evidenced that the higher mutational rate produced with the combined use of mutagenesis and experimental evolution could be a good strategy for obtaining phenotypes with significant improvements in this oenological trait, which is expected to be very polygenic.

The majority of laboratory strains of *S. cerevisiae* are either haploid or diploid, whereas industrial wine yeast strains are predominantly diploid or aneuploid, and occasionally polyploidy (Pretorius, 2003). This higher ploidy or aneuploidy of the industrial strains may make more difficult to obtain improved industrial strains than improving laboratory strains. However, in spite of this higher complexity, there are a few examples in the literature of improved wine strains by using evolutionary engineering (Cadière *et al.*, 2011; McBryde *et al.*, 2006). The strategy followed in this study also evidences the utility of the competition experiment for detecting the most competitive strains among a pool of commercial strains and the

evolutionary engineering as a non-recombinant technique to isolate commercial wine yeast strains that are highly tailored to the stressful conditions of a wine fermentation at low temperature.

Nevertheless the importance of obtaining improved strains for using during wine fermentations at industrial level, another important aim of this study was to detect the molecular basis underlying this improved phenotype. To this end, the genome of the evolved mutants and parent strain were sequenced and compared. As expected the number of mutations in P5-M is much larger than observed in P5-C because the EMS treatment. The majority of non-synonymous mutations observed in coding regions, in both strains, were related to general processes as protein fate, general transcription activities or translational control. However some genes as *MNS1* and YPR174C genes, 5' upstream mutated, are involved in process previously related with stress at low temperature. *MNS1* encodes an alpha-1,2-mannosidase that catalyzes the last step in glycoprotein maturation in the ER. Some mannoprotein genes were previously related to low temperature conditions (Abe, 2007; Abramova *et al.*, 2001; Rossignol *et al.*, 2003). Furthermore, YPR174C encodes a protein of unknown function which binds phosphatidylinositol and phosphatidylethanolamine. Several studies demonstrated that lipid metabolism plays a central role in low temperature adaptation (Rusell, 1990)

We also compared the transcriptome of the best fermenting strain at low temperature with its parental strain in a context mimicking wine fermentation. Detailed characterization of gene expression of P5-O and P5-M strains revealed that the main differences in MIPS categories of up-regulated genes in the improved strain were involved in "metabolism", "cell rescue, defense and virulence" and "interaction with the environment". The up-regulated genes classified as metabolism mainly were involved in C-compound and carbohydrate metabolism. This higher activity of genes encoding enzymes of the carbon metabolism

positively correlated with the higher fermentation rate of the evolved strain at 12 ºC.

Moreover the up-regulation of the functional category "cytokinesis (cell division) / septum formation and hydrolysis" in P5-M strain could be also related with the improved growth of this strain at low temperature. With respect to the down-regulated genes in the evolved strain, large percentage of these were classified into "transcription", "protein with binding function or cofactor requirement and mitotic" and "cell cycle control" MIPS categories. Within the "transcription" functional category mainly were down-regulated genes belonged to "RNA processing", especially rRNA processing. Furthermore the "ribosome biogenesis" was also down-regulated. Low temperature is know to induce the formation of secondary structure in RNA, affecting their stability and increases the inactivated ribosome (Jones and Inouye, 1996). Previous studies of transcriptional response to low temperature concluded that important and primary response is to increase ribosomal complex to compensate the reduced translational ability (Sahara *et al.*, 2002; Tai *et al.*, 2007). Our results showed that the evolved strain had down-regulated a set of genes related to RNA processing and ribosome biogenesis, suggesting that P5-M was in a less stressful situation.

The analysis of the most up-regulated genes in P5-M compared to the parental strain revealed a large induction of genes that encode cell wall mannoproteins. Within the 10 most overexpressed genes, four of those belonged to *DAN*/*TIR* family (*DAN1, TIR1, TIR4* and *TIR3*), furthermore *TIR2* and *TIP1* had also shown significant overexpression (Table S1). The overexpression of mannoprotein genes was correlated with an increase of mannoprotein content in cell wall yeast at the same point of fermentation and an increase of mannoprotein released at the end of fermentation. The induction of the subset *DAN*/*TIR* genes was previously related to hypoxia, high pressure and low temperature conditions (Abe, 2007; Abramova *et al.*, 2001; Rossignol *et al.*, 2003). Moreover, in a recent work, we detected that the

overexpression of *TIP1* and *TIR2* improved fermentation activity at 12 ºC (Chiva *et al*., 2012). The overexpression of *TIR2* also improved the growth and increased the population at low temperature compared with the control strain. Abramova *et al*. (2001) postulated either that this adaptation event is related to membrane fluidity: the cell wall proteins transit the membrane during the cell wall assembly, affecting the membrane properties or that some of these proteins play a role in transport of sterol.

On the other hand the 10 genes most down-regulated were involved in several biological process and had different molecular functions. We could highlight the repression of *GIT1* and *PUT1* genes in P5-M compared to P5-C. *GIT1* gene encodes a plasma membrane permease that mediates the uptake of glycerophosphoinositol and glycerophosphocholine as a source of the nutrients inositol and phosphate (Fisher *et al.*, 2005; Patton-Vogt and Henry, 1998). Incubation at low temperature increases the molecular order of membrane lipids by rigidification (Rusell, 1990). Thus, the lipid metabolism plays a central role in low temperature adaptation and a possible adaptive strategy might be the reshaping of the membrane composition. Finally the high repression of *PUT1* seems to play an important role in the adaptation at low temperature of P5-M. *PUT1* encodes a nuclear-encoded mitochondrial proline oxidase, involved in utilization of proline by degradation of this amino acid into glutamate. Proline is the most abundant source of nitrogen in grapes, but their utilization is limited to oxygen availability (Henscke and Jiranek, 1993). The intracellular accumulation of proline was related with an increased tolerance to freeze and ethanol stress (Takagi *et al*., 2000; Takagi *et al*., 2005).

In conclusion this study showed that competition experiments can provide essential information to select the best adapted yeast strain in different oenological contexts. We also demonstrated that evolutionary engineering is a good alternative to

recombinant techniques to improve commercial yeast strains that can be used in the wine industry.

ACKNOWLEDGMENTS

This work was financially supported by the grant from the Spanish government (project AGL2010-22001-C02-01) awarded to JMG. MLM also wants to thank to Spanish government for her FPI grant.

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UNIVERSITAT ROVIRA I VIRGILI
METABOLIC AND MOLECULAR ADAPTATION OF WINE YEASTS AT LOW TEMPERATURE FERMENTATION:
STRATEGIES FOR THEIR GENETIC IMPROVEMENT
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DL: T. 1275-2013

GENERAL DISCUSSION

More than 26 billion litres of wine are annually produced worldwide, and winemaking plays a major role in the economies of many nations. Over the last century, yeasts with optimal characteristics were selected to inoculate fermentations with pure yeast cultures. Consequently, wine production quality vastly improved (Pretorius, 2000). As the wine industry effectively controls fermentation temperatures, low temperature fermentation (10-15 ºC) is becoming more frequent in order to produce white and "rosé" wines with more pronounced aromatic profiles (Beltran *et al*., 2006; Llauradó *et al*., 2005; Molina *et al*., 2007; Torija *et al*., 2003). However, the optimal growth and fermentation temperature for *Saccharomyces cerevisiae* is 25-28 ºC. Thus, there are certain drawbacks to low temperature fermentations, such as reduced growth rate, a long lag phase, and sluggish or stuck fermentations. To avoid this problem, it is important to study the low temperature response, and to select or to develop well-adapted yeast strains.

S. cerevisiae is the predominant species responsible for alcohol fermentation and is the most widely used yeast in the wine industry. However, other species of the genus *Saccharomyces,* such as *Saccharomyces bayanus* var. *uvarum,* and natural interspecific *Saccharomyces* hybrids (between *S. cerevisiae* and *Saccharomyces kudriavzevii*, between *S. cerevisiae* and *S. bayanus*, as well as a triple hybrid *S. bayanus* x *S. cerevisiae* x *S. kudriavzevii*) have been isolated in wine fermentations (González *et al*., 2006; Masneuf-Pomarède *et al*., 2010; Naumov *et al*., 2000). *S. bayanus* var. *uvarum* and *S. kudriavzevii* are considered the most psychrotrophic species of the *Saccharomyces* genus. Several studies have focused on the characterization of the oenological properties at low temperature fermentation of these cryotolerant species and their hybrids. The results of these studies have revealed that these yeasts offer some advantages as compared to *S. cerevisiae* in terms of valuable organoleptic properties, such as greater glycerol production and lower ethanol production (Arroyo-López *et al*., 2010; Serra *et al*., 2005), modification of wine aroma (Gamero *et al*., 2011) and greater fructose

consumptions during low temperature fermentation (Tronchoni *et al*., 2011). Other studies have shed light on the different cold adaptation mechanisms of these yeasts, such as the up-regulation of the genes belonging to functional groups translation, amino acid metabolism and cold stress-related genes (Combina *et al*., 2012) or the modification of lipid composition (Tronchoni *et al*., 2012). Despite this information, our understanding of the interspecific metabolic differences of these species growing at 12 ºC is limited. Our results in Chapter 1 reveal that the main metabolic differences between the two cryotolerant species and *S. cerevisiae* growing at low temperature were carbohydrate metabolism, mainly fructose metabolism. However, these two species have developed different cold resistance strategies. *S. bayanus* var. *uvarum* presented elevated shikimate pathway activity and decreased lysine synthesis, while *S. kudriavzevii* displayed enhanced NAD^+ synthesis. Shikimate is an important precursor for aromatic amino acids tyrosine, phenylalanine and tryptophan. It is well-documented that tryptophan uptake is a limiting factor for yeast cell growth at low temperature (Abe and Horikoshi, 2000).

However as mentioned above, *S. cerevisiae* is the most widely used yeast in the wine industry. Many attempts have been made to elucidate the cold response in *S. cerevisiae* by a variety of high-throughput methodologies. Most of them, however, focused on cold shock on laboratory yeast strains (Homma *et al*., 2003; Murata *et al*; 2006; Sahara *et al*., 2002; Schade *et al*., 2004). In order to avoid the effect of the growth rate on transcriptional activity, Tai *et al.* (2007) analyzed gene expression during cold acclimation in a steady-state chemostat culture in which the growth rate was the same at both optimal and low temperatures. The results of these studies had some discrepancies, but they all showed that lipid metabolism was clearly regulated by low temperature. We aimed to elucidate the effects of temperature on commercial wine yeast QA23. Thus we compared its metabolome growing at 12 ºC and 28 ºC in a synthetic must in a steady-state chemostat culture. These metabolic changes might be due to temperature-mediated transcriptional

and/or to posttranscriptional effects on yeast. However, multiple genes may be involved in the synthesis and degradation of a single metabolite. This means that the metabolome is an appropriate level to study phenotypic responses. Our results in Chapter 1 show that the main differences between the metabolic profiling of *S. cerevisiae* growing at low and optimal temperatures was observed in redox homeostasis and lipid metabolism. Thus, these results confirm the relevance of lipid metabolism in adaptation at low temperature, which was previously described in transcriptional studies. The information obtained from the metabolic comparison can prove useful for further studies to link DNA-encoded processes with different phenotypes.

It is well-documented that biological membranes are the first barrier between the cell interior and its environment, and are a primary target for damage during cold stress. Incubation at low temperature increases the molecular order of membrane lipids by rigidification (Russell, 1990). Yeasts are known to have developed several strategies to maintain appropriate membrane fluidity. Previous studies have shown that temperature modifies the cellular lipid composition of yeast by increasing the degree of unsaturation at the beginning of fermentation and by shortening chain length as fermentation progresses (Beltran *et al*., 2008; Torija *et al*., 2003). One possible adaptation might be to reshape the plasma membrane by incubating yeast cells in the presence of different lipid compounds (Redón *et al*., 2009). Another strategy to redesign cellular lipid composition is to alter transcriptional activity by deleting or overexpressing key lipid metabolism genes. Recently, Redón *et al*. (2012) tested various phospholipid mutants from the EUROSCARF collection of *S. cerevisiae* BY4742 to ascertain whether the suppression of some genes could improve the fermentation vitality of cells at low temperature. We screened most of the mutants of laboratory strain BY4742 by encoding enzymes of the phospholipid, sterol and sphingolipid pathways in terms of their growth capacity at low temperature. The generation time of these mutant

strains was used to select those genes which were further deleted in a derivative industrial strain. In Chapter 2, the phenotypic differences between strains mutated in the same gene constructed in the laboratory and wine yeast reveal the importance of the genetic background and highlight the relevance of using a wine yeast strain (Pizarro *et al.,* 2008; Redón *et al.,* 2011). Our results demonstrate that the mutants of phospholipids pathway, *Δpsd1* and *Δopi3*, present not only the longest generation time when growing at low temperature on SC media, but also the most important modification of phospholipids composition. We also observed the strongly impaired growth of *Δerg3*. The overexpression of these genes (*PSD1, OPI3* and *ERG3*) reduced the generation time at low temperature. Similar results were obtained in synthetic must (Chapter 3). The deletion of some genes impaired the fermentation performance of the wine strain at low temperature. Moreover, the overexpression of *PSD1*, *LCB3*, *DPL1* and *OLE1* significantly improved fermentation activity at low temperature. Remarkably, the overexpression of *PSD1* and *OLE1* did not affect either the fermentation rate or generation time at 28 ºC. An improved phenotype in growth and fermentation activity at low temperature of an *OLE1* overexpressing strain has been previously reported by Kajiwara *et al*. (1999). Regarding lipid composition, the improved phenotypes correlated with PE and UFA increases, whereas the chain length of the FA shortened (conversion of C18 into C16). Redon *et al*. (2011) compared the lipid composition of those strains belonging to different *Saccharomyces* species which were isolated from different fermentative processes (wine, beer, bread) after growing at optimum (25 ºC) and low temperatures (13 ºC). A common change noted in all the strains under study was a lower PC/PE ratio.

Thus in Chapter 3, we developed four strains with improved growth and fermentation performance at low temperature by metabolic engineering. We know that the overexpression of non integrative plasmids has its limitations for industrial application. These recombinant strains usually require cultivation in the presence

of antibiotics or in a chemically defined medium in order to maintain the plasmid by selection pressure. Furthermore, chromosomal integration uses clean, safe integrating methods. Self- cloning has been described to be a genetic modification by gene transfer within the same species and it overcomes the major issue of the commercial application of genetically modified yeast. A previous study by our research group adopted this approach to generate new wine yeast strains (Chiva *et al*., 2012). Hence we believe that it would be a good strategy to overexpress the *PSD1*, *LCB3*, *DPL1* and *OLE1* genes by chromosomal integration into a wine yeast strain which could be used in the wine industry for future fermentations at low temperature. An alternative to recombinant approaches to develop improved wine yeast strains is evolutionary engineering. Strategies based on evolutionary engineering are appealing because they can generate improved strains that are not considered GMOs, and are most likely to have wider public acceptance. In Chapter 4, we isolated four evolved strains after 200 generations under selective pressure growth (12 ºC), with and without a mutagen, which showed improved growth and fermentation performance at low temperature. These four improved strains can be used by the wine industry, which cannot be said of those obtained by metabolic engineering. Another advantage of evolutionary engineering is that it does not require extensive biochemical and genetic information to develop new strains. Furthermore, identifying the underlying molecular mechanism for a derived phenotype by evolutionary engineering is difficult. However through a transcriptional and/or genomic analysis, we were able to attempt to decipher the molecular basis. In Chapter 4, the genome of the evolved mutants and parent strain were sequenced and compared and we did a comparative genome-wide expression analysis of the best evolved strains and their parental strain. The majority of non-synonymous mutations observed in coding regions, in both strains, were related to general processes. The results of gene expression analysis reveal that many genes of metabolism, specifically carbohydrate metabolism, were up-regulated in strain P5-M (the evolved strain), whereas many

of the genes involved in transcription were down-regulated. We highlight the repression of *GIT1* because it once again suggests that lipid metabolism is a vital key for low temperature adaptation. The most remarkable result obtained was that four genes belonged to the *DAN/TIR* family (*DAN1, TIR1, TIR4* and *TIR3*) showed the highest up-regulation in P5-M as compared to P5-O (the wild-type). The overexpression of mannoprotein genes was correlated with an increase of mannoprotein content in cell wall yeast at the same point of fermentation and an increase of mannoprotein released at the end of fermentation. Also we identified a 5' upstream SNPs in *MNS1* gene, which is involved in glycoprotein biosynthesis. The induction of these cell wall mannoproteins genes was previously related to hypoxia, high pressure and low temperature conditions (Abe, 2007; Abramova *et al*., 2001; Rossignol *et al*., 2003). Abramova *et al*. (2001) postulated that this adaptation event may be related to membrane fluidity because cell wall proteins transit the membrane during cell wall assembly.

While undertaking this work, we attempted to decipher some mechanisms related to adaptation to low temperature fermentation. The results obtained in this dissertation provide useful information for the wine industry. However, we are well aware that these results are a starting point for new studies to gain a better understanding of the mechanisms underlying low temperature adaptation.

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CONCLUSIONS

UNIVERSITAT ROVIRA I VIRGILI
METABOLIC AND MOLECULAR ADAPTATION OF WINE YEASTS AT LOW TEMPERATURE FERMENTATION:
STRATEGIES FOR THEIR GENETIC IMPROVEMENT
María López Malo
DL: T. 1275-2013

The main conclusions drawn from the results obtained in this thesis are:

1. The main metabolic differences between the two cryotolerant species, *Saccharomyces bayanus* var. *uvarum* and *Saccharomyces kudriavzevii*, and *Saccharomyces cerevisiae,* growing at low temperature, were carbohydrate metabolism, mainly fructose metabolism.
2. Different cold resistance strategies in *S. bayanus* var. *uvarum* and *S. kudriavzevii* were observed:
    a. *S. bayanus* var. *uvarum* presented elevated shikimate pathway activity and decreased lysine synthesis.
    b. *S. kudriavzevii* displayed enhanced NAD^+ synthesis and cell wall synthesis.
3. The main differences between the metabolic profiling of *S. cerevisiae* growing at low and optimal temperatures were observed in redox homeostasis and lipid metabolism.
4. To study the importance of certain genes in winemaking processes, it is relevant to construct the mutant and overexpressing strains in the genetic background of commercial wine yeast because many phenotypic differences were observed when compared with the laboratory strain.
5. The importance of the phospholipid pathway in growth at low temperature is that the deletion of the genes encoding the enzymes involved in PE and PC synthesis (*PSD1*, *CHO2* and *OPI3*) had a marked impact on growth at 12 ºC.
6. The overexpression of *PSD1*, *LCB3*, *DPL1* and *OLE1* significantly improved the fermentation activity at low temperature.

7. The lipid composition of the improved phenotypes suggests that the increase in the C16/C18 ratio and PE/PC content may be positive for growth and fermentation rates at low temperature.

8. We isolated four evolved strains after 200 generations by evolutionary engineering to show improved growth and fermentation performance at low temperature.

9. Cell wall mannoproteins genes (the *DAN/TIR* family) presented the highest up-regulation in the P5-M strain (evolved strain) as compared to the P5-O strain (parental strain), suggesting their implication in low temperature response.

Annex 1

Material and Methods

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1. YEAST STRAINS

The yeasts used in the present thesis are shown in Table 1. All strains are belonging to genus *Saccharomyces*. In this study QA23 and BY4742 were used as wine and laboratory model yeast strains and *S. bayanus* var. *uvarum* 12600 and *S. kudriavzevii* CR85 were used as cryotolerant model yeast species. Moreover, a collection of 27 commercial wine strains were used in this work.

Table 1. Strains used in this work.

Strain	Genotype	Source
BY4742	MATα; *his3Δ* 1; *leu2Δ* 0; *lys2Δ* 0; *ura3Δ* 0	EUROSCARF
BY-*cds1*	BY4742*cds1 Δ*::kanMX4	EUROSCARF
BY-*gde1*	BY4742*gde1 Δ*::kanMX4	EUROSCARF
BY-*ale1*	BY4742*ale1 Δ*::kanMX4	EUROSCARF
BY-*psd1*	BY4742*psd1 Δ*::kanMX4	EUROSCARF
BY-*muq1*	BY4742*muq1 Δ*::kanMX4	EUROSCARF
BY-*cho2*	BY4742*cho2 Δ*::kanMX4	EUROSCARF
BY-*cki1*	BY4742*cki1 Δ*::kanMX4	EUROSCARF
BY-*cpt1*	BY4742*cpt1 Δ*::kanMX4	EUROSCARF
BY-*opi3*	BY4742*opi3 Δ*::kanMX4	EUROSCARF
BY-*hnm1*	BY4742*hnm1 Δ*::kanMX4	EUROSCARF
BY-*git1*	BY4742*git1 Δ*::kanMX4	EUROSCARF
BY-*crd1*	BY4742*crd1 Δ*::kanMX4	EUROSCARF
BY-*pct1*	BY4742*pct1 Δ*::kanMX4	EUROSCARF
BY-*nte1*	BY4742*nte1 Δ*::kanMX4	EUROSCARF
BY-*erg2*	BY4742*erg2 Δ*::kanMX4	EUROSCARF
BY-*erg3*	BY4742*erg3 Δ*::kanMX4	EUROSCARF
BY-*erg4*	BY4742*erg4 Δ*::kanMX4	EUROSCARF
BY-*erg5*	BY4742*erg5 Δ*::kanMX4	EUROSCARF
BY-*erg6*	BY4742*erg6 Δ*::kanMX4	EUROSCARF

Table 1 (continuation)

Strain	Genotype	Source
BY-*erg24*	BY4742erg*24 Δ*::kanMX4	EUROSCARF
BY-*dpl1*	BY4742*dpl1 Δ*::kanMX4	EUROSCARF
BY-*lcb31*	BY4742*lcb3 Δ*::kanMX4	EUROSCARF
BY-*lcb4*	BY4742*lcb4 Δ*::kanMX4	EUROSCARF
BY-*lcb5*	BY4742 *lcb5 Δ*::kanMX4	EUROSCARF
BY-*ydc1*	BY4742*ydc1 Δ*::kanMX4	EUROSCARF
BY-*ypc1*	BY4742*ypc1 Δ*::kanMX4	EUROSCARF
BY-*sur1*	BY4742*sur1 Δ*::kanMX4	EUROSCARF
BY-*sur2*	BY4742*sur2 Δ*::kanMX4	EUROSCARF
BY-*ipt1*	BY4742*ipt1 Δ*::kanMX4	EUROSCARF
BY-*lac1*	BY4742*lac1 Δ*::kanMX4	EUROSCARF
BY-*scs7*	BY4742*scs7 Δ*::kanMX4	EUROSCARF
BY-*lag1*	BY4742*lag1 Δ*::kanMX4	EUROSCARF
BY-*csg2*	BY4742*csg2 Δ*::kanMX4	EUROSCARF
BY-*ysr3*	BY4742*ysr3 Δ*::kanMX4	EUROSCARF
QA23	Wine strain	Lallemand S.A. (Canada)
*ho*QA23	QA23*ho Δ*::kanMX4	Salvadó *et al.* (2012)
*ho*QA23	QA23*ho Δ*::loxP	Salvadó *et al.* (2012)
*ho*QA23*cho2*	QA23*ho Δ cho2 Δ*::kanMX4	This study
*ho*QA23*gde1*	QA23*ho Δ gde1 Δ*::kanMX4	This study
*ho*QA23*opi3*	QA23*ho Δ opi3 Δ*::kanMX4	This study
*ho*QA23*plb1*	QA23*ho Δ plb1 Δ*::kanMX4	This study
*ho*QA23*plb2*	QA23*ho Δ plb2 Δ*::kanMX4	This study
*ho*QA23*plb3*	QA23*ho Δ plb3 Δ*::kanMX4	This study
*ho*QA23*psd1*	QA23*ho Δ psd1 Δ*::kanMX4	This study
*ho*QA23*sct1*	QA23*ho Δ sct1 Δ*::kanMX4	This study
*ho*QA23*erg3*	QA23*ho Δ erg3 Δ*::kanMX4	This study
*ho*QA23*erg4*	QA23*ho Δ erg4Δ*::kanMX4	This study
*ho*QA23*erg6*	QA23*ho Δ erg6 Δ*::kanMX4	This study
*ho*QA23*erg24*	QA23*ho Δ erg24 Δ*::kanMX4	This study

Table 1 (continuation)

Strain	Genotype	Source
*ho*QA23*dpl1*	QA23*ho Δ dpl1Δ*::kanMX4	This study
*ho*QA23*ipt1*	QA23*ho Δ ipt1Δ*::kanMX4	This study
*ho*QA23*lac1*	QA23*ho Δ lac1Δ*::kanMX4	This study
*ho*QA23*lcb3*	QA23*ho Δ lcb3Δ*::kanMX4	This study
*ho*QA23*lcb4*	QA23*ho Δ lcb4Δ*::kanMX4	This study
*ho*QA23*ydc1*	QA23*ho Δ ydcΔ*::kanMX4	This study
*ho*QA23 pGREG	QA23*ho Δ*::loxP pGREG 505	Chiva *et al*. (2012)
pGREG *PSD1*	QA23*ho Δ*::loxP pGREG 505 *PSD1*	This study
pGREG *CHO2*	QA23*ho Δ*::loxP pGREG 505 *CHO2*	This study
pGREG *OPI3*	QA23*ho Δ*::loxP pGREG 505 *OPI3*	This study
pGREG *ERG3*	QA23*ho Δ*::loxP pGREG 505 *ERG3*	This study
pGREG *ERG6*	QA23*ho Δ*::loxP pGREG 505 *ERG6*	This study
pGREG *IDI1*	QA23*ho Δ*::loxP pGREG 505 *IDI1*	This study
pGREG *OLE1*	QA23*ho Δ*::loxP pGREG 505 *OLE1*	This study
pGREG *LCB3*	QA23*ho Δ*::loxP pGREG 505 *LCB3*	This study
pGREG *LCB4*	QA23*ho Δ*::loxP pGREG 505 *LCB4*	This study
pGREG *DPL1*	QA23*ho Δ*::loxP pGREG 505 *DPL1*	This study
CECT12600	*S. bayanus* var. *uvarum*	CECT[1]
CR85	*S. kudriavzevii*	Lopes *et al* (2010)

[1] Spanish culture collection

2. CULTURE MEDIA

2.1 Minimal medium. Synthetic complete drop out (SC) medium

Difco Yeast Nitrogen Base (w/o amino acids)	6.7 g
Glucose	20 g
*Drop-out mix	0.83 g
H_2O (distilled)	1 L
Agar (for solid media preparation)	20 g

* Synthetic complete drop-out mix (2 g Histidine, 4 g Leucine, 2 g Lysine and 1.2 g Uracil).

2.2 YEPD or YPD (Yeast Extract Peptone Dextrose) medium.

Glucose	20 g
Bacteriological peptone	20 g
Yeast extract	10 g
H_2O (distilled)	1 L
Agar (for solid media preparation)	15 g

2.3 Synthetic grape must (SM)

2.3.1 Synthetic grape must adapted from Riou et al. (1997)

To mimic the wine fermentation conditions synthetic must media was used that reproduces a standard natural must composition. This media is very useful to make laboratory micro-fermentations in a reproducible manner.

Media composition for 1 L

Sugars

Glucose	100 g
Fructose	100g

Organic acids

Malic acid	5 g
Citric acid	0.5 g
Tartaric acid	3 g

Mineral salts

KH_2PO_4	0.75 g
K_2SO_4	0.5 g
$MgSO_4$ $7H_2O$	0.250 g
$CaCl_2$ H_2O	0.155 g
NaCl	0.2 g

NH_4Cl	0.46 g

Weight the different substances and add distilled water (up to 1 L). Autoclave at 121º C for 20 min.

Add the previous prepared stocking solution of:

Amino acids	13.09 mL
Oligoelements	1 mL
Vitamins	10 mL

pH = 3.3 with pellets of NaOH

Filter the whole volume using a 0.22µm filter.

Aminoacids stocking solution (1 L)

Tyrosine (Tyr)	1.5 g
Tryptophan (Trp)	13.4 g
Isoleucine (Ile)	2.5 g
Aspartic Acid (Asp)	3.4 g
Glutamic Acid (Glu)	9.2 g
Arginine (Arg)	28.3 g
Leucine (Leu)	3.7 g
Threonine (Thr)	5.8 g
Glycine (Gly)	1.4 g
Glutamine (Gln)	38.4 g

Alanine (Ala)	11.2 g
Valine (Val)	3.4 g
Methionine (Met)	2.4 g
Phenylalanine (Phe)	2.9 g
Serine (Ser)	6 g
Histidine (His)	2.6 g
Lysine (Lys)	1.3 g
Cysteine (Cys)	1.5 g
Proline (Pro)	46.1 g

Keep at -20 ºC

Vitamins stocking solution (1 L)

Myo-inosito	12 g
Calcium pantothenate	15 g
Thiamine hydrochloride	0.025 g
Nicotinic acid	0.2 g
Pyridoxine	0.025 g
* Biotin	3 mL

*(stocking biotin solution 100 mg/L)

Keep at -20 ºC

Oligoelements stocking solution (1 L)

$MnSO_4$, H_20	4 g
Zn SO_4, $7H_2O$	4 g
$CuSO_4$, $5H_2O$	1 g
KI	1 g
$CoCl_2$, $6H_2O$	0.4 g
H_3BO_3	1 g
$(NH_4)6Mo_7O_{24}$	1 g

Keep at -20 ºC

2.3.2 Synthetic grape must adapted from Martínez-Moreno et al. (2012)

This synthetic must is very similar to the previous one described by Riou *et al.* (1997) with some variations that simplify its preparation.

Media composition for 1 L:

The medium composition included 100 g glucose and 100 g fructose, but only had two organic acids: 6 g malic acid and 6 g citric acid. The vitamins, oligoelements and mineral salts were substituted by 1.7 g YNB without ammonium and amino acids. This must also included anaerobic factors (0.015 g ergosterol, 0.005 g sodium oleate and 0.5 mL tween 80) and 0.006 g potassium disulfite. The assimilable nitrogen used was the same sources and amount (total amount of 300 mg N/L) than in the other synthetic must.

After weighting the different substances and adding distilled water (up to 1 L), the medium is autoclaved at 121° C for 20 min. Afterwards the anaerobic factors and amino acids were added. The pH was adjusted to 3.3 by pellets of NaOH and the whole volume was filtered by 0.22µm filter.

3. CULTURE TECHNIQUES

3.1 Batch cultures (serial dilution)

Batch cultures were performed at 12 ºC, with a continuous orbital shaking at 100 rpm. Batch cultures were done in laboratory-scale fermenters using 100 mL bottled filled with 60 mL of synthetic must and fitted with closures that enabled the carbon dioxide to escape and samples to be removed. The population inoculated in every flask reached an initial OD of approximately 0.2. The initial inoculums came from an overnight culture in YPD at 30 ºC.

Cultures were allowed to grow through a normal growth curve, with a weekly transfer of a small volume (the volume necessary to again inoculate an OD of 0.2) of the expanded culture into 60 mL of fresh medium.

3.2 Chemostat cultures (continuous cultures)

A chemostat is a growth vessel into which fresh medium is delivered at a constant rate and cells and spent medium overflow at the same rate. Thus, the culture is forced to divided to keep up with the dilution, and the system exists in a steady state where inputs match outputs (Dunham, 2010).

Continuous cultures were performance at 12 ºC and 28 ºC in 2 L chemostat (Biostat ® B, Braun Biotech International, Sartorius Group, Germany) with a working volume of 0.75 L. A temperature probe connected to a cryostat controlled the temperature cultures. pH was measured online and kept constant at 3.6 by the automatic addition of 2 M NaOH and 1 M HCl. The stirrer was set at 100 rpm. The population inoculated in the chemostat was approximately OD = 0.2. The initial inoculums came from an overnight culture in YPD at 30 ºC. Previously to start the continuous culture, cells were allowed to grow, at the same temperature than the continuous culture, through a normal growth curve to achieve enough biomass (batch phase). Yeast cells were grown at dilutions rates (D) between 0.03 and 0.04 h^{-1}.

3.3 Micro-fermentations

In order to simulate the wine fermentation conditions, a synthetic must media was used that reproduces a standard natural must composition. Fermentations were performed at 28 ºC and 12 ºC, with continuous orbital shaking at 100 rpm. Fermentations were done in laboratory-scale fermenters using 100 mL bottles filled with 60 mL of SM and fitted with closures that enabled the carbon dioxide to escape and samples to be removed. The population inoculated in the synthetic grape-must came from an overnight culture in YPD at 30 ºC. After counting under the microscope, the appropriate dilution of the overnight culture was transferred to synthetic must to achieve an initial cell concentration of 2 x 10^6 cells/mL. Fermentation was considered to be completed when density was below 998 g/L.

In the overexpressing strain fermentations, G418 Geneticin at 0.2 g/L concentration was added for assuring plasmid stability.

4. FERMENTATION MONITORING

Fermentations were sampling every day at 28 ºC and every two days at 12 ºC. In order to homogenize the fermentations, previously to take the sample, the fermentations were shaken.

4.1 Density measurements

Density measurement is a good fermentation monitoring approach, because the decrease density during the fermentation is directly proportional to sugar consumption. The must density usually is between 1070 and 1120 g /L and the wine density is between 990 and 998 g/L. The decrease of density is due to the transformation of sugars into ethanol.

To measure the density, cells were removed from the must by centrifugation. Afterwards samples were measured with Densito 30 PX densitometer (Mettler Toledo, Switzerland).

The fermentation kinetics were estimated by calculating the time needed to ferment 5% (T5), 50% (T50) and 100% (T100) of the sugars in the synthetic must. The fermentation kinetics were calculated by directly fitting density measurements versus time to the four-parameter logistic equation proposed by Speers *et al.* (2003). The estimation was done using Sigmaplot software (Systa Software Inc. USA).

$$Pt = P_D / \{1 + e^{[-B * (t - M)]}\} + P\infty$$

where P_t is the Plato values (density values) at time t, $P\infty$ is the Plato values at equilibrium, P_D represents the changes in the Plato during the fermentation (P_0 - $P\infty$), B is the fermentation rate and M is the time where the exponential

fermentation rate is maximal. When the data was fitted to four-parameter logistic equation we also obtained an estimation of time for every density value. These values were used to calculate the T5, T50 and T100 (Figure 1)

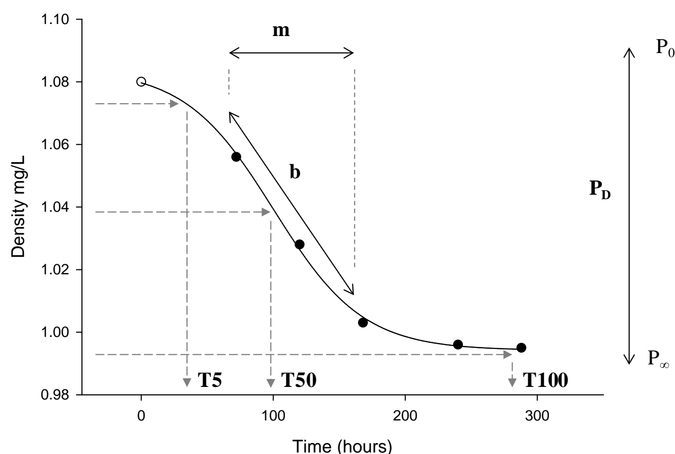

Figure 1 Estimation of fermentation parameters

4.2 Optical density measurements

During the fermentation the yeast growth was monitoring by optical density measurements. The absorbance measurement at 600 nm is directly proportional to the yeast cell biomass, between the ranges 0.1 and 0.8. The samples of the fermentation were properly diluted with distilled water, using as blank also distilled water.

4.3 Viable yeast counting

To calculate the viable yeast during the fermentation, yeast cells were plated on YPD agar at an adequate dilution and incubated for 2 days at 28 ºC.

4.4 HPLC analysis

Glucose, fructose, glycerol and ethanol were analyzed in supernatant samples. Analytical HPLC was carried out in a Surveyor Plus Chromatograph (Thermo Fisher Scientific, Waltham, MA) equipped with a refraction index detector, autosampler and a UV-Visible detector. Prior to injection, samples were centrifuged at 13300 rpm for 5 min, and samples were diluted 5 or 10-fold and filtered through 0.22-µm pore size nylon filters (Micron Analitica, Spain). A total volume of 25 µL was injected into a HyperREZ XP Carbohydrate H+8 µm column (Thermo Fisher Scientific) assembled to its correspondent guard. The mobile phase used was 1.5 mM H_2SO_4 with a flux of 0.6 mL/min and a column temperature of 50ºC. The concentration of each was calculated using external standards. Each sample was analyzed in duplicate.

5. GROWTH RATE ANALYSIS

Growth was monitored at 600 nm in a SPECTROstar Omega instrument (BMG Labtech, Offenburg, Germany) at 12 ºC and 28 ºC. Measurements were made every half h during 3 days after a pre-shaking of 20 sec at 28 ºC. However, at 12 ºC, the microplate had to be incubated outside the SPECTROstar spectrophotometer and then transferred into it to take the measurements every 8 h during lag phase and every 3 h during exponential phase. The wells of the microplate were filled with 0.25 mL of media (SC or SM), reaching an initial OD of approximately 0.2 (inoculum level of ~ 2 x 10^6 cells/mL). Uninoculated wells for each experimental series were also included in the microplate to determine, and consequently subtract, the noise signal. All experiments were carried out in triplicate.

Growth parameters were calculated from each treatment by directly fitting OD measurements versus time to the reparametized Gompertz equation proposed by (Zwietering *et al.*, 1990):

$$y=D*\exp\{-\exp[((\mu_{max}*e)/D)*(\lambda-t))+1]\}$$

where $y=\ln(OD_t/OD_0)$, OD_0 is the initial OD and OD_t is the OD at time t; $D=\ln(OD_t/OD_0)$ is the asymptotic maximum, μ_{max} is the maximum specific growth rate (h^{-1}), and λ the lag phase period (h) (Salvadó *et al.*, 2011).

The R code (statistical software R v.2.15 (R Development Core Team, 2010)) was used to fit the results to reparametized Gompertz equation. The script was developed by Guillem Salazar (Institut de Ciències del Mar, CSIC, Barcelona, Spain). Text accompanied by # sign is descriptive does not contribute to the code.

```
# The first row is where you have to write the path file where data are.

# Data have to be in a tab separated file where the first column is Time and the next are the ln(ODt/OD0) of each sample

# The first row at file has to be the variable name (time strain 1 ... strain N).

file<-file.choose()

data<-read.table(file,sep="\t",header=TRUE)


time<-data[,1]

results<-matrix(NA,ncol=4,nrow=dim(data)[2]-1)

colnames(results)<-c("A","m","l","squaredR")

rownames(results)<-colnames(data)[2:(dim(data)[2])]

for (i in 1:(dim(data)[2]-1)){


        dep.var<-data[,i+1]

        a<-try(nls(dep.var~D*exp(-exp(((umax*exp(1))/D)*(l-
```

```
time)+1)),start=list(D=2,umax=0.05,l=40),control=list(maxiter=1000)),silent=TRUE)

	if ((is.list(a))) mod<-nls(dep.var~D*exp(-exp(((umax*exp(1))/D)*(l-time)+1)),start=list(D=2,umax=0.05,l=40),control=list(maxiter=1000))

	if (is.list(a)) results[i,1:3]<-coefficients(mod)

	if (is.list(a)) RSS.p <- sum(residuals(mod)^2)

	if (is.list(a)) TSS <- sum((dep.var - mean(dep.var))^2)

	if (is.list(a)) squaredR<-1 - (RSS.p/TSS)

	if (is.list(a)) resultats[i,4]<-squaredR

	mod<-NULL

	squaredR<-NULL

	}

results

where.save<-choose.dir()

setwd(where.save) # Where you want to save the results!

write.table(resultats,file="results.txt",sep="\t")
```

Generation time was calculated using the equation $t_d=\ln2/\mu$. We normalised this value by making the quotient with its control. Values lower than 1 indicated lower generation time, whereas values greater than 1 indicated greater generation time compared to the control.

6. MOLECULAR TECNIQUES

6.1 DNA extraction

The extraction of yeast DNA was carried out from 3 mL overnight culture in YPD

at 30 ºC, using the method described by Querol *et al*. (1992). 1.5 mL of the cell culture was centrifuged at 10000 rpm for 2 min and the YPD was removed. The cell pellet was washed with 1 mL of distilled water and centrifuged at 10000 rpm for 2 min to remove the water. 0.5 mL of Buffer 1 (sorbitol 0.9 M, EDTA 0.1 M pH 7.5) and 30 µL of Zymolyase (Seikagaku Corporation, Japan) were added. The samples were vortexed and the tubes were incubated at 37 ºC for 20 min. Samples were centrifuged at 10000 rpm for 2 min to remove the supernatant. Protoplasts were re-suspended in 0.5 mL of Buffer 2 (Tris 50 mM pH 7.4, EDTA 20 mM). Afterwards 13 µL of SDS 10% was added and the tubes were incubated at 65 ºC for 5 min. After the incubation, 0.2 mL of potassium acetate was added, the samples were mixed and incubated in ice for 5 min. Samples were centrifuged 15 min at 12000 rpm at 4 ºC to ensure the elimination of SDS. Supernatant was added to 0.7 mL of isopropanol (v/v) and incubated at room temperature for 5 min. Samples were centrifuged 10 min at 12000 rpm at 4 ºC. Supernatant was removed and 0.5 mL of ethanol 70% was added. Samples were centrifuged 5 min at 12000 rpm at 4 ºC. Supernatant was removed and samples were dried with a vacuum pump. Finally DNA was re-suspended in 40 µL of TE (Tris 10 mM ph 7.4, EDTA 1 mM pH 8.0).

6.2 RNA extraction

Yeast RNA was extracted from frozen cell samples (10^8 cell/mL), harvested from fermentation, using the method described by Sierkstra *et al*. (1992). Previous to freeze the samples, cells were centrifuged at 4 ºC and washed with distilled water. The supernatant was removed and then the pellet was frozen with liquid nitrogen and then harvested at – 80 ºC until the RNA extraction.

Cells were defrost in ice and washed with 1 mL of Extraction Buffer (Tris-HCl 100 mM pH 7.4, LiCl 100 mM, EDTA 0.1 mM). Samples were centrifuged and then

0.5 mL of Vortex Buffer (LiCl 100 mM, EDTA 10 mM, Lithium Dodecyl Sulfate 0.5 % , pH 7.4) was added to the pellet. The suspension was added to 1 g of glass beads. Cells were broken vortexing vigorously with a mechanic shaker Mini Beadbeater-8 (BioSpec Products, USA) during 5 min, 30 sec shaking /30 sec incubating in ice cycles. Afterwards phenol:chloroform:isoamyl alcohol (25:24:1) (v/v) was added, then samples were centrifuged 5 min at 10000 rpm at 4 ºC and the aqueous phase was transferred to new tube (this step was repeated until non inter-phase was observed). The samples were centrifuged with chloroform-isoamyl alcohol (24:1, v/v), to extract the aqueous phase. The aqueous phase was transferred to new tube and 1/10 volume of 3 M NaAc (pH 5.6) and 2.5 volumes of ethanol 96% were added. Then it was precipitated for 15 min at – 80 ºC. The samples were centrifuged at 4 ºC for 30 min. Pellets were washed with ethanol 70% and samples were centrifuged 5 min at 12000 rpm at 4 ºC. Supernatant was removed and samples were dried with a vacuum pump. Finally RNA was re-suspended with 100 μL DEPC-treated water.

Total RNA suspensions were purified using the High Pure Isolation kit (Roche Applied Science, Germany) according to the manufacturer’s instructions. RNA concentrations were determined using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, USA), and the quality of the RNA was verified electrophoretically on a 0.8 % agarose gel.

Solutions and equipment were treated, so that they were RNase free, as outlined in Sambrook *et al.* (1989). All the solutions, with the exception of Tris- HCl, were treated with DEPC (Diethyl pyrocarbonate) to eliminate the possible RNases. For each 50 mL of solution 50 µL of DEPC was added, leaving work overnight and then was autoclaved (121 ºC for 20 min) to inactivate traces of DEPC. Plastic and glass materials were treated with RNAseZap (Ambion, Canada), which also inactivate the RNase, and then were autoclaved (121 ºC for 20 min).

6.3 Plasmid extraction

For the yeast plasmid extraction a modified protocol described by Robzyk and Kassir (1992) was used. Cells were growing in 5 mL overnight culture in YPD with G418 Geneticin at 0.2 g/L concentration in order to maintain the plasmid. 1.5 mL of the culture was transferred to new tube and centrifuged at 6000 rpm for 5 min. Supernatant was removed and then cells were resuspended in 100 µL of STET (8% sucrose, 5% Triton X-100, 50 mM Tris-HCl pH 8, 50 mM EDTA). 0.2 g glass beads (0.45 mm) were added, and then samples were mixed with a mechanic shaker Mini Beadbeater-8 (BioSpec Products, USA) during 5 min, 30 sec shaking /30 sec incubating in ice cycles. Another 100 µL of STET were added, samples were mixed briefly and were incubated in boiling water for 3 min. The samples were cooled in ice and then were centrifuged at 12000 rpm for 10 min at 4 ºC. 100 µL of the supernatant were transferred to new tube and 50 µL of ammonium acetate 7.5 M was added. Samples were centrifuged at 12000 rpm for 10 min at 4 ºC. 100 µL of supernatant was added to 200 µL of absolute ethanol, and then it was precipitated for 1 h at – 20 ºC. Samples were centrifuged at 12000 rpm for 15 min at 4 ºC. Pellets were washed with ethanol 70% and samples were centrifuged 5 min at 12000 rpm at 4 ºC. Supernatant was removed and samples were dried with a vacuum pump. Finally DNA plasmid was re-suspended with 20 µL distilled water.

6.4 Oligonucleotides

Oligonucleotides used in this study are shown in Table 2. Primer pairs were designed using the Web Primer tool and genome sequences from the *Saccharomyces* Genome Database (SGD) (http://www.yeastgenome.org/), with the exception of ACT1F and ACT1R, which was previously described by Beltran *et al.* (2004), rec2 and rec5 described by Jansen et al. (2005), K2 and K3 described by Güldener *et al.* (1996), and delta12 and delta21 described by Legras and Karst

(2003). The sequences were sent to Invitrogen to be synthesized. Primers were used to amplify DNA in PCR and RT-PCR.

Table 2. Primers used in this work.

Primer	Sequence 5' to 3'	Purpose
mCHO2F	TAATTTTATACGTTAGTTCAACCTAACAATCCAGGAT TTCATTAACAAGAGTACGCTGCAGGTCGACA	to amplify *KanMX* cassette to delete *CHO2*
mCHO2R	CATTAATTACAACAACATAACTACTTCTATTCAAAATGT TAACTTGAATACTAGTGGATCTGATATC	to amplify *KanMX* cassette to delete *CHO2*
mCRD1F	GTCAACCAACACTTCACAGTCATGTCTTCGAATCATTGT G CGGCGACTATGTACGCTGCAGGTCGACA	to amplify *KanMX* cassette to delete *CRD1*
mCRD1R	GGTGAATTGAATGACGGTATCTCTGTTAGACGATCTGGT A CTACGAACACACTAGTGGATCTGATATC	to amplify *KanMX* cassette to delete *CRD1*
mGDE1F	GACAAACTACTGAAACTGTAAGGTCTTTATATAATCAA AC AATATAATCAGTACGCTGCAGGTCGACA	to amplify KanMX cassette to delete *GDE1*
mGDE1R	CCATCTCTGCAGGTTGCGCCATTGCAAGGTGTGCATTGG G TGAAACCTTAACTAGTGGATCTGATATC	to amplify KanMX cassette to delete *GDE1*
mOPI3F	CACACATGCATCGTTGGTTTCTGTCCACTGCCACTGCAA T GACCACTGGAGTACGCTGCAGGTCGACA	to amplify KanMX cassette to delete *OPI3*
mOPI3R	AAGAAATTGCTTATGGAGCTATATAGAAACGGTAATAG CA TAGGCTTCTAACTAGTGGATCTGATATC	to amplify KanMX cassette to delete *OPI3*
mPLB1F	TCTAATATATAAAGACGCTTCCACCACATTTAACCAACG GAAACGTCTCTGTACGCTGCAGGTCGACA	to amplify KanMX cassette to delete *PLB1*

Table 2 (continuation)

Primer	Sequence 5' to 3'	Purpose
mPLB1R	AAGTACTGCATGAACATATCTGTATAGATAGATATATGTAGGGAAGACAC ACTAGTGGATCTGATATC	to amplify KanMX cassette to delete *PLB1*
mPLB2F	ATACCTTATATATGAGCACAATTAGTTATCTTTACAAGGCGTTAGAAATGGTACGCTGCAGGTCGACA	to amplify KanMX cassette to delete *PLB2*
mPLB2R	CTTTATAATAAGGAAGATATATCAATCCTTGAAGTTAGGCATCCTTCTACACTAGTGGATCTGATATC	to amplify KanMX cassette to delete *PLB2*
mPLB3F	TACGTGGTTTTACGAGTTCTAATATTTCACTTTTCAGCTTGGAATACGCCGTACGCTGCAGGTCGACA	to amplify *KanMX* cassette to delete *PLB3*
mPLB3R	AAGTTCCTTAAACATGGTCTTGCCCGAAAGGGATGATACTTCAATGCAAAACTAGTGGATCTGATATC	to amplify *KanMX* cassette to delete *PLB3*
mPSD1F	GTTCTTCACCTATAATATAAAACAGCTTACTCACAAAAGAGACGCCTAGAGTACGCTGCAGGTCGACA	to amplify *KanMX* cassette to delete *PSD1*
mPSD1R	GTATAATTTTACATACCTCCAAAAGAATTAAATCAAAGTGTGCAGATACTAGTGGATCTGATATC	to amplify *KanMX* cassette to delete *PSD1*
mSCT1F	ATGGCTTGTAGTTCATAACAGAGTGAATTAAGTGTAGGAAGCCCGGAATTGTACGCTGCAGGTCGACA	to amplify KanMX cassette to delete *SCT1*
mSCT1R	GATATACATAAACATTGAGCATTAATATTTACAACTCCCAATGAGCAATAACTAGTGGATCTGATATC	to amplify KanMX cassette to delete *SCT1*
mERG3F	CTTTACATTTGTCTTGTTCTTTGAAGTGGTTGCAGAGGAGGTCAGTTTGTGTACGCTGCAGGTCGACA	to amplify KanMX cassette to delete *ERG3*
mERG3R	AGCGCATATTGCACTAACGTGAGGTGTACATCATAATGATATGCGTATCTACTAGTGGATCTGATATC	to amplify KanMX cassette to delete *ERG3*

Primer	Sequence 5' to 3'	Purpose
mERG4F	TAGATAGGCAGATACGGATATTTACGTAGTGTACATAGA TTAGCATCGCTGTACGCTGCAGGTCGACA	to amplify KanMX cassette to delete *ERG4*
mERG4R	TGCTGAACAAGATTAACATACTTCCTGCCACAACATAAT GTGAAATCACAACTAGTGGATCTGATATC	to amplify KanMX cassette to delete *ERG4*
mERG6F	CATGCGTTTTATGCGAAGATTGGTGAGAAACCTCCAATA CTTGCTGTTGCGTACGCTGCAGGTCGACA	to amplify KanMX cassette to delete *ERG6*
mERG6R	CAAGTGGGATAAATTGTGGGGTAGTAAAGGCATCGGAC AGTCTGTTTGTAACTAGTGGATCTGATATC	to amplify KanMX cassette to delete *ERG6*
mERG24F	TGTGTGAAGGTTGTGCATATAAAGGGTTTTGCATAACGG ACGTTTTTCACGTACGCTGCAGGTCGACA	to amplify KanMX cassette to delete *ERG24*
mERG24R	TTCTTCTTCCTCATACTCACCCAATACATAACATGTATAC ACACATACATACTAGTGGATCTGATATC	to amplify KanMX cassette to delete *ERG24*
mDPL1F	ATGAGTGGAGTATCAAATAAAACAGTATCAATTAATGGT TGGTATGGCATGTACGCTGCAGGTCGACA	to amplify KanMX cassette to delete *DPL1*
mDPL1R	CGTGACATAGGATCAGGAATCACAATCATCACATTGCAC TCTCGTTCTT TACTAGTGGATCTGATATC	to amplify KanMX cassette to delete *DPL1*
mIPT1F	TTATCATTTCTGCTAAGAATCACCTAAAGTCTTTCAACGT CTAAGAAAGCGTACGCTGCAGGTCGACA	to amplify KanMX cassette to delete *IPT1*
mIPT1R	AAAAAGCCAATCTATATTATTTAAATTATCCGAAATTAC TTTTATTACATACTAGTGGATCTGATATC	to amplify KanMX cassette to delete *IPT1*

Table 2 (continuation)

Primer	Sequence 5' to 3'	Purpose
mLAC1F	CATACCTCCGGTAAACATTTAGATAGACACAGTATCAATAAACAAGAGCTGTACGCTGCAGGTCGACA	to amplify *KanMX* cassette to delete *LAC1*
mLAC1R	CACTGTATGTCTGGAAAAGAATACTATAAAAATACCTGTTTACATAAACCACTAGTGGATCTGATATC	to amplify *KanMX* cassette to delete *LAC1*
mLCB3F	AGCAGAGTTTAAGAAAGTTTGTTTACATACAACCATCACGTAGAGGAAATGTACGCTGCAGGTCGACA	to amplify *KanMX* cassette to delete *LCB3*
mLCB3R	ATTAATGATGATGACTTTAACCTATTCTTCTTTTGGGGTCCTGGTACGGTACTAGTGGATCTGATATC	to amplify *KanMX* cassette to delete *LCB3*
mLCB4F	CCCATCTGATACTTTCCCTTGTCTAACGTACTGATCCTGGAGGTTATCAAGTACGCTGCAGGTCGACA	to amplify KanMX cassette to delete *LCB4*
mLCB4R	ACTCATAATATTGTTACGTATTAATGATATATAACTGGGGATGTATAATTACTAGTGGATCTGATATC	to amplify KanMX cassette to delete *LCB4*
mYDC1F	TCTGCATTGTTCTTCGTTTTTCAATTCAAGAATTTAAGCAAAAGGATATAGTACGCTGCAGGTCGACA	to amplify KanMX cassette to delete *YDC1*
mYDC1R	ACGTTCGGTCAATTGCACGTACAACAATTTATATACATACATAACATATAACTAGTGGATCTGATATC	to amplify KanMX cassette to delete *YDC1*
cmCHO2F	TGAACGT AGCTATGCCA ATG	to check the deletion of *CHO2*
cmCHO2R	ACTCACATTAAGGTGTTATCAGAAT	to check the deletion of *CHO2*
cmCRD1F	ACGGCTCCTCCATTGAAATA	to check the deletion of *CRD1*

Primer	Sequence 5' to 3'	Purpose
cmCRD1R	CAAAGCCCGACACAGAAAAT	to check the deletion of *CRD1*
cmGDE1F	GCGATTTACTCGATCAAATGC	to check the deletion of *GDE1*
cmGDE1R	ATAGAGGATTTGGGCTGGTCT	to check the deletion of *GDE1*
cmOPI3F	GTGCCTCTTCTCTGCAAGAAT	to check the deletion of *OPI3*
cmOPI3R	TGCTTTTTCACGTTCTTGCT	to check the deletion of *OPI3*
cmPLB1F	GGAAATCAAATTCGCTTCAGC	to check the deletion of *PLB1*
cmPLB1R	TCACCAGTAGTGGACGACGAA	to check the deletion of *PLB1*
cmPLB2F	GGCAATGCTTTGGTGAATTAT	to check the deletion of *PLB2*
cmPLB2R	TTTGGACATGATGTCAAGTGC	to check the deletion of *PLB2*
cmPLB3F	GACCTGCCCTGATTCCAAG	to check the deletion of *PLB3*
cmPLB3R	AGCATTGGTTCGACGAAATAA	to check the deletion of *PLB3*
cmPSD1F	TCACATATCACATGCAGGGT	to check the deletion of *PSD1*
cmPSD1R	TGGCTAAAATTCTTCAGACGA	to check the deletion of *PSD1*

Table 2 (continuation)

Primer	Sequence 5' to 3'	Purpose
cmSCT1F	GACATAAAGGCAGTGCACCTAT	to check the deletion of *SCT1*
cmSCT1R	ACGTCTTCCGAATCTGTAGTAGAT	to check the deletion of *SCT1*
cmERG3F	ATCGTCGTCCTCCTGTTCAT	to check the deletion of *ERG3*
cmERG3R	CATATCAACATCTGGTGGCAA	to check the deletion of *ERG3*
cmERG4F	TTCCCATCACTTCCGCTATTA	to check the deletion of *ERG4*
cmERG4R	GACCAATACCGGATGCAAGA	to check the deletion of *ERG4*
cmERG6F	TATCCTCGCCATCACGTGTA	to check the deletion of *ERG6*
cmERG6R	GCTTGCATTTGTATGTCACGA	to check the deletion of *ERG6*
cmERG24F	TACCATCAACGGCACTAACAA	to check the deletion of *ERG24*
cmERG24R	CAGTTGTCGATGAACGTCAAA	to check the deletion of *ERG24*
cmDPL1F	GAATCTTCAAAAGCCGCA	to check the deletion of *DPL1*
cmDPL1R	AAGACAGAAGAAAGGATCGG	to check the deletion of *DPL1*
cmIPT1F	GGTAGAAGAAGGCTTGCTATAATTT	to check the deletion of *IPT1*
cmIPT1R	TCCTGATTACTCAATTTCTGAATGT	to check the deletion of *IPT1*

cmLAC1F	CTGTCGCTCTTATGATCCGTT	to check the deletion of *LAC1*
cmLAC1R	AAGACCGAACACGAAAGCA	to check the deletion of *LAC1*
cmLCB3F	ACCACCGATGAAGAGAACCA	to check the deletion of *LCB3*
cmLCB3R	GGCATGACTTCAGGGATCAA	to check the deletion of *LCB3*
cmLCB4F	CGTTCTCAAAGATGTAGGACCAT	to check the deletion of *LCB4*
cmLCB4R	AACAAGGTTGAAGAAGGTTCGA	to check the deletion of *LCB4*
cmYDC1F	TCGTGAAGATGAACGCGA	to check the deletion of *YDC1*
cmYDC1R	GCAAATGGTGGTTGGGAA	to check the deletion of *YDC1*
K3	CCTCGACATCATCTGCCC	to check the integration of *Kan*MX cassette
K2	GGGACAATTCAACGCGTCTG	to check the integration of *Kan*MX cassette
CHO2rec5F	AACAAAAGCTGGAGCTCGTTTAAACGGCGCGCCACCTGCCCAGATCCAAAGTT	to amplify *CHO2* overexpression cassette
CHO2rec2R	GCGTGACATAACTAATTACATGACTCGAGGTCGACCCAATGGCACTGTCGTATCAT	to amplify *CHO2* overexpression cassette

Table 2 (continuation)

Primer	Sequence 5' to 3'	Purpose
OPI3rec5F	AACAAAAGCTGGAGCTCGTTTAAACGGCGCGCCTAATCTGATCAACGCTACGCC	to amplify *OPI3* overexpression cassette
OPI3rec2R	GCGTGACATAACTAATTACATGACTCGAGGTCGACCGCAAGTGGGAGTTTAACCTT	to amplify *OPI3* overexpression cassette
PSD1rec5F	AACAAAAGCTGGAGCTCGTTTAAACGGCGCGCCT CATCATTGGTCAAGACCAC	to amplify *PSD1* overexpression cassette
PSD1rec2R	GCGTGACATAACTAATTACATGACTCGAGGTCGACACTTCTACTTTAGAATCTATCGCTTT	to amplify *PSD1* overexpression cassette
ERG3rec5F	AACAAAAGCTGGAGCTCGTTTAAACGGCGCGCCATTAGGGCAGCTCTGCCAT	to amplify *ERG3* overexpression cassette
ERG3rec2R	GCGTGACATAACTAATTACATGACTCGAGGTCGACCGAATAGCGCATATTGCACT	to amplify *ERG3* overexpression cassette
ERG6 rec5F	AACAAAAGCTGGAGCTCGTTTAAACGGCGCGCCAGTTGCCACCCTCCTACTCTT	to amplify *ERG6* overexpression cassette
ERG6 rec2R	GCGTGACATAACTAATTACATGACTCGAGGTCGACGCTGTAGGGGAGCAGTCAATA	to amplify *ERG6* overexpression cassette
IDI1 rec5F	AACAAAAGCTGGAGCTCGTTTAAACGGCGCGCCGCTTTTGCAGATTCCGGTAT	to amplify *IDI1* overexpression cassette
IDI1 rec2R	GCGTGACATAACTAATTACATGACTCGAGGTCGACACGCAATGGAATAGGCTAAGA	to amplify *IDI1* overexpression cassette

OLE1 rec5F	AACAAAAGCTGGAGCTCGTTTAAACGGCGCGCCTCTCCGTGACTATGGCTCTTT	to amplify *OLE1* overexpression cassette
OLE1 rec2R	GCGTGACATAACTAATTACATGACTCGAGGTCGACGATGCAGTAAGCCATCCCAT	to amplify *OLE1* overexpression cassette
DPL1 rec5F	AACAAAAGCTGGAGCTCGTTTAAACGGCGCGCCAGCTTGAAGAAGGAGCTGGAA	to amplify *DPL1* overexpression cassette
DPL1 rec2R	GCGTGACATAACTAATTACATGACTCGAGGTCGACGTTCCTGTTCGTGGCCTTTA	to amplify *DPL1* overexpression cassette
LCB3 rec5F	AACAAAAGCTGGAGCTCGTTTAAACGGCGCGCCCGGGTGTAAACACATTGTCGT	to amplify *LCB3* overexpression cassette
LCB3 rec2R	GCGTGACATAACTAATTACATGACTCGAGGTCGACTTGAGTGCCTCACTAGCTACCA	to amplify *LCB3* overexpression cassette
LCB4 rec5F	AACAAAAGCTGGAGCTCGTTTAAACGGCGCGCCGGTTCCTAAATGGGCTCGTACT	to amplify *LCB4* overexpression cassette
LCB4 rec2R	GCGTGACATAACTAATTACATGACTCGAGGTCGACCTTGAAGGACGCAACTTCCA	to amplify *LCB4* overexpression cassette
rec5F	AACAAAAGCTGGAGCTCGTTTAAACGGCGCGCCT	to check the overexpression
rec2R	GCGTGACATAACTAATTACATGACTCGAGGTCGAC	to check the overexpression cassette

Table 2 (continuation)

Primer	Sequence 5' to 3'	Purpose
CHO2F	GAAGCGGTATTAGGCGTTGTT	to determinate the transcriptional activity of *CHO2* by RT-PCR
CHO2R	TAACGGCAAAATTCGTCATCA	to determinate the transcriptional activity of *CHO2* by RT-PCR
OPI3F	GCCATTGTGTGCACGATGT	to determinate the transcriptional activity of *OPI3* by RT-PCR
OPI3R	ATTCTTGCAACGATGTTCCAA	to determinate the transcriptional activity of *OPI3* by RT-PCR
PSD1F	CTCTGAGCCTCATGATACGGA	to determinate the transcriptional activity of *PSD1* by RT-PCR
PSD1R	AATGATGGTAATCACCGGGA	to determinate the transcriptional activity of *PSD1* by RT-PCR
ERG3F	GTTGCATTGGCCAAGGGT	to determinate the transcriptional activity of *ERG3* by RT-PCR
ERG3R	AGATGCGAAAGGTGTGCAGA	to determinate the transcriptional activity of *ERG3* by RT-PCR
ERG6F	GCCTCGATAGCAAGACATGAA	to determinate the transcriptional activity of *ERG6* by RT-PCR
ERG6R	ACGTCGAGAACTAAATCGCCT	to determinate the transcriptional activity of *ERG6* by RT-PCR

Table 2 (continuation)

Primer	Sequence 5' to 3'	Purpose
LCB3F	AGGAATATGGTGCTCCAAGCT	to determinate the transcriptional activity of *LCB3* by RT-PCR
LCB3R	CTTGCATCCTCCAGATGTTGT	to determinate the transcriptional activity of *LCB3* by RT-PCR
LCB4F	CGCAATCACACCATATGTTCA	to determinate the transcriptional activity of *LCB4* by RT-PCR
LCB4R	ACAGGACAAGCAGCTCAACAA	to determinate the transcriptional activity of *LCB4* by RT-PCR
ACT1F	TGGATTCCGGTGATGGTGTT	to determinate the transcriptional activity of *ACT1* by RT-PCR
ACT1R	CGGCCAAATCGATTCTCAA	to determinate the transcriptional activity of *ACT1* by RT-PCR
delta12	TCAACAATGGAATCCCAAC	to amplify delta elements
delta21	CATCTTAACACCGTATATGA	to amplify delta elements

Underlined sequences correspond to sequences homologous to the plasmids. The plasmids used in this work were: pUG6 to amplify the deletion cassettes and pGREG 505 to construct the overexpression strains.

6.5 Polymerase chain reaction (PCR)

Standard DNA amplification was performed via PCR in a GenAmp PCR System 2700 (Applied Biosystems, USA) to synthesis the deletion and overexpression cassettes, to check the deletion and overexpresion and for typing by delta elements amplification. A typical 50 µL reaction was performed, contained 1-5 µL, 0.1 – 100 ng DNA, 2 µL 200 µM dNTPs, 5 µL 10 µM primers, 5 µL 10 x PCR buffer, 3-5 µL 50 mM $MgCl_2$, 1 µL of Taq polymerase, 0.03 U/µL and water to complete the 50 µL. PCR products were stored at – 20 ºC until required.

6.5 1Deletion cassette amplification

The deletion cassette was amplified from pUG6 plasmid. Primers used have 50-nucleotide extensions corresponding to regions upstream of the target gene start codon (forward primer) and downstream of the stop codon (reverse primer). The Taq polymerase EcoTaq (Ecogen, Spain) was used. Reactions were run with an initial denaturation period of 5 min at 94 ºC, then 30 cycles consisting of denaturation at 94 ºC for 30 sec, annealing at 51 ºC for 30 sec and elongation at 72 ºC for 2 min. The amplification terminated with an extended incubation at 72 ºC for 5 min and cooling to 4 ºC.

6.5.2 Deletion verification

The genomic DNA of possible deletion mutants was analyzed by PCR using primers upstream and downstream of the deleted region, and a combination with primers of the *Kan*MX gene (K2 and K3, Table 2). The Taq polymerase EcoTaq (Ecogen, Spain) was used. Reactions were run with an initial denaturation period of 5 min at 94 ºC, then 30 cycles consisting of denaturation at 94 ºC for 30 sec, annealing at 50-55 ºC (depending on the different primers) for 30 sec and

elongation at 72 ºC for 2- 5.5 min (depending on the different PCR product length) . The amplification terminated with an extended incubation at 72 ºC for 5 min and cooling to 4 ºC.

6.5.3 Overexpression cassette amplification

The overexpression cassettes were amplified from genomic DNA of wine yeast strain QA23. The Taq polymerase Expand Long High Fidelity (Roche Diagnostics, Germany) was used for extremely accurate amplifications. All genes were amplified from approximately 600 nucleotides upstream of the start codon to 400 nucleotides downstream of the stop codon to ensure that the promoter and terminator regions were included. The PCR protocol involved an initial denaturation at 94 ºC (2 min), followed by 30 cycles of 10 sec at 94 ºC, 30 sec at 49-50 ºC (depending on the different primers) and 3-4 min at 72 ºC (depending on the different PCR product length). The last cycle was followed by a final extension step of 10 min at 72 ºC. PCR fragments were generated with oligonucleotides that contained the short sequences rec5 (forward) and rec2 (reverse), which are homologous to the sequences in the plasmid (about 35 bp).

6.5.4 Overexpression plasmid verification

To verify the correct integration of the gene into the pGREG 505 vector, plasmids were checked by PCR using primers specified for sequences rec5 and rec2 (rec5F and rec2R, Table 2). The Taq polymerase EcoTaq (Ecogen, Spain) was used. Reactions were run with an initial denaturation period of 5 min at 94 ºC, then 35 cycles consisting of denaturation at 94 ºC for 30 sec, annealing at 52 ºC for 30 sec and elongation at 72 ºC for 4 min. The amplification terminated with an extended incubation at 72 ºC for 7 min and cooling to 4 ºC.

6.5.5 Delta elements amplification

Yeast typing was performance by delta elements amplification from genomic DNA. Specific primers delta 12 and delta 21 and Taq polymerase EcoTaq (Ecogen, Spain) were used. A 50 µL reaction was performed, contained 5 µL, 0.1 – 100 ng DNA, 1 µL 200 µM dNTPs, 1 µL 10 µM primers, 5 µL 10 x PCR buffer, 2.5 µL 50 mM $MgCl_2$, 1 µL BSA 200 µg/mL, 0.2 µL of Taq polymerase, and 33.3 µL water to complete the 50 µL. Reactions were run with an initial denaturation period of 4 min at 95 ºC, then 35 cycles consisting of denaturation at 95 ºC for 30 sec, annealing at 46 ºC for 30 sec and elongation at 72 ºC for 1.5 min. The amplification terminated with an extended incubation at 72 ºC for 10 min and cooling to 4 ºC.

6.6 Gene expression analysis by real-time quantitative PCR

Total RNA was reverse-transcribed with SuperscriptTM II RNase H- Reverse Transcriptase (Invitrogen, USA) in a GenAmp PCR System 2700 (Applied Biosystems, USA). The reaction contained 0.5 µg of Oligo (dT) 12-18 Primer (Invitrogen, USA) and 0.8 µg of total RNA as template in a total reaction volume of 20 µL. As directed by the manufacturer, after denaturation at 70 ºC for 10 min, cDNA was synthesized at 42 ºC for 50 min, and then the reaction was inactivated at 70 ºC for 15 min.

Real-Time Quantitative PCR was performed using LightCycler® 480 SYBR Green I Master (Roche, Germany). All amplicons were shorter than 100 bp, which ensured maximal PCR efficiency and the most precise quantification. The SYBR PCR reactions contained 2.5µM of each PCR primer, 5 µl cDNA and 10 µL of SYBR Green I Master (Roche, Germany) in a 20 µL reaction.
All PCR reactions were mixed in LightCycler® 480 Multiwell Plate 96 (Roche, Germany) and cycled in a LightCycler® 480 Instrument II, 96 well thermal cycler (Roche, Germany) using the following conditions: 95 ºC for 5 min, and 45 cycles

at 95 ºC for 10 sec, at 55 ºC for 10 sec and 72 ºC 10 sec. All samples were analyzed in triplicate with LightCycler® 480 Software, version 1.5 (Roche, Germany) and the expression values were averaged. Gene expression levels are shown as the concentration of the studied gene normalized to the concentration of the housekeeping gene, *ACT1* and referenced to the control.

6.7 Yeast transformation

Yeast transformation was performed using a lithium acetate protocol describe by Gietz *et al*. (2002). Transformation procedure was used to construct deletion and overexpressing strains. To construct the deletion strains *ho*QA23 was transformed with the deletion cassette, obtained by PCR. For the construction of overexpressing strains *ho*QA23 was co-transformed with the overexpression cassette, obtained by PCR, and the pGREG 505 plasmid previously linearized by *SalI* digestion and digested with *AslI* to avoid sticky ends and to make the recombination process easier.

Yeast cells were inoculated in 50 mL YPD, overnight at 30 ºC and 200 rpm. This culture was used to inoculate an OD of 0.2 in 50 mL YPD and was incubated at 30 ºC and 200 rpm until the cells were completed at least 3 divisions (OD of approximately 0.8). Then 5 mL culture was transferred to a new tube and centrifuged at 5000 rpm for 5 min. The media was poured off and cells were resuspended in 2.5 mL of sterile water and centrifuged again. The water was removed and cells were resuspended in 100 μL of 0.1 M LiAc. Cells were pellet a top speed for 15 sec and LiAc was removed with a micropipette. Cells were resuspended in 40 μL of 0.1 M LiAc, centrifuged and LiAc was removed again with a micropipette. Then transformation mix was added in this order: 240 μL of PEG (50% w/v), 36 μL of 1 M LiAc, 50 μL of ss-DNA 2 mg/mL (previously boiled for 5 min and quickly chilled in ice) and 34 μL of deletion cassette or 26 μL of overexpression cassette + 8 μL pGREG 505 plasmid. Each tube was vigorously

vortexed until the cell pellet had been completely mixed. Tubes were incubated at 30 ºC for 30 min, then 42 ºC for 15-30 min. Tubes were centrifuged at 7000 rpm for 15 sec and the transformation mix was removed with a micropipette. Into each tube 1 mL of water was added and pellet was resuspended by pipetting it up and down gently. Then tubes were centrifuged at 7000 rpm for 15 sec and water was removed. Pellet was resuspended in 500 μL YPD and incubated at 30 ºC for 3 h with a gentle shaking at 125 rpm. 200 μL (twice for each transformation) were plated onto selection plates (YPD with Geneticin G418, 0.2 g/L) and incubated at 30 ºC until colonies appeared (2-3 days).

6.8 EMS (Ethyl Methanesulfonate) mutagenesis of yeast

EMS mutagenesis of yeast was performed as described by Winston (2008). Spontaneous mutations occur at a low rate, so yeast cells are treated with mutagens to increase the frequency of mutants. Mutagenesis can increase the mutation frequency up to 100-fold per gene without excessive killing of the cells (within 10-50% of survival gives the highest proportion of mutants).

Yeasts cells were growing in 3 mL YPD overnight culture at 30 ºC and 200 rpm. The optical density of the cells was determinated and then was adjusted to 2 $x10^8$ cell/mL. Two lots were done, one acted as no EMS control. Cells were centrifuged at maximum speed at room temperature for 3 min. Supernatant was discarded and pellet was resuspended in 1 mL sterile water. Cells were centrifuged again at maximum speed at room temperature for 3 min. Supernatant was discarded and pellet was resuspended in 1.7 mL sterile 0.1 M sodium phosphate buffer, pH 7. 20 μL EMS were added to the cells and mixed by vortexing, and then cells were incubated at 30 ºC for 2 h. Cell suspension was transferred to a culture tube containing 8 mL sterile 5% sodium thiosulfate, which would stop the mutagenesis by inactivation of EMS. Cells were centrifuged at maximum speed at room

temperature for 3 min. Supernatant was discarded and pellet was resuspended in 1 mL sterile water. Cells were pelleted and then an appropriate dilution was transferred to synthetic must to achieve a cell concentration of 2 x 10^6 cells/mL and incubated during 200 generations with a selective pressure (low temperature). Yeast cells were also plated on YPD to do viable cell counts on two lots of cells.

7. LIPID ANALYSES

7.1 Determination of total yeast fatty acids

Yeast cells (5-10 mg dry mass) were placed in sealed tubes with a Teflon-lined screw cap and saponified using a 1 mL of 5% NaOH in 50% methanol/water (Rozès et al., 1992). The tubes were placed in a dry bath (100 ºC) for 5 minutes. Samples were vortexed and then the tubes were placed in a dry bath (100 ºC) for another 25 minutes. Then the saponified material was cooled to room temperature and 2 mL HCl 6M was added. Free fatty acids were extracted by adding 500 µL hexane: tert-Butyl methyl ether (MTBE) (1:1, v/v). Each tube was vortexed twice for 30 seconds. The organic phase was collected after centrifugation at 3000 rpm for 3 minutes.

Analytical gas chromatography was performed on a Hewlett-Packard 6850 (Agilent Technologies). 1µL of cellular extract was injected (splitless, 1 minute) into a FFAP-HP column (30m x 0,25mm x 0.25µm from Agilent) with an HP 6850 automatic injector. The initial temperature was set at 140 ºC and increased by 4 ºC/min up to 240 ºC. Injector and detector temperatures were 250 ºC and 280 ºC, respectively. The carrier gas (helium) was at a flow rate 1.4 mL/min. Heptanoic and heptadecanoic acids (10 and 40 mg/mL, respectively) were added as internal standards before cell saponification. Relative amounts of fatty acids were calculated from their respective chromatographic peaks. These values were related

to the dry weight of cells and expressed as a percentage of the total fatty acid extracted (Redón et al., 2009).

7.2 Lipid extraction

Prior to lipid extraction, a solution of 100 µL of cold methanol and 20 µL of EDTA 0.1 mM was added to the yeast cells (5-10 mg dry mass) with 1 g glass beads (0.5 mm, Biospec Products) in Eppendorf, and then mixed for 5 minutes in a Mini Beadbeater-8 (Biospec Products, Qiagen). Lipid extraction was performed in two steps with 1 mL chloroform/methanol (2:1, v/v, for 1 hour), one step with 1 mL chloroform/methanol (1:1, v/v, for 1 hour) and one step with 1 mL chloroform/methanol (1:2, v/v, for 1 hour). All the organic phases were transferred to a 15 mL glass screw tube and cleaned twice by adding KCl 0.88% (1/4 of a total volume of the extract). After vortexing and cooling at 4 ºC for 10 minutes, the samples were centrifuged at 3000 rpm for 5 minutes. The inferior organic phase was collected and finally concentrated to dryness under nitrogen stream. The residue was dissolve in 100 µL of chloroform/methanol (2:1, v/v).

7.3 Separation and quantification of the yeast lipids

7.3.1 Separation and quantification of the yeast phospholipids (PLs) by HPTLC

The yeast extract phospholipids were separated by one-dimensional HPTLC on silica gel 60F254 plates (10 x 20cm, 200 µm) with chloroform: acetone: methanol: glacial acetic acid: water (65:15:10:10:5, v/v/v/v/v). After charring the plate with 10% $CuSO_4$ in 8% H_3PO_4 and heating to 160 ºC for 4 min, the PLs were identified by known standards purchased from Sigma: phosphatidylinositol (PI),

phosphatidylserine (PS), phosphatidylcholine (PC), phosphatidylglycerol (PG), phosphatidylethanolamine (PE), cardiolipin (CL) and phosphatidic acid (PA). The plates were scanned and each spot of the image was quantified in terms of integrated optical densities (IOD) with ImageJ software (a public domain, Java-based image processing program developed at the National Institute of Health). Calibration curves were constructed by applying standards to each plate in the range of 1-4 µg/µL to quantify the PLs. These values were related to the dry weight of cells and expressed as a percentage of the total PLs extracted.

7.3.2 Yeast neutral lipid (NL) composition by thin-layer chromatography (TLC)

NL composition of yeast was separated by one-dimensional TLC on silica gel 60F254 (10 x 20 cm, 250 µm) (Merck, Germany). The plate was developed in three steps: the first step with hexane, tert-Butyl methyl ether (MTBE) and glacial acetic acid (50:50:2) to 35mm, the second step with hexane, tert-Butyl methyl ether (MTBE) and glacial acetic acid (80:20:1) to 60mm and the last step with hexane to 85mm. The standard lipids lanosterol, ergosterol, squalene, cholesteryl oleate, ethyl oleate, diolein, triolein were purchased from Sigma and were applied to every plate in the range of 1-4 µg/µL. After TLC, lipids were charred with 10% CuSO4 in 8% H3PO4 and heated to 160 ºC for 4 min on a TLC Plate Heater (CAMAG). Plates were scanned and each spot of the image was quantified as integrated optical densities (IOS) with ImageJ software (a public domain, Java-based image processing program developed at the National Institute of Health). Calibration curves were constructed by applying standards to each plate in the range of 1-4 µg/µL to quantify the NLs. These values were related to the cell dry weight and expressed as a percentage of total NLs extracted.

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Annex 2

Supplementary data of Chapter 1

UNIVERSITAT ROVIRA I VIRGILI
METABOLIC AND MOLECULAR ADAPTATION OF WINE YEASTS AT LOW TEMPERATURE FERMENTATION:
STRATEGIES FOR THEIR GENETIC IMPROVEMENT
María López Malo
DL: T. 1275-2013

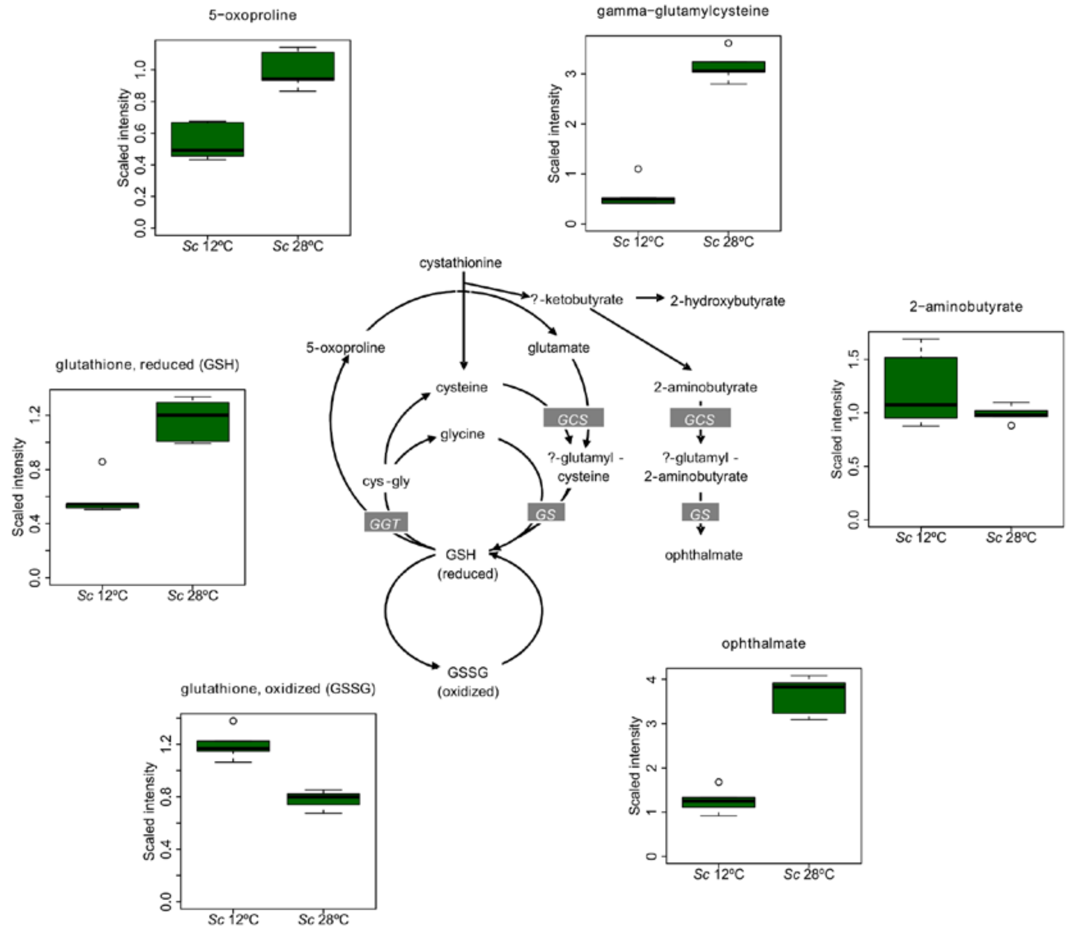

Figure S1. The homeostasis redox in *S. cerevisiae***.** Metabolic differences in the *Sc* growing at 12ºC and 28ºC. Box legend: bar inside the box represents the median value, upper bar represents maximum of distribution, lower bar represents minimum of distribution and the circle represents extreme data points.

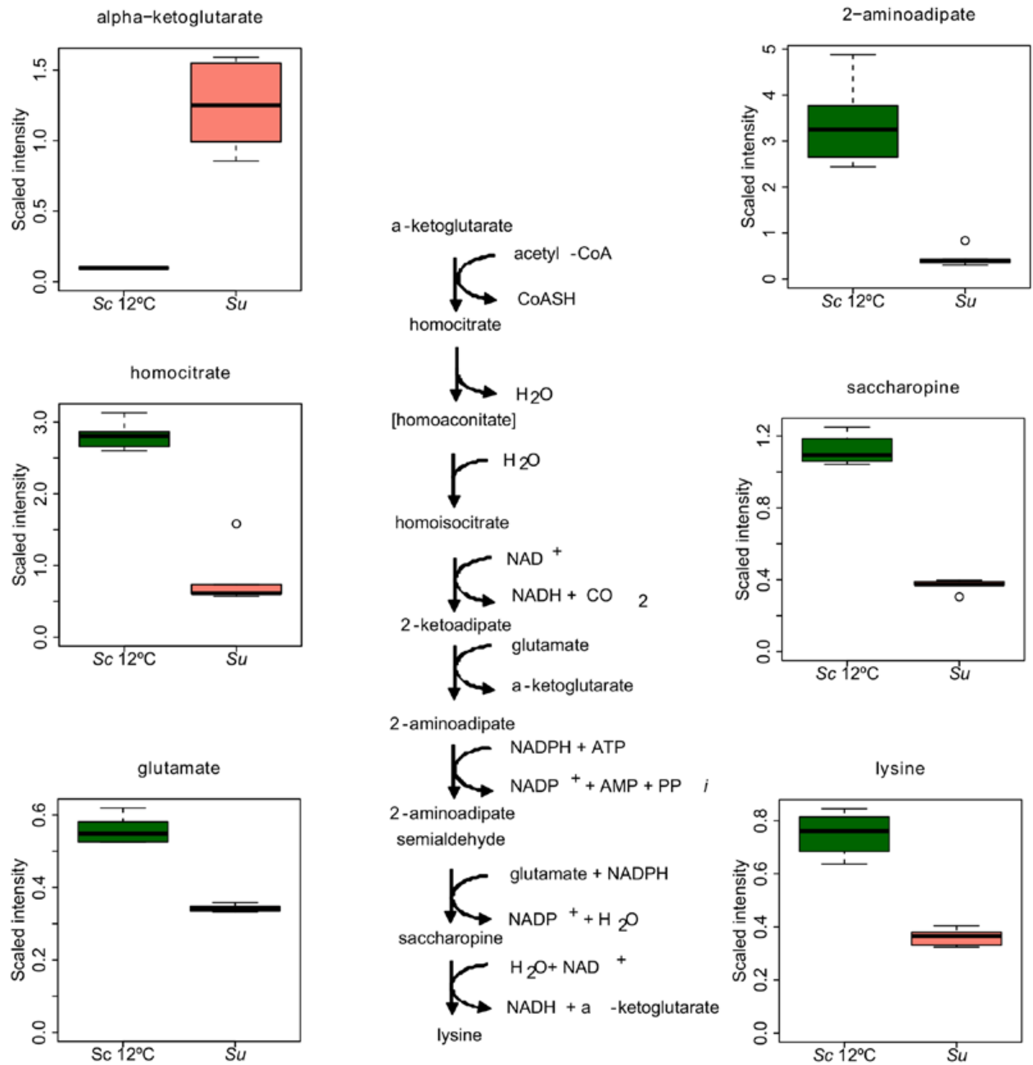

Figure S2. Lysine synthesis. Differentially produced metabolites within *Su* and *Sc*. Box legend: bar inside the box represents the median value, upper bar represents maximum of distribution, lower bar represents minimum of distribution and the circle represents extreme data points.

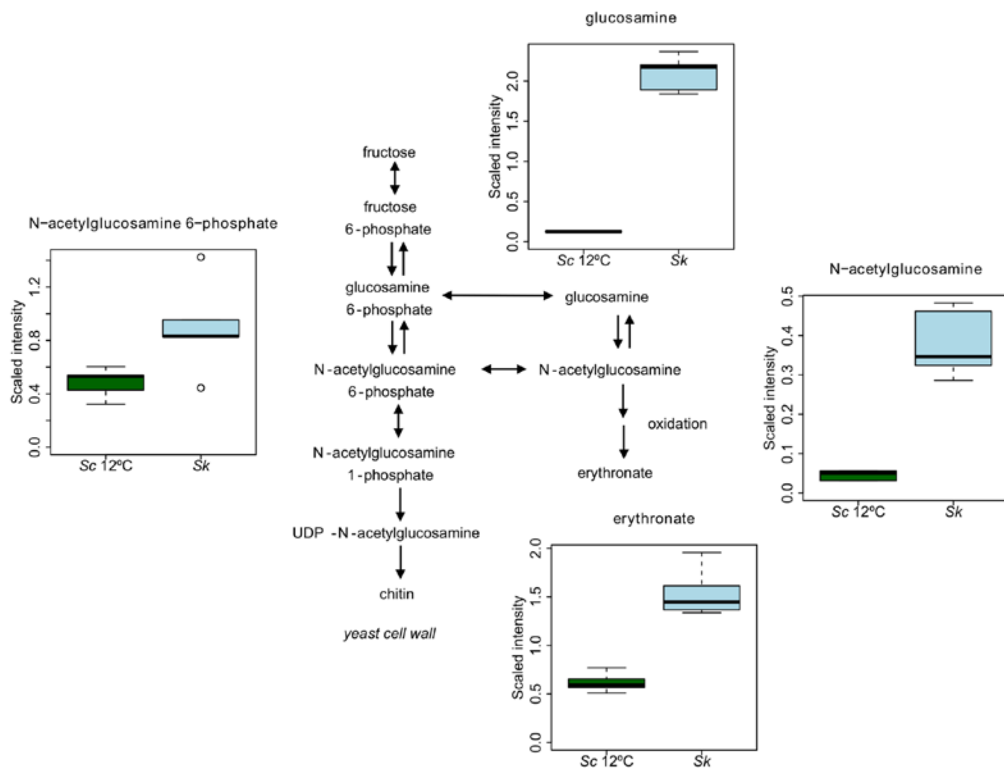

Figure S3. Cell wall synthesis. Differentially produced metabolites within *Sk* and *Sc*. Box legend: bar inside the box represents the median value, upper bar represents maximum of distribution, lower bar represents minimum of distribution and the circle represents extreme data points.

Table S2. Metabolic comparison between Sc QA23 growing at 28 ºC, Su 12600 and Sk CR85 and Sc QA23 growing at 12 ºC.

				Fold of Change			
				Sc QA23-28C / Sc QA23-12C	Su-12C / Sc QA23-12C	Sk-12C / Sc QA23-12C	Sk-12C / Su-12C
Super Pathway	**Sub Pathway**	**Biochemical Name**	**KEGG**				
Amino acid	Glycine, serine and threonine metabolism	Glycine	C00037	1,35	0,62	0,79	1,27
		N-acetylglycine		1,04	0,82	3,59	4,38
		beta-hydroxypyruvate	C00168	1	1	1,54	1,54
		Serine	C00065	1,88	1,31	1,18	0,9
		N-acetylserine		1,14	0,35	0,62	1,77
		Homoserine	C00263, C02926	2,19	0,71	2,39	3,37
		O-acetylhomoserine	C01077	1,6	0,3	0,08	0,27
		Threonine	C00188	2,41	0,69	1,48	2,13
		N-acetylthreonine	C01118	0,3	0,17	0,75	4,47
		allo-threonine	C05519	3,6	2,11	1,75	0,83
	Alanine and aspartate metabolism	Aspartate	C00049	5,52	1,83	2,5	1,37
		Asparagine	C00152	2,81	0,93	1,18	1,27
		Alanine	C00041	1,39	0,56	0,99	1,77
		N-carbamoylaspartate	C00438	1	1	1	1
	Glutamate metabolism	Glutamate	C00025	2,72	0,61	1,05	1,72
		glutamate, gamma-methyl ester		1,72	0,26	0,25	0,95
		glutamine	C00064	0,73	1,11	0,94	0,85
		gamma-aminobutyrate (GABA)	C00334	2,61	93,59	20,27	0,22
		N-acetylglutamate	C00624	4,57	1,38	1,72	1,24
	Histidine metabolism	histidine	C00135	0,29	0,27	0,44	1,63
		N-acetylhistidine	C02997	1	1	1	1
		imidazole lactate	C05568	0,09	0,16	0,05	0,33
	Lysine metabolism	glutarate (pentanedioate)	C00489	0,24	0,56	0,37	0,66
		lysine	C00047	1,52	0,48	1,17	2,43
		N6-carboxyethyllysine		2,52	0,63	0,63	1

		2-aminoadipate	C00956	0,43	0,14	0,36	2,66
		pipecolate	C00408	0,97	0,41	0,22	0,54
		N-6-trimethyllysine	C03793	1,81	0,99	0,8	0,81
		saccharopine	C00449	1,29	0,33	0,48	1,47
		N2-acetyllysine	C12989	1,4	0,45	0,91	2
		N6-acetyllysine	C02727	3,46	1,2	1,43	1,2
		2-aminopentanoate		1,3	0,32	0,48	1,51
	Phenylalanine & tyrosine metabolism	phenyllactate (PLA)	C05607	2,21	3,94	0,7	0,18
		phenylalanine	C00079	2,03	1,24	1,23	0,99
		tyrosine	C00082	2,43	0,79	0,85	1,08
		3-(4-hydroxyphenyl)lactate	C03672	3,81	1,92	0,89	0,46
		4-hydroxyphenylpyruvate	C01179	1,2	0,95	0,92	0,97
		N-acetylphenylalanine	C03519	3,64	1,48	0,69	0,46
		N-acetyltyrosine		2,23	0,34	0,34	1
	Tryptophan metabolism	xanthurenate	C02470	2,49	0,52	0,52	1
		kynurenate	C01717	1,45	0,04	0,02	0,57
		kynurenine	C00328	1,48	0,26	0,09	0,33
		tryptophan	C00078	7,16	11,74	27,5	2,34
		anthranilate	C00108	0,65	0,65	0,65	1
		3-hydroxykynurenine	C02794	1,65	0,15	1,45	10,02
		3-hydroxyanthranilate	C00632	0,62	1,24	1,42	1,15
	Valine, leucine and isoleucine metabolism	alpha-hydroxyisocaproate	C03264	1,53	1,04	1,32	1,27
		isoleucine	C00407	1,52	1,06	1,26	1,19
		leucine	C00123	1,88	1,14	1,09	0,95
		N-acetylleucine	C02710	1,4	1,54	1,01	0,66
		N-acetylisoleucine		0,77	0,91	1,3	1,44
		valine	C00183	1,54	1,16	1,23	1,06
		4-methyl-2-oxopentanoate	C00233	1,46	0,56	1,14	2,03
		alpha-hydroxyisovalerate		1,6	2,34	1,37	0,59
		citramalate	C02612, C00815	1,34	0,12	0,25	2,18
	Cysteine, methionine, SAM, taurine metabolism	cysteine	C00097	1,14	0,35	2,14	6,12
		cystathionine	C02291	0,47	0,13	1,21	9,19

		S-adenosylhomocysteine (SAH)	C00021	0,91	0,87	6,7	7,72
		methionine	C00073	1,77	1,31	1,12	0,86
		N-acetylmethionine	C02712	0,74	0,4	0,71	1,77
		homocysteine	C00155	1,5	0,72	2,84	3,93
	Urea cycle; arginine-, proline-, metabolism	dimethylarginine (SDMA + ADMA)	C03626	0,94	0,99	2,27	2,3
		arginine	C00062	0,44	0,36	2,44	6,81
		N-acetylarginine	C02562	0,66	0,55	2,25	4,12
		ornithine	C00077	1,53	0,11	0,27	2,37
		urea	C00086	0,46	0,33	0,21	0,64
		proline	C00148	1,17	1,16	2,32	1,99
		5-aminovalerate	C00431	0,08	0,28	0,18	0,64
		citrulline	C00327	15,84	5,62	1,06	0,19
		N-acetylornithine	C00437	1,73	1	1	1
		argininosuccinate	C03406	0,69	0,41	0,46	1,13
	Butanoate metabolism	2-aminobutyrate	C02261	0,81	0,21	0,26	1,2
	Polyamine metabolism	5-methylthioadenosine (MTA)	C00170	4,48	1,02	4,32	4,24
		putrescine	C00134	14,54	0,64	0,27	0,41
		N-acetylputrescine	C02714	0,58	0,41	0,44	1,07
		agmatine	C00179	5,99	0,62	0,56	0,9
		spermidine	C00315	2,95	0,74	1,53	2,07
	Guanidino and acetamido metabolism	4-guanidinobutanoate	C01035	0,82	0,46	0,96	2,09
	Glutathione metabolism	glutathione, reduced (GSH)	C00051	1,97	0,88	1,44	1,64
		5-oxoproline	C01879	1,84	1,58	2,91	1,84
		glutathione, oxidized (GSSG)	C00127	0,65	0,66	0,11	0,17
		ophthalmate		2,89	0,76	0,65	0,86
Peptide	Dipeptide	glycylvaline		0,7	0,75	8,3	11,08
		glycylglycine	C02037	1,52	0,34	0,87	2,6
		glycylproline		1,51	0,97	1,82	1,87
		glycylisoleucine		1,34	3,3	23,45	7,11
		glycylleucine	C02155	3,11	4,32	37,09	8,59
		glycylphenylalanine		2,63	3,07	19,81	6,45
		glycyltyrosine		2,56	3,45	18,39	5,32
		glycyltryptophan		2,36	2,56	41,34	16,17

		arginylvaline		0,88	3,3	39,56	12
		aspartylphenylalanine		4,05	4,23	17,97	4,25
		prolylglycine		0,55	2,03	4,92	2,42
		leucylproline		3,05	2,28	21,73	9,52
		aspartylvaline		0,81	1,88	10,71	5,7
		aspartylleucine		3,66	4,05	16,73	4,13
		histidylleucine		4,25	5,18	55,07	10,64
		histidylisoleucine*		1,04	0,97	12,18	12,58
		isoleucylalanine		3,45	4,57	13,71	3
		isoleucylarginine		1	1	1,55	1,55
		isoleucylglutamine		2,8	2,12	8,89	4,19
		isoleucylglycine		2,13	3,25	26,7	8,22
		leucylarginine		1	1	1,57	1,57
		leucylglycine		1,07	1,73	4,47	2,58
		valyllysine		0,81	2,66	21,95	8,24
	gamma-glutamyl	gamma-glutamylleucine		6,97	1,12	1,52	1,35
		gamma-glutamylisoleucine*		3,6	2,14	1	0,47
		gamma-glutamylcysteine	C00669	5,34	0,16	0,22	1,37
		gamma-glutamylglycine		49,98	2,18	6,39	2,94
		gamma-glutamylmethionine		1	1	1	1
		gamma-glutamylphenylalanine		2,75	0,73	0,73	1
		gamma-glutamyltyrosine		2,62	1,53	1	0,65
		gamma-glutamylalanine		18,95	1,02	1,42	1,39
	Polypeptide	pro-pro-pro		2,32	0,6	0,88	1,47
Carbohydrate	Aminosugars metabolism	glucosamine	C00329	1	1,13	16,77	14,88
		N-acetylglucosamine	C00140	3,86	62,3	8,45	0,14
		N-acetylglucosamine 6-phosphate	C00357	2,1	1,89	1,85	0,98
		erythronate*		0,99	2	2,49	1,24
	Fructose, mannose, galactose, starch, and sucrose metabolism	fructose	C00095	0,85	152,52	293,59	1,92
		isomaltose	C00252	1,71	1,85	4,86	2,63
		mannitol	C00392	0,89	0,05	0,33	7,02
		mannose-6-phosphate	C00275	1,46	110,32	306,85	2,78
		sorbitol	C00794	10,34	5,39	5,04	0,94
		trehalose	C01083	4,11	2,68	1,82	0,68
		isomaltotriose	C02160	2,84	2,82	0,24	0,08

	Glycolysis, gluconeogenesis, pyruvate metabolism	2-isopropylmalate	C02504	0,41	**2,9**	**5,56**	**1,92**
		glycerate	C00258	**0,47**	**0,57**	1,31	**2,31**
		glucose-6-phosphate (G6P)	C00668	1,11	**116,69**	**375,91**	**3,22**
		glucose 1-phosphate	C00103	1,22	**5,16**	**15,6**	**3,02**
		glucose	C00031	**0,04**	**2,89**	**92,45**	**32,01**
		fructose-6-phosphate	C05345	1	**96,87**	**225,52**	**2,33**
		fructose 1-phosphate	C01094	1	**3,04**	1,88	**0,62**
		Isobar: fructose 1,6-diphosphate, glucose 1,6-diphosphate, myo-inositol 1,4 or 1,3-diphosphate		0,75	**227,72**	**32,48**	**0,14**
		2-phosphoglycerate	C00631	**0,24**	**0,42**	0,91	**2,14**
		3-phosphoglycerate	C00597	**0,24**	0,64	**2,1**	**3,26**
		1,3-dihydroxyacetone	C00184	1,33	1,09	**4,09**	**3,75**
		phosphoenolpyruvate (PEP)	C00074	**0,26**	**0,09**	**0,13**	1,42
		pyruvate	C00022	**2,31**	**2,27**	**6,82**	**3,01**
		lactate	C00186	**2,36**	0,95	**1,35**	**1,42**
		2,3-butanediol	C03044	**4,42**	**0,51**	1,22	**2,39**
	Nucleotide sugars, pentose metabolism	6-phosphogluconate	C00345	1	**1,34**	**1,47**	1,09
		arabitol	C00474	**,33**	0,79	**0,26**	**0,33**
		ribitol	C00474	**4,8**	**1,62**	**1,81**	1,11
		sedoheptulose-7-phosphate	C05382	0,73	**11,12**	**50,03**	**4,5**
		gluconate	C00257	0,67	**26,66**	**10,98**	**0,41**
		ribose	C00121	1,15	1,2	**9,1**	**7,57**
		ribose 5-phosphate	C00117	1	**4,07**	**7,23**	**1,77**
		ribulose	C00309	1,38	**7,16**	**3,75**	**0,52**
		Isobar: ribulose 5-phosphate, xylulose 5-phosphate		1	**6,06**	**4,19**	0,69
		UDP-glucose	C00029	**2,31**	**6,9**	**3,58**	**0,52**
		xylulose	C00310	1,94	**7,81**	**16,91**	**2,17**
Energy	Krebs cycle	citrate	C00158	**8,16**	**39,97**	**75,48**	**1,89**
		homocitrate	C01251	**0,59**	**0,29**	**0,54**	**1,85**
		alpha-ketoglutarate	C00026	**8,6**	**12,82**	**7,71**	0,6
		succinate	C00042	**1,38**	0,97	1,26	**1,3**
		fumarate	C00122	**2,68**	**2,44**	**4,19**	**1,72**
		mesaconate	C01732	1,19	**1,78**	**0,38**	**0,21**

		(methylfumarate)					
		malate	C00149	1,13	**4,06**	**9,07**	**2,24**
	Oxidative phosphorylatio n	acetylphosphate	C00227	**1,61**	1,18	**1,31**	1,11
		phosphate	C00009	0,82	**0,62**	0,96	**1,56**
		pyrophosphate (PPi)	C00013	**1,8**	0,93	**0,04**	**0,04**
Lipid	Essential fatty acid	linoleate (18:2n6)	C01595	**2,8**	**2,46**	**2,43**	0,99
		linolenate [alpha or gamma; (18:3n3 or 6)]	C06427	**2,1**	**1,77**	**2,11**	1,19
	Medium chain fatty acid	caproate (6:0)	C01585	0,63	0,39	1,34	**3,46**
		heptanoate (7:0)	C17714	0,73	0,67	0,83	1,25
		caprylate (8:0)	C06423	**0,32**	0,81	0,93	1,15
		caprate (10:0)	C01571	0,79	**1,53**	0,77	**0,5**
	Long chain fatty acid	myristate (14:0)	C06424	0,79	**2,01**	1,38	**0,69**
		myristoleate (14:1n5)	C08322	**0,47**	**5,7**	1,66	**0,29**
		pentadecanoate (15:0)	C16537	1,05	**4,31**	1,4	**0,32**
		palmitate (16:0)	C00249	1,05	**2,11**	1,47	**0,7**
		palmitoleate (16:1n7)	C08362	**0,48**	**3,34**	**2,02**	**0,6**
		10-heptadecenoate (17:1n7)		1,04	**3,41**	**2,21**	**0,65**
		stearate (18:0)	C01530	1,27	**1,9**	**1,78**	0,94
		oleate (18:1n9)	C00712	0,99	**4,02**	**2,25**	**0,56**
		cis-vaccenate (18:1n7)	C08367	**1,67**	**2,02**	**1,9**	0,94
		conjugated linoleate (18:2n7; 9Z,11E)	C04056	**2,5**	**2,15**	**1,84**	0,85
		eicosenoate (20:1n9 or 11)		**1,63**	**1,49**	**2,2**	1,47
	Fatty acid, monohydroxy	4-hydroxybutyrate (GHB)	C00989	2,04	**16,28**	**5,47**	**0,34**
		2-hydroxypalmitate		**2,15**	**17,36**	**12,14**	**0,7**
	Fatty acid, dicarboxylate	2-hydroxyglutarate	C02630	0,97	**2,54**	**5,68**	**2,23**
	Fatty acid metabolism	isovalerate	C08262	1	1	1	1
	Glycerolipid metabolism	ethanolamine	C00189	1,54	**13,27**	**2,84**	**0,21**
		phosphoethanolamine	C00346	1,25	**3,11**	**2,5**	0,8
		glycerophosphoethan olamine	C01233	**2,06**	**5,18**	2,3	**0,44**
		glycerol	C00116	1,08	1,26	**1,7**	**1,35**
		glycerol 3-phosphate (G3P)	C00093	**3,28**	**2,21**	1,12	**0,51**
		glycerophosphorylchol ine (GPC)	C00670	**3,21**	**2,87**	**3,25**	1,13

		cytidine 5'-diphosphocholine		1,55	0,64	0,46	0,72
	Inositol metabolism	myo-inositol	C00137	1,07	1,46	0,77	0,53
		inositol 1-phosphate (I1P)		0,7	2,2	1,83	0,83
	Ketone bodies	3-hydroxybutyrate (BHBA)	C01089	1,07	0,91	0,88	0,96
		1,2-propanediol	C00717,C02912, C00583,C01506, C02917	3,15	1,83	1,27	0,69
	Lysolipid	1-myristoylglycerophosphoethanolamine		0,34	0,49	0,79	1,62
		1-palmitoylglycerophosphoethanolamine		1,95	0,25	0,98	3,88
		2-palmitoylglycerophosphoethanolamine*		3,69	0,53	0,72	1,38
		1-palmitoleoylglycerophosphoethanolamine*		2,53	0,21	1,25	6,07
		2-palmitoleoylglycerophosphoethanolamine*		1,54	0,28	1,18	4,2
		1-stearoylglycerophosphoethanolamine		1,1	1,11	1,29	1,16
		1-oleoylglycerophosphoethanolamine		3,53	0,14	0,54	3,97
		2-oleoylglycerophosphoethanolamine*		1,95	0,12	1,01	8,34
		1-linoleoylglycerophosphoethanolamine*		2,86	0,44	1,43	3,23
		2-linoleoylglycerophosphoethanolamine*		1,96	1,02	2,65	2,61
		1-myristoylglycerophosphocholine		4,04	0,78	3,89	4,96
		1-palmitoylglycerophosphocholine		8,51	0,67	5,13	7,66
		2-palmitoylglycerophosphocholine*		10,33	1,02	2,61	2,57
		1-palmitoleoylglycerophosphocholine*		2,31	0,42	3	7,22
		2-palmitoleoylglycerophosphocholine*		1,92	0,29	2,42	8,44
		1-stearoylglycerophosphocholine		22,85	0,82	5,4	6,58
		1-oleoylglycerophosphocholine		9,8	0,11	1,19	10,83

		2-oleoylglycerophosphocholine*		4,87	0,17	1,41	8,08
		1-linoleoylglycerophosphocholine	C04100	16,58	0,73	1,6	2,2
		2-linoleoylglycerophosphocholine*		5,28	1	1,01	1,01
		1-palmitoylglycerophosphoinositol*		4,14	4,87	4,63	0,95
		1-palmitoleoylglycerophosphoinositol*		0,48	1,57	1,36	0,87
		2-palmitoleoylglycerophosphoinositol*		0,41	0,55	2,47	4,5
		1-stearoylglycerophosphoinositol		1,96	3,03	3,48	1,15
		1-oleoylglycerophosphoinositol*		1,68	0,3	1	3,32
		2-oleoylglycerophosphoinositol*		1,43	0,18	2,41	13,7
		1-oleoylglycerophosphoserine		1,86	0,5	0,61	1,22
		2-oleoylglycerophosphoserine*		1,59	0,21	1,25	6,02
	Monoacylglycerol	1-palmitoylglycerol (1-monopalmitin)		1,33	3,81	3,05	0,8
	Diacylglycerol	1,3-dipalmitoylglycerol		0,34	5,13	1,8	0,35
	Sphingolipid	sphinganine	C00836	16,91	2,63	18,44	7,01
		phytosphingosine	C12144	11,12	2,72	5,82	2,15
	Mevalonate metabolism	3-hydroxy-3-methylglutarate	C03761	2,68	0,8	0,8	1
		mevalonate	C02104	0,89	0,72	0,34	0,48
	Sterol/Steroid	squalene	C00751	1,78	1,74	3,45	1,98
		lanosterol	C01724	1,98	0,58	1,24	2,15
		ergosterol	C01694	0,56	1,49	2,08	1,4
Nucleotide	Purine metabolism, (hypo)xanthine/inosine containing	xanthine	C00385	0,85	1,57	1,46	0,93
		xanthosine	C01762	1	1	1	1
		hypoxanthine	C00262	1,38	0,52	2,43	4,68
		inosine		1,41	3,43	3,05	0,89
	Purine	adenine	C00147	4,48	0,96	7,87	8,18

	metabolism, adenine containing						
		adenosine	C00212	0,11	0,3	0,58	1,9
		1-methyladenine	C02216	6,23	1,1	1,32	1,2
		N1-methyladenosine	C02494	0,91	0,84	2,4	2,87
		2'-deoxyadenosine	C00559	2,08	2,68	1,69	0,63
		adenosine 3'-monophosphate (3'-AMP)	C01367	0,77	2,61	6,56	2,52
		adenosine 5'-monophosphate (AMP)	C00020	0,78	0,14	1,24	8,69
		adenosine 5'-diphosphate (ADP)	C00008	0,97	3,35	3,89	1,16
		adenosine-2',3'-cyclic monophosphate	C02353	0,54	1,01	1,9	1,87
		adenylosuccinate	C03794	1,15	0,13	1,93	15,08
	Purine metabolism, guanine containing	guanine	C00242	0,21	0,21	0,56	2,73
		7-methylguanine	C02242	0,55	0,8	3,11	3,89
		guanosine	C00387	0,43	0,85	1,07	1,26
		guanosine 5'-monophosphate (5'-GMP)		0,17	0,02	0,82	35,36
		guanosine-2',3'-cyclic monophosphate	C06194	1,63	1,35	3,34	2,48
		2'-O-methylguanosine	C04545	1,8	0,64	2,65	4,13
		N6-carbamoylthreonyladenosine		3,63	0,36	0,35	0,96
	Pyrimidine metabolism, cytidine containing	cytidine	C00475	0,81	1,63	2,79	1,71
		cytidine 5'-monophosphate (5'-CMP)	C00055	0,33	0,04	0,61	16,47
	Pyrimidine metabolism, orotate containing	orotate	C00295	0,24	0,18	3,31	18,29
	Pyrimidine metabolism, thymine containing	thymine	C00178	0,66	0,79	1,39	1,75
		thymidine	C00214	2,1	2,34	0,63	0,27
		thymidine 5'-monophosphate	C00364	1,55	0,2	0,55	2,79
	Pyrimidine metabolism, uracil	uracil	C00106	1,09	1,27	1,45	1,14

	containing						
		uridine	C00299	1,56	1,24	2,84	2,3
		pseudouridine	C02067	0,9	0,76	1,09	1,45
		uridine monophosphate (5' or 3')		0,49	0,03	1,11	33,96
	Purine and pyrimidine metabolism	methylphosphate		1,17	1,52	1,6	1,06
Cofactors and vitamins	Hemoglobin and porphyrin metabolism	5-aminolevulinate	C00430	1,77	0,73	1,19	1,62
	Nicotinate and nicotinamide metabolism	nicotinamide	C00153	0,62	0,97	0,95	0,97
		nicotinamide ribonucleotide (NMN)	C00455	0,3	1,85	1,74	0,94
		nicotinamide adenine dinucleotide (NAD+)	C00003	0,67	1,26	2,15	1,7
		nicotinamide adenine dinucleotide reduced (NADH)	C00004	1,23	3,75	7,12	1,9
		quinolinate	C03722	1,21	0,08	0,06	0,8
		nicotinate	C00253	0,48	0,66	12,97	19,8
		nicotinate ribonucleoside*		1,88	3,9	31,87	8,18
	Pantothenate and CoA metabolism	pantothenate	C00864	1,02	9,75	7,24	0,74
		phosphopantetheine	C01134	1,3	0,25	0,55	2,2
		coenzyme A	C00010	2,09	1	2,66	2,65
		3'-dephosphocoenzyme A	C00882	1,32	1,11	2,49	2,24
		acetyl CoA	C00024	1,58	0,73	5,13	7,02
		2,3-dihydroxyisovalerate	C04039	0,75	10,25	42,65	4,16
	Pyridoxal metabolism	pyridoxal	C00250	1,66	2,67	1,74	0,65
	Riboflavin metabolism	flavin adenine dinucleotide (FAD)	C00016	1,47	0,54	0,47	0,87
		riboflavin (Vitamin B2)	C00255	1	1	1	1
		flavin mononucleotide (FMN)	C00061	0,79	1,22	0,75	0,62
	Thiamine metabolism	thiamin (Vitamin B1)		0,65	2,08	5,6	2,69
		thiamin diphosphate	C00068	0,6	1,17	1,21	1,04
	Vitamin B6 metabolism	pyridoxine (Vitamin B6)	C00314	1,8	1,32	1,41	1,07
Xenobiotics	Benzoate metabolism	p-aminobenzoate (PABA)	C00568	1,02	1,2	0,57	0,48
	Chemical	glycolate (hydroxyacetate)	C00160	1,12	1,54	4,54	2,96

		glycerol 2-phosphate	C02979,D01488	2,6	2,57	2,19	0,85
		2-pyrrolidinone		1,32	14,06	6,62	0,47
		oxamate	C01444	1,25	1,15	0,96	0,84
	Food component/Plant	allose	C01487	5,74	2,67	0,67	0,25
		quinate	C00296	0,44	5,2	9,61	1,85
		shikimate	C00493	3,48	6,87	0,31	0,05
	Sugar, sugar substitute, starch	trehalose 6-phosphate	C00689	1	2,48	1,17	0,47
		erythritol	C00503	3,01	4,8	6,87	1,43
	Bacterial	3-deoxyoctulosonate		0,96	1,3	1,13	0,87
N/A	N/A	glucosylglycerol		1,12	1,18	4,59	3,89

Annex 3

Supplementary data of Chapter 2

UNIVERSITAT ROVIRA I VIRGILI
METABOLIC AND MOLECULAR ADAPTATION OF WINE YEASTS AT LOW TEMPERATURE FERMENTATION:
STRATEGIES FOR THEIR GENETIC IMPROVEMENT
María López Malo
DL: T. 1275-2013

Table S1. Percentage of phospholipids (PI, PS, PC, PE, CL, PA and MM-PE), neutral lipids (DG and TG), sterols (squalene, lanosterol, ergosterol and sterol esters) and fatty acids (C14:1, C16, C16:1, C18 and C18:1) expressed as the mean ± SEM (standard error of the mean) of total cellular concentration of these compounds. *Significant differences compared with their respective control strains (*ho*QA23 and *ho*QA23 pGREG).

	PHOSPHOLIPIDS							NEUTRAL LIPIDS	
Strains	**PI**	**PS**	**PC**	**PE**	**CL**	**PA**	**MM-PE**	**DG**	**TG**
*ho*QA23	17.89 ± 1.16	6.11 ± 0.86	33.70 ± 2.72	25.25 ± 1.47	12.82 ± 2.83	4.23 ± 0.42	-	20.46 ± 2.43	79.54 ± 2.43
Δ*psd1*	38.10 ± 2.56*	1.50 ± 0.61*	13.54 ± 4.34*	-	39.05 ± 9.25*	7.81 ± 1.74*	-	39.59 ± 1.19*	60.41 ± 1.19*
Δ*opi3*	59.86 ± 1.39*	0.94 ± 0.13*	-	5.42 ±1.89*	10.23 ± 0.96	2.02 ± 0.43*	21.53 ± 1.39*	32.25 ± 8.07	67.75 ± 8.07
Δ*erg3*	12.49 ± 0.01*	4.66 ± 0.34*	28.34 ± 2.96	21.26 ± 3.97	18.45 ± 0.70*	14.80 ± 1.37*	-	18.25 ± 4.38	81.75 ± 4.38
Δ*erg6*	19.72 ± 0.28	4.46 ± 0.63	20.65 ± 0.32*	17.84 ± 0.90*	27.69 ± 0.57*	9.65 ± 0.92*	-	30.50 ± 6.66	69.50 ± 6.66
Δ*dpl1*	8.42 ± 2.11*	7.02 ± 0.19	39.74 ± 2.30*	23.38 ± 1.53	nq	nq	-	20.62 ± 1.08	79.38 ± 1.08
Δ*lcb3*	10.89 ± 5.47	7.24 ± 1.15	39.15 ± 4.69	26.28 ± 3.25	nq	nq	-	22.51 ± 4.54	77.49 ± 4.54
Δ*lcb4*	13.51 ± 1.03*	6.09 ± 0.41	41.94 ± 3.77*	20.70 ± 3.53	nq	nq	-	29.96 ± 1.84*	70.04 ± 1.84*
*ho*QA23 pGREG	10.34 ± 1.27	6.78 ± 0.76	48.61 ± 3.12	22.16 ± 1.57	3.07 ± 2.79	9.04 ± 1.84	-	29.06 ± 2.67	70.94 ± 2.67
pGREG *PSD1*	7.69 ± 0.61	7.31 ± 0.80	46.70 ± 3.25	27.30 ± 2.96	4.31 ± 0.36	6.70 ± 0.25	-	29.24 ± 2.43	70.76 ± 2.43
pGREG *OPI3*	16.15 ± 4.32	7.64 ± 0.26	28.51 ± 3.46*	24.34 ± 3.69	20.25 ± 5.50	3.10 ± 0.68	-	44.46 ± 1.75*	55.54 ± 1.75*
pGREG *CHO2*	3.46 ± 0.03*	8.85 ± 0.07*	35.79 ± 2.07*	31.98 ± 2.21*	18.30 ± 6.67*	3.25 ± 0	-	29.70 ± 4.70	70.30 ± 4.70
pGREG *ERG3*	10.10 ± 0.55	6.87 ± 0.25	42.27 ± 6.42	19.58 ± 3.51	13.84 ± 1.68*	7.34 ± 0.42	-	31.53 ± 5.45	68.47 ± 5.45
pGREG *ERG6*	15.73 ± 0.28*	6.38 ± 0.63	40.49 ± 5.74	30.58 ± 0.91	4.65 ± 0.57	2.17 ± 0.92*	-	23.51 ± 4.25	76.49 ± 4.25
pGREG *IDI1*	8.87 ± 3.27	5.05 ± 0.66*	40.55 ± 0.96*	28.39 ± 2.83*	4.05 ± 0.71	13.09 ± 0.87*	-	21.83 ± 3.27	78.17 ± 3.27
pGREG *OLE1*	23.01 ± 6.77*	8.00 ± 0.64	46.55 ± 4.90	6.41 ± 1.50*	9.91 ± 0.59*	6.11 ± 0.32	-	12.51 ± 0.27*	87.49 ± 0.27*
pGREG *DPL1*	12.05 ± 1.79	8.82 ± 0.44	53.26 ± 2.99	9.96 ± 0.38*	nq	nq	-	17.88 ± 3.63*	82.12 ± 3.63
pGREG *LCB3*	3.16 ± 0.73*	5.24 ± 0.19*	36.63 ± 2.65*	41.48 ± 2.66*	nq	nq	-	29.50 ± 3.67	70.50 ± 3.67
pGREG *LCB4*	7.58 ± 0.00*	5.79 ± 0.33*	37.83 ± 3.92*	36.35 ± 9.85*	nq	nq	-	30.80 ± 7.31	69.20 ± 7.31*

(cont.)	STEROLS				FATTY ACIDS				
Strains	**Squalene**	**Lanosterol**	**Ergosterol**	**Sterol esters**	**C14:1**	**C16**	**C16:1**	**C18**	**C18:1**
*ho*QA23	6.69 ± 1.07	4.09 ± 0.69	30.80 ± 4.30	58.43 ± 4.68	-	13.62 ± 0.64	32.55 ± 0.19	3.66 ± 0.51	50.18 ± 0.90
Δ*psd1*	1.69 ± 0.09*	8.98 ± 0.25*	33.92 ± 2.28	55.41 ± 1.94	-	13.18 ± 0.88	33.07 ± 1.47*	6.57 ± 2.88	47.19 ± 2.60
Δ*opi3*	12.19 ± 3.95	25.00 ± 4.19*	36.86 ± 2.83	25.94 ± 6.16*	-	16.53 ± 0.37*	43.44 ± 1.54	2.66 ± 2.35	37.37 ± 0.54*
Δ*erg3*	4.73 ± 1.08	5.36 ± 2.47	45.16 ± 5.55	44.74 ± 7.41	-	17.08 ±1.37*	41.92 ± 1.20*	3.23 ± 1.15	35.78 ± 1.43*
Δ*erg6*	10.15 ± 0.72	1.88 ± 2.62	45.78 ± 10.35	42.20 ± 13.69	12.39 ± 4.95*	15.22 ± 0.99	33.45 ± 1.73*	2.35 ± 0.37	34.61 ± 2.78*
Δ*dpl1*	4.19 ± 0.98	3.11 ± 0.65	34.46 ± 1.89	58.24 ± 2.06	-	17.93 ± 1.09*	35.14 ± 1.08*	3.78 ± 0.24	42.62 ± 0.15*
Δ*lcb3*	10.71 ± 3.03	2.85 ± 0.75	30.05 ± 3.07	56.39 ± 4.22	-	19.30 ± 3.31	41.62 ± 2.53*	-	39.08 ± 0.78*
Δ*lcb4*	7.47 ± 2.95	3.60 ± 1.55	43.47 ± 3.49*	45.45 ± 3.01*	-	22.06 ± 1.46*	37.37 ± 1.06*	2.22 ± 1.92	38.35 ± 2.48*
*ho*QA23 pGREG	6.50 ± 1.08	-	15.95 ± 0.12	77.55 ± 1.20	-	22.53 ± 1.02	33.88 ± 1.70	5.37 ± 1.87	35.81 ± 1.09
pGREG *PSD1*	9.59 ± 2.39	-	15.39 ± 3.06	75.02 ± 0.67	-	22.43 ± 0.93	32.89 ± 0.84	4.13 ± 0.10	38.24 ± 1.57
pGREG *OPI3*	3.73 ± 0.67*	1.72 ± 0.11*	24.11 ± 1.21*	70.44 ± 1.99	-	17.65 ± 1.25*	41.50 ± 2.12*	1.05 ± 1.81*	39.81 ± 0.36*
pGREG *CHO2*	3.69 ± 1.44	2.56 ± 0.90*	20.19 ± 4.31	73.57 ± 6.42	-	17.60 ± 0.87*	36.45 ± 1.07	3.31 ± 0.22	42.65 ± 0.03*
pGREG *ERG3*	3.68 ± 0.48*	0.30 ± 0.16	12.95 ± 1.87	83.08 ± 1.94*	-	17.11 ± 0.29*	37.19 ± 1.16*	3.34 ± 0.32	40.30 ± 1.62
pGREG *ERG6*	2.85 ± 0.00*	-	14.44 ± 2.03	82.71 ± 2.03	-	18.35 ± 0.99*	35.51 ± 0.67	3.12 ± 3.03	43.02 ± 2.83*
pGREG *IDI1*	3.97 ± 0.32*	-	11.26 ± 2.83	84.77 ± 2.88*	-	20.29 ± 0.99*	36.47 ± 0.67*	3.31 ± 3.03	39.93 ± 2.83
pGREG *OLE1*	4.61 ± 1.10	2.28 ± 0.52	13.14 ± 1.39	79.98 ± 3.02	-	12.25 ± 0.82*	41.93 ± 0.39*	3.95 ± 0.30	41.88 ± 1.36*
pGREG *DPL1*	3.18 ± 0.60*	0.89 ± 0.12*	10.05 ± 0.46	85.87 ± 0.30*	-	16.80 ± 0.34*	35.77 ± 1.74	3.96 ± 0.35	43.20 ± 1.39*
pGREG *LCB3*	8.01 ± 2.52	9.34 ± 4.55	12.57 ± 1.70	70.08 ± 8.70	-	19.91 ± 0.34	36.37 ± 1.74	3.97 ± 0.35	39.75 ± 1.39*
pGREG *LCB4*	1.90 ± 0.36*	2.23 ± 0.95	11.45 ± 1.89	84.42 ± 2.05*	-	21.35 ± 0.44*	33.58 ± 2.41	5.06 ± 1.40	38.90 ±1.80*

(-) not detected, (nq) not quantified

Annex 4

Supplementary data of Chapter 4

Table S1: Non-synonymous single nucleotide polymorphism (SNPs) identified in coding regions in P5-M strain in comparison to parental strain (P5-O).

Chromosome	Reference base	Alternate base	Systematic name	Standard name	Change
chrIV	G	A	YDR283C	*GCN2*	R371C
chrXIV	G	A	YNL189W	*SRP1*	A201T
chrVIII	C	T	YHR023W	*MYO1*	T901M
chrXII	C	T	YLR088W	*GAA1*	T108I
chrXIII	G	A	YML006C	*GIS4*	P201S
chrXIV	G	A	YNL027W	*CRZ1*	S231N
chrVII	C	A	YGR231C	*PHB2*	A294S
chrVIII	G	A	YHL016C	*DUR3*	A107V
chrV	G	A	YER172C	*BRR2*	T1809I
chrXIV	C	T	YNL166C	*BNI5*	D302N
chrVIII	A	T	YHR187W	*IKI1*	T97S
chrIX	C	T	YIL129C	*TAO3*	G1654D
chrVI	C	T	YFR002W	*NIC96*	Q280*
chrV	C	T	YER060W-A	*FCY22*	A403V
chrXV	C	T	YOR284W	*HUA2*	T90I
chrV	C	T	YER045C	*ACA1*	G41S
chrIV	C	T	YDR259C	*YAP6*	E318K
chrXVI	G	A	YPR117W		G194S
chrX	A	G	YJR062C	*NTA*	C102R
chrIV	G	A	YDR175C	*RSM24*	P181S
chrXIV	C	G	YNL132W	*KRE33*	S549C
chrIV	G	A	YDL190C	*UFD2*	S547F
chrIV	C	T	YDR484W	*VPS52*	D429D
chrXVI	G	A	YPR002W	*PDH1*	D428N
chrVIII	G	A	YHR077C	*NMD2*	P630L
chrXII	A	G	YLR016C	*PML1*	M29T
chrXVI	C	T	YPL209C	*IPL1*	G263R
chrX	G	A	YJL089W	*SIP4*	D464N
chrXIII	G	A	YML084W		G28E
chrIV	G	A	YDR081C	*PDC2*	A678V
chrXIII	G	C	YMR230W	*RPS10B*	L80F
chrVII	G	A	YGL021W	*ALK1*	R717K
chrIV	A	T	YDR036C	*EHD3*	F121I
chrVII	C	T	YGR002C	*SWC4*	D388N
chrX	C	T	YJR137C	*MET5*	S801N
chrXII	C	T	YLR084C	*RAX2*	S1036N
chrXVI	A	T	YPL125W	*KAP120*	Q297L
chrVIII	A	T	YHR109W	*CTM1*	E149V
chrV	C	T	YER123W	*YCK3*	A291V
chrII	T	C	YBR097W	*VPS15*	I860T

Table S2: Non-synonymous single nucleotide polymorphism (SNPs) identified in coding regions in P5-C strain in comparison to parental strain (P5-O).

Chromosome	Reference base	Alternate base	Systematic name	Standard name	Change
chrXVI	C	T	YPR010C	*RPA135*	A488T
chrXI	G	A	YKL010C	*UFD4*	T301M
chrIV	T	A	YDR083W	*RRP8*	L3I
chrIV	A	G	YDR257C	*RKM4*	L435P

Table S3: Significant different gene expression list, with at least two fold differences in transcript levels (log2 fold were ≤-1 or ≥ 1), between P5-O and P5-M at low temperature fermentation.

Gene	Molecular function	Ratio
SFC1	Mitochondrial succinate-fumarate transporter	-3.52
GIT1	glycerophosphodiester transmembrane transporter	-3.20
PUT1	Proline oxidase	-2.69
ZEO1	Molecular_function unknown	-2.42
SPS100	Molecular_function unknown	-2.37
CHA1	Catabolic L-serine (L-threonine) deaminase	-2.31
HMX1	ER localized, heme-binding peroxidase	-2.19
NCA3	Molecular_function unknown	-2.12
PRM5	Pheromone-regulated protein	-2.08
HPA2	Tetrameric histone acetyltransferase	-1.94
RLP7	rRNA binding	-1.87
CHS1	Chitin synthase I	-1.83
RPF1	rRNA primary transcript binding	-1.80
KRR1	Molecular_function unknown	-1.77
CRG1	Putative S-adenosylmethionine-dependent methyltransferase	-1.75
DRS1	ATP-dependent RNA helicase activity	-1.69
SMC3	ATPase activity	-1.66
ENP2	Molecular_function unknown	-1.57
PRY2	Molecular_function unknown	-1.53
POG1	Molecular_function unknown	-1.52
BFR2	Molecular_function unknown	-1.49
MND1	Double-stranded DNA binding	-1.48
TRM11	RNA binding	-1.46
MTW1	Molecular_function unknown	-1.45
MOD5	tRNA dimethylallyltransferase activity	-1.45
FPR4	Peptidyl-prolyl cis-trans isomerase (PPIase) (proline isomerase)	-1.45
MDN1	ATPase activity	-1.42
MGA1	DNA binding	-1.42
YPS3	Aspartic-type endopeptidase activity	-1.40
REX4	3'-5' exonuclease activity	-1.39
TOD6	Sequence-specific DNA binding	-1.39
CIC1	Protein binding, bridging	-1.39
SRN2	Protein binding	-1.38

JEN1	Secondary active monocarboxylate transmembrane transporter	-1.35
DFG5	Mannosidase activity	-1.34
NDE1	NADH dehydrogenase	-1.34
SLK19	Molecular_function unknown	-1.34
PDC6	Pyruvate decarboxylase	-1.33
YSY6	Molecular_function unknown	-1.32
PRM4	Molecular_function unknown	-1.30
OGG1	Oxidized purine nucleobase lesion DNA N-glycosylase	-1.30
SCW10	glucosidase activity	-1.29
CSE2	RNA polymerase II transcription coactivator	-1.29
CWC21	Molecular_function unknown	-1.29
NOP14	snoRNA binding	-1.26
BUR6	Transcription coactivator	-1.26
MUD2	pre-mRNA branch point binding	-1.25
PEX13	Protein binding, bridging	-1.25
RET1	Contributes_to DNA-directed RNA polymerase activity	-1.25
LTV1	Molecular_function unknown	-1.25
MRD1	Molecular_function unknown	-1.24
OYE3	NADPH dehydrogenase	-1.23
PES4	Molecular_function unknown	-1.23
PSK2	Protein serine/threonine kinase	-1.23
NSA1	Molecular_function unknown	-1.23
URB1	Molecular_function unknown	-1.21
UBP5	Ubiquitin-specific protease	-1.20
TMA10	Molecular_function unknown	-1.19
GRX6	Glutathione-disulfide reductase activity	-1.19
HCA4	ATP-dependent RNA helicase activity	-1.19
HAL5	Protein kinase activity	-1.17
CSR2	Ubiquitin protein ligase binding	-1.17
LHP1	RNA binding	-1.16
KAR3	Minus-end-directed microtubule motor;protein heterodimerization activity	-1.15
PWP1	Molecular_function unknown	-1.15
LHS1	Adenyl-nucleotide exchange factor activity; ATP binding	-1.15
PDS5	Structural molecule	-1.14
ACS1	Acetate-CoA ligase	-1.12

ICT1	Lysophosphatidic acid acyltransferase	-1.12
NOP2	RNA methyltransferase	-1.12
ALB1	Molecular_function unknown	-1.12
RLP24	Molecular_function unknown	-1.11
CRH1	Transferase activity, transferring glycosyl groups	-1.11
GYP7	Rab GTPase activator	-1.11
ROK1	ATP-dependent RNA helicase	-1.11
SLX9	Molecular_function unknown	-1.10
IBD2	Molecular_function unknown	-1.10
PSH1	ubiquitin-protein ligase	-1.10
PTR2	Di and tripeptide transporter	-1.10
SMP1	DNA binding, bending	-1.09
CBF2	Centromeric DNA binding	-1.09
KRE11	Contributes_to Rab guanyl-nucleotide exchange factor activity	-1.09
PEP12	SNAP receptor	-1.08
GRC3	Polynucleotide 5'-hydroxyl-kinase	-1.08
ADI1	Acireductone dioxygenase (Ni2+-requiring)	-1.08
JJJ1	ATPase activator	-1.08
PRM10	Molecular_function unknown	-1.08
CDC7	Protein serine/threonine kinase activity	-1.07
RTS3	Molecular_function unknown	-1.07
ECM16	RNA helicase activity	-1.07
PTP2	Protein tyrosine phosphatase activity	-1.06
RCR1	Molecular_function unknown	-1.06
YIP3	Molecular_function unknown	-1.06
STU1	Structural constituent of cytoskeleton	-1.06
ERO1	Protein disulfide isomerase activity	-1.05
PHO84	Inorganic phosphate transmembrane transporter activity	-1.05
LCB5	D-erythro-sphingosine kinase activity	-1.05
RAD4	Damaged DNA binding	-1.05
ATG11	Protein complex scaffold	-1.04
DBP10	ATP-dependent RNA helicase	-1.04
RBA50	Molecular_function unknown	-1.03
NMD3	Ribosomal large subunit binding	-1.02
SGD1	Molecular_function unknown	-1.02
NOC2	Molecular_function unknown	-1.02

RIO2	Nucleocytoplasmic transporter	-1.01
CTF4	Chromatin binding; DNA binding	-1.01
GFD2	Molecular_function unknown	-1.01
SRP40	Molecular_function unknown	-1.00
IMD2 /// IMD3	Chromatin binding;IMP dehydrogenase	1.03
PCL9	Cyclin-dependent protein kinase regulator	1.03
PER1	Molecular_function unknown	1.04
ERS1	L-cystine transmembrane transporter	1.06
AIM20	Molecular_function unknown	1.07
DSD1	D-serine ammonia-lyase	1.07
TIP1	Lipase activity;structural constituent of cell wall	1.07
UTR2	Transferase activity, transferring glycosyl groups	1.09
HPA3	D-amino-acid N-acetyltransferase	1.10
RPS29A	Structural constituent of ribosome	1.10
COX18	Membrane insertase	1.11
CTS1	Endochitinase activity	1.12
FLO1 /// FLO5 /// FLO9	Mannose binding	1.13
SDP1	MAP kinase tyrosine phosphatase	1.14
DSF1	Molecular_function unknown	1.14
SMK1	MAP kinase	1.15
YPS6	Aspartic-type endopeptidase activity	1.15
POR2	Voltage-gated anion channel	1.15
ELO1	Fatty acid elongase activity	1.16
MUC1	Molecular_function unknown	1.16
DAK2	Glycerone kinase	1.19
SUT1	RNA polymerase II repressing transcription factor binding; positive regulation of sterol import by positive regulation of transcription from RNA polymerase II promoter	1.19
HOF1	Cytoskeletal protein binding	1.20
TNA1	Nicotinamide mononucleotide transmembrane transporter	1.20
RPS1A	Structural constituent of ribosome	1.21
ASG7	Molecular_function unknown	1.26
NFT1	ATPase activity, coupled to transmembrane movement of substances	1.29
AMN1	Protein binding	1.33

DSE2	Glucosidase	1.38
ATF2	Alcohol O-acetyltransferase	1.39
BNA4	Kynurenine 3-mono oxigenase	1.40
SCW11	Glucan endo-1,3-beta-D-glucosidase	1.41
PRY3	Molecular_function unknown	1.42
SUN4	Glucosidase	1.44
AUS1	Sterol-transporting ATPase	1.49
PHO3	Acid phosphatase activity	1.52
AAC3	ATP:ADP antiporter	1.58
NRT1	Nicotinamide riboside transmembrane transporter activity	1.62
ANB1	Ribosome binding; RNA binding; translation elongation factor	1.70
TIR2	Molecular_function unknown	1.70
DSE4	Glucan endo-1,3-beta-D-glucosidase	1.73
SUC2	Beta-fructofuranosidase	1.87
TIR3	Molecular_function unknown	1.93
TIR4	Molecular_function unknown	2.00
IMD2	Chromatin binding;IMP dehydrogenase	2.07
EGT2	cellulase activity	2.16
IMD1	Molecular_function unknown	2.22
VEL1	Molecular_function unknown	2.23
TIR1	Structural constituent of cell wall	2.35
HPF1	Glucosidase	2.64
DAN1	Molecular_function unknown	4.17