UNIVERSITAT ROVIRA i VIRGILI

DEPARTAMENT DE BIOQUÍMICA i BIOTECNOLOGIA

FACULTAT D'ENOLOGIA

EFFECT OF LOW TEMPERATURE FERMENTATION AND NITROGEN CONTENT ON WINE YEAST METABOLISM

Memoria presentada per

GEMMA BELTRAN i CASELLAS

per optar al grau de Doctora per la Universitat Rovira i Virgili

sota la direcció del Dr. José Manuel Guillamón i el Dr. Nicolas Rozès

Tarragona, 2005

UNIVERSITAT
ROVIRA I VIRGILI

Dept.Bioquímica i Biotecnologia
FACULTAT D'ENOLOGIA
C/Marcel·lí Domingo s/n
43007 Tarragona
Tel. 34-977558442 / 34-977558280
Fax. 34-977558232
E-mail: josemanuel.guillamon@urv.net
nicolasrozes@urv.net

Els sotasignants, Dr José Manuel Guillamón Navarro, Professor Titular d'Universitat de Nutrició i Bromatologia del Departament de Bioquímica i Biotecnologia de la Universitat Rovira i Virgili, i

El Dr Nicolas Rozès, Professor Titular d'Escola Universitària de Nutrició i Bromatologia del Departament de Bioquímica i Biotecnologia de la Universitat Rovira i Virgili

FAN CONSTAR,

Que el present treball, amb títol '**Effect of low temperature and nitrogen content on wine yeast metabolism**' que presenta la **Srta Gemma Beltran Casellas**, per optar al Grau de Doctora per la Universitat Rovira i Virgili ha estat realitzat sota la nostra direcció, i que tots els resultats obtinguts són fruit dels experiments duts a terme per l'esmentada doctoranda.

I perquè se'n prengui coneixement i tingui els efectes que correspongui, signem aquesta certificació.

Dr. José Manuel Guillamón   Dr. Nicolas Rozès

Tarragona, 23 de desembre de 2004

Als meus pares

A l'Adrià

AGRAÏMENTS

Voldria agrair a totes les persones que d'una manera o altra han contribuït a que aquesta tesi s'hagi dut a terme, i que m'han mostrat suport durant tots aquests anys.

En primer lloc als meus directors de tesi, Dr. José Manuel Guillamón i Dr. Nicolas Rozès, sense els quals no hauria estat possible la realització d'aquest treball. Als dos voldria agrair la seva dedicació i suport, així com haver-me transmès el seus coneixements però sobretot el seu entusiasme, característica indispensable en qualsevol investigador i que intentaré guardar sempre amb mi.

Al Dr. Albert Mas, per confiar en mi des d'un primer moment, i oferir-me la possibilitat d'incorporar-me al seu grup de treball, i d'aquesta manera permetre'm descobrir el món dels llevats i la fermentació alcohòlica, tant desconegut per a mi en un principi, però que poc a poc ha arribat a captivar-me.

A tots membres del tribunal de la tesi, per acceptar formar-ne part del mateix, així com a Jean Luc Parrou i Paolo Guidicci, per acceptar fer l'informe d'avaluació de la tesi. És realment un honor i un plaer per a mi. Moltes gràcies.

A tots els professors i persones del Departament de Bioquímica i Biotecnologia, així com als de la Facultat d'Enologia que m'han donat suport i m'han ajudat en que ha fet falta durant tots aquest anys. A les secretaries del departament, Mireia i Cristina, per la seva eficiència i disposició, en especial aquests últims mesos, i també a la Montse Poblet, Isabel Baiges i Santiago Moreno, que m'han aconsellat i ajudat a obtenir el que he necessitat per la realització dels experiments.

A tots els meus companys i persones que he tingut a prop durant aquest temps. En especial voldria agrair la seva amistat a la Xus i la Maite (mis angeles) i com no, a la Sumpta, companyes i amigues, que han estat sempre al meu costat, tant dins com fora del laboratori, gràcies per ser-hi, pels savis consells i per tants bons moments, que espero continuin durant molt de temps. A la resta de companys del laboratori, amb els que he compartit poiata, tertúlies al pigsa, cine, i molts altres moments, gràcies a tots, tant als d'abans (la vieja guardia), ja doctors: Miriam, Josep, Ramon, Cristina, Pep, Judit,... que hem van fer sentir molt ben acollida des d'un primer moment i em van transmetre la seva experiència com a investigadors, com els d'ara: Núria, Rosanna, Mireia, Angel, Marian, Zoel, Roser, Carmen, els analítics Marc, Pili i Montse, i tots els que han estat de pas, per fer del petit convent del Ramon y Cajal nº70 quelcom més que un simple lloc de treball.

A Emilia Matallana per acollir-me en el seu laboratori del IATA a València durant una breu estada i permetre'm realitzar i aprendre la tècnica de Northerns-blot. Gràcies en especial a la Geno, la meva mestra durant 2 setmanes, amb qui va ser un plaer treballar i conversar, i a la resta de companys, Agustín, Marcel·lí, Roberto, Puri, Elena i Aurora, que, tot i que per poc temps, em van fer sentir com a casa.

Je voudrais remercier à Jean Maire François pour m'avoir accueilli dans son labo et me permettre réaliser et apprendre l'analyse de puces de DNA, aussi lui remercier sa confiance en moi et m'avoir donné l'opportunité de travailler dans son équipe pendant une année. C'était pour moi un vrai plaisir travailler avec Jean Luc, un modèle à suivre au niveau scientifique et aussi personnel, je voudrais lui remercier sa patience et son

bon encadrement, et m'avoir faire connaître la tréhalose chez les levures. Au personnel de la plate-forme, Véronique, Adilia, Sergueï et Delphine, qui m'ont aidé avec l'analyse et interprétation des puces, merci pour votre aide et patience. Je voudrais aussi remercier au reste de copains du labo: Marie-Ange, Matthieu, Olivier, Marlene, Helène C., Helène M., Lidwine, Celine, Fadi, et tout l'équipe JMF en général, pour son accueil, aide et conseils quand il a fallu. Y una mención especial a la parte hispana de Toulouse (Filipa, Angelica, Carles, Juan Carlos, Maria, Maite y Xus) por su apoyo y sus risas, y con los que la estancia en Francia fue muy agradable. Y como no, a Sandra, Juan y Santi, amigos ya para siempre, por hacernos sentir como una gran familia y demostrarnos que aún existe gente con el corazón tan grande como ellos.
Je voudrais bien evidentment remercier à Tristan, pour une très bonne concurrance scientiphique, pour son soutien et aide, même en la distance.

Gràcies a tots els que d'alguna manera m'heu ajudat aquest últims mesos en la escriptura i realització de la tesi. Al Xavi, per ajudar-me amb el disseny i elaboració de la portada, així com pel seu suport i amistat durant tot aquest temps; a la Cristina, Tristan i els companys del servei lingüístic per ajudar-me i aconsellar-me en bona part de les correccions lingüístiques de la tesi; a la Xus, la Maite, el Josep, la Rosanna per ajudar-me a resoldre aspectes i dubtes tant tècnics com científics, a la Susanna per la seva paciència, consells i aliments i a la Sumpta, per escoltar-me i ajudar-me a desconnectar i desestressar-me quan m'ha fet falta.

Als meus amics de sempre, per ser-hi i estar sempre al meu costat.

I per acabar voldria agrair de manera molt especial als meus pares, àvia i germans, Susanna, Mari i Xavier, així com a la resta de família, per haver-me donat suport incondicional en tot moment i haver acceptat i entès les meves inquietuds. Al petit Adrià, que sols la seva presència transmet alegria i il·lusió, i m'ha ajudat a superar de manera més amena aquests difícils mesos d'escriptura.

Gràcies a tots i molta sort.

INDEX

OBJECTIVES & OUTLINE OF THESIS ____ **1**

INTRODUCTION ____ **7**

CHAPTER 1 ____ **63**

"Effect of fermentation temperature and culture media on the lipid composition and volatile compounds in wine"

CHAPTER 2 ____ **87**

"Integration of transcriptomic and metabolomic analyses for understanding the global responses of low temperature winemaking fermentations."

CHAPTER 3 ____ **123**

"Nitrogen Catabolite Repression in *Saccharomyces cerevisiae* during wine fermentation."

CHAPTER 4 ____ **139**

"Influence of the timing of nitrogen additions during wine fermentations on the fermentation kinetics and nitrogen consumption."

CHAPTER 5 ____ **161**

"Effect of low temperature fermentation on yeast nitrogen metabolism."

CONCLUSIONS AND PERSPECTIVES ____ **173**

ANNEXES ____ **187**

Annex1: Materials and Methods 189
Annex2: Supplementary data of yeast functional genomics at low temperature 221
Annex3: "Fermentaciones a bajas temperaturas" 235

OBJECTIVES

&

OUTLINE OF THESIS

OBJECTIVES

The winemaking industry is facing continuous challenges in the present global market. Those involving the quality and reproducibility of the product, which depend heavily on yeast metabolism, are of particular interest. Finding ways to improve fermentation performance, wine flavour and other sensory qualities are becoming important objectives for wine producers.

When I joined the research group into Enological Biotechnology at the Department of Biochemistry and Biotechnology (Faculty of Enology, Rovira i Virgili University, Tarragona, SPAIN), the research objectives focused on the characterization of yeast metabolism in low temperature fermentations. Low temperature fermentations have interesting applications in the wine industry, especially in the production of wines with more pronounced aromatic profile. Our research group had already collaborated with the cellar Torres S.A (Spain) and the yeast producers Lallemand S.A. (Toulouse, France) to further our knowledge of low temperature fermentations, particularly in terms of changes in yeast metabolism and improvements in fermentation performance. This work was supported by grants from the Spanish government (FEDER 2FD97-1854, AGL2000-0205-P4-0) and the France-Spanish Picasso Integrated Project (*Acciones Integradas* HF2001-0015).

In modern winemaking, consumer olfactory profiling is becoming increasingly important as a guide for production decisions. Today the wine industry manages to control the fermentation temperature effectively, and a low temperature is becoming more common for white and rosé fermentation. Wines produced at low temperatures (10-15ºC) are known to develop certain characteristics of taste and aroma. A first hypothesis was that these improved sensorial properties were generally due to a greater retention of volatile substances at low temperature. However, the results obtained in previous studies by our research group (PhD. Thesis M.J. Torija and J.M. Llauradó) demonstrated that not only is there a greater retention of aromatic substances but also a greater production. The fermentation temperature also affects yeast lipid metabolism, which is related to cell development, membrane integrity and the production of several by-products, especially those directly related to wine aroma.

Fermenting at low temperature also has some disadvantages, however. These include a longer process and a greater risk of stuck or sluggish fermentation. Controlling nutritional requirements is important for avoiding problematic fermentations. Nitrogen deficiencies are one of the main causes of stuck or sluggish fermentations and nitrogen uptake patterns also influence the production of some aromatic compounds.

Despite major advances in knowledge of the genome of the yeast *S. cerevisiae,* our understanding of the gene expression in wine yeast during industrial fermentations is limited. Little information about the metabolism of nitrogen and its regulation in winemaking conditions is known. One way to solve problems related to nitrogen deficiency is to add nutritional supplements (usually inorganic forms of nitrogen such as ammonium salts) to grape must prior to fermentation. Most nitrogen additions in winemaking are made empirically and do not take into account the nitrogen needs of the cell during wine fermentation, the proper timing for these additions or the source of the nitrogen added. It is important to know the nitrogen content of grape juice and the nitrogen requirements of yeast to achieve optimal fermentation performance.

To study some of these important aspects for the correct performance of alcoholic fermentation and the quality of wine, we established the following objectives:

➤ **To study wine yeast metabolism at low temperature fermentation and its influence on:**

- **Fermentation kinetics and yeast growth**
- **Yeast lipid metabolism**
- **Production of aromatic compounds**
- **Global yeast gene expression**

➤ **To study the nitrogen metabolism of wine yeast in alcoholic fermentation**

We aimed to determine the precise role of nitrogen metabolism in wine fermentation at both temperatures and in nitrogen supplementation. This is important for accurately controlling fermentation performance and yeast nitrogen requirements. These results will be applied to improving fermentations at low temperature.

OUTLINE OF THE THESIS

Effect of fermentation temperature and culture media on the lipid composition and volatile compounds in wine

To characterize low temperature fermentations and to study how they affect several aspects of yeast metabolism and fermentation performance, we carried out fermentations on the laboratory and industrial scale with two culture media (synthetic must and natural grape must) and at two fermentation temperatures (13ºC and 25ºC). We then analysed how culture media and temperature affected:

- Fermentation kinetics and yeast growth
- Lipid cell composition: fatty acids, sterols and phospholipids
- Production of aromatic compounds such as volatile fatty acids and their corresponding ethyl esters, and higher alcohols and their corresponding acetate esters.

These results are reported in Chapter 1.

Integration of transcriptomic and metabolomic analyses for understanding the global responses of low temperature winemaking fermentations

To determine the molecular mechanisms encoding the low temperature impacts on wine flavours and lipid metabolism, we used the powerful DNA microarrays technology to examine the global gene response of yeast during industrial wine making processes at 25 and 13°C. Moreover, to make it easier to biologically interpret transcriptomic data, we also measured intracellular and extracellular parameters such as the lipid composition of the cell, fermentation by-products and flavours. These results are summarized and discussed in Chapter 2. Supplementary data are reported in the annex of this Thesis and at **http://biopuce.insa-tlse.fr/jmflab/winegenomic/**.

Nitrogen Catabolite Repression in *S. cerevisiae* during wine fermentation

We studied the nitrogen metabolism of wine yeast during winemaking conditions, and specifically the role of different nitrogen transporters, by fermenting synthetic must at several nitrogen concentrations: control (300 mg/l YAN), high nitrogen content (1200 mg/l YAN) and low nitrogen content (60 mg/l YAN). We then studied how these nitrogen concentrations affected the following aspects of yeast and fermentation:

- Fermentation performance and kinetics

- Regulation of some nitrogen permeases: gene expression of ammonia (Mep1p, Mep2p, Mep3p) and general amino acid (Gap1p) permeases along the fermentation.
- Uptake of nitrogen compounds (ammonia and amino acids) along the fermentation.
- Arginase activity along the fermentation as a nitrogen deficiency marker.

These results are presented and discussed in Chapter 3.

Influence of the timing of nitrogen additions during wine fermentations on the fermentation kinetics and nitrogen consumption

We analysed the effect of these additions (mixture of ammonia and amino acids) at several stages of nitrogen-deficient fermentations on:

- Fermentation kinetic and yeast growth.
- Gene expression of ammonia (Mep1p, Mep2p, Mep3p) and general amino acid (Gap1p) permeases.
- Uptake of nitrogen compounds along the fermentation.
- Production of secondary metabolites and aromatic compounds such as volatile fatty acids and their corresponding ethyl esters, and higher alcohols and their corresponding acetate esters.

These results are reported in Chapter 4.

Effect of low temperature fermentation on yeast nitrogen metabolism

We analysed the influence of low temperature on the nitrogen regulation and, therefore, on nitrogen consumption. We carried out wine fermentations at 25ºC and 13ºC in laboratory scale using synthetic media, and we estimated the effect of temperature on:

- nitrogen consumption (ammonia and amino acid uptake)
- the Nitrogen Catabolite Repression (NCR) system (expression of permeases Gap1p and Mep2p).

These results are reported in Chapter 5.

INTRODUCTION

1. YEAST AND ALCOHOLIC FERMENTATION 10
1.1 General characteristics of yeast 10
1.2. Ecology of yeast population... 13
1.2.1. Taxonomy 13
1.2.2. Ecology in grape and must fermentation 14
1.2.3. Spontaneous *vs* inoculated fermentations 15
1.3. Alcoholic Fermentation 17
1.3.1. Causes of stuck or sluggish fermentations.. 18
1.4. Yeasts and the post-genomic era.. 19
1.4.1. DNA chips in the study of wine yeasts 19
1.4.2. Improvement of wine-yeast strains 20
2. LOW TEMPERATURE FERMENTATIONS 22
2.1. Effect of temperature on yeast ecology and growth 22
2.1.1 The importance of inoculum: cryotolerant yeasts.................................. 24
2.2. Effect of temperature on fermentation kinetic 25
2.3. Effect of temperature on yeast metabolism 25
2.3.1. Effect of temperature on alcohol and by-products synthesis 26
2.3.2. Influence of temperature on volatile compounds.................................. 27
2.4. Effect of temperature on membrane fluidity and lipid composition............ 28
2.4.1. Yeast membrane composition... 28
2.4.2. Effect of temperature on biological membranes 31
2.4.3. Yeast lipid composition in whine-making conditions 32
2.5. Effect of temperature on transport and nitrogen up-take 34
2.6. Stress and low temperature 34
2.6.1. General stress response 34
2.6.2. Yeast Stress response in wine fermentations 35
2.6.3. Low temperature gene response... 36
2.7. Role of reserve carbohydrates.. 38
3. NITROGEN METABOLISM... 39
3.1. Grape must composition 40
3.2. Central Nitrogen Metabolism 40
3.3. Nitrogen Catabolite Repression 42
3.4. Nitrogen compounds uptake 44
3.4.1. Factors affecting nitrogen assimilation... 45
3.4.2. Nitrogen supplements 46
3.4.3. Transport of nitrogen compounds... 47
3.5. Nitrogen and Yeast Flavour Compounds.. 49
REFERENCES 52

1. YEAST AND ALCOHOLIC FERMENTATION

Yeasts are predominant in the ancient and complex process of winemaking. In 1863, Louis Pasteur revealed for the first time the hidden world of microbial activity during wine fermentation. Although previously observed by Cagniard de la Tour (1830), Pasteur proved conclusively that yeast is the primary catalyst in wine fermentation. Since Pasteur, yeasts and alcoholic fermentation have incited a considerable amount of research, making use of progress in microbiology, biochemistry and now genetics and molecular biology. Although many genera and species of yeast are found in musts, the genus *Saccharomyces*, and mainly the species *S. cerevisiae*, is responsible for biotransformation. Because of this, *S. cerevisiae* is referred as “the wine yeast” (Pretorius, 2000).

1.1 General characteristics of yeast

Yeast is the most simple of the eukaryotes. The yeast cell contains cellular envelopes, a cytoplasm with various organelles, and a nucleus surrounded by a membrane and enclosing the chromosomes (Figure 1.1). Like all plant cells, the yeast cell has two cellular envelopes: the cell wall and the membrane. The periplasm is the space between the cell wall and the membrane. The cytoplasm and the membrane make up the protoplasm.

The yeast **cell wall** is made up of two principal constituents: β-glucans (60%) and mannoproteins (25-50 %), its principal function is to protect the cell. Its composition and functions evolve during the life of the cell, in response to environmental factors. Its first function is to protect the cell, but it is also the site for molecule recognition, which determines certain cellular interactions such as sexual union, flocculation, and the killer factor. Finally, a number of enzymes, generally hydrolases, are connected to the cell wall or situated in the periplasmic space.

The **plasma membrane** constitutes a stable, hydrophobic barrier between the cytoplasm and the environment outside the cell. Like all biological membranes, the yeast plasma membrane is principally made up of lipids (essentially phospholipids and sterols, forming a lipid bilayer) and proteins. The integral proteins are strongly associated to the non-polar region of the bilayer, and the peripheral proteins are linked to the integral proteins. The membrane composition in fatty acids and its proportion in sterols control its fluidity.

Between the plasma membrane and the nuclear membrane, the **cytoplasm** contains a cytoplasmic substance, or cytosol, which is a buffered solution, with a pH between 5 and 6, containing soluble enzymes, reserve carbohydrates and ribosomes. The organelles (endoplasmatic reticulum, Golgi apparatus, vacuole and mitochondria) are isolated from cytosol by membranes.

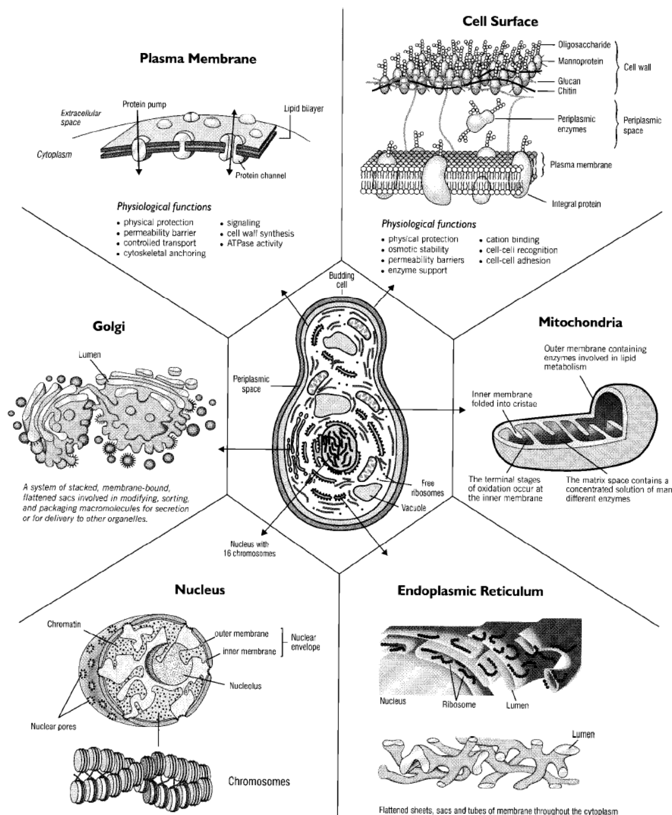


Figure 1.1
A schematic representation of the subcelular compartmentalization of a wine yeast cell (Pretorius, 2000).

Yeasts are defined as unicellular fungi that reproduce predominantly asexually by budding (only *Schyzosaccharomyces* genera reproduce by binary fission). Yeasts form a complex and heterogeneous group found in three classes of fungi, characterized by their reproduction mode: *Ascomycetes*, *Basidomycetes* and the imperfect fungi or *Deuteromycetes*. The yeast found on the surface of the grape and in the wine belongs to *Ascomycetes* and *Deuteromycetes*. Like other sporiferous yeast belonging to the class *Ascomycetes*, *S. cerevisiae* can multiply either asexually by vegetative multiplication or sexually by forming ascospores (Figure 1.2). Under optimal nutritional and cultural conditions *S. cerevisiae* doubles its mass every 90 min. The cell division cycle in vegetative multiplication consists of four phases (Figure 1.3): G_1 (period preceding DNA synthesis), S (DNA synthesis), G_2 (period preceding the mitosis) and M (mitosis

and cytokinesis). The cell cycle of the yeast *Saccharomyces cerevisiae* is characterised by the formation of a bud as the cell enters the S phase. During the rest of the cell cycle, this bud will grow until a viable daughter cell has formed that is separated from the mother cell at the end of the M phase. In contrast to other eukaryotic cells, division on *S. cerevisiae* is an asymmetric process in which the newly formed cell is smaller than the mother cell.

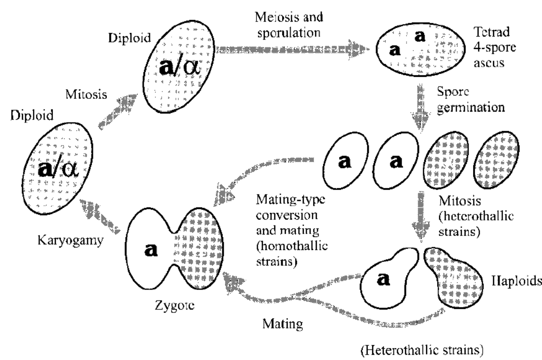


Figure 1.2
A schematic representation of the life cycle of heterothalic and homothallic wine yeast strains. (Pretorius, 2000)

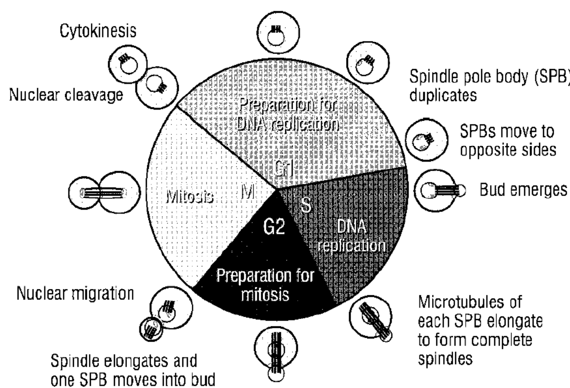


Figure 1.3
A schematic representation of the cell cycle of a budding yeast cell. (Pretorius, 2000)

Upon nutritional starvation, the diploid cell undergoes meiosis, generating four haploid ascospores (two MATa and two MATα ascospores) encapsulated within an ascus (Figure1.2). When released from ascus, the ascospores germinate to commence new rounds of haploid existence. Strains that can be maintained stably for many generations as haploids are termed **heterothallic**. Strains in which sex reversals, cell fusion and diploid formation occur are termed **homothallic**. Wine yeast, both wild and selected, do not sporulate easily, and when they do they often produce non-viable spores.

S. cerevisiae has a relatively small compact genome, a large number of chromosomes, little repetitive DNA and a few introns (Pretorius 2000). Haploid strains contain approximately 12-13 Mb of nuclear DNA, distributed along 16 linear chromosomes (varying in length from 200 to 2200 Kb). The genome of a laboratory strain of *S. cerevisiae* has been completely sequenced and found to contain roughly 6000 protein-

encoding genes (Goffeau *et al.*, 1996; Goffeau *et al.*, 1997). Most laboratory-bred strains of *S. cerevisiae* are either haploid or diploid. However, industrial wine yeast strains are predominantly diploid or aneuploid, and occasionally polyploid (Bakalinsky and Snow, 1990; Barre *et al.*, 1993), and show a high level of chromosome length polymorphism (Bidenne *et al.*, 1992; Rachidi *et al.*, 2000). It is not yet clear whether polyploidy in industrial yeast strains is advantageous. The ploidy of the wine yeast may confer advantages to adapt to variable external environments or, perhaps, is a way to increase dosage of some genes important in fermentation (Bakalinsky and Snow, 1990; Salmon, 1997). Apart of genomic features, wine strains of *S.cerevisiae* are homothallic and highly heterozygous (Barre *et al.*, 1993; Codon *et al.*, 1995).

1.2. Ecology of yeast population

1.2.1. Taxonomy

Yeasts constitute a vast group of unicellular fungi taxonomically heterogenous and very complex. According to the current classification, yeasts belonging to *Ascomycetes*, *Basidomycetes* and imperfect fungi are divided into 99 genera representing 770 different species (Kurzman and Fell, 1998). Taxonomists first delimited yeast species using morphological and physiological criteria. Over the last 15 years researchers have developed a molecular taxonomy based on the following tests: DNA recombination, the similarity of DNA base composition, the similarity of enzymes, ultrastructure characteristics and cell wall composition. The DNA recombination tests have proven to be effective for delimiting yeast species.

The evolution of species classification for the genus *Saccharomyces* have often changed since the beginning of taxonomic studies. Presently the species of *Saccharomyces* are divided into three groups. The first group, *Saccharomyces sensu stricto,* is formed by *S. cerevisiae, S. bayanus, S. paradoxus* and *S. pastorianus* (which replaced the name *S. calsbergensis*). A second group, *Saccharomyces sensu lato*, is made up of the species *S. exiguous, S. castelli, S. servazzii* and *S. unisporus*. The third group consists only of the species S. *kluveri*. Only the first group comprises species having an enological interest: *S. cerevisiae* and *S. bayanus.* (Ribéreau-Gayon *et al.*, 2000)

Genetic methods are indispensable for identifying wine yeasts. In the late 1980s, owing to the development of genetics, certain techniques of molecular biology were successfully applied to characterize wine yeast strains. They are based on the clonal polymorphism of the mitochondrial and genomic DNA of *S.cerevisiae* (Querol *et al.*,

1992b). Different methods of DNA analysis should be combined to identify wine yeast strains.

1.2.2. Ecology in grape and must fermentation

Until recently, a large amount of research has been focused on the description and ecology of wine yeasts. It concerned the distribution and succession of species found on the grape and then in wine during fermentation and conservation.

The diversity, composition and evolution of yeast flora in grape musts depend on a variety of environmental and technological factors: geographic location (Versavaud *et al.*, 1995; Van der Westhuizen *et al.*, 2000; Torija *et al.*, 2001), climatic conditions (Fleet *et al.*, 1984; Parrish and Carrol, 1985; Longo *et al.*, 1991), age of the vineyard and grape variety (Martini *et al.*, 1980; Rosini *et al.*, 1982; Beltran *et al.*, 2002b), sulphite addition (Constanti *et al.*, 1998), presence of yeast starters (Heard and Fleet, 1985; Constanti *et al.*, 1998; Beltran *et al.*, 2002b) and fermentation temperature (Sharf and Margalith, 1983; Heard and Fleet, 1988; Torija *et al.*, 2003b).

The number of yeast on the grape berry, just before the harvest, is between 10^3 and 10^5 CFU ml^{-1}. *Kloeckera apiculata* and *Hanseniaspora uvarum* are the predominant species on the surface of grape berries, 50-75 % of the total yeast population (Fleet and Heard, 1993; Fleet, 1998). Numerically less prevalent than these apiculate yeasts are species of *Candida* (e.g. *C. stellata* and *C. pulcherrima*), *Brettanomyces, Cryptococcus, Kluyveromyces, Metschnikowia, Pichia* and *Rhodotorula* (Fleet and Heard, 1993; Fleet, 1998). Contrary to popular belief, fermentative species of *Saccharomyces* (e.g. *S. cerevisiae*) occur at extremely low populations on healthy, undamaged grapes and are rarely isolated from intact berries and vineyard soils (Martini, 1993). In fact, the origin of *S. cerevisiae* is quite controversial; one school of thought claims that the primary source of this industrially important yeast is the vineyard, and the presence or absence of *S. cerevisiae* differs with each plant and grape cluster (Mortimer and Polsinelly, 1999). Others believe a direct association of *S. cerevisiae* 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). Every vintage, the surfaces of winery equipment are exposed to billions of yeast cells, so they are easily colonised and become locations for the development of a resident or winery yeast microbiota. These *Saccharomyces cerevisiae* strains resident on the winery surfaces are much more abundant than those that might come from the grapes or vineyard and it is very probable that they will predominate during the spontaneous

fermentations or play a role in other fermentations inoculated with pure yeast culture (Rosini, 1984; Martini *et al.*, 1996; Constanti *et al.*, 1998; Beltran *et al.*, 2002b). The extent of the development of a winery yeast flora, usually comprising species of *Saccharomyces, Candida* and *Brettanomyces*, depends upon the nature of the surface and the degree to which it has been cleaned and sanitized (Fugelsang, 1997; Pretorius, 2000).

1.2.3. Spontaneous *vs* inoculated fermentations

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 the high sugar content of grape must exert strong selective pressure on the microorganisms, such that only few yeast and bacterial species can proliferate. Yeast species with lower fermentative activity than *Saccharomyces cerevisiae*, such as *Hanseniaspora, Candida* and *Pichia*, grow during the first period of spontaneous fermentations but then the population size of non-*Saccharomyces* species decreases progressively, leaving the most ethanol-tolerant species of *Saccharomyces cerevisiae* to take over the fermentation (Heard and Fleet, 1985; Torija *et al.*, 2001; Beltran *et al.*, 2002b) (Figure 1.4).

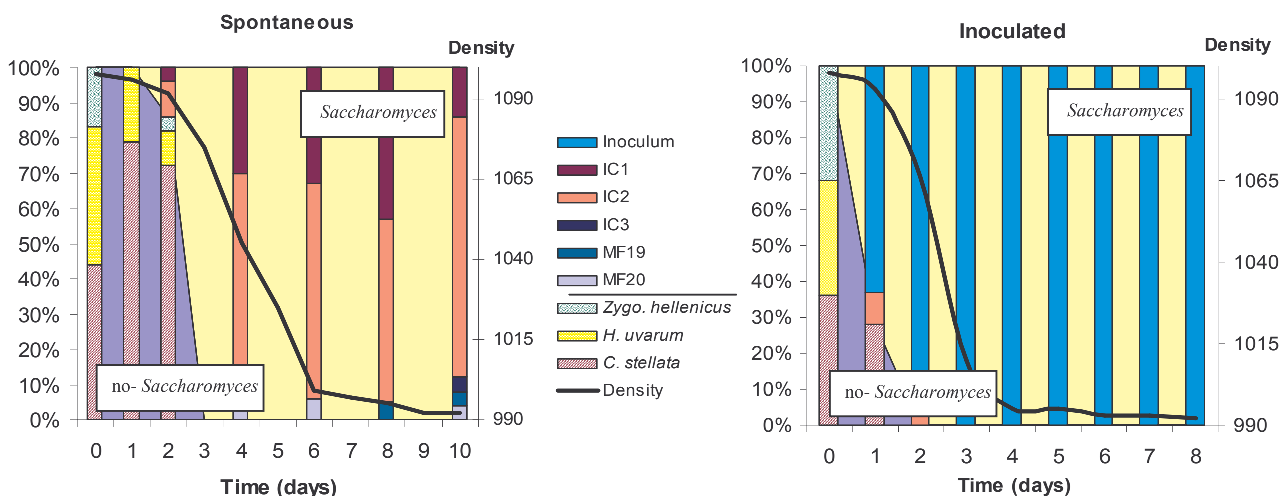


Figure 1.4 Yeast ecology on spontaneous and inoculated Garnatxa grape fermentations (Beltran *et al.*, 2002b). IC strains are commercial inocula used on other fermentations (resident yeast flora), and MF are strains of *Saccharomyces* autochthones.

The use of active dry yeast in fermentation is becoming one of the most common practices in winemaking because it ensures a reproducible product and reduces the lag phase and the risk of wine spoilage (Figure 1.5). However, the winemaking community is still widely divided about this practice because of a widespread belief that native

yeast strains give a distinctive style and quality to wine (Mateo et al., 1991; Fugelsang, 1996; Heard, 1999).

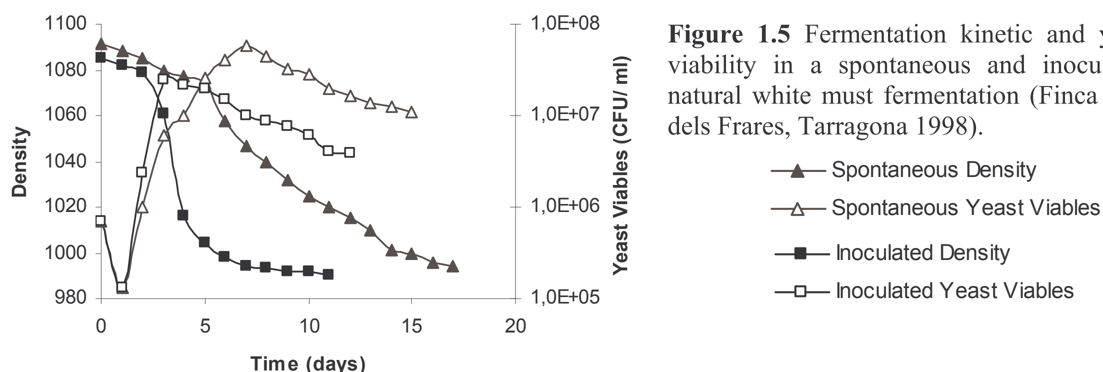


Figure 1.5 Fermentation kinetic and yeast viability in a spontaneous and inoculated natural white must fermentation (Finca Mas dels Frares, Tarragona 1998).

In fact, the use of active dry yeast reduces the number of different indigenous *Saccharomyces cerevisiae* strains due to the starter imposition (Figure 1.6), but does not completely prevent them from growing until several days after the inoculation (Figure 1.4). During this time, wild strains may have an important effect on wine flavour and characteristics (Querol *et al.*, 1992a).

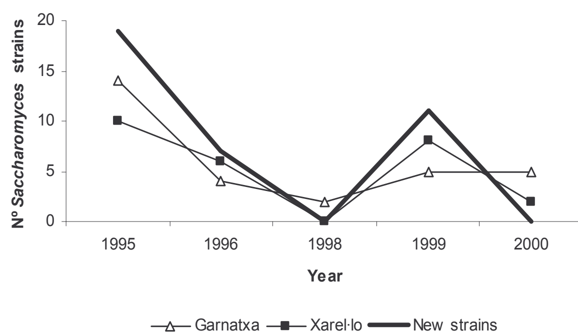


Figure 1.6 Number of indigenous *Saccharomyces cerevisiae* strains found in spontaneous fermentations of Xarel·lo and Garnatxa musts, from 1995 to 2000. (Beltran *et al.*, 2002b)

Taula 1.1 Desirable characteristics of wine yeasts (Pretorius, 2000)

Fermentation properties	**Technological properties**
Rapid initiation of fermentation	High genetic stability
High fermentation efficiency	High sulphite tolerance
High ethanol tolerance	Low sulphite binding activity
High osmotolerance	Low foam formation
Low temperature optimum	Flocculation properties
Moderate biomass production	Compacts sediment
Flavour characteristics	Resistance to desiccation
Low sulphite/DMS/thiol formation	Zymocidal (killer) properties
Low volatile acidity production	Genetic marking
Low higher alcohol production	Proteoytic activity
Liberation of glycosylated flavour precursors	Low nitrogen demand
High glycerol production	**Metabolic properties with health implications**
Hydrolytic activity	Low sulphite formation
Enhanced autolysis	Low biogenic amine formation
Modified esterase activity	Low ethyl carbamate (urea) formation

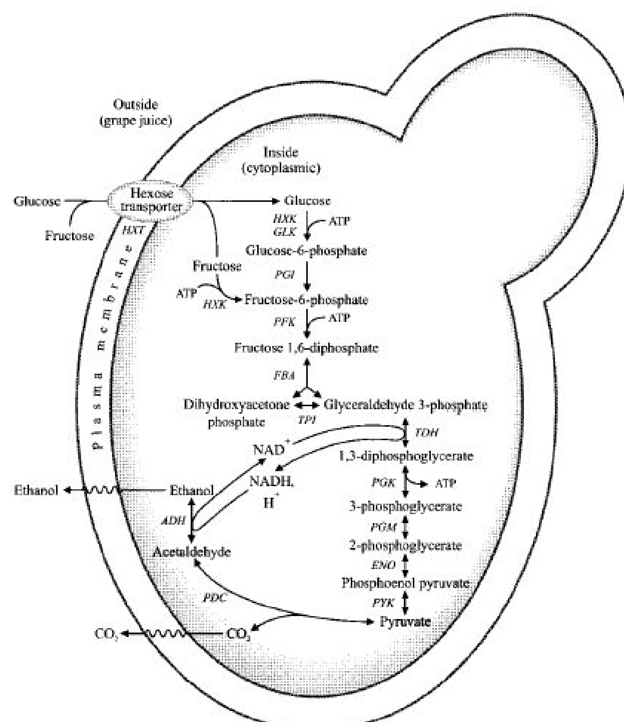
Pure cultures have been obtained from natural isolates all around the world. In addition to the primary role of wine yeast to catalyse the efficient and complete conversion of grape sugars to alcohol without the development of off-flavours, starter culture strains of *S. cerevisiae* must now possess a range of other properties, such as listed in Table 1.1. Analysis of stress resistance has been recently proposed as a suitable criterion for wine yeast selection (Zuzuarregui and del Olmo, 2004a; 2004b).

1.3. Alcoholic Fermentation

The principal metabolic process in winemaking is the alcoholic fermentation, which consists in the biotransformation of grape sugars (glucose and fructose) into ethanol and carbon dioxide. The principal responsible for this transformation is the yeast.

For high glucose concentrations (approximately above 9g/l), *S. cerevisiae* only metabolises sugars by the fermentative pathway. Even in the presence of oxygen, respiration is blocked. This phenomenon, firstly named Crabtree effect, is also known as catabolic repression by glucose or the Pasteur contrary effect (Ribéreau-Gayon *et al.*, 2000). So, in *S. cerevisiae* glucose and fructose are metabolised to pyruvate via glycolitic pathway (Figure 1.7). Pyruvate is decarboxylated to acetaldehyde, which is then reduced to ethanol. During wine yeast glycolysis, one molecule of glucose or fructose yields two molecules of ethanol and carbon dioxide. However, the theoretical conversion of 180 g sugar into 92 g ethanol and 88 g carbon dioxide could only be expected in the absence of any yeast growth, production of other metabolites and loss of ethanol as vapour. In a standard fermentation, about 95% of the sugar is converted into ethanol and carbon dioxide, 1% into cellular material and 4% into other products such as glycerol.

Figure 1.7
Enzymatic steps of the glycolitic pathway in wine yeast (Pretorius, 2000).



1.3.1. Causes of stuck or sluggish fermentations

One of the very important objectives during most winemaking processes is the achievement of completing alcoholic fermentation, so that the residual fermentable sugar in the wine is less than 2-4 g/l. The completion of fermentation may help avoid problems not only with acetic acid bacteria but also with lactic acid bacteria, which could metabolise residual sugars to increase volatile acidity and also in the formation of abnormal esters and perhaps alter the pattern of diacetyl formation (O'Connor-Cox and Ingledew, 1991).

The study of the factors responsible for the occurrence of stuck and sluggish fermentations is receiving increased interest because of the economic impact of fermentation problems. Many factors such as vitamin, magnesium, nitrogen and oxygen deficiencies or presence of ethanol, toxic fatty acids, acetic acid or sulphites may be involved (Table 1.2). The effects related to these factors are numerous and include decrease in pH, inhibition of key enzyme activities, and alteration of the plasma membrane. These may induce decrease in the metabolism of the yeast cell and consequently decreases in biomass production, cell viability and fermentation rate. Furthermore, occurrence of stuck and sluggish fermentation could be the result of interaction of these factors (for reviews see Alexandre and Charpentier, 1998; Bisson, 1999).

Table 1.2 Principal causes of stuck and sluggish fermentations

Nutritional deficiencies	- Nitrogen - Minerals - Vitamins - Oxygen - Ergosterol & unsaturated fatty acid
Inhibitory substances	- Ethanol - Toxic Acids (MCFA) - Acetic acid - Sulphites - Pesticide residues
Antagonism between microorganisms	- Fungi, acetic and lactic bacteria and "killer" yeasts
Enological practices	- Level of must clarification - Extreme temperatures (too high or too low)
High sugar concentration	

1.4. Yeasts and the post-genomic era

The completion of the sequence of the *S.cerevisiae* genome leads the development of new tools for the analysis of the expression of the entire genome (transcriptome). The DNA microarray hybridization analysis (DeRisi *et al.*, 1997;Wodicka *et al.*, 1997) has been used to investigate relevant changes in gene expression during a biological process.

The yeast *S. cerevisiae* is one of the most extended model organisms. It was the first eukaryote whose genome was sequenced (Goffeau *et al.*, 1996;Goffeau *et al.*, 1997). Since then, many functional analysis projects have been dedicated to the investigation of its molecular biology. Yeast was selected as the ideal organism to develop the DNA array technology for several reasons: the sequence of the entire genome accomplished in 1997, the relatively small genome size, the considerable genetic information available and the powerful set of tools that are available for its analysis. However, *S. cerevisiae* is not only a model organism. It is also an important industrial food microorganism.

To date many studies have been made analysing the global gene expression of *S. cerevisiae* strains. Comprehensive studies carried out with yeast laboratory strains characterized the genes that are differentially expressed in different biological conditions: during the diauxic shift (DeRisi *et al.*, 1997;Kuhn *et al.*, 2001), during the sporulation (Chu *et al.*, 1998), the cell cycle (Spellman *et al.*, 1998;Cho *et al.*, 1998), or the consequences of over-expression or deletion of both known and poorly defined genes (DeRisi *et al.*, 1997;Hughes *et al.*, 2000;Bianchi *et al.*, 2001).

Moreover, the yeast gene response and adaptation to several stress conditions has also been analysed (Gasch *et al.*, 2000;Causton *et al.*, 2001). Some of these stress conditions are frequently found in alcoholic fermentation, such as nutrient limitation (Boer *et al*. 2003), anaerobic conditions (ter Linde *et al.*, 1999;Kwast *et al.*, 2002), ethanol stress (Alexandre *et al.*, 2001) or osmotic stress (Yale and Bohnert, 2001).

1.4.1. DNA chips in the study of wine yeasts

Although the use of laboratory culture conditions facilitates the analysis of the yeast, it does not efficiently reproduce the natural environment for wine yeast i.e. high sugar concentration, acidic pH and variable nitrogen conditions. In general, as concluded by different studies, the genes involved in amino acid biosynthesis, as well as in purine biosynthesis, generally showed higher expression levels in the enological yeast strains

than in laboratory yeast strains (Cavalieri *et al.*, 2000;Backhus *et al.*, 2001;Hauser *et al.*, 2001), indicating a high replicative activity in wine yeasts.
The knowledge of genetic features as well as the specific expression profiles of wine yeast strains under different growth conditions could help us to better understand the biological process of fermentation at the molecular level and how the gene expression is regulated in relation to changes in the physical and chemical properties of the growth medium (for a review, see Perez-Ortín *et al*., 2002).
Some studies done with wine yeast strains analysed the gene response of yeasts to different nitrogen concentrations (Backus *et al*., 2001) and nitrogen additions (Marks *et al.*, 2003), to high sugar concentrations (Erasmus *et al.*, 2003), and the gene expression changes throughout the alcoholic fermentation (Rossignol *et al*., 2003). Most of these studies mimicked enological environment by growing the wine yeast strain on a synthetic must. These results mainly showed that the entry into the stationary phase triggered major transcriptional changes, being a key physiological that was followed by a general stress response (Backus *et al*., 2001, Rossignol *et al*., 2003).

1.4.2. Improvement of wine-yeast strains

The knowledge of how genes in *S. cerevisiae* are expressed during wine fermentations facilitate our ability to improve wine yeasts by breeding or genetic engineering, or to control fermentation conditions to maximize aroma or minimize spoilage compounds in wines. Recent development of techniques to monitor gene expression in *S. cerevisiae* on a genomic scale represents a huge step towards the application of functional genomics in industrial fermentations (DeRisi *et al.*, 1997;Wodicka *et al.*, 1997). The application of DNA microarray technology combined with proteomics will revolutionize research n enology next decade.
Wine producers and consumers ultimately dictate the direction of targeted strain development, which in turn depends upon an understanding of the complex mechanisms by which yeast cells adapt to the continuously changing environment during winemaking. Improved wine yeast starter culture strains are developed to enhance cost-effectiveness, efficiency and sustainability in the production of high-quality wine.
Despite very vocal opposition to genetically modified organisms (GMOs) and products, in certain countries, the wine industry increasingly is focussing on genetic improvement of the grapevine and the wine yeast, the two main organisms involved in the production of wine (for reviews see Pretorius and Bauer, 2002; Pretorius, 2003). Table 4.1

summarises the progress made in developing wine yeasts that improve fermentation, processing and biopreservation, and increase the wholesomeness and sensory quality of wine (Pretorius and Bauer, 2002).

Table 4.1. Targets for the genetic improvement of wine-yeast strains (Pretorious and Bauer, 2002)

Desirable properties	Focus areas	Examples of potential target genes[a]
Improved fermentation performance		
Improved general resilience and stress tolerance	Stress response, sterol, glycogen and trehalose accumulation	Modification of glycogen or trehalose metabolism (e.g. acting on *GSY1* and *GSY2* , *TPS1* and *TPS2*)
Improved efficiency of sugar utilization	Hexose transporters, hexose kinases	Overexpression and modification of *HXT1-HXT18*, *SNF3*, *FSY1* and use of heterologous transporters and kinases
Improved efficiency of nitrogen assimilation	Improved utilization of less efficient N sources	Proline catabolism (*PUT1* and *PUT2*) and use of heterologous catabolic genes
Improved ethanol tolerance	Sterol formation, membrane ATPase activity	Modification of the expression of *PMA1* and *PMA2* (ATPase), sterol anabolic sterol anabolic genes
Increased tolerance to antimicrobial compounds	Resistance to killer toxins, sulfur dioxide, agrochemicals	Inclusion of *KIL2*, overexpression of *CUP1*
Reduced foam formation	Cell-surface proteins	Deletion of *FRO1* and *FRO2*
Improved processing efficiency		
Improved protein clarification	Proteases	Overexpression of *PEP4* and secretion of other proteases
Improved polysaccharide clarification	Glucanases, pectinases, xylanases, arabinfuranosidases	Overexpression of *END1*, *EXG1*, *CEL1*, *BGL1*, *PEL5* and *PEH1*, *XYN1–5*, *ABF2*
Controlled cell sedimentation and flocculation	Flocculins	Late expression of flocculation genes (*FLO1, FLO5, MUC1*/*FLO11)* under control of promoters (*HSP30*) imparting desired expression
Controlled cell flotation and flor formation	Cell-wall hydrophobic proteins	Late expression of *MUC1*/*FLO11* under control of promoters (*HSP30*) imparting desired expression pattern
Improved biological control of wine spoilage microorganisms		
Wine yeasts producing antimicrobial enzymes	Lysozyme, glucanases, chitinases	Expression of *HEL1*, *CTS1*, *EXG1* and other antimicrobial enzymes
Wine yeasts producing antimicrobial peptides	Bacteriocins	Expression of *PED1*, *LCA1* and other heterologous bacteriocin and zymocin genes
Wine yeasts producing sulfur dioxide	Sulfur metabolism and sulfur dioxide formation	Overexpression of *MET14* and *MET16* and deletion of *MET10*
Improved wine wholesomeness		
Increased production of resveratrol	Stilbene synthesis	Expression of *4CL9/216*, *VST1*
Reduced formation of ethyl carbamate	Amino acid metabolism, urea formation	Deletion of *CAR1* or expression of *URE1*
Reduced formation of biogenic amines	Bacteriolytic enzymes, bacteriocins	Expression of *HEL1*, *PED1*, *LCA1* and other bacteriocins
Decreased levels of alcohol	Carbon flux, glycerol metabolism and glucose oxidation	Overexpression of *GPD1* and *GPD2*, modification of *FPS1*, expression of *GOX1*
Improved wine flavour and other sensory qualities		
Enhanced liberation of grape terpenoids	Glycosidases, glucanases, arabinofuranosidases	Overexpression of *END1*, *EXG1*, *CEL1*, *BGL1*, *PEL5* and *PEH1*, *ABF2*
Enhanced production of desirable volatile esters	Esterases	Modified expression of *ATF1* and other alcohol transferases, *IAH1* and other esterases
Optimized fusel oil production	Amino acid metabolism	Deletion of the *ILE*, *LEU* and *VAL* genes
Enhanced glycerol production	Glycerol metabolism	Overexpression of *GPD1* and *GPD2*, *FPS1*, and deletion of *ALD6*
Bioadjustment of wine acidity	Maloethanolic and malolactic fermentation, lactic acid production	Expression of *MAE1*, together with *MAE2* or *mleS*, or *LDH1*
Optimization of phenolics	Phenolic acid metabolism	Modified expression of *PAD1*, *pdc, padc*
Reduced sulfite and sulfide production	Sulfur metabolism, hydrogen sulfide formation	Deletion of *MET14* and *MRX1*

[a]Abbreviations : *4CL9/216*, coenzyme A ligase; *ABF2*, arabinofuranosidase; *ATF2*, alcohol acetyl transferase; *BGL1*, β-glucosidase, cellobiase; *CAR1*, arginase; *CEL1*, cellodextrinase; *CTS1*, chitinase; *CUP1*, copper chelatin; *END1*, endoglucanase; *EXG1*, exoglucanase; *FPS1*, glycerol transport facilitator; *FRO*, froth protein; *GOX1*, glucose oxidase; *GPD*, glycerol-3-phosphate dehydrogenase; *GSY*, glycogen synthase; *HEL1*, hen egg white lysozyme; *IAH1*, esterase; *KIL2*, zymocin and immunity factor; *LCA1*, leucocin; *LDH1*, lacticodehydrogenase; *MAE1*, malate permease; *MAE2*, malic enzyme; *MRX1*, methionine sulfoxide reductase; *MET10*, sulfite reductase; *MET14*, adenosylphosphosulfate kinase; *MET16*, phospho adenosylphosphosulfate reductase; *mleS*, malolactic enzyme; *PAD1*, phenyl acrylic acid decarboxylase; *padc*, phenolic acid decarboxylase; *pdc*, ρ-coumaric acid decarboxylase; *PED1*, pediocin; *PEH1*, polygalacturonase; *PEL5*, pectate lyase; *PEP4*, protease A; *PUT1*, proline oxidase; *PUT2*, pyrroline-5-carboxylate dehydrogenase; *TPS1*, trehalose-6-phosphate synthase; *TPS2*, trehalose-6-phosphate phosphatase; *URE1*, urease; *VST1*, stilbene synthase; *XYN*, xylanases.

2. LOW TEMPERATURE FERMENTATIONS

The single most important factor in winemaking is the organoleptic quality of the final product. Many variables contribute to the distinctive flavours of wine and others grape-derived alcoholic beverages. Enological practices, including the yeast and fermentation conditions, have a primary effect on the primary and secondary flavours (Lambrechts and Pretorius, 2000). With the effective control of fermentation temperature by the wine industry, low temperature alcoholic fermentations are becoming more frequent due to the aim to produce white and "rosé" wines with more pronounced aromatic profile. Wines produced at low temperatures (10-15ºC) are known to develop certain characteristics of taste and aroma (Feuillat *et al.*, 1997; Llauradó *et al.*, 2002; Beltran *et al.*, 2002a; Torija *et al.*, 2003a; Novo *et al.*, 2003a; Novo *et al.*, 2003b).

The volatile profile of wines is dominated by those components that are formed and retained mostly during fermentation (Lambrechts and Pretorius, 2000). Low temperatures increase not only the retention but also the production of some volatile compounds (Killian and Ough, 1979). Another interesting aspect is that low temperatures notably reduce the growth of acetic and lactic acid bacteria and they can facilitate the control of alcoholic fermentation (Ribéreau-Gayon *et al.*, 2000). However, the optimal growth temperature for *S. cerevisiae* is 25ºC, while 13ºC is restrictive and increases the risk of stuck and sluggish fermentations.

Some notably effects of these low temperatures are:

- Modification of microorganism populations.
- Long lag-phase with the risk of prevailing non-*Saccharomyces* strains.
- Decrease in the rate of sugar consumption.
- Longer fermentations with the risk of stuck and sluggish fermentation.
- Modification in the metabolic activity of yeast, with different production of secondary metabolites.
- Modification in lipid membranes, with the consequent modification in the transport of compounds.

2.1. Effect of temperature on yeast ecology and growth

Yeast species with lower fermentative activity than *Saccharomyces cerevisiae*, such as *Hanseniaspora, Candida* and *Pichia*, grow during the first period of spontaneous fermentations but then the population size of non-*Saccharomyces* species decrease progressively, allowing the most ethanol-tolerant species of *Saccharomyces cerevisiae* to take over the fermentation (Heard and Fleet, 1985; Beltran *et al.*, 2002b).

The temperature of fermentation directly affects the **microbial ecology** of the grape must and the biochemical reactions of the yeasts (Fleet and Heard, 1993). Several authors have suggested that some species of non-*Saccharomyces*, such as *Kl. apiculata* and *C. stellata*, have a better chance of growing at low temperatures than *Saccharomyces* (Sharf and Margalith, 1983; Heard and Fleet, 1988) because they can increase their tolerance to ethanol (Gao and Fleet, 1988). These studies observed that in fermentations conducted at 10 ºC, *Kl. apiculata* and *C. stellata* remained at high populations throughout the fermentation (10^7-10^8 cfu/ml), and in some cases *Kl. apiculata* replaced *S. cerevisiae* as the dominant species. Also both the must temperature and the fermentation temperature condition the number of different species as well as their endurance during the fermentation. These changes determine the chemical and organoleptic qualities of the wine (Fleet and Heard, 1993).

Torija *et al.* (2003b) determined the influence of fermentation temperature on a mixed *Saccharomyces* strain population, and the result was that different strains were better or less suited for different temperatures. Moreover, the number of strains who conducted the fermentation increased at low temperatures (Fig 2.1.). This capability to ferment at different temperatures has been currently used to select active dry yeast (Torija *et al.*, 2003b).

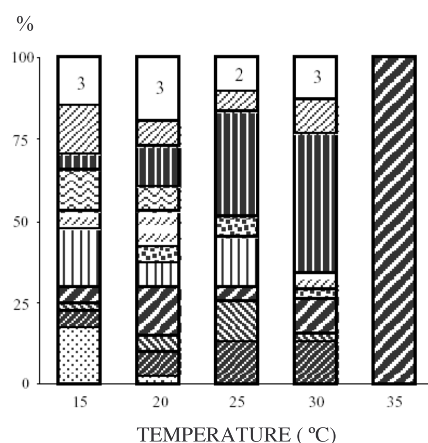


Figure 2.1

Percentages of *Saccharomyces cerevisiae* strains present at the end of fermentation at different temperatures of fermentation. Numbers on top bars represent the numbers of different minoritary strains, <10%. (Torija *et al.* 2003b)

Temperature affects also to **yeast viability**, which decreases as the temperature increases (Ough, 1966; Casey *et al.* 1984; Torija *et al*. 2003b). At low temperatures, maximal population remained constant throughout the alcoholic fermentation. On the other hand, viable cells decreased at high temperatures, especially at 35ºC (Lafon-Laourcade, 1983; Torija *et al*. 2003b). This high yeast mortality may also induce a lower end of fermentation and can produce stuck fermentations with higher sugars contents.

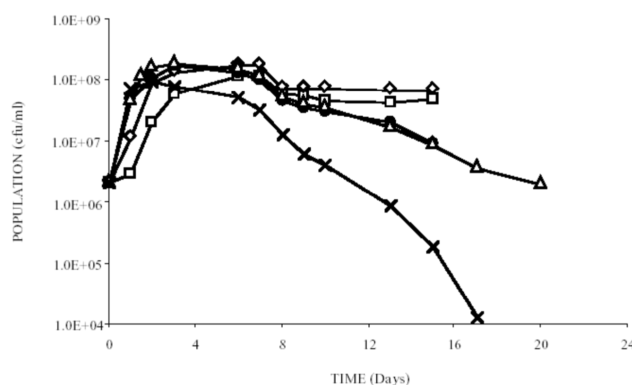


Figure 2.2 Variation in population size during alcoholic fermentation at different temperatures. (Torija *et al*. 2003b)

□ 15ºC ◇ 20ºC ● 25ºC △ 30ºC × 35ºC

2.1.1 The importance of inoculum: cryotolerant yeasts

Using active dry yeast ensures a reproducible product and reduces the lag phase. The use of active dry yeast reduces the number of different indigenous non-*Saccharomyces* and *Saccharomyces cerevisiae* strains due to the starter imposition (Figure 1.4). Therefore, the use of a good yeast starter is crucial for fermentation performed at low temperatures, where the non-*Saccharomyces* strains remained viable long-time throughout the fermentation (Sharf and Margalith, 1983; Heard and Fleet, 1988).

As already shown (see *Item* 1.2.3), the characteristics of yeast starters differ with the type and style of wine to be made and the technical requirements of the winery. The selection of yeast able to ferment at low temperatures is of great interest for winemaking industry (Castellari *et al.*, 1994; Giudici *et al.*, 1998; Massoutier *et al.*, 1998). These yeasts, named cryotolerants, are species of *S. cerevisiae* and *bayanus*, although they are sometimes named *S. uvarum* using the old nomenclature. The yeast selection was carried out in different wine-growing regions in the world, with the aim to find cryotolerant strains (Naumov *et al.*, 2000).

Cryotolerants strains have the important trait of being high producers of glycerol and succinic acid, low producers of acetic acid and synthesizers of malic acid (Table 2.1, Castellari *et al.* 1994).

Table 2.1 Comparison of major fermentative products formed by cryo and non-cryotolerant *S.cerevisiae* strains (Castellari *et al.*, 1994;Feuillat *et al.*, 1997)

	Cryotolerant *S.cerevisiæ* p.r. *uvarum*	Non-cryotolerant *S. cerevisiæ*
Fermentation temperature	6 – 30ºC	12 – 36ºC
Ethanol tolerance	< 14%	≥ 14%
Fermentation capacity	high	medium
Malic acid	Synthesis 0,5 – 1,5 g/l	Degradation 10 – 30%
Acetic acid	30 – 300 mg/l	100 – 800 mg/l
Succinic acid	1 – 2 g/l	0,5 – 1,5 g/l
Glycerol	4 – 8 g/l	3,5 – 6,5 g/l

2.2. Effect of temperature on fermentation kinetic

Temperature has an impact on yeast development and on fermentation kinetics (Fleet and Heard, 1993; Ribéreau-Gayon *et al.*, 2000). Temperature affects the rate of fermentation, with lower temperatures yielding slower fermentation rates and longer fermentations. The duration of the lag phase and the delay before the initiation of fermentation become shorter as the temperature increases (Figure 2.3). The maximal fermentation rate increases also with the temperature (Table 2.2). For musts with higher sugar concentrations, the fermentation becomes more limited as the temperature increases (e.g. 35ºC); in fact, fermentation can stop, leaving sugar in the medium.

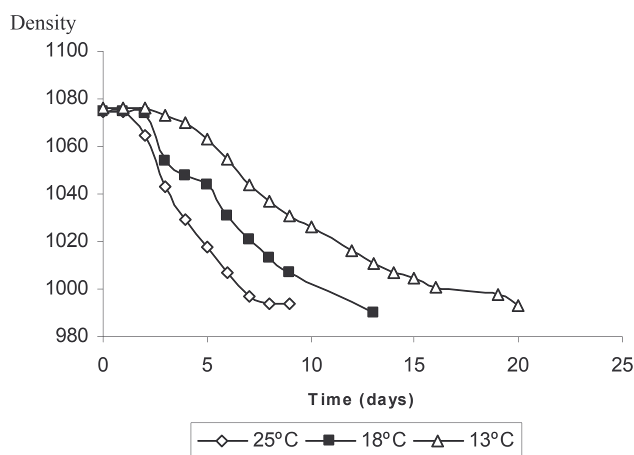


Figure 2.3
Fermentations of Muscat grape at 13, 18 and 25ºC, inoculated with QA23 (Lallemand) (Finca Mas dels Frares, Tarragona 2002)

Table 2.2 Effect of fermentation temperature on yeast population and rate of fermentation (Torija *et al.* 2003b)

	Day of maximal population	Maximal population reached (cfu ml $^{-1}$)	Length of fermentation (days)	Maximal fermentation rate (g l^{-1} day^{-1})
15°C	6	1.18×10^8	15	9.41
20°C	3	1.46×10^8	15	20.87
25°C	3	1.73×10^8	15	52.87
30°C	3	1.95×10^8	20	63.23
35°C	2	0.97×10^8	20	69.69

2.3. Effect of temperature on yeast metabolism

In addition to the influence on fermentation ecology and growth rate, temperature also affects biochemical activities of yeast and, as a result, the production of ethanol, secondary metabolites such as glycerol, acetic acid, succinic acid, and aromatic compounds such as fusel alcohols, acetate esters and fatty acid ethyl esters. These changes could determine the chemical composition and sensory quality of the wine (Fleet and Heard, 1993).

2.3.1. Effect of temperature on alcohol and by-products synthesis

The alcohol yield is generally lower at elevated temperatures, which has been related to a drop in the ethanol yield and a reduction of substrate (Ribéreau-Gayon *et al.*, 1975; Llauradó, 2002; Torija *et al.*, 2003b). A possible cause of this reduction on the final alcohol concentration at high temperatures is the increase of products of other metabolic pathways such as glycerol, acetic acid or acetaldehyde (Table 2.3). In table 2.4, the effect of temperature in the final composition of wines can be also seen, although these compounds are also influenced by the grape must variety (Llauradó *et al.*, 2002).

Table 2.2 Alcohol formation (%vol.) according to fermentation temperature and initial sugar content (Ribéreau-Gayon *et al.*, 2000)

Sugar concentracion	Potential alcohol	Alcohol produced at			
(g/l)	(% vol)	9ºC	18ºC	27ºC	36ºC
127	7.2	7.0	6.9	6.9	4.2
217	12.4	11.8	11.0	9.4	4.8
303	17.3	9.9	9.9	7.7	5.1

Table 2.3 Chemical analyses of wine fermented at different temperatures (Torija *et al.*, 2003b).

Concentration (g l^{-1})	15°C	20°C	25°C	30°C	35°C
Ethanol	93.60 ±0.56[a]	93.04 ±0.88	90.00 ±0.56	89.60 ±0.00	79.52 ±1.84
Glycerol	6.05 ±0.11	6.59 ±0.07	6.91 ±0.11	7.18 ±0.02	7.38 ±0.08
Acetaldehyde	0.05 ±0.00	0.09 ±0.01	0.04 ±0.00	0.04 ±0.00	0.02 ±0.00
Succinic acid	0.74 ±0.06	0.89 ±0.04	0.77 ±0.06	0.92 ±0.08	0.70 ±0.03
Acetic acid	0.08 ±0.01	0.13 ±0.01	0.14 ±0.00	0.13 ±0.01	0.22 ±0.04
Sum of products	100.52	100.74	97.86	97.87	87.84
CO_2	89.53	88.99	86.08	85.70	76.06
Products + CO_2	190.05	189.73	183.94	183.57	163.90
Ethanol yield[b]	47.51	47.23	45.68	45.48	40.36

[a] Mean ±SD
[b] Ethanol produced (g l^{-1}) ×100 / initial sugars (g l^{-1})

Table 2.4 The effect of fermentation temperature on the final composition of wines (Llauradó *et al.*, 2002)

	T	Chardonnay	Garnacha Blanca	Riesling	Cariñena	Pinot Noir
Ethanol (%, v/v)	17 °C	12.5 ± 0.1	12.3 ± 0.0	13.9 ± 0.4	12.0 ± 0.2	12.5 ± 0.0
	13°C	12.4 ± 0.2	11.9 ± 0.4	14.6 ± 0.2	12.0 ± 0.1	12.3 ± 0.1
Acetic acid (mg/L)	17 °C	305 ± 6	360 ± 11	581 ± 49	1545 ± 185	252 ± 46
	13 °C	260 ± 11	377 ± 56	466 ± 23	1276 ± 258	179 ± 24
Acetaldehyde (mg/L)	17 °C	33 ± 8	27 ± 3	99 ± 11	23 ± 4	113 ± 22
	13 °C	26 ± 1	23 ± 1	64 ± 3	32 ± 6	94 ± 8
Ethyl acetate (mg/L)	17 °C	n.d.	21	22 ± 1	34 ± 17	19.3 ± 3.7
	13 °C	12.5 ± 0.3	5.3 ± 1.5	24 ± 10	18 ± 5	11.6 ± .8
Glycerol (g/L)	17 °C	7.6 ± 0.2	8.5 ± 0.5	9.5 ± 0.3	9.6 ± 0.4	6.8 ± 0.4
	13 °C	7.5 ± 0.6	8.0 ± 0.1	9.4 ± 0.5	9.7 ± 0.2	6.2 ± 0.4
Succinic acid (g/L)	17 °C	0.8 ± 0.1	0.8 ± 0.0	1.0 ± 0.1	0.6 ± 0.1	0.7 ±0.1
	13 °C	0.8 ± 0.1	0.9 ± 0.1	0.8 ± 0.1	1.1 ± 0.5	0.7 ± 0.1

Each value represents mean ± standard deviation of 3 independent samples.
T = fermentation temperature
Two way anova (variety/temperature) was performed and significance level set at $p \leq 0.05$

2.3.2. Influence of temperature on volatile compounds

The aromatic complexity of wines varies according to the primary or varietal flavour (flavour compounds originating from the grapes), the secondary or fermentative flavour (produced by yeast and bacteria during alcoholic and malolactic fermentation) and tertiary or post-fermentative flavour (compounds that appear during the ageing process) (Boulton *et al.*, 1996; Rapp, 1998; Lambrechts and Pretorius, 2000).

The main groups of compounds that form the "fermentation bouquet" are the organic acids, higher (fusel) alcohols and esters, and to a lesser extent, aldehydes (Rapp and Versini, 1991). Fermentation increases the chemical and flavour complexity of wine by assisting in the extraction of compounds from solids present in grape must, modifying some grape derived compounds, and producing a substantial amount of yeast metabolites. The formation of aroma compounds by yeast, i.e. short and medium-chain fatty acids and their corresponding ethyl esters, higher alcohols, and their corresponding acetate esters, is intrinsically linked to the metabolism of yeast cells (Figure 2.4)

Table 2.5 Principal volatile compounds present in wines (Lambrechts and Pretorius, 2000)

	Conc. in wine (mg/l)	Threshold (mg/l)	Odour
MCFA	Traces - 50	10	Fatty, sour, rancid, cheese,...
EEFA	Traces – 4	0.08 – 0.58	Floral, fruity
Acetate of HA	0.01 – 8.1	0.26	Banana, rose, fruity
Higher alcohols (HA)	15 - 500	300	Marzipan
Phenylethyl alcohol	10 - 180	8	Floral, rose

MCFA: Medium Chain Fatty Acid; EEFA: Ethyl Ester Fatty Acids

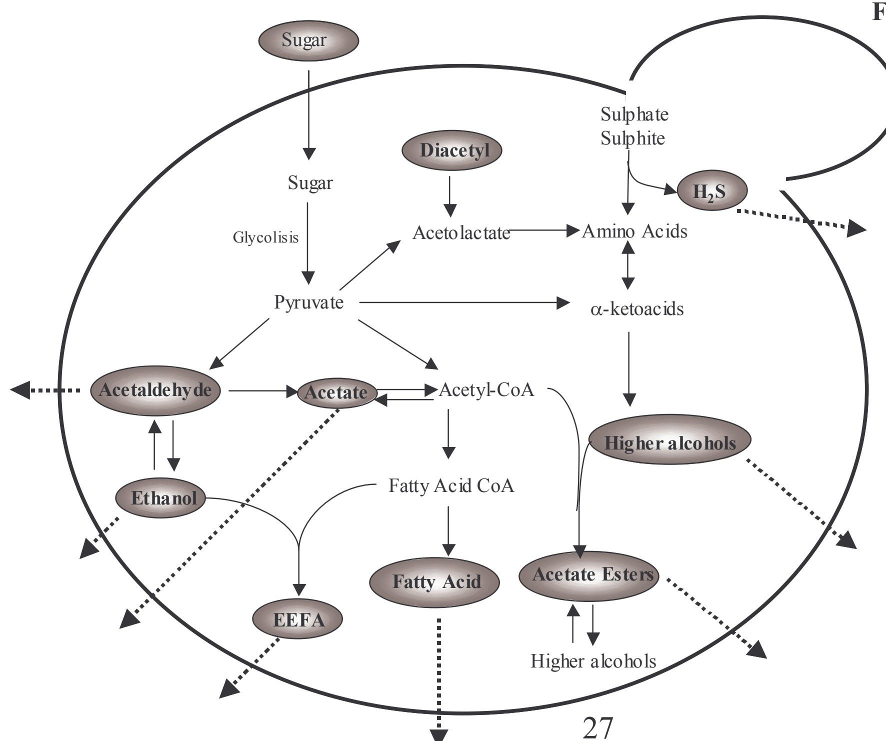


Figure 2.4 A schematic representation of the formation of aroma compounds by yeast (adapted from Lambrechts and Pretorius, 2000)

Some studies done with fermentations at low temperatures observed an increase of ethyl and acetate esters and overall medium-chain fatty acids (MCFA), although this increase is strain dependent (Torija *et al.*, 2003a) (Table 2.6). On the other hand, the production of fusel alcohols decrease with the temperature (Castellari *et al.*, 1995;Llauradó *et al.*, 2002), whatever the variety analysed, confirming the consistence of these values (Table 2.7).

Table 2.6 Content of volatile compounds in wines fermented with *S.cerevisiae* and *S.bayanus* strains at 13ºC and 25ºC (Torija *et al.*, 2003a).

	S.cerevisiae		***S.bayanus***	
	25ºC	13ºC	25ºC	13ºC
Phenyl-2-ethanol	13.91	15.48	49.22	61.55
Σ Acetates	0.23	3.43	0.51	1.15
Σ Ethyls	0.21	1.17	0.14	0.32
Σ MCFA	6.77	14.92	5.57	12.04

Table 2.7 Effect of fermentation temperature on the higher alcohol composition of final wines (mg/L) (Llauradó et al. 2002).

	Chardonay	**Garnacha blanca**	**Riesling**	**Cariñena**	**Pinot Noir**
17ºC	411 ± 96	197 ± 5	262 ± 0	159 ± 42	688 ± 43
13ºC	320 ± 18	161 ± 45	305 ± 51	169 ± 0	423 ± 45

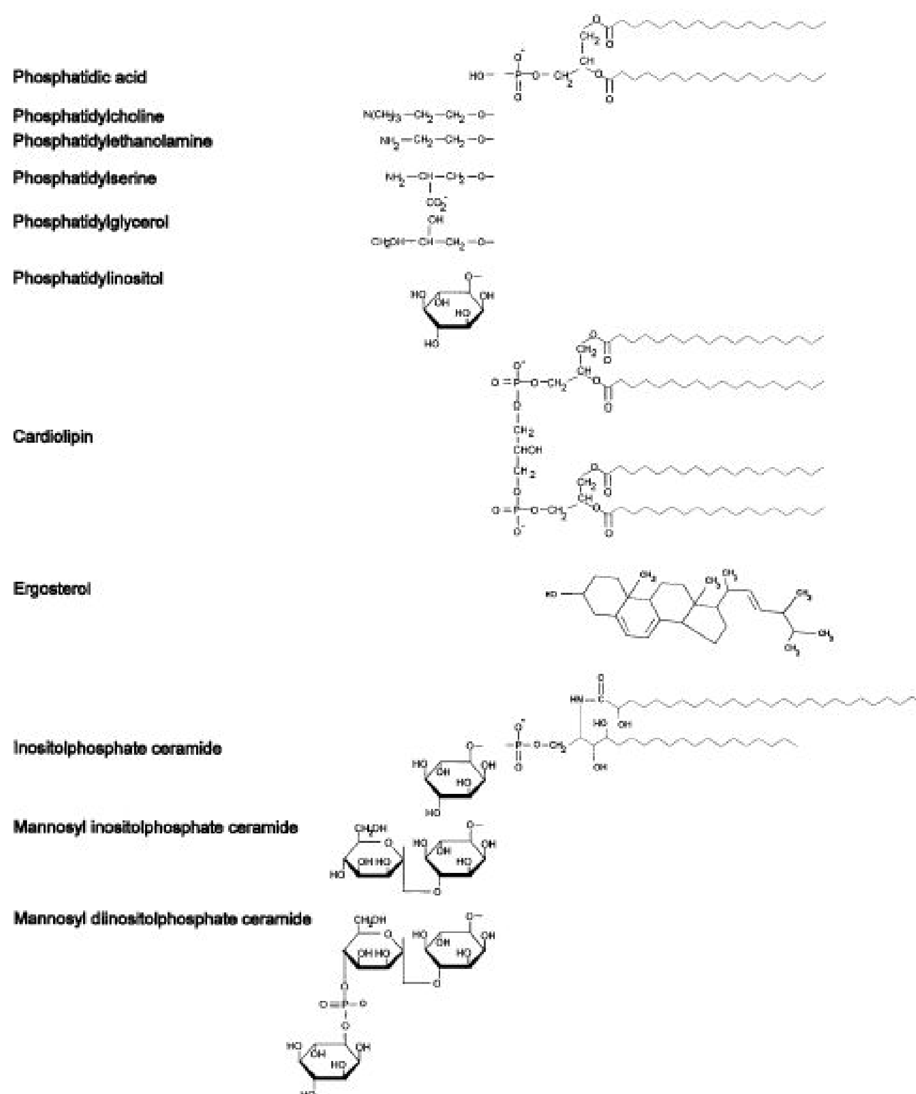
2.4. Effect of temperature on membrane fluidity and lipid composition

Another mechanism in response to cold is the adaptation of the membrane fluidity. Dynamic and structural characteristics of membranes can be changed, either by changing the environmental conditions, by changing the molecular composition of the membrane, or by adding foreign molecules that interact with membrane constituents. This explains why changes in membrane fluidity are observed in response to many environmental stresses, and why cells, in contrast, can control their fluidity by the modulation of their membrane composition, in order to maintain an optimal level of fluidity within the lipid matrix (Beney and Gervais, 2001).

2.4.1. Yeast membrane composition

Major lipid components of yeast membranes are phospholipids, sterols, sphingolipids and glycerolipids (reviewed in Daum *et al.*, 1998).

Figure 2.5. Structure of lipids found in *S.cerevisiae* membrane (van der Rest *et al.* 1995)



Phospholipids (PL), 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 (mostly saturated) and sn-2 (mostly unsaturated) positions, and a phosphate group in the sn-3 position. The most abundant fatty acids in *S. cerevisiae* PL are C-16:0 (palmitic acid), C16:1 (palmitoleic acid), C18:0 (stearic acid) and C18:1 (oleic acid). Various molecules such as choline (in PC), ethanolamine (in PE), serine (in PS), *myo*-inositol (in PI) and glycerol (in PG) can be linked to the phosphoril group (Figure 2.5). The inner plasma membrane is enriched in PE, PI, and PS, while the external is enriched in PC and sphingolipids. The pathway of glycerophospholipid formation in yeast is shown in Figure 2.6.

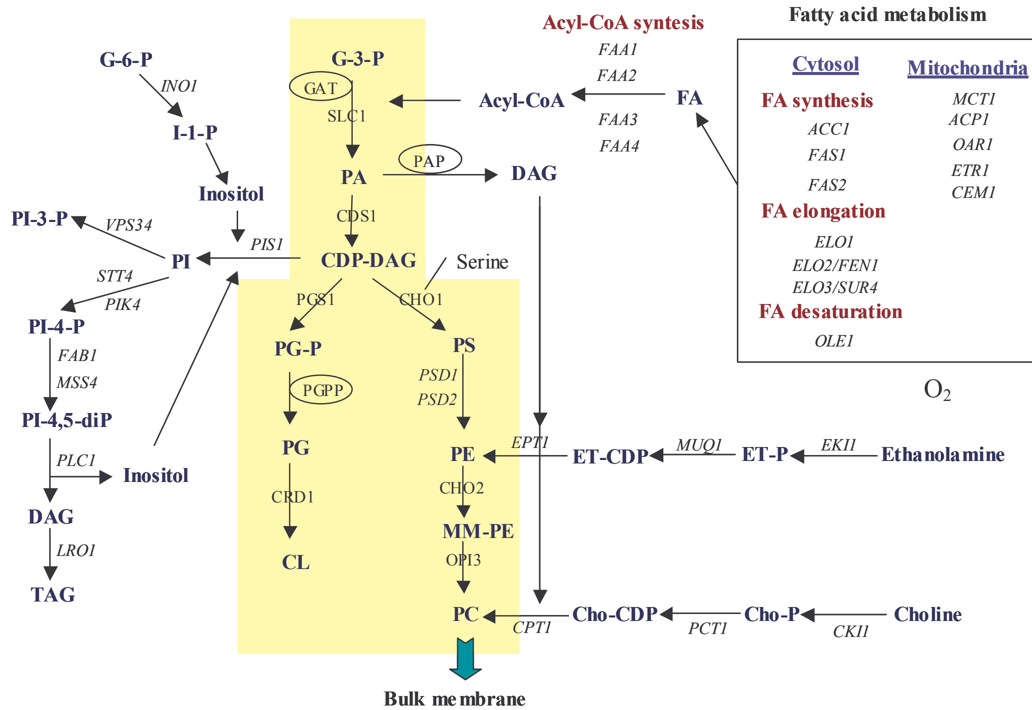


Figure 2.6 Phospholipid biosynthetic pathway in *S.cerevisiae* (adapted from van der Rest *et al.* 1995)
CL: Cardiolipine; DAG: Diacylglicerid; FA: Fatty acid; G-3-P: Glycerol 3P; PA: Phosphatidic acid; PC: Phosphatidylcholine; PE: Phosphatidylethanolamine; PG: Phosphatidylglycerol; PI: Phosphatilylinositol; PS: Phosphatidylserine; TAG: Triacylglicerid

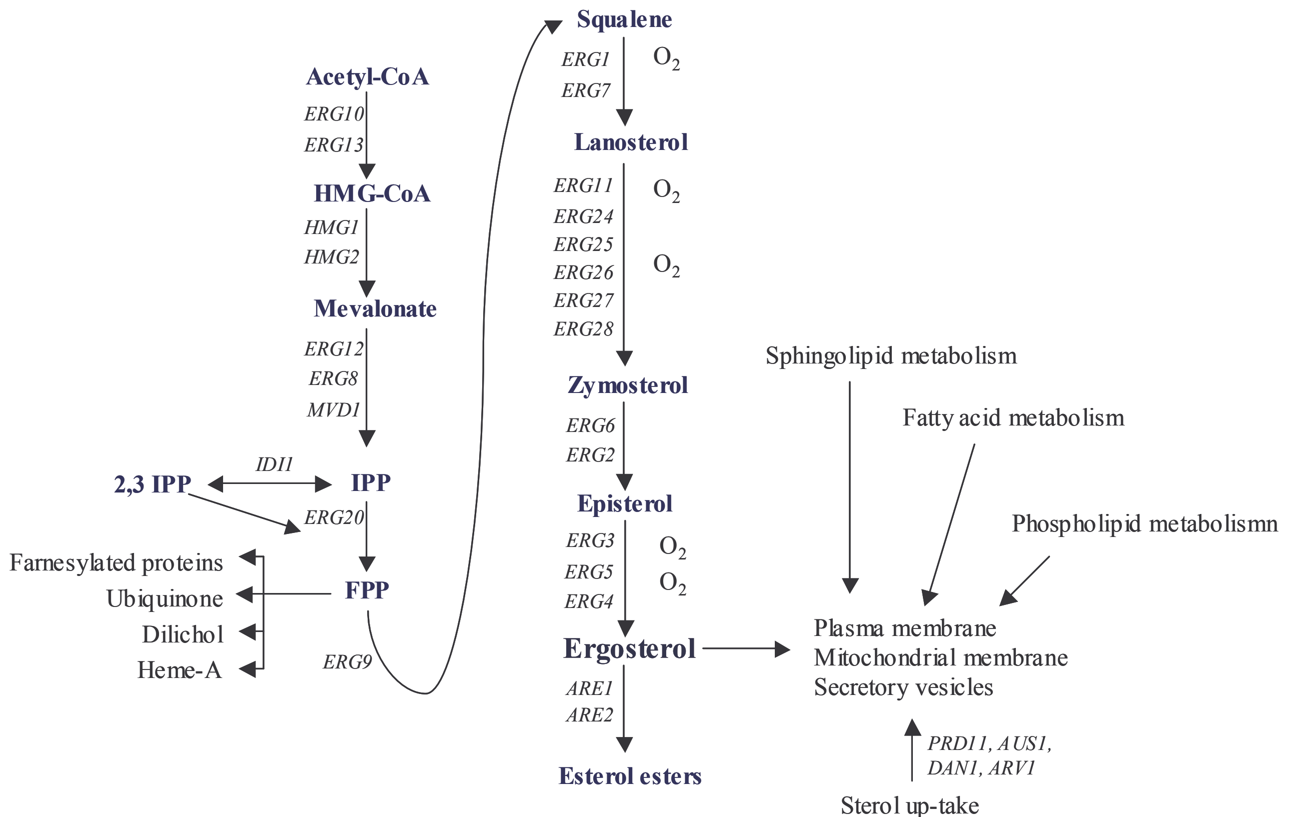


Figure 2.7 Sterol biosynthetic pathway in S.cerevisiae (adapted from Kwast *et al.*, 2002)

Sphingolipids have a ceramide backbone, which is composed of a long-chain base, phytosphingosine (D-erythro-2-amino-octadecane-1, 3, 4-triol) in *S. cerevisiae*. *S. cerevisiae* have mainly three major sphingolipids: inositol phosphate ceramide, mannosyl-inositolphosphate-ceramnide, and mannosyl-diinositolphosphate-ceramide. More than 90% of sphingolipids are located in the plasma membrane. The sphingolipids constitute about 30% of the total phospholipid content (Patton and Lester, 1991).

Sterols are compact rigid hydrophobic molecules with a polar hydroxyl group. Yeast plasma membrane contains mainly ergosterol and in less concentration zymosterol (Zinser *et al.*, 1991; van der Rest *et al.*, 1995). Sterols are essential lipid components of eukaryotic membranes and are important regulators of membrane permeability and fluidity. The sterols determine to a large extent the rigidity of the plasma membrane, which, in turn may affect the lateral movement and the activity of membrane proteins. Figure 2.7 shows the ergosterol biosynthetic pathway in yeast.

2.4.2. Effect of temperature on biological membranes

Membrane lipids are the only structural elements of the cell to be reshaped as environmental temperature changes. Temperature determines both the rates of molecular motion of membrane constituents and the phase state and order of membrane lipids. Changes in either of these features of the membrane organization can significantly disturb the membrane function. (Hunter and Rose, 1972)

Reduced temperatures cause a decrease in membrane fluidity. This is compensated by the increased production of unsaturated fatty acids. Decreasing length of the fatty acyl chains and increasing extent of unsaturation leads to a less ordered structure and an increase in fluidity. Psychrophilic yeasts have a degree of unsaturation higher than mesophilic and thermophilic yeasts (Watson, 1987).

In addition to their role in adaptation to low temperatures, membranes with unsaturated fatty acids also contribute to an organism's ability to adapt to other environmental stresses. For example, tolerance of yeast to ethanol has been shown to be affected by membrane lipid composition and membrane fluidity, with higher unsaturation correlating with higher ethanol tolerance (Alexandre *et al.*, 1994b).

Phospholipids (PLs) are polymorphic, assuming a variety of different phase organizations depending on temperature, pressure, degree of hydratation and the chemical composition of the phospholipid itself. Three phases have been identified in phospholipids (Figure 2.8).

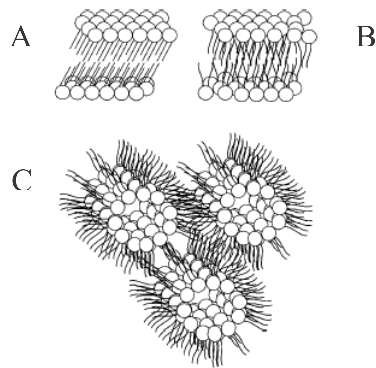


Figure 2.8 Organization of phospholipids (Beney and Gervais, 2001).

A) bilayer in the gel phase (L_β),
B) bilayer in the liquid crystalline phase (L_α),
C) hexagonal (non-bilayer) (H_{II}) phase

A decrease in temperature or an increase in pressure lead to a decrease in the membrane fluidity, and at a typical temperature (T_m), the membrane could change from the fluid crystalline phase to the gel phase (Beney and Gervais, 2001). Several factors affect the transition temperature (T_m) of phospholipids, but the most important is the nature of the fatty acids present in the molecule. The phase transition temperature of fatty acids increases with the chain length while the degree of unsaturation and the position of the double bond mainly located in the centre of the chain decreases the T_m. Also, the nature, size and charge of the polar head groups of PLs have an effect on the transition temperatures and the physical state of the membrane. Lipids such as PC, PS, PI and sphingolipids, which have head groups and acyl chains with comparable cross-sectional area, are cylindrical and organize easily in bilayers. Lipids which have smaller head groups than acyl chains, such as PE, CL and sterols, are cone shaped, and at high concentrations in the membrane may locally induce a high membrane curvature and membrane-packing defect (van der Rest *et al.*, 1995).

The maintenance of the dynamic characteristics of the membrane is also regulated by others classes of molecules (van der Rest *et al.*, 1995). The sterols, determine to a large extent the rigidity of the plasma membrane, which, in turn may affect the lateral movement and the activity of membrane proteins.

2.4.3. Yeast lipid composition in wine-making conditions

The fatty acid composition of a cell can also be influenced by the environment's lipid composition since it can include fatty acids from the medium in its own phospholipids (Thurston *et al.*, 1981; Rosi and Bertuccioli, 1992; Benchekroun and Bonaly, 1992). In grapes, unsaturated fatty acids (UFA) represent 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 saturated fatty acids (SFA), palmitic acid (C16) is the most abundant (Bertrand and Miele, 1984; Castela *et al.*,

1985). However the initial fatty acid content of the must depends on the technological procedures applied to the grapes and musts such as pressing, maceration or clarification (Bertuccioli and Rosi, 1984; Delfini and Costa, 1993). Under industrial conditions, natural musts have low sterol contents among of which ß-sitosterol is the main phytosterol (Le Fur *et al.*, 1994).

In white wine production the absence of oxygen suppresses the fatty acid desaturation and sterol biosynthesis by yeast and consequently the capacity to synthesize ergosterol essential to protect the yeast against ethanol stress (Alexandre *et al.*, 1994a; Alexandre *et al.*, 1994b) is reduced. So, in wine fermentation, ergosterol synthesis can occur if oxygen is added during fermentation but the efficiency of oxygen additions decreases as the fermentation progresses (Sablayrolles *et al.*, 1996). However, the yeasts can grow in the absence of oxygen incorporating exogenous sterols, such as β-sitosterol (Ness *et al.*, 1998; Luparia *et al.*, 2004) and unsaturated fatty acids (Chen, 1980).

Table 2.8 Percentages of cell membrane individual fatty acids at the end of fermentation. Values are expressed as percentages of total fatty acids. (Torija *et al.*, 2003a).

	S. cerevisiae A		***S. cerevisiae* B**		***S. bayanus* C**	
	25°C	**13°C**	**25°C**	**13°C**	**25°C**	**13°C**
C8:0	0.94 ± 0.18	1.18 ± 0.35^{bc}	1.51 ± 0.35	2.12 ± 0.40^{ac}	1.36 ± 0.33	$3.12\pm0.45^{*ab}$
C10:0	9.54 ± 2.47^{b}	8.64 ± 1.81^{bc}	13.59 ± 0.80^{ac}	14.67 ± 1.16^{ac}	9.70 ± 0.92^{b}	$19.21\pm1.37^{*ab}$
C12:0	5.14 ± 2.05^{bc}	2.15 ± 0.56^{bc}	8.08 ± 0.42^{ac}	$5.20\pm0.58^{*ac}$	11.08 ± 1.32^{ab}	$13.76\pm0.70^{*ab}$
ΣMCFA	$\mathbf{15.61\pm4.70}^{bc}$	$\mathbf{11.97\pm2.69}^{bc}$	$\mathbf{23.18\pm1.14}^{a}$	$\mathbf{21.99\pm1.32}^{ac}$	$\mathbf{22.13\pm2.56}^{a}$	$\mathbf{36.09\pm2.21}^{*ab}$
C14:0	0.32 ± 0.18^{c}	0.39 ± 0.02^{c}	0.67 ± 0.15^{c}	0.28 ± 0.13^{c}	1.94 ± 0.19^{ab}	2.16 ± 0.44^{ab}
C16:0	35.31 ± 2.51^{bc}	29.73 ± 2.60^{b}	43.61 ± 3.55^{a}	39.80 ± 3.31^{ac}	43.13 ± 1.16^{a}	$33.82\pm2.61^{*b}$
C18:0	20.29 ± 1.71^{c}	$11.55\pm0.29^{*c}$	17.46 ± 0.99	$10.17\pm1.15^{*c}$	14.72 ± 1.54^{a}	$8.37\pm0.89^{*ab}$
ΣSFA	$\mathbf{55.93\pm1.22}^{bc}$	$\mathbf{41.67\pm2.34}^{*b}$	$\mathbf{61.73\pm2.48}^{a}$	$\mathbf{50.25\pm2.41}^{*ac}$	$\mathbf{59.78\pm0.59}^{a}$	$\mathbf{44.34\pm1.81}^{*b}$
C14:1	0	$0.29\pm0.07^{*bc}$	0	$0.18\pm0.04^{*ac}$	0	$0.46\pm0.02^{*ab}$
C16:1	16.64 ± 2.60^{bc}	$26.10\pm1.09^{*bc}$	7.25 ± 0.57^{a}	$18.28\pm1.80^{*ac}$	9.54 ± 0.55^{a}	$12.22\pm0.52^{*ab}$
C18:1	11.82 ± 2.03^{bc}	$19.98\pm3.91^{*bc}$	7.83 ± 1.33^{a}	9.30 ± 1.46^{a}	8.47 ± 1.56^{a}	6.88 ± 0.95^{a}
ΣUFA	$\mathbf{28.46\pm4.22}^{bc}$	$\mathbf{46.37\pm4.93}^{*bc}$	$\mathbf{15.09\pm1.64}^{a}$	$\mathbf{27.76\pm2.01}^{*ac}$	$\mathbf{18.09\pm2.04}^{a}$	$\mathbf{19.57\pm1.14}^{ab}$

* significantly different to 25°C ($P \leq 0.05$)

a, b, c significantly different respect to the indicated strain at the same temperature ($P \leq 0.05$)

It is well established that environmental temperature affects the unsaturation degree (Suutari and Laakso, 1994), however this might not be a universal response in yeast (Suutari et al., 1990). In the case of wine yeast, Torija *et al.* (2003a) observed that the optimal membrane fluidity at low temperatures was modulated by changes in the unsaturation degree in *S. cervisiae* strains, showing a higher percentage of UFA at low temperatures. However, in *S. bayanus* strains temperature only had a significant effect

on the medium chain fatty acids (MCFA) (Table 2.8). The increased synthesis of MCFA could also modulate membrane fluidity (Torija *et al.*, 2003a).

Moreover, some authors observed that a decrease in temperature in the exponential phase induced variations in the phospholipid content (Hunter and Rose, 1972) whereas the sterol content of *S. cerevisiæ* remained high (Rozès *et al.* 1988).

2.5. Effect of temperature on transport and nitrogen up-take

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). The membrane permeases are highly temperature-dependent, because changes in temperature produce conformational changes in these molecules (Entian and Barnett, 1992).

Both nitrogen and temperature has an influence on the fermentation kinetics (Figure 2.8). Higher Yeast Assimilable Nitrogen (YAN) content and higher fermentation temperature yield higher fermentation rates (Llauradó 2000, 2002).

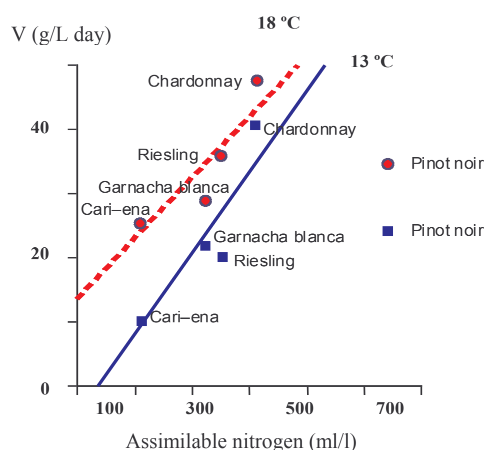


Figure 2.8
The effect of fermentation temperature (13ºC and 18ºC) on the correlation between maximum fermentation rate and assimilable nitrogen. (Llauradó *et al.* 2002)

Nitrogen assimilation has been shown to depend on fermentation temperature. Thus, even if the overall pattern of amino acid consumption remains constant over the range of temperatures used in wine production, low temperatures result in a lower rate of amino acid assimilation (Lopez *et al.*, 1996), consistent with lower rates of fermentation and yeast growth.

2.6. Stress and low temperature

2.6.1. General stress response

All environmental changes that elicit an adaptive response are qualified of “stress”. Consequently, the molecular and physiological response of an organism to changes in

the environment is referred to as "stress response", while the ability to withstand unfavourable or changing external conditions is defined as "stress resistance" or "stress tolerance".

The molecular basis of response to many different stresses has been extensively studied in *S. cerevisiae* (Gasch *et al.*, 2000; Hohmann and Mager, 2003). In response to various stresses the transcription of a common set of genes is changed; this defines the "general stress response" (Ruis and Schüller, 1995). Genome-wide transcriptional profiling has shown that about 10% of the genome is induced or repressed in this response, and the genes involved are defined as the environmental stress response, ESR (Gasch *et al.*, 2000), or common stress response, CER (Causton *et al.*, 2001). Induced ESR genes are involved in a variety of cellular functions such as protein folding and degradation, transport and carbohydrate metabolism. Repressed ESR genes are generally involved in cell growth-related processes, including RNA metabolism, nucleotide biosynthesis, secretion and ribosomal performance (Gasch, 2003). The regulation of the ESR is determined by the function of the two transcription factors, Msn2p and Msn4p that bind to stress response elements (STREs) in the promoters of their target genes (Martinez-Pastor *et al.*, 1996; Schmitt and McEntee, 1996; Gorner *et al.*, 1998). Under specific stress conditions, other transcription factors are also involved in the modulation of the gene expression.

Most heat-shock proteins (Hsps) are induced in response to most of the investigated stress conditions, suggesting that Hsps can be classified as general stress-protection proteins (Ruis and Schüller, 1995; Piper, 1997). However, the data show that this "general" response is complemented by molecular adaptations, which are specific to each type of stress (Ruis and Schüller, 1995; Mager and Hohmann, 1997).

2.6.2. Yeast Stress response in wine fermentations

Numerous factors influence the fermentation performance of wine yeast. Following a successful inoculation of grape must with an appropriate starter culture strain, the ability of wine yeast to adapt to and cope with the hostile environment and stress conditions prevailing in grape juice fermentation are of vital importance to fermentation performance. There is a direct correlation between fermentation efficiency and stress resistance, which refers to the ability of a yeast strain to adapt efficiently to a changing environment and unfavourable growth conditions (Ivorra *et al.*, 1999).

The most vexing problems of wine production, particularly stuck and sluggish fermentation and the production of off-flavours by the yeast, are usually associated with the inability of yeast strains to respond and adapt to unfavourable, stressful growth conditions (Attfield, 1997; Bisson, 1999). The best-studied stress responses include: (a) the temperature (heat or cold) shock stress response; (b) the response to the limitation of essential nutrients; (c) responses to changes in osmotic pressures (hyper- or hypoosmotic shock); and (d) ethanol toxicity, all of which are of obvious importance during fermentation (reviewed in Bauer and Pretorius, 2000; Hohmann and Mager, 2003).

Analysis of stress resistance has been recently proposed as suitable criterion for wine yeast selection (Zuzuarregui and del Olmo, 2004a; 2004b). In wine fermentation the stress response is primarily associated with the stationary phase. This stage is usually reached by nitrogen source limitation, high ethanol concentrations, or a combination of both (Fleet and Heard, 1993; Boulton *et al.*, 1996). So, many stress genes are induced on entry in stationary phase or shortly afterwards (Riou *et al.*, 1997; Backhus *et al.*, 2001; Perez-Ortin *et al.*, 2002; Rossignol *et al.*, 2003).

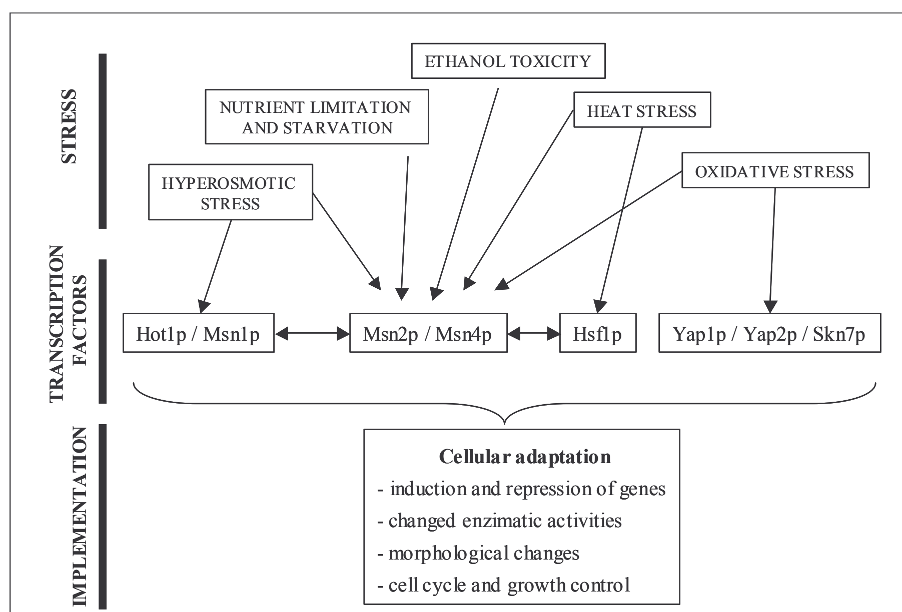


Figure 2.9 Representation of the best studied and most frequently encountered stress conditions and the most relevant transcription factors involved in the control of the expression of stress responsive genes in *S.cerevisiae*. (adapted from Bauer and Pretorius, 2000).

2.6.3. Low temperature gene response

Little is known about the mechanisms responsible for growth and survival at low temperature. In *S.cerevisiae*, several cold-inducible genes have been identified. *TIP1*

(temperature inducible protein) and other members of its family (*TIR1* and *TIR2*) are induced by cold shock (Kondo and Inouye, 1991; Kowalski *et al.*, 1995). These genes encoding a serine- and alanine-rich cell wall protein may be involved in maintaining cell wall integrity during stress. *NSR1*, is required for normal pre-rRNA processing and cell growth upon exposure to cold shock (Kondo *et al.*, 1992). The fatty acid desaturase gene *OLE1* has been shown also to be also induced at low temperature (Nakagawa *et al.*, 2001), as observed in other eukaryotic organisms such as plants (Browse and Xin, 2001), dimorphic fungi (Laoteng *et al.*, 1999) and prokaryotes (Thieringer *et al.*, 1998; Aguilar *et al.*, 1999), suggesting that membrane fluidity is a common response to cold.
These cold-inducible genes are thought to play an important role in acclimation to low temperature. Although some studies in yeast gene expression at low temperatures have been done (Sahara *et al.*, 2002; Homma *et al.*, 2003) the entire mechanisms of low temperature response and low temperature-dependent gene expression are still unclear in yeast. Major problems that result from cold shock are the reduction of membrane fluidity, impaired protein synthesis and stabilization of secondary structures of RNA and DNA. As a result, ribosomes have more difficult translating mRNA.
Sahara *et al.* (2002) analysed the expression states of yeast cells exposed at low temperature (10ºC) up to 8 hours. They found several phases according to the expression profile: (i) in early phase, genes involved in RNA polymerase I and rRNA processing are up-regulated; (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. Through these phases, yeast cells may improve reduced efficiency of translation and enhance cell protection mechanism to survive under low temperature conditions.
These results agree with others done by Schade *et al.* (2004), who studied the cold adaptation of yeast cells exposed up to 60h at low temperatures (10ºC). *S.cerevisiae* initiates different expression programs during the response to cold, and their regulation is gene- and time-specific. Two distinct cold responses were identified: early cold response (ECR, < 2h) and late cold response (LCR, >12h). ECR includes the induction of genes implicated in RNA metabolism and lipid metabolism, whereas genes induced during the LCR mainly encode proteins that are involved in protecting the cell against a variety of stresses. The environmental stress response that occurs during the late cold response is mediated via the transcriptional activators Msn2p and Msn4p. In contrast, the transcriptional response of the ECR genes was Msn2p /Msn4p-independent.

2.7. Role of reserve carbohydrates

Glycogen and trehalose are the main reserve carbohydrates in yeast cells (reviewed by Francois and Parrou, 2001) and recent extensive work is proving how important these carbohydrates are for the viability, vitality and physiological activity of yeast. 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 (Ribeiro *et al.*, 1994; De Virgilio *et al.*, 1994; Gounalaki and Thireos, 1994) and its accumulation correlate 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 the carbohydrate reserves trehalose and glycogen is induced during late cold response. Exogenous trehalose restored the viability of yeast cells during freezing by possible protection of the cellular membrane (Diniz-Mendes *et al.*, 1999). At high temperature, trehalose can protect cells by acting as a “chemical chaperone”, which reduces heat-induced denaturation and aggregation of proteins in yeast cells (Welch and Brown, 1996). Trehalose could protect yeast cells at low temperature by similar mechanisms (Sahara *et al.*, 2002).

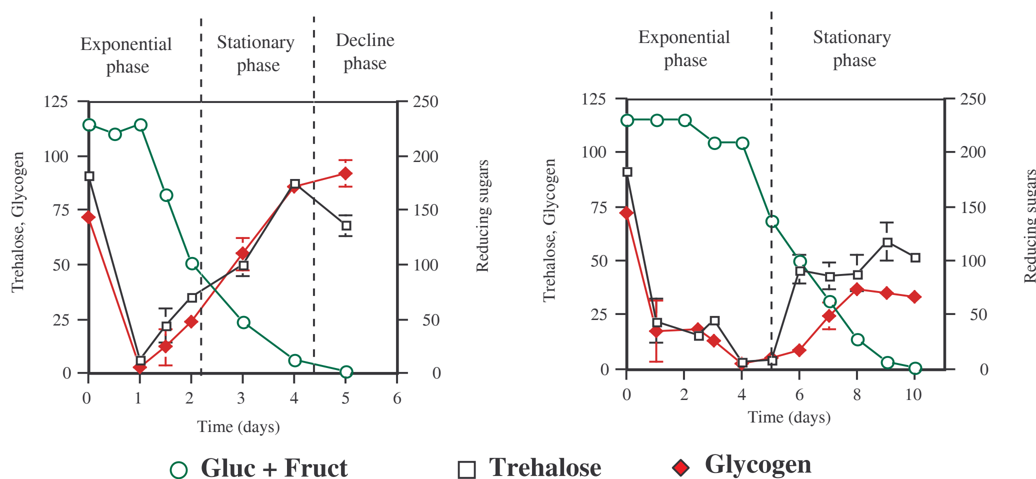


Figure 2.10 Effect of fermentation temperature (13 and 25ºC) on the changes of trehalose and glycogen intracellular levels in QA23 yeast strain. Trehalose and glycogen are expressed in mg 10^{-7} cells; reducing sugars in g/l. (Novo *et al.*, 2003c)

In fermentation conditions yeast cells began to accumulate storage carbohydrates from growth arrest (Fig 2.10) (Rossignol *et al.*, 2003; Novo *et al.*, 2003c). Genes encoding proteins involved in metabolism of glycogen and trehalose were induced during growth phase and their expression peaked in the stationary phase (Rossignol *et al*., 2003). Novo *et al.* (2003c) observed a correlation with the initial degradation of trehalose and glycogen by yeast and the timing of lag phase. On the other hand, a similar accumulation profile of both reserve carbohydrates was found along the fermentation at 13ºC and 25ºC.

3. NITROGEN METABOLISM

Nitrogen compounds are essential to the growth and metabolism of yeast. Of all the nutrients assimilated by yeasts during fermentation of grape must, nitrogen is, quantitatively, the most important after carbon-compounds. A wide variety of nitrogen-containing compounds are present in grape juice. These depend on the variety of grape and the time of harvest, as well as upon several aspects of vineyard management, including nitrogen fertilization, berry maturation, vine water status, soil type and fungal infection (Henschke and Jiranek, 1992).

The nitrogen composition of grapes affects the growth and metabolism of yeast, the fermentation rate and the completion of fermentation (Bisson, 1991). Llauradó *et al.* (2002) showed that maximum fermentation rates were correlated with the available nitrogen of different grape varieties.

In fact, nitrogen impacts yeast cells in two ways, one by increasing the biomass production and the other by stimulating the rate of sugar utilization. Deficiencies in the supply of nitrogenous compounds remain the most common causes of poor fermentative performance and sluggish or stuck fermentation (Bisson, 1991; Bisson, 1999). Such problematic and incomplete fermentations occur in part because nitrogen depletion irreversibly arrests hexose transport (Bely *et al.*, 1990a). Other problems related to the nitrogen composition of grape must include the formation of reduced-sulphur compounds, in particular hydrogen sulfide (Henschke and Jiranek, 1991; Jiranek *et al.*, 1995b), and the potential formation of ethyl carbamate from metabolically produced urea (Ough, 1991). In a positive context, there is potential for manipulating the fermentation bouquet of wine by controlling the amount and type of nitrogen source (Rapp and Versini, 1991; Lambrechts and Pretorius, 2000).

3.1. Grape must composition

The total nitrogen content of grape juices ranges from 60 to 2400 mg/l. Agenbach (1977) established a minimal amount of 140 mg N/l to avoid stuck fermentations, although the nitrogen exigencies and preferences are strain dependent (Jiranek *et al.*, 1991; Manginot *et al.*, 1998). Amino acids, ammonium, peptides and proteins are, quantitatively, the most important nitrogen compounds. *S.cerevisiae* is unable of hydrolyzing grape proteins to supplement nitrogen-deficient musts, and relies therefore on the ammonium and amino acids present in the juice, which constitute the Yeast Assimilable Nitrogen (YAN). Proline and arginine are the most common nitrogenous compounds in grape juice (30-65 % of total amino-acid content), followed by alanine, glutamine, glutamate, serine and threonine, while ammonium ion levels may also be high (up to 40 %), depending on grape variety and vineyard management. (Llauradó et al. 2002)

Table 3.1. Individual amino acid composition of some Spanish grape musts (Llauradó *et al*. 2002)

Amino Acid		Chardonnay	Garnacha Blanca	Riesling	Cariñena	Pinot Noir
	His	n.d.	n.d.	n.d.	21	40
	Arg	437	354	514	243	1202
Basic	Lys	0.8	1.7	1.6	4.0	3.1
	Ala	183	30	72	61	233
	Val	16	4.1	26	10	35
	Leu	12	5.9	28	8.3	37
	Ile	9.1	2.2	19	6.5	23
	Pro	176	25	18	14	26
	Phe	15	7.5	17	4.3	17
	Trp	11	5.5	19	5.3	10
	Met	22	1.2	5.8	2.3	13
Proteic Neutral	Gly	11	2.1	6.3	5.5	10
	Asn	n.d.	5.8	5.3	1.7	12
	Gln	369	114	264	67	n.d.
	Ser	99	16	58	28	82
	Thr	83	20	57	22	115
	Tyr	22	12	11	5.5	15
	Asp	20	13	15	6.4	19
Acid	Glu	45	40	35	15	112
	Orn	2.0	2.6	1.5	1.7	13
No	Cit	7.9	5.4	12.0	1.6	30
Proteic	Aba[a]	2.5	1.5	n.d.	0.1	1.3
	Gaba[b]	79	29	144	113	160
	Tau	2.2	1.6	0.1	n.d.	0.6
	Total	1625	700	1330	647	2209

[a]á–aminobutyric acid
[b]ã–aminobutyric acid
n.d.: not detected

3.2. Central Nitrogen Metabolism

Nitrogen-containing compounds in grape might meet one of three fates (Henschke and Jiranek, 1992): 1) Without modification, utilized as that compound directly in biosynthesis; 2) Converted to a related compound and utilized in biosynthesis; or 3) Degraded releasing nitrogen either as free ammonium ion or as bound nitrogen via a

transamination reaction. In this case, the skeleton of the nitrogen-containing compound would be a waste product.

Yeasts are able to use a wide variety of nitrogen sources for growth. Essentially, all nitrogen compounds accumulated are degraded to one of two end-products, ammonium or glutamate (Fig. 3.1). Glutamate together with glutamine plays an essential role in the nitrogen metabolism, and the interconversion of ammonia, glutamate and glutamine is called **Central Nitrogen Metabolism (CNM)** (Fig 3.1). From these two amino acids all other nitrogen containing compounds in the cell are produced (Fig 3.2).

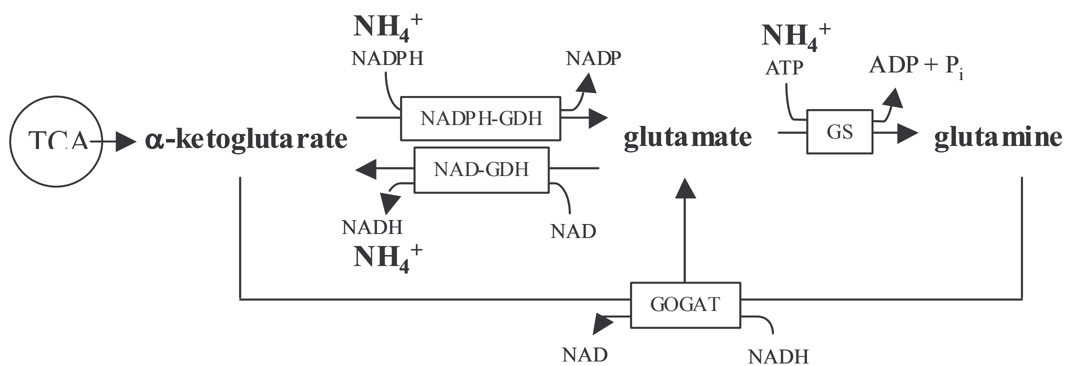


Figure 3.1 Central Nitrogen Metabolism of *S.cerevisiae* (adapted from ter Schure *et al.* 2000)

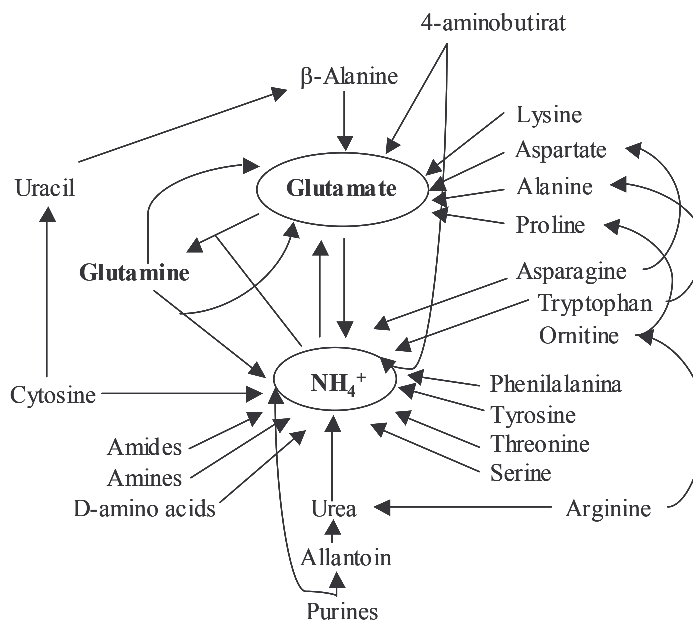


Figure 3.2 A schematic representation of the degradation of nitrogenous compounds by wine yeast (adapted from Pretorius 2000).

Both glutamate and glutamine can be synthesized directly using ammonia as amino donor. The NADPH dependent Glutamate DeHydrogenase (NADPH-GDH) converts

ammonia and α-ketoglutarate into glutamate and the Glutamine Synthetase (GS) produces glutamine out of ammonia and glutamate, at the cost of one ATP. Glutamate synthase (GOGAT), which is NAD dependent in *S. cerevisiae*, converts one molecule of glutamine and one molecule of α-ketoglutarate into two molecules of glutamate. The NAD dependent Glutamate DeHydrogenase (NAD-GDH) degrades glutamate into α-ketoglutarate and ammonia. This reaction is active under amino acid catabolism, resulting in a net flux of C5 carbon skeletons towards the TCA-cycle.

3.3. Nitrogen Catabolite Repression

S.cerevisiae selects nitrogen sources that enable the best growth by a mechanism called Nitrogen Catabolite Repression (NCR). When good nitrogen sources are present in the medium, the transcription of some genes involved in the utilization of the poorer nitrogen sources is repressed, and their corresponding products are inactivated and degraded (for reviews see ter Schurre *et al*., 2000; Magasanik and Kaiser,2000).

Yeasts have developed molecular mechanisms in order to select the best out of a large diversity of available nitrogen sources. These mechanisms consist of a sensing mechanism and a regulatory mechanism, which includes induction of the needed systems, and repression of systems that are not beneficial. The first step in nitrogen metabolism is the uptake of the nitrogen compounds via more or less specific permeases. Hence this is the first level of regulation. Next step is the degradation of the nitrogen source via the nitrogen metabolic pathways. The activation of specific degradation routes is also regulated depending on the availability of a particular nitrogen source.

NCR operates at different levels. At first there is a short term response which will lead to direct inactivation of existing enzymes, followed by their degradation (Grenson, 1983). Secondly there is a more long-term effect, which prevents the synthesis of new enzymes at the level of gene expression (Magasanik and Kaiser, 2002).

a) Operation of NCR at the protein level: Inactivation, internalisation and degradation of permeases

The principal mechanism of post-transcriptional regulation is inactivation of permeases, which transport poor nitrogen sources (Gap1p, Put4p, Dal5p, etc.). This mechanism mainly operates by the phosphorylation/dephosphorylation of the protein by specific

kinases/phosphatases. Addition of ammonia, or compound(s) derived from it, to nitrogen catabolite derepressed cells causes dephosphorylation and subsequent inactivation of the permease. This process is well-documented for Gap1p. The presence of a good nitrogen source causes dephosphorilation of Gap1p. Subsequently dephosphorylated permease becomes a target for Npi1p which probably ubiquitinates the permease inducing its degradation in the vacuole. (Fig 3.3) In addition to permeases other enzymes involved in nitrogen metabolism are also regulated post-transcriptionally.

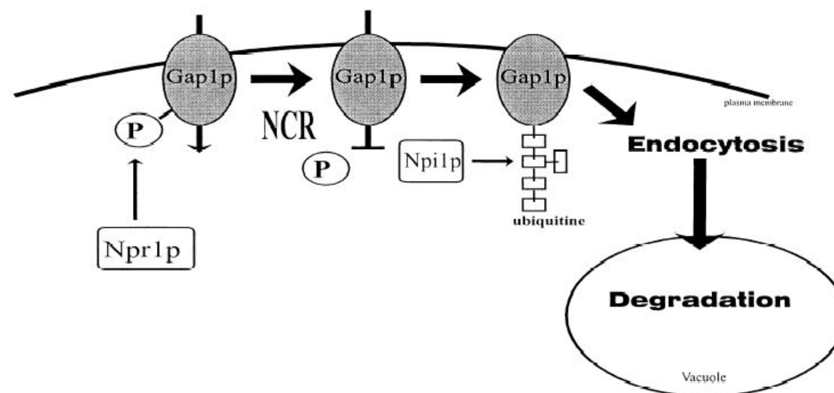


Figura 3.3 Posttranscriptional regulation of the general amino acid permease Gap1p. (ter Schure *et al.*, 2000)

b) Transcriptional repression

Nitrogen catabolite repression has been shown to be mediated by inhibitors of, among others, transcriptional factors that recognize UAS_{NTR}. The nitrogen regulated activation sequence UAS_{NTR} consists of two separate dodecanucleotide sites with the sequence GAT(T/A)A at their code.

Until now, five key players have been identified to participate in NCR at the level of transcription. These include four GATA transcription factors, two positive (activators): Gln3p, Gat1p/Nil1p and two negative (repressors): Dal80p and Deh1/Gzf3p. The fifth player is the regulatory protein Ure2p (Fig 3.4).

Gln3p can be considered to be the initial activator. Under nitrogen-rich conditions, the GATA family transcriptional activators, Gln3 and Gat1, form complexes with Ure2, which retains them in the cytoplasm, and avoid their entrance in the nucleus. The lack of activators in the nucleus decreases NCR-sensitive expression. Under nitrogen-limiting conditions, Gln3 and Gat1 are dephosphorylated, move from the cytoplasm to the nucleus, and increase expression of NCR-sensitive genes.

Ter Schure *et al.* (1998) showed that the URE2 gene is essential for ammonium specific pathway. The general transcription activator Gln3 stimulates the transcription of genes encoding some permeases as GAP1 and MEP2 and also of the genes of the central nitrogen metabolism GDH2, GLN1, GLT1 and probably GDH1. In the regulation of the CNM two signals exist, one glutamine and one ammonia-derived signal. (Fig 3.5).

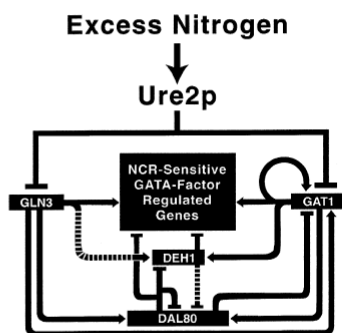
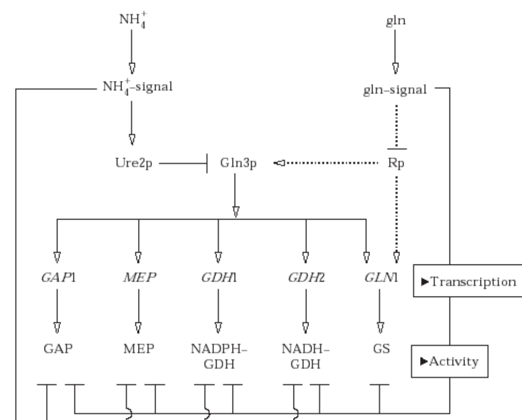


Figure 3.4 Model of reciprocal regulation of GATA factor gene expression and GATA factor regulation of NCR-sensitive gene expression. Arrowheads and bars designate positive and negative regulation, respectively. (Cooper, 2002)

Figure 3.5 Schematic overview of two levels of regulation incorporated in the model of the CNM. (…) indicate hypothetical relations. Rp: a hypothetical (transcription) regulator of the glutamine signalling pathway, corresponding to Ure2p in the ammonia signalling pathway.



3.4. Nitrogen compounds uptake

Selective accumulation of various amino acids and ammonium occurs in *S.cerevisiae* (Table 3.2). During fermentation, these compounds are taken up at the beginning of *S. cerevisiae* growth and stored in the vacuole to be used, as it is needed. *Saccharomyces* can accumulate large intracellular pools of amino acids. Vacuolar sequestration allows the cells to rapidly consume all available nitrogen, store the nitrogen in the vacuole, then utilize compounds as they are needed by regulating the release of amino acids from the vacuole to the cytoplasm (Boulton *et al.*, 1996).

Saccharomyces is able to use different nitrogen sources for growth, but not all nitrogen sources support growth equally well. Good nitrogen sources are ammonium, glutamine and asparagine whereas proline and urea are qualified as poor nitrogen sources. The presence of good nitrogen sources decreases the level of enzymes and permeases required for the utilization and uptake of poorer nitrogen sources, this phenomenon is known as **nitrogen catabolite repression (NCR)** (ter Schure *et al.*, 2000)(See Item 3.3).

The sequence of amino acid uptake is influenced by the presence and relative abundance of various nitrogen compounds, especially the ammonium ion, and possibly, the total nitrogen concentration (Henschke and Jiranek, 1992). Using a chemical-defined grape juice medium, Jiranek *et al.* (1995a) investigated the kinetics of amino acid utilization in the presence and (or) absence of ammonium ions by wine yeast under semi-anaerobic conditions. In the absence of ammonium ions, they distinguished a sequential pattern of amino acid utilization (divided in four groups, Table 3.2.). Initiation of uptake and removal of Group A constituents was rapid, while that of Group B was delayed. Initial uptake of Group C compounds coincided with the depletion of those of Group A, and rarely attained completion. These observations were similar to those made in wort fermentation (Jones and Pierce, 1964). A major difference relates to the timing of ammonium accumulation, which was rapid under enological conditions but fell into group C under brewing conditions. The group B and C amino acids were especially susceptible to delayed/reduced uptake in response to ammonium supplementation. The accumulation of alanine and arginine by some *S.cereviciae* strains was most delayed by ammonium (Jiranek *et al.*, 1995a).

Table 3.2. Kinetic of absorption of nitrogen compounds during fermentation of Chemical-Defined Grape Juice medium (Jiranek *et al.*, 1995a) and Wort fermentation (Jones and Pierce, 1964).

Groups	Kinetic of absorption	Grape-Juice Medium	Wort
A	Fast	Arg, Asp, Asn, Gln, Ile, Leu, Lis, Ser, Thr, NH_4^+	Arg, Asp, Asn, Glu, Gln, Lys, Ser, Thr
B	Slow	Glu, Ala, His, Met, Phe, Val	His, Ile, Leu, Val
C	Assimilated when A and B are depleted	Gly, Trp, Tyr	Ala, Gly, Phe, Trp, Tyr, NH_4^+
D	Not taken up	Pro	

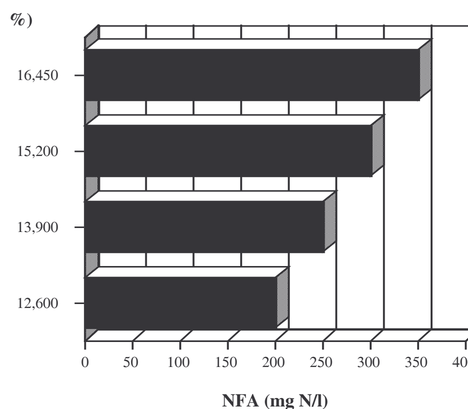
3.4.1. Factors affecting nitrogen assimilation

Many factors affect the assimilation of nitrogen during the fermentation, these include culture conditions, medium composition and yeast strain.

The presence and relative abundance of **good nitrogen sources** as ammonium affect the sequence of amino acid accumulation, repressing the utilization of the poorer nitrogen sources by NCR (Henschke and Jiranek, 1992; Jiranek *et al.*, 1995a) (See Item 3.3).

The **must sugar concentration** clearly affects the nitrogen requirements of yeast. The higher the sugar concentration is, the higher nitrogen content is needed to complete the alcoholic fermentation (Bisson and Butzke, 2000)(Fig 3.6).

Figura 3.6 – Relation between alcohol content attained (% vol.) and requeriments of Yeast Assimilable Nitrogen (NFA). (Bisson and Butzke, 2000).



Industrial **yeast strains** significantly have different nitrogen requirements. These differences are strain specific and mostly appear during the stationary phase (Jiranek *et al.*, 1991; Henschke and Jiranek, 1992; Manginot *et al.*, 1998). Henschke and Jiranek (1992) reported that different *S. cerevisiae* strains fermenting grape must assimilated quantities of nitrogen varying from 392 to 473 mg/l at 20ºC. This study also showed that **temperature** increases nitrogen assimilation. The effect of temperature on nitrogen consumption was also reported in other studies (Ough *et al.*, 1991; Lopez *et al.*, 1996; Llauradó *et al.*, 2002). **Oxygen**, however, has the most important effect on nitrogen assimilation. Yeasts have long been known to use considerably more nitrogen in the presence of oxygen (Ribéreau-Gayon *et al.*, 1975). The influence of oxygen and the **addition of ammonium** to must on fermentation kinetics seem to be complex. It depends on the timing of these two operations (Sablayrolles *et al.*, 1996).

3.4.2. Nitrogen supplements

There are two basic strategies to circumvent problems linked to nitrogen deficiency: prevention of nitrogen deficiency in grape juice by optimising vineyard fertility, and more commonly, supplementation with ammonium salts such as diammonium phosphate (DAP). Additions of DAP up to 300 mg/l are permissible in accordance with European Union law whereas in the USA up to 950 mg/l may be added. In Australia, the maximum addition is indirectly limited by a maximum wine phosphate concentration of 400 mg of Pi/l. The standard dose is between 100 and 200 mg/l.

The addition of nitrogen increases yeast biomass and fermentation speed in nitrogen-limited grape musts. Bely *et al.* (1990a) found that the addition of nitrogen is effective

when the concentration of assimilable nitrogen (ammonium and amino acid except proline) is lower than 130 mg/l, but it is not necessary for concentrations greater than 300 mg/l.

Excessive nitrogen additions may lead to the presence of non-assimilated residual nitrogen at the end of fermentation, leading to microbial instability and ethyl carbamate accumulation in wine. Therefore, the knowledge of the nitrogen content of grape juice and the requirement for nitrogen by yeast are important considerations for optimal fermentation performance and the production of wines that comply with the demands of regulatory authorities and consumers.

The timing of the addition of ammonium salts appears also to be important in nitrogen assimilation and fermentation kinetics. Ribéreau-Gayon (1975) had suggested their addition in must before the initiation of fermentation. Yeasts react best to stimuli during the growth phase in a medium containing little ethanol. Nitrogen additions performed during cell growth period resulted in maximum cell populations. Additions made later during the stationary phase had no effects on cell population level, but significantly reduced the fermentation length by increasing the specific fermentation rate (Bely *et al.*, 1990a; Bely *et al.*, 1990b; Mendes-Ferreira *et al.*, 2004). Sablayrolles *et al.* (1996) demonstrated that nitrogen added at mid-fermentation at the same time as aeration gave the best results. This dual operation has more effect on fermentation kinetics than aeration alone.

3.4.3. Transport of nitrogen compounds

a) Ammonium transport: Mep proteins

The first step in the use of most nitrogen sources is its uptake via more or less specific permeases. *S. cerevisiae* has three specific ammonium transporters, Mep1p, Mep2p and Mep3p. The Mep2p displays the highest affinity for NH_4^+ (Km 1.4 to 2.1 μM), followed closely by Mep1p (Km 5 to 10 μM) and finally by Mep3p, whose affinity is much lower (Km 1.4 to 2.1 mM). The MEP genes are subjected to nitrogen control. In presence of a good nitrogen source, all three MEP genes are repressed. On a poor nitrogen source, MEP2 expression is much higher than that of MEP1 and MEP3. (Marini *et al.*, 1997).

Table 3.3 Yeast ammonia permeases family.

Gene	Name	Gene Product	Reference
YGR121c	*MEP1*	Medium –affinity, high-capacity ammonium permease	Marini *et al.*, 1994
YNL142w	*MEP2*	High-affinity, low-capacity ammonium permease, ammonium sensor	Marini *et al*. 1997, Lorenz and Heitman 1998
YPR138c	*MEP3*	Low-affinity, high-capacity ammonium permease	Marini *et al.* 1997

Table 3.4 Yeast Amino Acid permeases family (AAPs) (Yeast Transport Proteins database: YTPdb) (Andre, 1995)

Gene name	ORF name	Brief description	Substrates
ClusterI			
AGP1	YCL025C	Broad-specificity amino-acid permease - inductible by most neutral amino acids	L-cysteine ;L-alanine ;L-asparagine ;L-glutamine ;L-glycine ;L-histidine ;L-isoleucine ;L-leucine ;L-methionine ;L-phenylalanine ; L-serine ;L-threonine ;L-tryptophan ;L-tyrosine ;L-valine
BAP2	YBR068C	Broad-specificity amino-acid permease - inductible by most neutral amino acids	L-tryptophan ;L-tyrosine ;L-phenylalanine ;L-isoleucine ;L-leucine ;L-valine ;L-cysteine ;L-methionine
BAP3	YDR046C	Broad-specificity amino-acid permease - inductible by most neutral amino acids	L-tryptophan ;L-phenylalanine ;L-tyrosine ;L-isoleucine ;L-leucine ;L-valine ;L-alanine ;L-cysteine ;L-methionine ;L-threonine
GNP1	YDR508C	Broad-specificity amino-acid permease	L-leucine ;L-cysteine ;L-methionine ;L-serine ;L-threonine ;L-glutamine ;L-asparagine
TAT1	YBR069C	Broad-specificity amino-acid permease - inductible by most neutral amino acids	L-tryptophan ;L-tyrosine ;L-valine
TAT2	YOL020W	Broad-specificity amino-acid permease - inductible by most neutral amino acids	L-phenylalanine ;L-tryptophan ;L-tyrosine
Cluster II			
GAP1	YKR039W	General amino acid permease	D-histidine ;L-glutamate ;L-aspartate ;L-arginine ;L-lysine ;L-cysteine ;L-alanine ;L-asparagine ;L-glutamine ;L-glycine ; L-histidine ;L-isoleucine ;L-leucine ;L-methionine ;L-ornithine ;L-phenylalanine ;L-proline ;L-serine ;L-threonine ;L-tryptophan ; L-tyrosine ;L-valine ;gamma-aminobutyrate ;L-citrulline
HIP1	YGR191W	Histidine permease	D-histidine ;manganese
MMP1	YLL061W	s-methylmethionine permease	S-methylmethionine
SAM3	YPL274W	S-adenosylmethionine permease	S-adenosylmethionine
Cluster III			
APL1	YNL270C	Protein of the amino-acid permease family - unknown biological function	unknown
CAN1	YEL063C	Arginine permease	L-arginine
LYP1	YNL268W	Lysine permease	L-lysine
Unclustered			
AGP2	YBR132C	Carnitine permease	carnitine
AGP3	YFL055W	Protein of the amino-acid permease family - unknown biological function	unknown
PUT4	YOR348C	Proline permease	L-alanine ;L-glycine ;L-proline ;gamma-aminobutyrate
DIP5	YPL265W	Glutamate and aspartate permease - able to mediate transport of other amino acids	L-glutamate ;L-aspartate ;L-alanine ;L-glycine ;L-serine ;L-glutamine ;L-asparagine
BIO5	YNR056C	7-keto 8-aminopelargonic acid permease	keto-aminopelargonate
SSY1	YDR160W	Permease-like sensor of external amino acids	L-citrulline ;L-cysteine ;L-alanine ;L-asparagine ;L-glutamine ;L-glycine ;L-histidine ;L-isoleucine ;L-leucine ;L-methionine ; L-ornithine ;L-phenylalanine ;L-serine ;L-threonine ;L-tryptophan ;L-tyrosine ;L-valine

b) Amino Acid transport

Amino acids are transported into the cell by general and specific transport systems. These polytopic transport proteins are members of the conserved amino acid permease (AAP) family that includes 19 core members (Andre, 1995; Nelissen *et al.*, 1997; Forsberg and Ljungdahl, 2001) (Table 3.4).

Each of the AAP family members has defined substrate specificities and transport capacities. The genes encoding AAPs are differently regulated. The high capacity permeases, AGP1, GAP1 and PUT4, are nitrogen-regulated and become down-regulated at the transcriptional, as well as post-transcriptional level, in response to high quality nitrogen sources like ammonium. The more specific permeases, with lower transport capacities, may not to be able to support growth when amino acids are the sole nitrogen source; but they seem to supply amino acids for protein synthesis to the cell. The expression of genes encoding the ClusterI permeases, AGP1, GNP1, BAP2, BAP3, TAT1 and TAT2, is induced by the presence of amino acids in the growth media. This induced expression requires the SSY1, PTR3 and SS5 gene products, which are considered sensors of extracellular amino acids (SPS sensor complex) (Forsberg and Ljungdahl, 2001). Amino acid induction of more distantly related AAPs genes, i.e., HIP1, GAP1, CAN1, ALP1, LYP1, DIP5 and PUT4, has not been demonstrated.

3.5. Nitrogen and Yeast Flavour Compounds

The yeast flavour compounds can be placed into three groups: the major volatile products of fermentation; the trace “fermentation bouquet” compounds and the undesirable or “negative” aroma compounds.

The major volatile products of yeast metabolism, ethanol, glycerol and carbon dioxide are only indirectly affected by nitrogen metabolism. These compounds make a relatively small, but, nonetheless, fundamental contribution to wine flavour.

The main groups of compounds that form the “fermentation bouquet”, are the organic acids, higher alcohols and esters and, at lesser extent, aldehydes, and are influenced to various degrees by the nitrogen source (Rapp and Versini, 1991).

Higher alcohols are quantitatively the largest group of aroma compounds in alcoholic beverages, and are secondary products of alcoholic fermentation. Although they exhibit unpleasant aroma, at the concentrations generally found in wines (bellow 300 mg/l) they contribute to the desirable complexity of wine. Yeasts produce higher alcohols during alcoholic fermentation through the conversion of the branched chain amino acids

present in the medium (catabolic or Ehrlich pathway): valine, leucine, isoleucine threonine and phenylalanine (Table 3.5), or they are also produced *de novo* from a sugar substrate (anabolic pathway) (Figure 3.7). Ammonium and amino acid deficiencies in must lead to an increase formation of higher alcohols. The amino acids in a medium are among the most important factors influencing fusel alcohol formation. They are able to alter the yield of higher alcohols in several different ways. Despite the fact that low amounts of amino acids were present with respect to the quantity of corresponding higher alcohols formed (via the Ehrlich reaction), the amino acids may play a role in controlling the pathways of their own formation and thus influence the anabolic formation of the higher alcohols (Lambrechts and Pretorius, 2000).

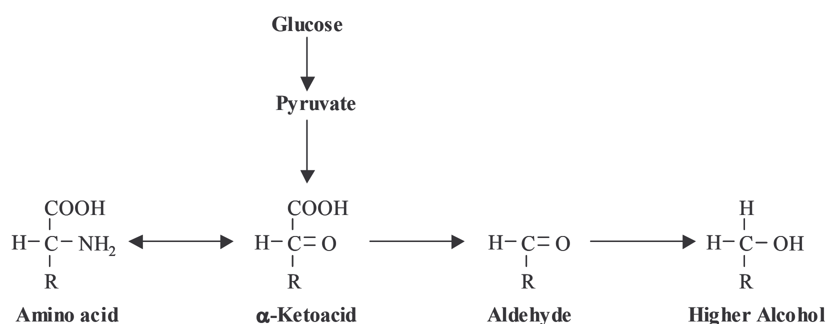


Figure 3.7 Generalized pathway for formation of higher alcohols from amino acids (Ehrlich pathway) and from a sugar substrate (anabolic pathway)

Table 3.5 Some higher alcohols produced by yeast and their concentrations, threshold values and odours in wine (Lambrechts and Pretorious, 2000)

Alcohol	Amino acid	Conc. in wine (mg/l)	Threshold value (mg/l)	Odour
Propanol	Threonine, 2-Amino-butyric acid	9 - 68	500	Stupefying
Butanol	-	0.5 – 8.5		Fusel odour
Isobutyl alcohol	Valine	9 – 28	500	Alcoholic
Active amyl alcohol	Isoleucine	15 - 150	75.0	Marzipan
Isoamyl alcohol	Leucine	45 – 490	300	Marzipan
Hexanol	-	0.3 – 12		
Tyrosol	Tyrosine			Bees wax, Honey-like
Tryptophol	Tryptophan			
Phenethyl alcohol	Phenylalanine	10 -180		Floral, rose

The most "negative" aroma compounds are the reduced sulphur compounds, hydrogen sulphide, organic sulphides and thiols (mercaptans). **Hydrogen sulphide** has an unpleasant aroma with a low sensory threshold (10-100 μg/ml). Its formation is regulated by nitrogen availability (Henschke and Jiranek, 1991; Jiranek *et al*. 1995b).

Wineries commonly observe two phases of H_2S production: the first phase occurs during active fermentation and is responsive to supplementation with assimilable nitrogen, the second phase is most frequently observed near to depletion of sugars from the must and proceeds in the presence of assimilable nitrogen.

The formation of the carcinogen compound **ethyl carbamate** is also correlated with nitrogen concentration. In musts containing higher concentrations of assimilable nitrogen, yeasts tend to show an incomplete or delayed uptake of arginine, and the urea-producing strains excrete urea into the fermentation medium. Urea can react with ethanol in acidic conditions to form ethyl carbamate (Ough, 1991; Rapp and Versini, 1991).

REFERENCES

1. Aguilar,P.S., Lopez,P., de Mendoza,D. (1999). Transcriptional control of the low-temperature-inducible des gene, encoding the delta5 desaturase of Bacillus subtilis. J Bacteriol. *181*, 7028-7033.

2. Alexandre,H., Charpentier,C. (1998). Biochemical aspects of stuck and sluggish fermentation in grape must. J.Ind.Microbiol.Biotechnol. *20*, 20-27.

3. Alexandre,H., Heintz,D., Chassagne,D., Guilloux-Benatier,M., Charpentier,C., Feuillat,M. (2001). Protease A activity and nitrogen fractions released during alcoholic fermentation and autolysis in enological conditions. J.Ind.Microbiol.Biotechnol. *26*, 235-240.

4. Alexandre,H., Rousseaux,I., Charpentier,C. (1994a). Ethanol adaptation mechanisms in Saccharomyces cerevisiae. Biotechnol.Appl.Biochem. *20*, 173-183.

5. Alexandre,H., Rousseaux,I., Charpentier,C. (1994b). Relationship between ethanol tolerance, lipid composition and plasma membrane fluidity in Saccharomyces cerevisiae and Kloeckera apiculata. FEMS Microbiol.Lett. *124*, 17-22.

6. Andre,B. (1995). An overview of membrane transport proteins in Saccharomyces cerevisiae. Yeast *11*, 1575-1611.

7. Attfield,P.V. (1997). Stress tolerance: the key to effective strains of industrial baker's yeast. Nat.Biotechnol. *15*, 1351-1357.

8. Backhus,L.E., DeRisi,J., Brown,P.O., Bisson,L.F. (2001). Functional genomic analysis of a commercial wine strain of Saccharomyces cerevisiae under differing nitrogen conditions. FEMS Yeast Res. *1*, 111-125.

9. Bakalinsky,A.T., Snow,R. (1990). The chromosomal constitution of wine strains of Saccharomyces cerevisiae. Yeast *6*, 367-382.

10. Barre,P., Vézinhet,F., Dequin,S., Blondin,B. (1993). Genetic Improvement of Wine Yeast. In: Wine Microbiology and Biotechnology, ed. G.H.Fleet Harbor Academic Reading, 421-447.

11. Bauer,F.F., Pretorius,I.S. (2000). Yeast stress response and fermentation efficiency: how to survive the making of wine - a review. S.Afr.J.Enol.Vitic ***21***, 27-51.

12. Beltran,G., Novo,M., Torija,M.J., Poblet,M., Rozes,N., Guillamon,J.M., Mas,A. (2002a). Fermentaciones a bajas temperaturas. Tecnología del vino *1*, 73-76.

13. Beltran,G., Torija,M.J., Novo,M., Ferrer,N., Poblet,M., Guillamon,J.M., Rozes,N., Mas,A. (2002b). Analysis of yeast populations during alcoholic fermentation: a six year follow-up study. Syst.Appl.Microbiol. *25*, 287-293.

14. Bely,M., Sablayrolles,J.M., Barre,P. (1990a). Automatic detection of assimilable nitrogen deficiencies during alcoholic fermentation in oenological conditions. J.Ferment.Bioeng. *70*, 246-252.

15. Bely,M., Sablayrolles,J.M., Barre,P. (1990b). Description of alcoholic fermentation kinetics: its variability and significance. American Journal of Enology and Viticulture *41*, 319-324.

16. Benchekroun,K., Bonaly,R. (1992). Physiological properties and plasma membrane composition of Saccharomyces cerevisiae grown in sequential batch culture and in presence of surfactant. Appl.Microbiol.Biotechnol. *36*, 673-678.

17. Beney,L., Gervais,P. (2001). Influence of the fluidity of the membrane on the response of microorganisms to environmental stresses. Appl.Microbiol.Biotechnol. *57*, 34-42.

18. Bertrand,A., Miele,A. (1984). Influence of clarification du moût de raisin sur sa teneur en acides gras. Conn.Vigne Vin *48*, 293-297.

19. Bertuccioli,M., Rosi,I. (1984). Progress in Flavour Research. In: Developments in Food Science, ed. J.AddaAmsterdam: Elsevier, 387.

20. Bianchi,M.M., Ngo,S., Vandenbol,M., Sartori,G., Morlupi,A., Ricci,C., Stefani,S., Morlino,G.B., Hilger,F., Carignani,G., Slonimski,P.P., Frontali,L. (2001). Large-scale phenotypic analysis reveals identical contributions to cell functions of known and unknown yeast genes. Yeast *18*, 1397-1412.

21. Bidenne,C., Blondin,B., Dequin,S., Vezinhet,F. (1992). Analysis of the chromosomal DNA polymorphism of wine strains of Saccharomyces cerevisiae. Curr.Genet. *22*, 1-7.

22. Bisson,L.F. (1991). Influence of Nitrogen on Yeast and Fermentation of Grapes. In: Proceedings of the International Sumposium on Nitrogen in Grapes and Wine, ed. J.M.RanzDavis, CA: Am. Soc. Enol. Vitic., 172-184.

23. Bisson,L.F. (1999). Stuck and sluggish fermetnations. American Journal of Enology and Viticulture *50*, 107-119.

24. Bisson,L.F., Butzke,C.E. (2000). Diagnosis and rectifications of stuck and sluggish fermentations. Am.J.Enol.Vitic. *51*, 168-177.

25. Boer,V.M., de Winde,J.H., Pronk,J.T., Piper,M.D. (2003). The genome-wide transcriptional responses of Saccharomyces cerevisiae grown on glucose in aerobic chemostat cultures limited for carbon, nitrogen, phosphorus, or sulfur. J Biol.Chem *278*, 3265-3274.

26. Boulton,R.B., Singleton,V.L., Bisson,L.F., Kunkee,R.E. (1996). Principles and Practices of Winemaking. New York: Chapman and Hall.

27. Browse,J., Xin,Z. (2001). Temperature sensing and cold acclimation. Curr.Opin.Plant Biol. *4*, 241-246.

28. Castela,P.M., Mesías,J.L., Maynar,J.I. (1985). Changes in contents of total, polar and neutral lipids in Macabeo grapes during development. Sci.Aliments *5*, 587-597.

29. Castellari,L., Ferruzzi,M., Magrini,A., Giudici,P. (1994). Unbalanced wine fermentation by cryotolerant *vs.* non-cryotolerant *Saccharomyces* strains. Vitis *33*, 49.

30. Castellari,L., Magrini,A., Passarelli,P., Zambonelli,C. (1995). Effect of must fermentation temperature on minor products formed by cryo and non-cryotolerant *Saccharomyces cerevisiae* strains. Ital.J.Food Sci *2*, 125-132.

31. Causton,H.C., Ren,B., Koh,S.S., Harbison,C.T., Kanin,E., Jennings,E.G., Lee,T.I., True,H.L., Lander,E.S., Young,R.A. (2001). Remodeling of yeast genome expression in response to environmental changes. Mol.Biol.Cell *12*, 323-337.

32. Cavalieri,D., Townsend,J.P., Hartl,D.L. (2000). Manifold anomalies in gene expression in a vineyard isolate of Saccharomyces cerevisiae revealed by DNA microarray analysis. Proc.Natl.Acad.Sci.U.S.A *97*, 12369-12374.

33. Chen,E.C.H. (1980). Utilization of wort fatty acids by yeast during fermentation. Ann.Soc.Brew.Chem. *38*, 148-153.

34. Cho,R.J., Campbell,M.J., Winzeler,E.A., Steinmetz,L., Conway,A., Wodicka,L., Wolfsberg,T.G., Gabrielian,A.E., Landsman,D., Lockhart,D.J., Davis,R.W. (1998). A genome-wide transcriptional analysis of the mitotic cell cycle. Mol.Cell *2*, 65-73.

35. Chu,S., DeRisi,J., Eisen,M., Mulholland,J., Botstein,D., Brown,P.O., Herskowitz,I. (1998). The transcriptional program of sporulation in budding yeast. Science *282*, 699-705.

36. Codon,A.C., Gasent-Ramirez,J.M., Benitez,T. (1995). Factors which affect the frequency of sporulation and tetrad formation in Saccharomyces cerevisiae baker's yeasts. Appl.Environ.Microbiol. *61*, 630-638.

37. Constanti,M., Reguant,C., Poblet,M., Zamora,F., Mas,A., Guillamon,J.M. (1998). Molecular analysis of yeast population dynamics: effect of sulphur dioxide and inoculum on must fermentation. Int.J.Food Microbiol. *41*, 169-175.

38. Cooper,T.G. (2002). Transmitting the signal of excess nitrogen in Saccharomyces cerevisiae from the Tor proteins to the GATA factors: connecting the dots. FEMS Microbiol.Rev. *26*, 223-238.

39. Daum,G., Lees,N.D., Bard,M., Dickson,R. (1998). Biochemistry, cell biology and molecular biology of lipids of Saccharomyces cerevisiae. Yeast *14*, 1471-1510.

40. De Virgilio,C., Hottiger,T., Dominguez,J., Boller,T., Wiemken,A. (1994). The role of trehalose synthesis for the acquisition of thermotolerance in yeast. I. Genetic evidence that trehalose is a thermoprotectant. Eur.J.Biochem. *219*, 179-186.

41. Delfini,C., Costa,A. (1993). Effects of the grape must lees and insoluble materials on the alcoholic fermentation rate and the production of acetic acid, pyruvic acid, and acetaldehyde. Am.J.Enol.Vitic. *44*, 86-92.

42. DeRisi,J.L., Iyer,V.R., Brown,P.O. (1997). Exploring the metabolic and genetic control of gene expression on a genomic scale. Science *278*, 680-686.

43. Diniz-Mendes,L., Bernardes,E., de Araujo,P.S., Panek,A.D., Paschoalin,V.M. (1999). Preservation of frozen yeast cells by trehalose. Biotechnol.Bioeng. *65*, 572-578.

44. Entian,K.D., Barnett,J. (1992). Regulation of sugar utilization by Saccharomyces cerevisiae. Trends Biochem.Sci. *17*, 506-510.

45. Erasmus,D.J., van der Merwe,G.K., van Vuuren,H.J. (2003). Genome-wide expression analyses: Metabolic adaptation of Saccharomyces cerevisiae to high sugar stress. FEMS Yeast Res. *3*, 375-399.

46. Feuillat,M., Charpentier,C., Massoutier,C. (1997). Intérêt oenologique des souches de levures Saccharomyces cryotolérantes. Revue des Oenologues *85*, 18-21.

47. Fleet,G.H. (1998). The Microbiology of Alcoholic Beverages. In: Microbiology of Fermented Foods Vol1, ed. B.WoodGalsgow: Blackie Academic and Professional, 217-262.

48. Fleet,G.H., Heard,G.M. (1993). Yeast Growth During Fermentation. In: Wine Microbiology and Biotechnology, ed. G.H.FleetSwitzerland: Harwood Academic Publishers, 27-54.

49. Fleet,G.H., Lafon-Lafourcade,S., Ribéreau-Gayon,P. (1984). Evolution of yeasts and lactic acid bacteria during fermentation and storage of Bordeaux wines. Appl.Environ.Microbiol. *48*, 1034-1038.

50. Forsberg,H., Ljungdahl,P.O. (2001). Sensors of extracellular nutrients in Saccharomyces cerevisiae. Curr.Genet. *40*, 91-109.

51. Francois,J., Parrou,J.L. (2001). Reserve carbohydrates metabolism in the yeast Saccharomyces cerevisiae. FEMS Microbiol.Rev. *25*, 125-145.

52. Fugelsang,K.C. (1997). Wine Microbiology. New York.

53. Gao,C., Fleet,G.H. (1988). The effect of temperature and pH on the ethanol tolerance of the wine yeasts *Saccharomyces cerevisiae*, *Candida stellata* and *Kloeckera apiculata*. J.Appl.Bacteriol. *65*, 405-409.

54. Gasch,A.P. (2003). The Environmental Stress Response: a Common Yeast Response to Diverse Environmental Stresses. In: Yeast Stress Responses, ed. S.Hohmann, W.H.MagerBerlin Heidelberg: Springer-Verlag, 11-70.

55. Gasch,A.P., Spellman,P.T., Kao,C.M., Carmel-Harel,O., Eisen,M.B., Storz,G., Botstein,D., Brown,P.O. (2000). Genomic expression programs in the response of yeast cells to environmental changes. Mol.Biol.Cell *11*, 4241-4257.

56. Giudici,P., Caggia,C., Pulvirenti,A., Rainieri,S. (1998). Karyotyping of Saccharomyces strains with different temperature profiles. J Appl Microbiol *84*, 811-819.

57. Goffeau,A., Barrell,B.G., Bussey,H., Davis,R.W., Dujon,B., Feldmann,H., Galibert,F., Hoheisel,J.D., Jacq,C., Johnston,M., Louis,E.J., Mewes,H.W., Murakami,Y., Philippsen,P., Tettelin,H., Oliver,S.G. (1996). Life with 6000 genes. Science *274*, 546, 563-546, 567.

58. Goffeau,A., Park,J., Paulsen,I.T., Jonniaux,J.L., Dinh,T., Mordant,P., Saier,M.H., Jr. (1997). Multidrug-resistant transport proteins in yeast: complete inventory and phylogenetic characterization of yeast open reading frames with the major facilitator superfamily. Yeast *13*, 43-54.

59. Gorner,W., Durchschlag,E., Martinez-Pastor,M.T., Estruch,F., Ammerer,G., Hamilton,B., Ruis,H., Schuller,C. (1998). Nuclear localization of the C2H2 zinc finger protein Msn2p is regulated by stress and protein kinase A activity. Genes Dev. *12*, 586-597.

60. Gounalaki,N., Thireos,G. (1994). Yap1p, a yeast transcriptional activator that mediates multidrug resistance, regulates the metabolic stress response. EMBO J. *13*, 4036-4041.

61. Grenson,M. (1983). Inactivation-reactivation process and repression of permease formation regulate several ammonia-sensitive permeases in the yeast Saccharomyces cerevisiae. Eur.J Biochem. *133*, 135-139.

62. Hauser,N.C., Fellenberg,K., Gil,R., Bastuck,S., Hoheisel,J.D., Pérez-Ortín,J.E. (2001). Whole genome analysis of a wine yeast strain. Comp.Func.Genom. *2*, 69-79.

63. Heard,G.M., Fleet,G.H. (1985). Growth of natural yeast flora during the fermentation of inoculated wines. Appl.Environ.Microbiol. *50*, 727-728.

64. Heard,G.M., Fleet,G.H. (1988). The effects of temperature and pH on the growth of yeast species during the fermentation of grape juice. J.Appl.Bacteriol. *65*, 23-28.

65. Henschke,P.A., Jiranek,V. (1991). Hydrogen Sulphide Formation During Fermentation: Effect of Nitrogen Composition in Model Grape Must. In: Proceedings of the International Sumposium on Nitrogen in Grapes and Wine, ed. J.RantzDavis, CA: Am. Soc. Enol. Vitic., 172-184.

66. Henschke,P.A., Jiranek,V. (1992). Yeast. Metabolism of Nitrogen Compounds. In: Wine Microbiology and Biotechnology, ed. G.H.FleetChur, Switzerland: Harwood Academic Publishers, 77-169.

67. Hohmann,S., Mager,W.H. (2003). Yeast Stress Responses. Berlin Heidelberg: Springer-Verlag.

68. Homma,T., Iwahashi,H., Komatsu,Y. (2003). Yeast gene expression during growth at low temperature. Cryobiology *46*, 230-237.

69. Hughes,T.R., Marton,M.J., Jones,A.R., Roberts,C.J., Stoughton,R., Armour,C.D., Bennett,H.A., Coffey,E., Dai,H., He,Y.D., Kidd,M.J., King,A.M., Meyer,M.R., Slade,D., Lum,P.Y., Stepaniants,S.B., Shoemaker,D.D., Gachotte,D., Chakraburtty,K., Simon,J., Bard,M.,

Friend,S.H. (2000). Functional discovery via a compendium of expression profiles. Cell *102*, 109-126.

70. Hunter,K., Rose,A.H. (1972). Lipid composition of Saccharomyces cerevisiae as influenced by growth temperature. Biochim.Biophys.Acta *260*, 639-653.

71. Ivorra,C., Perez-Ortin,J.E., del Olmo,M. (1999). An inverse correlation between stress resistance and stuck fermentations in wine yeasts. A molecular study. Biotechnol.Bioeng. *64*, 698-708.

72. Jiranek,V., Langridge,P., Henschke,P.A. (1991). Yeast Nitrogen Demand: Selection Criterion for Wine Yeasts for Fermenting Low Nitrogen Musts. In: Proceedings of the International Symposium on Nitrogen in Grapes and Wine, ed. J.M.RanzDavis, CA: Am.Soc.Enol.Vitic., 266-269.

73. Jiranek,V., Langridge,P., Henschke,P.A. (1995a). Amino acid and ammonium utilization by Saccharomyces cerevisiae wine yeasts from a chemical defined medium. American Journal of Enology and Viticulture *46*, 75-83.

74. Jiranek,V., Langridge,P., Henschke,P.A. (1995b). Regulation of hydrogen sulfide liberation in wine-producing Saccharomyces cerevisiae strains by assimilable nitrogen. Appl.Environ.Microbiol. *61*, 461-467.

75. Jones,M., Pierce,J.S. (1964). Absorption of amino acids from wort by yeasts. J.Inst.Brew. *70*, 307-315.

76. Kandror,O., DeLeon,A., Goldberg,A.L. (2002). Trehalose synthesis is induced upon exposure of Escherichia coli to cold and is essential for viability at low temperatures. Proc.Natl.Acad.Sci.U.S.A *99*, 9727-9732.

77. Killian,E., Ough,C.S. (1979). Fermentation esters-formation and retention as affected by fermentation temperature. Am.J.Enol.Vitic. *30*, 301-305.

78. Kondo,K., Inouye,M. (1991). TIP 1, a cold shock-inducible gene of Saccharomyces cerevisiae. J.Biol.Chem. *266*, 17537-17544.

79. Kondo,K., Kowalski,L.R., Inouye,M. (1992). Cold shock induction of yeast NSR1 protein and its role in pre-rRNA processing. J.Biol.Chem. *267*, 16259-16265.

80. Kowalski,L.R., Kondo,K., Inouye,M. (1995). Cold-shock induction of a family of TIP1-related proteins associated with the membrane in Saccharomyces cerevisiae. Mol.Microbiol. *15*, 341-353.

81. Kuhn,K.M., DeRisi,J.L., Brown,P.O., Sarnow,P. (2001). Global and specific translational regulation in the genomic response of Saccharomyces cerevisiae to a rapid transfer from a fermentable to a nonfermentable carbon source. Mol.Cell Biol. *21*, 916-927.

82. Kurzman,C.P., Fell,J.W. (1998). *The Yeast: A Taxanomic Study*. Amsterdam: Elservier Science Publishers.

83. Kwast,K.E., Lai,L.C., Menda,N., James,D.T., III, Aref,S., Burke,P.V. (2002). Genomic analyses of anaerobically induced genes in Saccharomyces cerevisiae: functional roles of Rox1 and other factors in mediating the anoxic response. J.Bacteriol. *184*, 250-265.

84. Lambrechts,M.G., Pretorius,I.S. (2000). Yeast and its importance to wine aroma. S.Afr.J.Enol.Vitic *21*, 97-129.

85. Laoteng,K., Anjard,C., Rachadawong,S., Tanticharoen,M., Maresca,B., Cheevadhanarak,S. (1999). Mucor rouxii delta9-desaturase gene is transcriptionally regulated during cell growth and by low temperature. Mol.Cell Biol.Res.Commun. *1*, 36-43.

86. Le Fur,Y., Hory,C., Bard,MH., Olsson,A. (1994). Evolution of phytosterols in Chardonnay grape berry skins during last stages of ripening. Vitis *33*, 127-131.

87. Llauradó, J. M. Avaluació dels condicionants del most en el desenvolupament de la fermentació alcohòlica a baixes temperatures. 2002. Universitat Rovira i Virgili, Tarragona (Spain).

88. Llauradó,J.M., Rozes,N., Bobet,R., Mas,A., Constantí,M. (2002). Low temperature alcoholic fermentation in high sugar concentration grape must. J.Food Science *67*, 268-273.

89. Longo,E., Cansado,J., Agrelo,D., Villa,T.G. (1991). Effect of climatic conditions on yeast diversity in grape musts from northwest Spain. Am.J.Enol.Vitic. 141-144.

90. Lopez,R., Santamaria,P., Gutierrez,A., Iñiguez,M. (1996). Changes on amino acids during the alcoholic fermentation of grape juice at different temperatures. Sci.Aliments *16*, 529-535.

91. Lucero,P., Penalver,E., Moreno,E., Lagunas,R. (2000). Internal trehalose protects endocytosis from inhibition by ethanol in Saccharomyces cerevisiae. Appl.Environ.Microbiol. *66*, 4456-4461.

92. Luparia,V., Soubeyrand,V., Berges,T., Julien,A., Salmon,J.M. (2004). Assimilation of grape phytosterols by Saccharomyces cerevisiae and their impact on enological fermentations. Appl.Microbiol.Biotechnol. *65*, 25-32.

93. Magasanik,B., Kaiser,C.A. (2002). Nitrogen regulation in Saccharomyces cerevisiae. Gene *290*, 1-18.

94. Mager,W.H., Hohmann,S. (1997). Stress Response Mechanisms in the Yeast *Saccharomyces Cerevisiae*. In: Yeast stress response, ed. S.Hohmann, W.H.MagerAustin: R.G.Landes Company, 1-5.

95. Manginot,C., Roustan,J.L., Sablayrolles,J.M. (1998). Nitrogen demand of different yeast strains during alcoholic fermentation. Importance of the stationary phase. Enzyme Microb.Technol. *23*, 511-517.

96. Marini,A.M., Soussi-Boudekou,S., Vissers,S., Andre,B. (1997). A family of ammonium transporters in Saccharomyces cerevisiae. Mol.Cell Biol. *17*, 4282-4293.

97. Marini,A.M., Vissers,S., Urrestarazu,A., Andre,B. (1994). Cloning and expression of the MEP1 gene encoding an ammonium transporter in Saccharomyces cerevisiae. EMBO J. *13*, 3456-3463.

98. Marks,V.D., van der Merwe,G.K., van Vuuren,H.J. (2003). Transcriptional profiling of wine yeast in fermenting grape juice: regulatory effect of diammonium phosphate. FEMS Yeast Res. *3*, 269-287.

99. Martinez-Pastor,M.T., Marchler,G., Schuller,C., Marchler-Bauer,A., Ruis,H., Estruch,F. (1996). The Saccharomyces cerevisiae zinc finger proteins Msn2p and Msn4p are required for transcriptional induction through the stress response element (STRE). EMBO J *15*, 2227-2235.

100. Martini,A. (1993). Origin and domestication of the wine yeast *Saccharomyces cerevisiae*. J.Wine Res. *3*, 165-176.

101. Martini,A., Ciani,M., Scorzzetti,G. (1996). Direct enumeration and isolation of wine yeasts from grape surfaces. Am.J.Enol.Vitic. *47*, 435-440.

102. Martini,A., Federici,F., Rosini,G. (1980). A new approach to the study of yeast ecology of natural substrates. Can.J Microbiol. *26*, 856-859.

103. Massoutier,C., Alexandre,H., Feuillat,M., Charpentier,C. (1998). Isolation and characterization of cryotolerant *Saccharomyces* strains. Vitis *37*, 55-69.

104. Mendes-Ferreira,A., Mendes-Faia,A., Leao,C. (2004). Growth and fermentation patterns of Saccharomyces cerevisiae under different ammonium concentrations and its implications in winemaking industry. J.Appl.Microbiol. *97*, 540-545.

105. Mortimer,R.K., Polsinelly,M. (1999). On the origins of wine yeast. Microbiol.Res. *150*, 199-204.

106. Nakagawa,Y., Sugioka,S., Kaneko,Y., Harashima,S. (2001). O2R, a novel regulatory element mediating Rox1p-independent O(2) and unsaturated fatty acid repression of OLE1 in Saccharomyces cerevisiae. J.Bacteriol. *183*, 745-751.

107. Naumov,G.I., Masneuf,I., Naumova,E.S., Aigle,M., Dubourdieu,D. (2000). Association of Saccharomyces bayanus var. uvarum with some French wines: genetic analysis of yeast populations. Res.Microbiol *151*, 683-691.

108. Nelissen,B., De Wachter,R., Goffeau,A. (1997). Classification of all putative permeases and other membrane plurispanners of the major facilitator superfamily encoded by the complete genome of *Saccharomyces cerevisiae*. FEMS Microbiol.Rev. *21*, 113-134.

109. Ness,F., Achstetter,T., Duport,C., Karst,F., Spagnoli,R., Degryse,E. (1998). Sterol uptake in Saccharomyces cerevisiae heme auxotrophic mutants is affected by ergosterol and oleate but not by palmitoleate or by sterol esterification. J.Bacteriol. *180*, 1913-1919.

110. Novo,M., Beltran,G., Torija,M.J., Poblet,M., Guillamon,J.M., Mas,A., Rozes,N. (2003a). Fermentaciones a bajas temperatras: análisis químico y sensorial. Tecnología del vino *2*, 51-55.

111. Novo,M., Torija,M.J., Beltran,G., Rozes,N., Mas,A., Guillamon,J.M. (2003b). Revisión sobre las fermentaciones vínicas a baja temperatura. Perspectivas de futuro. Tecnología del vino *3*, 38-43.

112. Novo,M.T., Beltran,G., Torija,M.J., Poblet,M., Rozes,N., Guillamon,J.M., Mas,A. (2003c). Changes in wine yeast storage carbohydrate levels during preadaptation, rehydration and low temperature fermentations. Int.J.Food Microbiol. *86*, 153-161.

113. O'Connor-Cox,E.S., Ingledew,W.M. (1991). Alleviation of the effects of nitrogen limitation in high gravity worts through increased inoculation rates. J.Ind.Microbiol. *7*, 89-96.

114. Ough,C.S. (1991). Influence of Nitrogen Compounds in Grapes in Ethyl Carbamate Formation in Wines. In: Proceedings of the International Sumposium on Nitrogen in Grapes and Wine, ed. J.M.RanzDavis, CA: Am.Soc.Enol.Vitic., 165-171.

115. Ough,C.S., Huang,D.A., Stevens,D. (1991). Amino acid uptake by four commercial yeasts at two different temperatures of growth and fermentation: Effects on urea excretion and re-adsorption. American Journal of Enology and Viticulture *41*, 26-40.

116. Parrish,M.E., Carrol,D.E. (1985). Indigenous yeasts associated with muscadine (*Vitis rotundifolia*) grapes and musts. Am.J.Enol.Vitic. *12*, 3-31.

117. Patton,J.L., Lester,R.L. (1991). The phosphoinositol sphingolipids of Saccharomyces cerevisiae are highly localized in the plasma membrane. J.Bacteriol. *173*, 3101-3108.

118. Perez-Ortin,J.E., Garcia-Martinez,J., Alberola,T.M. (2002). DNA chips for yeast biotechnology. The case of wine yeasts. J.Biotechnol. *98*, 227-241.

119. Piper,M.D. (1997). The Yeast Heat Shock Response. In: Yeast stress responses, ed. S.Hohmann, W.H.MagerAustin: R.G.Landes Company, 75-99.

120. Piper,P. (1998). Differential role of Hsps and trehalose in stress tolerance. Trends Microbiol. *6*, 43-44.

121. Pretorius,I.S. (2000). Tailoring wine yeast for the new millennium: novel approaches to the ancient art of winemaking. Yeast *16*, 675-729.

122. Pretorius,I.S. (2003). The Genetic Analysis and Tailoring of Wine Yeasts. In: Topics in current genetics, Vol.2: Functional Genetics of Industrial Yeasts, ed. J.H.de WindeBerlin Heidelberg: Springer-Verlag, 99-142.

123. Pretorius,I.S., Bauer,F.F. (2002). Meeting the consumer challenge through genetically customized wine-yeast strains. Trends Biotechnol. *20*, 426-432.

124. Querol,A., Barrio,E., Huerta,T., Ramon,D. (1992a). Molecular monitoring of wine fermentations conducted by dry yeast strains. Appl.Environ.Microbiol. *58*, 2948-2952.

125. Querol,A., Barrio,E., Ramon,D. (1992b). A comparative study of different methods of yeast strain characterization. Syst.Appl.Microbiol. *21*, 315-323.

126. Rachidi,N., Barre,P., Blondin,B. (2000). Examination of the transcriptional specificity of an enological yeast. A pilot experiment on the chromosome-III right arm. Curr.Genet. *37*, 1-11.

127. Rapp,A. (1998). Volatile flavour of wine: correlation between instrumental analysis and sensory perception. Nahrung *42*, 351-884.

128. Rapp,A., Versini,G. (1991). Influence of Nitrogen Compounds in Grapes on Aroma Compounds of Wines. In: Proceedings of the International Symposium on Nitrogen in Grapes and Wine, ed. J.M.RanzDavis, CA: Am. Soc. Enol. Vitic. 156-164.

129. Ribeiro,M.J., Silva,J.T., Panek,A.D. (1994). Trehalose metabolism in Saccharomyces cerevisiae during heat-shock. Biochim.Biophys.Acta *1200*, 139-147.

130. Ribéreau-Gayon,J., Peynaud,E., Ribéreau-Gayon,P., Sudraud,P. (1975). Sciences Et Techniques Du Vin. In: Traité d'Oenology IIParis: Dunod.

131. Ribéreau-Gayon,P., Dubourdieu,D., Donèche,B., Lonvaud,A. (2000). Handbook of Enology. The Microbiology of Wine and Vinifications Vol. I. West Sussex, England: Wiley.

132. Riou,C., Nicaud,J.M., Barre,P., Gaillardin,C. (1997). Stationary-phase gene expression in Saccharomyces cerevisiae during wine fermentation. Yeast *13*, 903-915.

133. Rosi,I., Bertuccioli,M. (1992). Influences of lipid addition on fatty acid composition of Saccharomyces cerevisiae and aroma characteristics of experimental wines. J.Inst.Brew. *98*, 305-314.

134. Rosini,G. (1984). Assesment of dominance of added yeast in wine fermentation and origin of *Saccharomyces cerevisiae* in wine-making. J.Gen.Appl.Microbiol. *30*, 249-256.

135. Rosini,G., Federici,F., Martini,A. (1982). Yeast flora of grape berries during ripening. Microbiol.Ecol. *8*, 83-89.

136. Rossignol,T., Dulau,L., Julien,A., Blondin,B. (2003). Genome-wide monitoring of wine yeast gene expression during alcoholic fermentation. Yeast *20*, 1369-1385.

137. Rozes,N., Larue,F., Ribéreau-Gayon,P. (1988). Effect of a variation of grape must temperature on the fermentative ability and the neutral lipid content. Biotechnol.Lett. *10*, 821-824.

138. Ruis,H., Schüller,C. (1995). Stress signaling in yeast. Bioessays *17*, 959-965.

139. Sablayrolles,J.M., Dubois,C., Manginot,C., Roustan,J.L., Barre,P. (1996). Efectiveness of combined ammoniacal nitrogen and oxygen additions for completion of sluggish and stuck fermentations. J.Ferment.Bioeng. *82*, 337-381.

140. Sahara,T., Goda,T., Ohgiya,S. (2002). Comprehensive expression analysis of time-dependent genetic responses in yeast cells to low temperature. J.Biol.Chem. *277*, 50015-50021.

141. Salmon,J.M. (1997). Enological fermentation kinetics of an isogenic ploidy series derived form an industrial *Saccharomyces cerevisiae* strain. J.Ferment.Bioeng. *83*, 253-260.

142. Schmitt,A.P., McEntee,K. (1996). Msn2p, a zinc finger DNA-binding protein, is the transcriptional activator of the multistress response in Saccharomyces cerevisiae. Proc.Natl.Acad.Sci U.S.A *93*, 5777-5782.

143. Sharf,R., Margalith,P. (1983). The effect of temperature on spontaneous wine fermentation. Eur.J.Appl.Microbiol.Biotechnol. *17*, 311-313.

144. Singer,M.A., Lindquist,S. (1998a). Multiple effects of trehalose on protein folding in vitro and in vivo. Mol.Cell *1*, 639-648.

145. Singer,M.A., Lindquist,S. (1998b). Thermotolerance in Saccharomyces cerevisiae: the Yin and Yang of trehalose. Trends Biotechnol. *16*, 460-468.

146. Spellman,P.T., Sherlock,G., Zhang,M.Q., Iyer,V.R., Anders,K., Eisen,M.B., Brown,P.O., Botstein,D., Futcher,B. (1998). Comprehensive identification of cell cycle-regulated genes of the yeast Saccharomyces cerevisiae by microarray hybridization. Mol.Biol.Cell *9*, 3273-3297.

147. ter Linde,J.J., Liang,H., Davis,R.W., Steensma,H.Y., van Dijken,J.P., Pronk,J.T. (1999). Genome-wide transcriptional analysis of aerobic and anaerobic chemostat cultures of Saccharomyces cerevisiae. J Bacteriol. *181*, 7409-7413.

148. ter Schure,E.G., van Riel,N.A., Verrips,C.T. (2000). The role of ammonia metabolism in nitrogen catabolite repression in Saccharomyces cerevisiae. FEMS Microbiol.Rev. *24*, 67-83.

149. Thieringer,H.A., Jones,P.G., Inouye,M. (1998). Cold shock and adaptation. Bioessays *20*, 49-57.

150. Thurston,P.A., Taylor,R., Avhenainen,J. (1981). Effects of linoleic acid supplements on the synthesis by yeast of lipids and acetate esters. J.Inst.Brew. *87*, 92-95.

151. Torija,M.J., Beltran,G., Novo,M., Poblet,M., Guillamon,J.M., Mas,A., Rozes,N. (2003a). Effects of fermentation temperature and Saccharomyces species on the cell fatty acid composition and presence of volatile compounds in wine. Int.J.Food Microbiol. *85*, 127-136.

152. Torija,M.J., Rozes,N., Poblet,M., Guillamon,J.M., Mas,A. (2001). Yeast population dynamics in spontaneous fermentations: comparison between two different wine-producing areas over a period of three years. Antonie Van Leeuwenhoek *79*, 345-352.

153. Torija,M.J., Rozes,N., Poblet,M., Guillamon,J.M., Mas,A. (2003b). Effects of fermentation temperature on the strain population of Saccharomyces cerevisiae. Int.J Food Microbiol. *80*, 47-53.

154. van der Rest,M.E., Kamminga,A.H., Nakano,A., Anraku,Y., Poolman,B., Konings,W.N. (1995). The plasma membrane of Saccharomyces cerevisiae: Structure, Function and Biogenesis. Microbiol.Rev. *59*, 304-322.

155. Van der Westhuizen,T.J., Augustyn,O.P.H., Pretorius,I.S. (2000). Geographical distribution of indigenous *Saccharomyces cerevisiae* strains isolated from vineyards in the Coastal Regions of the Western Cape in South Africa. S.Afr.J.Enol.Vitic *21*, 3-9.

156. Vaughan-Martini,A., Martini,A. (1995). Facts, myths and legends on the prime industrial microorganism. J Ind.Microbiol. *14*, 514-522.

157. Versavaud,A., Courcoux,P., Poulland,C., Dulau,L., Hallet,J.N. (1995). Genetic diversity and geographical distribution of wild *Saccharomyces cerevisiae* strains from the wine-producing area of Charentes, France. Appl.Environ.Microbiol. *61*, 3521-3529.

158. Vigh,L., Maresca,B., Harwood,J.L. (1998). Does the membrane's physical state control the expression of heat shock and other genes? Trends Biochem.Sci. *23*, 369-374.

159. Watson,K. (1987). Temperature Relations. In: The yeast. Yeast and Environment, ed. A.H.Rose, J.S.HarrisonLondon: Academic Press, 41-71.

160. Welch,W.J., Brown,C.R. (1996). Influence of molecular and chemical chaperones on protein folding. Cell Stress Chaperones *1*, 109-115.

161. Wodicka,L., Dong,H., Mittmann,M., Ho,M.H., Lockhart,D.J. (1997). Genome-wide expression monitoring in *Saccharomyces cerevisiae*. Nat.Biotechnol. *15*, 1359-1367.

162. Yale,J., Bohnert,H.J. (2001). Transcript expression in Saccharomyces cerevisiae at high salinity. J Biol.Chem *276*, 15996-16007.

163. Zinser,E., Sperka-Gottlieb,C.D., Fasch,E.V., Kohlwein,S.D., Paltauf,F., Daum,G. (1991). Phospholipid synthesis and lipid composition of subcellular membranes in the unicellular eukaryote Saccharomyces cerevisiae. J.Bacteriol. *173*, 2026-2034.

164. Zuzuarregui,A., del Olmo,M. (2004a). Analyses of stress resistance under laboratory conditions constitute a suitable criterion for wine yeast selection. Antonie Van Leeuwenhoek *85*, 271-280.

165. Zuzuarregui,A., del Olmo,M.L. (2004b). Expression of stress response genes in wine strains with different fermentative behavior. FEMS Yeast Res. *4*, 699-710.

CHAPTER 1

Effect of fermentation temperature and culture media on the lipid composition and volatile compounds in wine

Gemma Beltran, Maite Novo, José M. Guillamón, Albert Mas and Nicolas Rozès

Unitat d'Enologia del Centre de Referència de Tecnologia d'Aliments. Dept. Bioquímica i Biotecnologia. Facultat d'Enologia de Tarragona. Universitat Rovira i Virgili. Marcel·lí Domingo s/n. 43007 Tarragona, Spain

(*Submitted for publication*)

ABSTRACT
We investigated the relationships between fatty acids, the sterol and phospholipid composition of yeast cells and the flavour compounds of wines in industrial conditions (grape must) and laboratory conditions (synthetic medium) at 13 and 25ºC. In white wine production, the growth yeast, the lipid composition of cells and the aromatic intensity of wines were affected by temperature and the industrial process. Our results show that yeast viability is better at 13ºC than at 25ºC whichever growth medium is used, but that the complexity of the grape must enables cells to reach higher values. Viability also seems to be related to the incorporation of linoleic acid and ß-sitosterol, which are present in the grape must, and the high content of phosphatidyl inositol in the cells. We also found that medium-chain fatty acids, mainly dodecanoic acid, were present in the cell phospholipids. Wines obtained from industrial must were fruitier and more aromatic and had a lower volatile acidity content than those of the laboratory medium. The low temperature emphasized this feature.

Keywords: fatty acid, sterol, phospholipid, low temperature, aromatic compounds, alcoholic fermentation, grape must.

INTRODUCTION

Low temperature alcoholic fermentations are becoming more frequent due to the winemaker's tendency to produce wines with more pronounced aromatic profile. Wines produced at low temperatures (10-15ºC) develop improved characteristics of taste and aroma (*17*). The aromatic complexity of wines varies according to the primary or varietal flavour (flavour compounds originating from the grapes), the secondary or fermentative flavour (produced by yeast and bacteria during alcoholic and malolactic fermentation) and tertiary or post-fermentative flavour (compounds that appear during the ageing process) (9*;45*). The temperature of fermentation affects both the retention of some varietal compounds and the production of fermentative metabolites. The improved quality of wines produced at low temperatures can be attributed to a greater retention of terpens, a reduction in higher alcohols and an increase in the proportion of ethyl and acetate esters in the total volatile compounds (*31;35;56*).

However, the optimal growth temperature for *Saccharomyces cerevisiae* is 25ºC and 13ºC is a restrictive temperature that increases the risk of stuck or sluggish fermentations (*8*). Low temperatures increase the duration of alcoholic fermentation, decrease the rate of yeast growth and modify the ecology of wine fermentation (*11;18;57*). Therefore, although low temperature fermentation has interesting applications in the enological industry, it also has an adverse effect on cell growth because it increases the yeast stress during wine production.

Wine yeast strains have been selected for their ability to efficiently ferment grape-juice sugars under rather stressful conditions (high concentration of sugar and ethanol, low pH and often limited nitrogen, lipids, vitamins, added sulphites and anaerobic conditions). The survival of cells can depend on their ability to adapt quickly to the changing environment. Changes in plasma membrane composition may be an adaptative response by the yeast since it is highly variable and clearly influenced by environmental factors such as temperature, oxygen, nutrient limitation and growth rate (*25;46*). Ethanol mainly affects membrane structural integrity and membrane permeability (*9*), and the defensive adaptation of wine yeasts ranges from alterations in membrane fluidity to synthesis of detoxification enzymes (*9;43*).

The main lipid components of eukaryotic membranes are phospholipids, sterols, sphingolipids and glycerolipids. Alterations in fatty acid, phospholipid and sterol levels are needed to maintain ethanol tolerance (*8*). The membrane fatty-acyl composition of yeast, like that of many other microorganisms, also changes with temperature: the lower

the temperature, the more unsaturated the membrane fatty-acyl composition (*56;59*). However, the fatty acid composition of a cell can also be influenced by the environment's lipid composition since it can include fatty acids from the medium in its own phospholipids (*5;49;55*).

In grapes, unsaturated fatty acids (UFA) represent the main component of total lipids. The most abundant of these is linoleic acid, followed by oleic, linolenic and palmitoleic acid (*10*). Of the saturated fatty acids (SFA), palmitic acid is the most abundant (*6;10*). However, the initial fatty acid content of the must depends on which technological procedures, such as pressing, maceration or clarification, are applied to the grapes and musts (*7;15*).

Under industrial conditions, natural musts have low sterol contents. The main phytosterol in these musts was ß-sitosterol (*33*). In white wine production, the absence of oxygen suppresses fatty acid desaturation and the sterol biosynthesis by yeast, which reduces the capacity to synthesize ergosterol that is essential for protecting the yeast against ethanol stress (*1;2*). In wine fermentation, therefore, ergosterol synthesis can occur if oxygen is added during fermentation, but the efficiency of oxygen additions decreases as the fermentation progresses (*52*). The yeasts can grow in the absence of oxygen, however, by incorporating exogenous sterols (*36;40*) and unsaturated fatty acids (*12*).

On the other hand, the lower oxygen presence during the fermentation induces the synthesis of medium-chain fatty acids (C6 to C14) and their corresponding ethyl esters by the yeasts, which are in synergy with ethanol, which is toxic to the cells themselves (*30*).

The fermentation temperature therefore affects the lipid metabolism, which is related to cell development, membrane integrity and the production of several by-products, especially those directly related to wine aroma.

Moreover, both the culture medium (*35*) and the industrial process used in fermentation (*42*) also influence the fermentation performance, the yeast metabolism and the final quality of the wines. Synthetic media for simulating natural musts in laboratory fermentations are widely used to study several aspects of wine fermentation and wine yeast metabolism (*3;27;47;50*). However, wines produced from natural grape must have varietal and fermentative flavours, whereas wines from synthetic must only have fermentative flavours.

The aim of this study is to estimate how the fermentation temperature (13ºC *vs* 25ºC) and culture media (natural grape must *vs*. synthetic must) affect fermentation kinetics, the yeast lipid composition and the production and/or retention of wine flavours, which are the main factors affected by fermentation temperature. To do this, we carried out fermentations at 25 and 13ºC in the laboratory and on an industrial scale using synthetic and natural grape must, respectively, and analysed these parameters.

MATERIALS AND METHODS

Yeast strain and culture conditions

A commercial *Saccharomyces cerevisiæ* wine strain (QA23, Lallemand S.A., Toulouse, France) was used to an initial population of 2 x 10^6 cell mL^{-1} of dry yeast rehydrated in water at 37ºC for 30 min prior to inoculation according to the manufacturer's instructions.

Two media were used in this experiment. One was Moscatell grape must (obtained from the experimental fields of the Faculty of Enology in Tarragona, Spain). This was clarified by natural settling for 12 hours at 20ºC to separate the clear juice from the sediments and corrected with diammonium sulphate up to 300 mg L^{-1} of yeast assimilable nitrogen (YAN). The other was a synthetic grape must (*47*) with 200 g L^{-1} of reducing sugars (100 g L^{-1} Glucose and 100 g L^{-1} Fructose), 300 mg YAN L^{-1} and without anaerobic factors.

In the wine cellar, the fermentations were done in 100-liter tanks filled with 80 l of grape must. In the laboratory the fermentations were done in 2-liter bottles filled with 1.8 l of the medium and fitted with closures that enabled the carbon dioxide to escape. The fermentations were performed at 13ºC and 25ºC for both laboratory scale and industrial assays.

Yeast growth and fermentation kinetics

Yeast growth was calculated by counting the viable yeast after plating it on YPD agar (Glucose, 20 g L^{-1}; Peptone Bacteriological (Cultimed, Panreac, Barcelona, Spain), 20 g L^{-1}; Yeast Extract (Cultimed), 10 g L^{-1}; Agar Bacteriological American Type (Cultimed), 20 g L^{-1}) at a suitable dilution for 2 days at 28ºC.

The density was measured every day by weighing 5 ml of medium in the laboratory scale fermentations and with a Baumé hydrometer in the industrial fermentations. The fermentation kinetic along the process was calculated as dDensity/dt. Final fermentation

was considered when no residual sugars were left (< 2 g L^{-1}). Residual sugars (Glucose and Fructose) were assayed using Boehringer Mannheim enzymatic kits.

Cell samples were harvested in triplicate by centrifugation (5000 x 5min at 4°C) at three periods during the process. These were initial fermentation (IF, density ≈ 1060), mid-fermentation (MF, density ≈ 1025) and final fermentation (FF, density ≈ 1000). The samples were quickly frozen in liquid nitrogen and stored at -80°C.

Determination of cell fatty acid composition

Cells (5-10 mg Dry Weight) were saponified and fatty acids were extracted according to the method used by (*51*). Analytical GC was carried out on a Hewlett Packard 5890 connected to an HP Vectra computer with the ChemStation software (Agilent Technologies, Wilmington, DE, USA). The extract (2 µl) was injected (splitless, 1 minute) into an FFAP-HP column of 30 m x 0.25 mm and 0.25 mm phase thickness (Agilent) with an HP automatic injector (Agilent). The temperature program was 140ºC x 4ºC min^{-1} to 240 ºC (13 min.). Injector and detector temperatures were 250 and 280ºC, respectively. The carrier gas was helium at 1.2 ml min^{-1}. Heptanoic and heptadecanoic acids (1 mg mL^{-1} and 41 mg mL^{-1}, respectively) were added as internal standards. Relative amounts of given fatty acids were calculated from their respective chromatographic peak areas. These amounts were related to the dry weight and later transformed into percentages of the total fatty acids obtained. Mean fatty acid chain length (CL) was calculated as: $CL = \sum(P \times C)/100$, where P is the percentage of fatty acids and C is the number of carbons.

Determination of cell sterol composition

Yeast cells (5-10 mg Dry Weight) were collected by centrifugation and stored at –20 ºC at different points of the fermentation process for the analysis of cell sterol composition. Cells in the presence of 5-α-cholestane (10µg μL^{-1}), internal standard, were saponified by 15% KOH in methanol at 90ºC for 30 min and sterols were extracted with 300 µl of hexane. Analytical GC was carried out on a Hewlett-Packard 5890 connected to an HP Vectra computer with the ChemStation software (Agilent Technologies, Wilmington, DE, USA). The extract after concentration under nitrogen gas was injected (2 µl) (splitless, 1 min) into an SAC-5 column of 15 m x 0.25 mm and 0.25 µm phase thickness (Supelco, Bellefonte, PA, USA) with an HP 7673 automatic injector

(Agilent). The temperature program was 140 x 4 ºC min^{-1} to 240 ºC (13 min). Injector and detector (FID) temperatures were 250 and 280 ºC, respectively. The carrier gas was helium at 1.2 mL min^{-1}. Relative amounts of given sterols were calculated from their respective chromatographic peak areas.

From the same extracts, GC/MS of sterols was conducted on an HP5890 series II coupled to an HP5972 mass selective detector. Electron impact GC/MS (70eV, scanning from 42 to 600 atomic mass units at 1-sec intervals) was performed by the following conditions: SAC-5 column (30 m x 0.25 mm and 0.25 µm film thickness), He as carrier gas (30 cm sec^{-1}), detector temperature 250ºC, column temperature 125-250ºC (125ºC for 2 min, 20ºC min^{-1} to 250ºC for 60min), injector temperature 250ºC. All injections were run in splitless mode. The major sterols (squalen, lanosterol, ergosterol, stigmasterol and ß-sitosterol) were identified from their GC peak retention times relative to sterol standards (Supelco). Confirmation of phytosterol assimilation was determined by comparison of GC retention times and mass spectra.

Lipid extraction and separation

Prior to lipid extraction, 10 µl EDTA 0.1 mM, 100 µl cold methanol and glass beads (0.5 mm, Biospec Products, USA) were added to yeast cells (5-10mg Dry Weight) in 2.0 ml conical screw cap microtubes (Porex Bio Products, USA), then mixed for 5 min in a minibeadbeater-8 (Biospec Products). Lipid extraction was performed with chloroform: methanol (2: 1, v/v, two times and 1:1, one time). All the inferior organic phases were transferred in a 15 ml glass screw tube in the presence of KCl 0.88% (a quarter of the total volume of the extract). After vortexing and cooling in ice for 15 min, samples were centrifuged 10 min at 3,000 rpm. The organic phase was collected and dried through a Na_2SO_4 column, and then concentrated to dryness with nitrogen. The extract was dissolved in chloroform: methanol (2: 1, v/v).

Individual lipid classes were separated by thin-layer chromatography (TLC) on silica gel $60F_{254}$ plates (10 x 20cm, 250µm, Merck, Germany) with solvent systems as follows: sterol, sterol ester (SE), diacylglycerol (DAG), triacylglycerol (TAG), fatty acid ethyl ester (FAEE) and squalene in hexane: *tert*-butylmethyl ether (MTBE): acetic acid glacial (70: 30: 2, v/v); phosphatidylinositol (PI), phosphatidylserine (PS), phosphatidylcholine (PC), phosphatidylglycerol (PG), phosphatidylethanolamine (PE), cardiolipin (CL) and phosphatidic acid (PA) in chloroform: aceton: methanol: acetic acid glacial: water (50: 15: 10: 10: 5, v/v). The standard lipids lanosterol, ergosterol,

squalene, cholesterol oleate (SE), ethyl oleate (EEAG), diolein (DAG), triolein (TAG), PI, PS, PC, PG, PE, CL and PA were obtained from Sigma. Calibration was performed using standard solutions (0.5-4 µg/µl). Lipids on TLC plates were detected with 10% $CuSO_4$ in 8% H_3PO_4 and heated at 120ºC for 20 min. The TLC plate showing brown spots was filmed with a Kodak DC290 Zoom digital camera. For lipid quantification, each spot of the image of the TLC plate was processed with the Quantity One software (Biorad, USA).

Determination of phospholipid fatty acid composition

Phospholipids (PL) separated from neutral lipids on TLC plates with hexane: *tert*-butylmethyl ether (MTBE): acetic acid glacial (70: 30: 2, v/v) were scraped off after visualization by iodine vapours. PLs dissolved in 100µl of MTBE were transmethylated with 200µl of 0.2N sodium methylate in methanol for 2 min at room temperature. After the addition of 60 µl H_2SO_4 N and 400 µl water, samples were vortexed for 1 min. The fatty acid methyl esters (FAME) in MTBE were collected after centrifugation at 3000 rpm for 2 min. The extract (3 µl) was injected (splitless, 1 minute) into an FFAP-HP column of 30 m x 0.25 mm and 0.25 mm phase thickness (Agilent) with an HP automatic injector (Agilent). The temperature program was 100ºC x3ºC min^{-1} to 240 ºC (13 min.). Injector and detector temperatures were 250 and 280ºC, respectively. The carrier gas was helium at 1.2 ml min^{-1}. 5µl of heptadecanoic acid methyl ester (1 µg μ^{-1}) was added as internal standard. FAMEs were identified from their retention times relative to appropriate standards (Supelco) prepared according to the proposed method. Relative amounts of given fatty acids were calculated from their respective chromatographic peak areas.

Determination of by-products and volatile compounds of wines

Wine samples (100 ml) were taken at the end of the fermentation process to measure acetic acid and ethanol according to the method described in (*20*).

Other by-products, including fusel alcohols, short-chain and medium chain aliphatic esters, terpens and free short and medium aliphatic fatty acids were extracted by liquid/liquid extraction with 200 µl of 1,1,2- Trichlorotrifluoroethane (Fluka) and 0.5 g NaCl using as internal standard n-decanol (0.2 mg l^{-1}) according to the method of (*37*). After 2 min agitation and centrifugation, the organic phase was extracted and 2 µl of it was injected onto a gas chromatograph HP 6890N using the automatic injector HP 7683

and a column TR-WAX (60m x 0.25mm x 0.25 μm) equipped with a FID detector. Aromatic volatile compounds were identified and quantified by comparison with standards.

Correlation matrix

Matrix correlation between fermentation conditions were calculated using the application software package (*21*), which allows multivariable data analysis with Microsoft Excel v. 2001. Statistical analyses were performed using the Statview v. 4.0 (Abacus Concepts, Berkeley, USA) program to detect differences between temperatures and media.

RESULTS

Wine fermentations were carried out at 25 and 13ºC in laboratory and industrial scales using synthetic and natural grape must, respectively, in order to estimate how temperature and culture media affect fermentation kinetics, yeast lipid composition and the production of wine flavours.

Effect of temperature and culture media on fermentation kinetic and yeast growth

Wine fermentations were carried out at 25 and 13ºC in laboratory and industrial scales using synthetic and natural grape must, respectively, in order to estimate how temperature and culture media affect fermentation kinetics, yeast lipid composition and the production of wine flavours.

Both temperature and media affected the fermentation kinetics and yeast growth. The low temperature produced slower fermentations, with lower fermentation rates and Vmax. The Vmax/maximal fermentation rate was roughly twice as high at 25ºC fermentation (18.6 and 23 at 25ºC vs. 8.3 and 10.9 at 13ºC) and reached higher values in industrial ones. Moreover, the industrial fermentations finished more rapidly (9 and 20 days at 25 and 13ºC, respectively) than the laboratory fermentations (14 and 25 days).

At 25ºC yeast grew quickly, reaching higher values of maximal cell population than at 13ºC. However, after entering the stationary phase, their viability began to decrease. At 13ºC, on other hand, yeast grew slowly but its viability remained more or less constant until the end of fermentation. This cell growth profile was similar in both fermentation media, but the laboratory fermentations reached lower values of cell population than industrial ones.

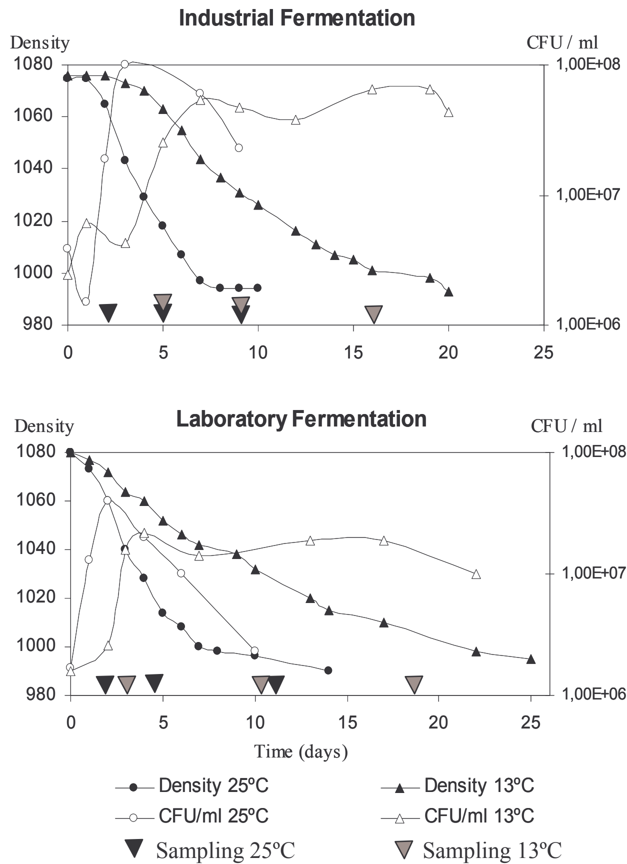


Figure 1
Effects of temperature fermentation and culture media on fermentation kinetics and yeast growth.

Effect of temperature and culture media on lipid cell composition

Total fatty acid, sterol and phospholipid cell composition were analysed at the initial (IF), middle (MF) and final phase of fermentation (FF) at both fermentation temperatures and in both fermentation media.

Total fatty acid composition of cells

Fig. 2 shows the changes, during fermentation, in the total fatty acid composition of yeast cells grown in grape must and synthetic medium at both temperatures.

In both conditions the medium-chain fatty acid (MCFA, C6 to C14) represented about 30% of the total percentage of FA in whole cells. However, in industrial fermentations at 25ºC this percentage decreased and the percentage of long-chain fatty acid (LCFA, C16 and C18) increased. On the other hand, in laboratory fermentations at 13ºC, the percentage of MCFA increased dramatically while that of LCFA decreased.

At both temperatures the percentage of unsaturated fatty acid (UFA, C16:1 and C18:1) was higher in industrial fermentations (29.4 ± 4.8) than in laboratory fermentations (17.3 ± 5.6). This UFA level remained constant at 25ºC but decreased at 13ºC. The

same occurred in yeast cells from laboratory fermentations, though the levels were lower. We only detected the presence of linoleic acid (C18:2) in cells grown in grape must.

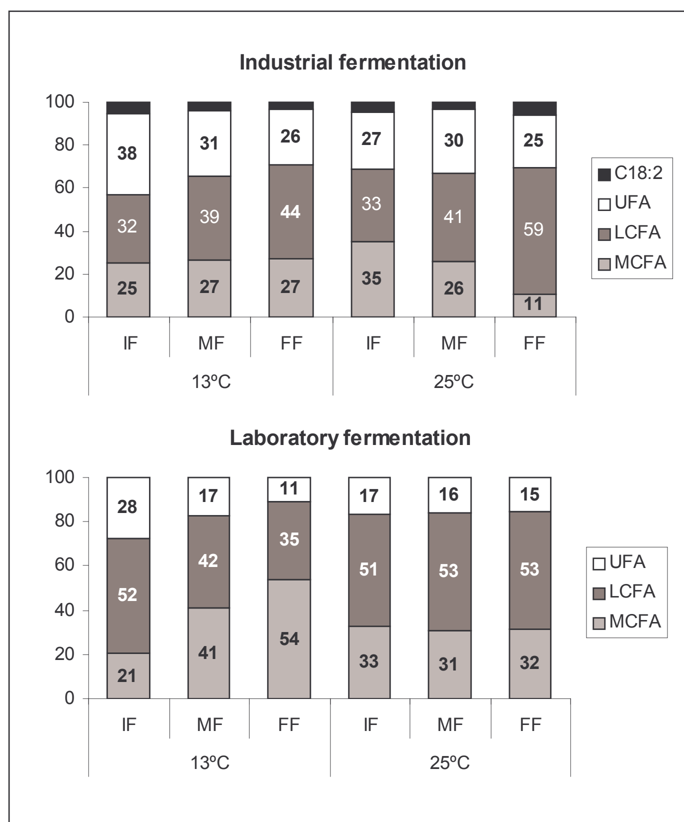


Figure 2. Effects of temperature fermentation and culture media on total fatty acid composition (%) of cells. UFA: Unsaturated Fatty Acids (C16:1 + C18:1); LCFA: Long Chain Fatty Acids (C16-C18); MCFA: Medium Chain Fatty Acids (C6-C14).

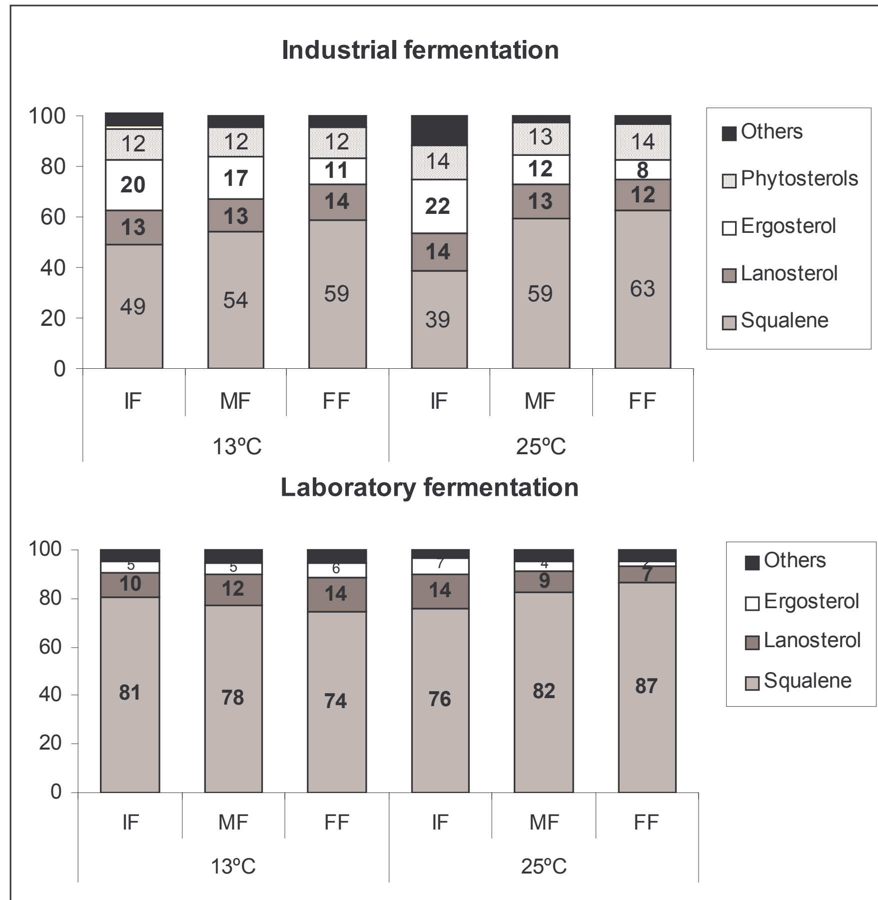
Sterol composition of cells

The sterol composition of yeast cells determined by gas chromatography is shown in Fig. 3. In all experimental conditions, the main sterol present in wine yeast cells was squalene, which is the precursor for the ergosterol synthesis via the formation of lanosterol. Squalene represented more than 50 % of total sterols in the cell in industrial fermentations and 80% in laboratory fermentations. Its proportion was higher at 13ºC at the initial fermentation point but, as fermentation progressed, the proportion of squalene increased more clearly at 25ºC and was higher at this temperature in the final stages of fermentation. In compensation, at the final point of fermentation at 13ºC, the proportions of lanosterol and ergosterol were higher than at the final point of fermentation at 25ºC. The lanosterol contents for both media were the same, whereas the ergosterol content was higher in yeast cells grown in grape must (14.8 ± 5.6%) than in yeast cells grown in synthetic medium (4.8 ± 1.5%).

The main difference between the fermentation media was the presence of phytosterols (stigmasterol and β-sitosterol) in yeast cells fermenting grape must. The incorporation

of these phytosterols (mainly ß-sitosterol) was similar at both temperatures (13.4 ± 0.7%).

Figure 3. Effects of temperature fermentation and culture media on sterol composition (%) of cells.



Phospholipid composition of cells and fatty acid composition of PL

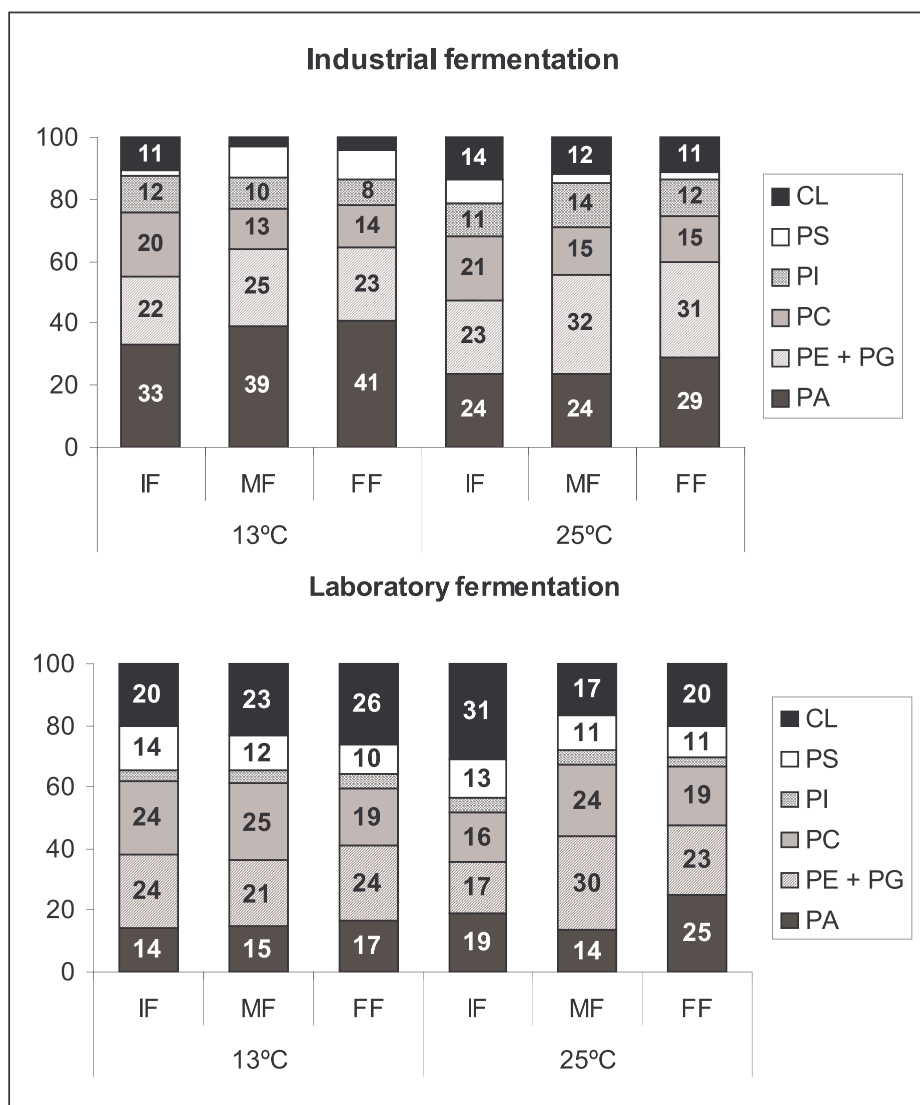
The phospholipid composition of cells determined by TLC was PI, PS, PC, PG+PE (not separated in our conditions), CL and PA. Fig. 4 shows how the fermentation temperature and the culture media affected the phospholipid percentages of yeast cells. In industrial fermentations, the main PLs, in decreasing order, were: PA> PE+PG> PC> PI, CL> PS. In laboratory fermentations, all PLs except PS and PI were close to 20%. There were significant differences in CL, PS, PI and PA between the industrial and the laboratory fermentations. PI and PA contents were higher in cells grown in grape must whereas CL and PS contents were higher in cells grown in the synthetic medium.

There were no important differences between fermentations at 13ºC and fermentations at 25ºC.

When we analyzed the fatty acid composition of total PL, we found that MCFA (mainly C12 (> 15%)) were incorporated into the PL in all experimental conditions. C18:2 (ca

1.5%) was detected only at the initial stage of the industrial fermentations at both temperatures. Unexpected UFA percentages (about 26%) were found at both temperatures in both media. Palmitic acid (C16:0) was the most abundant saturated fatty acids in industrial fermentations (data not shown).

Figure 4. Effect of temperature fermentation and culture media on phospholipid composition (%) of cells. CL:Cardiolipin; PS: Phosphatidylserine; PI: Phosphatilylinositol; PC: Phospahtidylcholine; PE: Phosphatidylethanolamine; PG: Phosphatidylglycerol; PA: Phosphatidic acid



Effect of temperature and culture media on volatile compounds of wine

We analysed flavour compounds such as fusel alcohols and their corresponding acetate esters, volatile fatty acids and their corresponding ethyl esters, which arose from yeast metabolism (see Table 1).

Table 1. Effects of temperature fermentation and culture media on ethanol content, volatile acidity and volatile compounds (mg l^{-1}) of wines.

	Industrial fermentation		Laboratory fermentation	
	13ºC	25ºC	13ºC	25ºC
Volatile acidity (g acetic acid l^{-1})	0.36	0.45	1.03	1.19
Hexanol	0.4	0.4	-	-
Isoamylic alcohols	140	182	45.5	54.5
Phenyl-2-ethanol	21.2	35.2	5.6	11.8
∑ Fusel Alcohols	161.6	217.6	51.1	66.3
Isoamyl acetate	1.48	1.14	1.00	0.55
Hexyl acetate	0.12	0.08	-	-
Phenyl-2-ethanol acetate	0.14	0.24	0.15	0.23
∑ Fusel Alcohol Acetates	1.74	1.45	1.15	0.78
Ethyl butyrate	0.38	0.21	0.25	0.14
Ethyl isovalerate	0.01	0.01	-	-
Ethyl hexanoate	1.30	0.93	0.90	0.79
Ethyl octanoate	1.24	0.96	0.50	0.48
Ethyl decanoate	0.31	0.36	0.20	0.20
∑ EEAG [a]	3.24	2.47	1.85	1.61
Isobutyric acid	0.05	0.05	0.35	0.41
Butyric acid	0.90	0.56	0.67	0.38
Isovaleric acid	0.29	0.23	0.12	0.01
Valeric acid	0.27	0.28	0.33	0.46
Hexanoic acid	2.47	1.21	2.26	1.76
Octanoic acid	4.56	2.51	3.12	2.62
Decanoic acid	1.27	0.93	1.25	0.84
Dodecanoic acid	0.12	0.05	0.02	0.01
∑ Fatty acid	9.93	5.82	8.12	6.49

[a]: Ethyl Ester of Fatty Acids; - : Not detected.

The concentrations of volatile compounds were higher in natural must, possibly due to the media used. However, temperature affected the industrial and laboratory fermentations in the same way: the low temperature increased the proportion of fusel alcohol acetate esters, fatty acids and their corresponding ethyl esters, and decreased volatile acidity and fusel alcohol concentrations.

The highest concentrations of fatty acids were those of hexanoic, octanoic and decanoic acids. Consequently, their corresponding ethyl esters, ethyl hexanoate and ethyl octanoate, also had the highest concentrations (the content of ethyl octanoate was twice as high in natural fermentations than in synthetic fermentations).

With regard to fusel alcohols, isoamylic alcohol was more than three times higher in industrial fermentations than in synthetic fermentations. Hexanol, a compound produced during grape processing by the oxidation of the grape's unsaturated fatty acids, was only present in industrial fermentations. Its corresponding acetate ester (hexyl acetate) was also detected in natural fermentation. Interestingly, the ratio of esterification between fusel alcohols and their corresponding acetate esters was always higher (two-fold) at 13ºC than at 25ºC. It was also higher in wines from synthetic media than in wines from industrial media.

In industrial fermentations there was a greater retention of terpens, which are compounds not synthesized by yeasts, at 13ºC than at 25ºC (data not shown).

The acetic acid content of wines represented by volatile acidity decreased at the low temperature in both fermentation media but its production was higher in synthetic fermentations.

DISCUSSION

Wines produced at low temperatures (10-15ºC) develop characteristics of taste and aroma (*17;35;56*) by integrating varietal, fermentative and sometimes post-fermentative flavours (*9;45*). In common white winemaking, in which hypoxic conditions and natural settling lead to a growth medium deficiency, yeast stress increases through the alcoholic fermentation and leads to a sluggish and sometimes stuck fermentation (*8*). We have previously reported that low-temperature fermentations carried out in a laboratory-scale medium enabled us to increase the aromatic profile of wines (*56*) and show that the nitrogen content of the growth medium could play an important role in improving yeast capacity during fermentation (*35*). We also observed in previous reports that the viability of cells at 13ºC was better than at 25ºC (*35;57*). To better understand low-temperature fermentation, therefore, we have now studied the effect of both media (synthetic and natural must) at both temperatures (13 and 25ºC) on the growth and lipid metabolism of yeasts and the aromatic volatile compounds of wines.

Temperature clearly affects yeast growth and fermentation kinetics. As expected, fermentation rates and maximal yeast population were better at 25ºC than at 13ºC in

both media, but viability gradually decreased at 25ºC when the cells reached the stationary phase. Also, fermentations carried out with natural grape must were shorter at both temperatures. This could be because the membrane composition of yeast fermenting natural must is different, mainly due to the incorporation of some sterols and UFA from the medium. The correlation matrix in Table 2 shows that yeast viability was positively related to phytosterol (overall β-sitosterol) and phosphatidyl inositol (PI) contents. All these compounds were more present in yeast fermenting natural must. Squalene was negatively related to ergosterol (-0.961) and ß-sitosterol (-0.855), and PS was negatively related to PI (-0.870).

Table 2. Correlation matrix calculated from the PCA analysis

	Ergosterol	UNSAT	PI	ß-Sitosterol	PS	**Squalen**	CL	Viability
Ergosterol	1.000							
UNSAT	**0.797**	1.000						
PI	**0.746**	**0.813**	1.000					
ß-Sitosterol	**0.726**	**0.826**	**0.935**	1.000				
PS	-0.534	-0.638	**-0.870**	**-0.797**	1.000			
Squalen	**-0.961**	**-0.796**	**-0.836**	**-0.855**	0.629	1.000		
CL	-0.586	**-0.756**	-0.678	**-0.802**	0.480	0.643	1.000	
Viability	0.480	0.580	**0.765**	**0.730**	-0.489	-0.583	-0.540	1.000

UNSAT: Sum of unsaturated fatty acids (C16:1 + C18:1 + C18:2); PI: PhosphatidylInositol; PS: PhosphatidylSerine; CL: Cardiolipin.

In grape must with a high sugar concentration in white winemaking (i.e. hypoxic conditions), the fermentative metabolism of the yeast was directly linked to its cellular sterol content (*32*). The incorporation of phytosterol into the cell membrane could alleviate this deficiency in the yeast cells during the decline phase and improve the exchange between the cells and the medium. Although this incorporation of exogenous sterol seems to be similar at 13 and 25ºC, cell viability was better at 13ºC. As Luparia et al. (*36*) demonstrated by supplying defined amounts of phytosterols in a laboratory medium, at 24ºC cell viability also decreased during the stationary phase and this can lead to a stuck fermentation. Although the sterol effect on viability and fermentation ability is both concentration-dependent (*13;34*) and structure-dependent (*48*), at low

temperature and in a complex medium such as grape must, other factors could play an important role.

As observed in previous studies (*26;28*), however, yeast fermenting synthetic media, in the absence of both anaerobic factors and oxygen, accumulated a large amount of squalene in membranes (74–87 % of total sterols) and small amounts of ergosterol (2-7 %) and its intermediates. The viability of the cells was also extremely low. The previously reported (*55*) decrease in squalene in cells with increasing amounts of C18:2 in the fermentation medium was confirmed by the fact that the ergosterol content in cells grown in grape must was three times higher than in cells grown in synthetic medium.

Yeasts fermenting at low temperature increased the unsaturated fatty-acyl composition of the membrane (*56;59*). However, the fatty acid composition of a cell can also be influenced by the environment's lipid composition, since it can include fatty acids from the medium in its own phospholipids (*5;49*) such as linoleic acid from grape must. We found that the percentage of UFA in yeast cells was higher at the beginning of fermentation at 13ºC in both media, and that this percentage was higher in yeasts fermenting natural must. However, although C18.2 was detected throughout the grape must fermentation in cells at both temperatures, it seems that the C18:2 acylation into PLs was only efficient at the initial stage of fermentation. These results are consistent with the work of Thurston (*55*), who found 98% of C18:2 in esterified form in cells grown in C18:2-supplemented medium. Similarly, some oleic acid could be taken up from the medium and incorporated into the cells. Several studies have shown that the increase in fatty acid unsaturation in yeast cells improves ethanol tolerance and increases cell viability (*1;2*). The yeast strain overexpressing *OLE1* gene was more fluid than the wild type strain (*29*), and the increase in the membrane fluidity of *S. cerevisiae* was essential for increasing the cell viability.

Moreover, MCFA production is directly related to the fermentative metabolism of yeasts promoted by hypoxic conditions (*4;30;53*). In our conditions, whether in the presence of exogenous lipid or not, the MCFA content in the cells is always high. At low temperature, however, cells supported a large MCFA content that increased in the absence of exogenous lipids. Because unsaturated fatty acids cannot be synthesized in hypoxic conditions, this MCFA synthesis enables the cell to adjust its structural and functional membrane integrity. On the other hand, Bardi *et al*. (*4*) suggested that MCFA were not immobilized in cell structures but were mainly released into the medium and

could therefore be regarded as an effect and not the cause of the sluggish or stuck fermentation. In our conditions the presence of C18:2 and ß-sitosterol in cells growing in grape must decreased the MCFA content of PLs and whole cells, enabling better growth at 13ºC and 25ºC and a lower final time of fermentation for the same temperature.

We found that the values of PI were higher in industrial grape must fermentations. Interestingly, phosphatidylinositol (PI) is an essential phospholipid for yeast, probably not because of its structural requirement for membrane assembly but because of its roles in cellular signaling and as a membrane sensor and because of its viability (*14*). Its biosynthesis increases as a response to ethanol toxicity.

The alteration of the lipid composition of yeast may change the fermentative capacity of the cells (*38;58*) and the final aroma characteristics of the wines (*7;24*). Low temperature obtained wines with increasing floral (fatty acid ethyl esters) and fruity (fusel alcohol acetates) yeast aromas and maintained a high level of varietal aromas (terpens). Also, the volatile acidity and fusel alcohol concentration in fermentations at 13ºC were lower than those at 25ºC. In industrial must fermentation, there was a large increase in isoamylic alcohols and therefore also in isoamyl acetate. We also observed the presence of hexanol (and its ester acetate). Although the fusel alcohol acetate content was higher in industrial wines than in synthetic wines, the conversion of fusel alcohols into esters seemed to be lower. Our results are consistent with previous reports which showed that the alcohol acetyltransferase (AATase) activity (*37*) and the *ATF1* transcription (*19*) are repressed by oxygen and C18:2.

The content of fatty acids in natural and synthetic musts was very similar. However, the EEFA were higher in fermented natural must. Generally, the ester synthesis increases when the cell division in the fermentation process stops (*44*). A large proportion of ethyl esters is observed in sublethally or lethally damaged cells. A dramatic accumulation of ethyl esters of UFA, released by phospholipids, is observed in yeast cells subjected to multiple stresses (ethanol and pressure) (*22*).

The specific rates at which ethyl acetate and isoamyl-acetate are produced by yeast increase markedly at the point in fermentation where syntheses of lipids stop. The specific rates of ester synthesis are likely to be correlated with changes in the formation of lipids, since both utilise acetyl-CoA (*41;54;55*). Adding linoleic acid (50mg/l) suppresses the induction of ester synthesis and reduces the overall formation of ethyl and isoamyl acetates (*55*).

Interestingly, the volatile acidity decreased as the temperature decreased and the use of the industrial medium led to a further decrease. The complexity of the grape must influences the yeast metabolism and leads to low acetic acid levels (*39*). Unsaturated fatty acids (*55*), ß-sitosterol (*36*), light clarification of must (*15;39*) and the presence of lees (*23*) reduces the volatile acidity.

In conclusion, in white wine production low temperatures increase the flavour-active compounds and decrease unpleasant ones such as acetic acid and fusel alcohols. Further research using molecular biological techniques and biochemical studies is needed to determine the underlying mechanisms involved in ester synthesis in a complex medium such as grape must. A more extensive study may also be necessary to better understand the relationship between the presence of exogenous lipids on the one hand and phospholipid metabolism and cell viability on the other.

ACKNOWLEDGEMENTS

This work was supported by grants (AGL2000-0205-P4-03) from the Spanish Government. We thank Torres S.A. for analyzing the volatile compounds of wines. We thank Kevin Costello from the Languages Services of the Rovira i Virgili University for revising the manuscript.

REFERENCES

1. Alexandre,H.; Rousseaux,I.; Charpentier,C. Ethanol adaptation mechanisms in Saccharomyces cerevisiae. *Biotechnol.Appl.Biochem.* **1994**, *20*, 173-183.

2. Alexandre,H.; Rousseaux,I.; Charpentier,C. Relationship between ethanol tolerance, lipid composition and plasma membrane fluidity in Saccharomyces cerevisiae and Kloeckera apiculata. *FEMS Microbiol.Lett.* **1994**, *124*, 17-22.

3. Backhus,L.E.; DeRisi,J.; Brown,P.O.; Bisson,L.F. Functional genomic analysis of a commercial wine strain of Saccharomyces cerevisiae under differing nitrogen conditions. *FEMS Yeast Res.* **2001**, *1*, 111-125.

4. Bardi,L.; Cocito,C.; Marzona,M. Saccharomyces cerevisiae cell fatty acid composition and release during fermentation without aeration and in absence of exogenous lipids. *Int.J Food Microbiol.* **1999**, *47*, 133-140.

5. Benchekroun,K.; Bonaly,R. Physiological properties and plasma membrane composition of Saccharomyces cerevisiae grown in sequential batch culture and in presence of surfactant. *Appl.Microbiol.Biotechnol.* **1992**, *36*, 673-678.

6. Bertrand,A.; Miele,A. Influence of clarification du moût de raisin sur sa teneur en acides gras. *Conn.Vigne Vin* **1984**, *48*, 293-297.

7. Bertuccioli,M.; Rosi,I. Progress in Flavour Research. In *Developments in Food Science*; Adda,J., Ed.; Elsevier: Amsterdam, 1984.

8. Bisson,L.F. Stuck and sluggish fermetnations. *American Journal of Enology and Viticulture* **1999**, *50*, 107-119.

9. Boulton,R.B.; Singleton,V.L.; Bisson,L.F.; Kunkee,R.E. *Principles and practices of winemaking*, Chapman and Hall: New York, 1996.

10. Castela,P.M.; Mesías,J.L.; Maynar,J.I. Changes in contents of total, polar and neutral lipids in Macabeo grapes during development. *Sci.Aliments* **1985**, *5*, 587-597.

11. Charoenchai,C.; Fleet,G.H.; Henschke,P.A. Effects of temperature, pH, and sugar concnetration on the growth rates and cell biomass of wine yeasts. *American Journal of Enology and Viticulture* **1998**, *49*, 283-288.

12. Chen,E.C.H. Utilization of wort fatty acids by yeast during fermentation. *Ann.Soc.Brew.Chem.* **1980**, *38*, 148-153.

13. Dahl,C.; Biemann,H.P.; Dahl,J. A protein kinase antigenically related to pp60v-src possibly involved in yeast cell cycle control: positive in vivo regulation by sterol. *Proc.Natl.Acad.Sci.U.S.A* **1987**, *84*, 4012-4016.

14. Daum,G.; Lees,N.D.; Bard,M.; Dickson,R. Biochemistry, cell biology and molecular biology of lipids of Saccharomyces cerevisiae. *Yeast* **1998**, *14*, 1471-1510.

15. Delfini,C.; Costa,A. Effects of the grape must lees and insoluble materials on the alcoholic fermentation rate and the production of acetic acid, pyruvic acid, and acetaldehyde. *Am.J.Enol.Vitic.* **1993**, *44*, 86-92.

16. Ferreira,V.; Sharman,M.; Cacho,J.; Dennis,J. New and efficient microextraction / solid-phase extraction method for the gas chromatographic analysis of wine volatiles. *J.Chromatogr.A* **1996**, *731*, 247-259.

17. Feuillat,M.; Charpentier,C.; Massoutier,C. Intérêt oenologique des souches de levures Saccharomyces cryotolérantes. *Revue des Oenologues* **1997**, *85*, 18-21.

18. Fleet,G.H.; Heard,G.M. *Wine Microbiology and Biotechnology*, Harwood Academic Publishers: Switzerland, 1993.

19. Fujiwara,D.; Kobayashi,O.; Yoshimoto,H.; Harashima,S.; Tamai,Y. Molecular mechanism of the multiple regulation of the Saccharomyces cerevisiae ATF1 gene encoding alcohol acetyltransferase. *Yeast* **1999**, *15*, 1183-1197.

20. García Barceló,J. *Técnicas analíticas para vinos*, GAB: Barcelona, 1990.

21. Georgin,J.P. Analyse des données (ACP, AFC) avec Excel 2000. Théorie et pratique. *Presses Universitaires de Rennes, France* **2002**.

22. Guerzoni,M.E.; Ferruzzi,M.; Gardini,F.; Lanciotti,R. Combined effects of ethanol, high homogenization pressure, and temperature on cell fatty acid composition in Saccharomyces cerevisiae. *Can.J.Microbiol.* **1999**, *45*, 805-810.

23. Guilloux-Benatier,M.; Feuillat,M. Incidence de la clarification des moûts de raisin sur les fermentescibilités alcoolique et malolactique. *J.Int.Sci.Vigne Vin* **1993**, *27*, 299-311.

24. Houtman,A.C.; Marais,J.; Du Plessis,C.S. Factors affecting the reproducibility of fermentation of grape juice and of the aroma composition of wines. I. Grape maturity, sugar, inoculum concentration, aeration, juice turbidity and ergosterol. *Vitis* **1980**, *19*, 37-54.

25. Hunter,K.; Rose,A.H. Lipid composition of Saccharomyces cerevisiae as influenced by growth temperature. *Biochim.Biophys.Acta* **1972**, *260*, 639-653.

26. Jahnke,L.; Klein,H.P. Oxygen requirements for formation and activity of the squalene epoxidase in Saccharomyces cerevisiae. *J.Appl.Microbiol.* **1983**, *155*, 488-492.

27. Jiranek,V.; Langridge,P.; Henschke,P.A. Amino acid and ammonium utilization by Saccharomyces cerevisiae wine yeasts from a chemical defined medium. *American Journal of Enology and Viticulture* **1995**, *46*, 75-83.

28. Jollow,D.; Kellerman,G.M.; Linnane,A.W. The biogenesis of mitochondria III The lipid composition of aerobically and anaerobically grown Saccharomyces cerevisiae as relatedto the membrane systems of the cells. *J.Cell.Biol.* **1968**, *37*, 221-230.

29. Kajiwara,S.; Aritomi,T.; Suga,K.; Ohtaguchi,K.; Kobayashi,O. Overexpression of the OLE1 gene enhances ethanol fermentation by Saccharomyces cerevisiae. *Appl.Microbiol.Biotechnol.* **2000**, *53*, 568-574.

30. Lafon-Lafourcade,S.; Geneix,C.; Ribéreau-Gayon,P. Inhibition of alcoholic fermentation of grape must by fatty acids produced by yeast. Their elimination by yeast ghosts. *Appl.Environ.Microbiol.* **1984**, *47*, 1246-1249.

31. Lambrechts,M.G.; Pretorius,I.S. Yeast and its importance to wine aroma. *S.Afr.J.Enol.Vitic* **2000**, *21*, 97-129.

32. Larue,F.; Lafon-Lafourcade,S.; Ribéreau-Gayon,P. Relationship between the sterol content of yeast cells and their fermentation activity in grape must. *Appl.Environ.Microbiol.* **1980**, *39*, 808-811.

33. Le Fur,Y.; Hory,C.; Bard,MH.; Olsson,A. Evolution of phytosterols in Chardonnay grape berry skins during last stages of ripening. *Vitis* **1994**, *33*, 127-131.

34. Lees,N.D.; Bard,M.; Kemple,M.D.; Haak,R.A.; Kleinhans,F.W. ESR determination of membrane order parameter in yeast sterol mutants. *Biochim.Biophys.Acta* **1979**, *553*, 469-475.

35. Llauradó,J.M.; Rozes,N.; Bobet,R.; Mas,A.; Constantí,M. Low temperature alcoholic fermentation in high sugar concentration grape must. *J.Food Science* **2002**, *67*, 268-273.

36. Luparia,V.; Soubeyrand,V.; Berges,T.; Julien,A.; Salmon,J.M. Assimilation of grape phytosterols by Saccharomyces cerevisiae and their impact on enological fermentations. *Appl.Microbiol.Biotechnol.* **2004**, *65*, 25-32.

37. Malcorps,P.; Dufour,J.P. Short-chain and medium-chain aliphatic-ester synthesis in Saccharomyces cerevisiae. *Eur.J Biochem.* **1992**, *210*, 1015-1022.

38. Mauricio,J.C.; Guijo,S.; Ortega,J.M. Relationship between the phospholipid and sterol contents in *Saccharomyces cerevisiae* and *Torulaspora delbrueckii* and their fermentation activity in grape musts. *Am.J.Enol.Vitic.* **1993**, *42*, 301-308.

39. Moruno,E.G.; Delfini,C.; Pessione,E.; Giunta,C. Factors affecting acetic acid production by yeasts in strongly clarified grape musts. *Microbios* **1993**, *74*, 249-256.

40. Ness,F.; Achstetter,T.; Duport,C.; Karst,F.; Spagnoli,R.; Degryse,E. Sterol uptake in Saccharomyces cerevisiae heme auxotrophic mutants is affected by ergosterol and oleate but not by palmitoleate or by sterol esterification. *J.Bacteriol.* **1998**, *180*, 1913-1919.

41. Nordström,K. Formation of ethyl acetate in fermentation with brewer's yeast IV: metabolism of acetyl coenzyme A. *J.Inst.Brew.* **1963**, *69*, 142-153.

42. Novo,M.T.; Beltran,G.; Torija,M.J.; Poblet,M.; Rozes,N.; Guillamon,J.M.; Mas,A. Changes in wine yeast storage carbohydrate levels during preadaptation, rehydration and low temperature fermentations. *Int.J.Food Microbiol.* **2003**, *86*, 153-161.

43. Pretorius,I.S. Tailoring wine yeast for the new millennium: novel approaches to the ancient art of winemaking. *Yeast* **2000**, *16*, 675-729.

44. Quain,D.E. Studies on yeast physiology-impact on fermentation performances and product quality. *J.Inst.Brew.* **1988**, *95*, 315-323.

45. Rapp,A.; Versini,G. Influence of nitrogen compounds in grapes on aroma compounds of wines. In *Proceedings of the International Symposium on Nitrogen in Grapes and Wine*; Ranz,J.M., Ed.; Am. Soc. Enol. Vitic. Davis, CA, 1991.

46. Ratledge,C.; Evans,C.T. Lipids and their Metabolism. In *The Yeast*; Rose,A.H.; Harrison,J.S., Eds.; Academic Press: London, 1989.

47. Riou,C.; Nicaud,J.M.; Barre,P.; Gaillardin,C. Stationary-phase gene expression in Saccharomyces cerevisiae during wine fermentation. *Yeast* **1997**, *13*, 903-915.

48. Rodriguez,R.J.; Low,C.; Bottema,C.D.K.; Parks,L.W. Multiple functions for sterols in S.cerevisiae. *Biochim.Biophys.Acta* **1985**, *837*, 336-343.

49. Rosi,I.; Bertuccioli,M. Influences of lipid addition on fatty acid composition of Saccharomyces cerevisiae and aroma characteristics of experimental wines. *J.Inst.Brew.* **1992**, *98*, 305-314.

50. Rossignol,T.; Dulau,L.; Julien,A.; Blondin,B. Genome-wide monitoring of wine yeast gene expression during alcoholic fermentation. *Yeast* **2003**, *20*, 1369-1385.

51. Rozès,N.; García-Jares,C.; Larue,F.; Lonvaud-Funel,A. Differentiation between fermenting and spoilage yeasts in wine by total free fatty acid analysis. *J.Sci.Food Agric.* **1992**, *59*, 351-357.

52. Sablayrolles,J.M.; Dubois,C.; Manginot,C.; Roustan,J.L.; Barre,P. Efectiveness of combined ammoniacal nitrogen and oxygen additions for completion of sluggish and stuck fermentations. *J.Ferment.Bioeng.* **1996**, *82*, 337-381.

53. Taylor,G.T.; Kirsop,B. The origin of the medium chain length fatty acids present in beer. *J.Inst.Brew.* **1977**, *83*, 241-243.

54. Thurston,P.; Quain,D.E.; Tubb,R.S. Lipid metabolism and the regulation of volatile synthesis in *Saccharomyces cerevisiae*. *J.Inst.Brew.* **1982**, *88*, 90-94.

55. Thurston,P.A.; Taylor,R.; Avhenainen,J. Effects of linoleic acid supplements on the synthesis by yeast of lipids and acetate esters. *J.Inst.Brew.* **1981**, *87*, 92-95.

56. Torija,M.J.; Beltran,G.; Novo,M.; Poblet,M.; Guillamon,J.M.; Mas,A.; Rozes,N. Effects of fermentation temperature and Saccharomyces species on the cell fatty acid composition and presence of volatile compounds in wine. *Int.J.Food Microbiol.* **2003**, *85*, 127-136.

57. Torija,M.J.; Rozes,N.; Poblet,M.; Guillamon,J.M.; Mas,A. Effects of fermentation temperature on the strain population of Saccharomyces cerevisiae. *Int.J Food Microbiol.* **2003**, *80*, 47-53.

58. Valero,E.; Millan,C.; Ortega,J.M. Influence of Pre-Fermentative Treatment on the Fatty Acid Content of *Saccharomyces cerevisiae J.Biosci.Bioeng.* **2001**, *91*, 117-122.

59. Watson,K. Temperature relations. In *The yeast. Yeast and Environment*; Rose,A.H.; Harrison,J.S., Eds.; Academic Press: London, 1987.

CHAPTER 2

Integration of transcriptomic and metabolomic analyses for understanding the global responses of low temperature winemaking fermentations.

Gemma Beltran[1,2], Maite Novo[1,2], Véronique Leberre[2,3], Sergei Sokol[3], Delphine Labourdette[3], José-Manuel Guillamón[1], Alberto Mas[1], Jean François*[2,3] and Nicolas Rozès[1]

[1]Departament de Bioquímica i Biotecnologia, Facultat d'Enologia de Tarragona, Universitat Rovira i Virgili, C/ Marcel·lí Comingo s/n, 43007 Tarragona, Spain
[2]Centre de Bioingénierie Gilbert Durand, UMR-CNRS 5504, UMR-INRA 792
[3]Biochips platform of Genopole Toulouse- Midi-Pyrénées, 135 Avenue de Rangueil, 31077 Toulouse, France.

Supplementary data in Annex 2 and http://biopuce.insa-tlse.fr/jmflab/winegenomic/

(*Submitted for publication*)

ABSTRACT

Conducting wine fermentation at 13°C instead of 25°C has several industrial applications such as the improvement of wine tasting quality. To investigate the effect of temperature on the industrial winemaking, the expression programs at three equivalent phases during the fermentation process at 13ºC and 25°C were compared and tentatively correlated with biochemical data including cell's viability, fermentation by-products and lipid content of the cells. From 535 open reading frames that were significantly differentially expressed between 13ºC and 25°C-fermentation, two important transcription programs were identified. A cold-stress response was activated in the initial phase of fermentation at 13°C, followed by up-regulation of genes belonging to cell fate, growth control, and maintenance in the middle and late phases of the fermentation at 13ºC with respect to 25ºC. These expression changes correlated with a higher cell's viability at low temperature. Another relevant difference was that at 13ºC there was a down-regulation of genes involved in the cytosolic fatty acid synthesis, which was consistent with a change in the membrane fluidity and a greater resistance to ethanol at low temperature. Conversely, genes involved in the mitochondrial short-chain fatty acids synthesis were up-regulated together with genes involved in the formation of the F1 unit of the F0F1-ATP synthase. Interestingly, these transcriptional changes correlated with a higher production of short-chain (C4-C8) fatty acids and their corresponding esters at 13°C than at 25°C. While increased of fatty acids ethyl esters may account in part for the higher tasting quality of the wines fermented at 13°C, it is still unclear how the esterification of fatty acids takes place. But, based on its strong up-regulation at 13°C, we propose the possible role in this process of *IAH1* encoding an esterase/ester synthase activity.

Keywords: DNA chips, transcript profiling, wine fermentation, flavours, yeast

INTRODUCTION

The wine yeast strains were selected for their ability to efficiently ferment grape-must sugars under a rather stressful enological environment. These conditions, which are substantially different from the laboratory conditions are, among others, a high sugar content (160 - 250 g/l), a low pH (< 3.5), a high alcohol production (> 10 % v/v) and a strong oxygen-limitation (hypoxia). Moreover, the grape musts are often unbalanced, with nitrogen sources, lipids and vitamins as limiting components (4, 5, 53). All these conditions lead to gene expression changes and structural modifications of yeast cells to adapt under these extreme conditions (3, 11, 54, 58).

Another important parameter that can dramatically alter the winemaking process is temperature. While the optimal process of wine fermentation by *Saccharomyces cerevisiae* takes place at around 25ºC, there is increasing interest to conduct this process at lower temperatures. In fact, most white wine fermentations are carried out at 18ºC. Lowering the fermentation temperature of the process to 13°C or even below has several disadvantages, including an increase in the duration of the process and a greater risk of stuck and sluggish fermentation (8). Moreover, a low temperature induces cold-responsive genes (36-38, 52, 57, 61, 63), whose role in the winemaking process has not yet been addressed. On the other hand, a low temperature of fermentation results in tasting advantages (12, 24, 72), such as a restructuring of the flavor profiles with very promising industrial applications (23). The aromatic complexity of a wine depends on primary flavors (those originating from the grapes), secondary or fermentative flavors (those that are produced by yeasts and bacteria during alcoholic and malolactic fermentation) and tertiary or post-fermentative flavors (those appearing during the ageing process) (9, 55, 65). Low temperatures of 10 - 15ºC mainly affects the retention of primary and secondary flavors. The better sensorial quality and taste of wines produced at low temperatures can therefore be attributed to a major retention of terpenoids, an increase in the production of volatile esters and C_6-C_{10} medium-chain fatty acid esters and a reduction of higher alcohols and volatile acidity (40, 44, 72).

To determine the molecular mechanisms behind the impact of low temperature on wine flavors, we used the powerful DNA microarrays technology to examine the global gene response of yeast during the wine making process at 25ºC and 13°C and compared the transcriptional profiles at these two fermentation temperatures. Our yeast species used in this work was a pure wine strain that was originally isolated in a Portuguese vineyard, but this should not pose any problem for hybridization analysis on yeast

microarrays bearing 6000 ORFs from the laboratory-sequenced strain S288c, because it was shown that the sequence homology between the wine and laboratory strains was over 98%, and that the major differences were in the intergenic regions (32). Moreover, to make any biological interpretation of transcriptomic data easier, we measured metabolic parameters such as cell's viability, fermentation by-products and flavors, and cellular lipid composition during the wine process at both temperatures. The two data sets (transcriptomics and metabolomics) obtained at two levels of the cellular function were quite helpful as we were able to identify many correlations between expression changes and physiological modifications between 13ºC and 25°C. This global and integrated functional analysis is the first study carried out under rigorous industrial fermentations, since previous studies have been carried out under simulated enological conditions (3, 46, 58).

MATERIALS AND METHODS

Strain and Fermentation conditions

The *Saccharomyces cerevisiae* wine strain QA23 (commercialized by Lallemand S.A., Canada) was cultured in 100 L steel vat containing a Muscat grape must (this medium was supplied by Mas dels Frares, Tarragona, Spain and contained about 160 - 200 g.l^{-1} sugar) under a controlled temperature set at 25ºC or at 13ºC. Before starting the fermentation, the must medium was clarified by natural settling to separate the clear juice from the sediments and sulfur dioxide (50 mg l^{-1}) was added to the culture media. The fermentation was started with 2 x 10^6 cell ml^{-1} of dry yeast cells rehydrated in water at 37ºC for 30 min prior to inoculation according to the manufacturer's instructions. Yeast growth was monitored by counting the viable cells after plating them on YEPD agar medium (Yeast Extract, 1 % [w/v]; bactoPeptone 2 % [w/v]; glucose 2 % [w/v]; Difco agar2 % [w/v]) at an adequate dilution for 2 days at 28ºC. The values were expressed in colonies forming unit. ml^{-1} (CFU. ml^{-1}). The consumption of sugars during fermentation was monitored by change of the medium density, which is correlated to levels of reducing sugar content as described in Ribéreau-Gayon *et al*. (56). Fermentation was considered to be finished when the reducing sugars measured by enzymatic assay using an enzymatic kit from Roche Applied Science (Germany) were below 2 g l^{-1}. The absence of any other wild yeast in the wine fermentation was verified by RFLPs methods as described in (7).

Preparation of mRNA and microarray analysis

Cell samples, corresponding to ~10^8 UFC from the fermentation tank were harvested in triplicate by centrifugation (5000 x 5 min at 4°C) at three periods during the process, namely initial fermentation (IF), mid-fermentation (MF) and final fermentation (FF). The cell pellet was then immediately frozen in liquid nitrogen, and stored at - 80°C until use. Total RNA was extracted using a commercial kit (RNAeasy mini kit, Qiagen). The quantity and control-quality of the extracted RNA were checked by micro capillarity electrophoresis using a Bioanalyzer 2100 (Agilent).

The DNA chips were manufactured at the Biochip platform, Toulouse - Genopole on dendrislides (42) using 70 mers oligonucleotides representing ~99 % of the yeast genome purchased from Operon Inc (list of corresponding genes is described at http://biopuce.insa-tlse.fr/yeastdendrichips). Fluorescent-labeled cDNA was synthesized with 25 µg of total RNA using the CyScribe™ first strand cDNA labeling kit (Amersham Bioscience). Labeled cDNA was purified using CyScribe™ GFX™ Purification kit (Amersham Pharmacia). Hybridization was carried out in an automatic hybridation chamber (Discovery™, Ventana). Microarrays were prehybridized in a solution of 1 % BSA, 2 x SCC, 0.2 % SDS for 30 min at 42ºC and a mixture containing 200 µl of RiboHybe™ (Ventana), 10 µl of Cy3-labelled cDNA and 10 µl of Cy5-labelled cDNA was added. After 14 hr of hybridization at 42ºC, the DNA chips were washed for 5 min in 2 x SSC, 0,1 % SDS at room temperature, and four times in 0.1 x SSC buffer for 2 min each at room temperature. The hybridization signal was detected by scanning using GenePix 4000B laser Scanner (Axon Instruments), and the signal quantification was transformed to numerical values using the integrated GenePix software version 3.01. Experiments (from RNA extraction to image analysis) were repeated by swapping the fluorescent dye CY3 and CY5 to reduce the false positive/negative values due to dye effects.

Data acquisition and data treatments

All raw data are presented at http://biopuce.insa-tlse.fr/jmflab/winegenomic/, which provides full details of normalization and statistical regimes using our home-made Bioplot software. This software is an online web service available to all users of Biochips platform. A complete user's guide is available at http://biopuce.insa-toulouse.fr/ExperimentExplorer/doc/BioPlot/. Raw intensities were corrected from the

background, log transformed and normalized by the mean log-intensity of all spots. Log-ratios of normalized intensities from duplicate samples were tested for statistical significance using Student's paired bi-tailed *t*-test. To reduce false discovery rate, we tested genes with at least a 2-fold variation, and the p-value threshold in the Student's *t*-test was set at ≤ 0.05. To determine the degree at which the transcription of a particular gene was regulated under a given condition (MF and FF), the normalized value from that condition was divided by the corresponding value from the other condition (IF), and converted to $\log_{10}$. Positive values and negative values define up and down-regulated genes under the conditions studied. The regulated genes were placed into the 16 functional classifications as defined by the Munich Information Center for Protein Sequences Yeast Genome database (http://mips.gsf.de/proj/yeast/). Genespring version 4.2 software (Silicon genetics Inc, USA) was used for visualization and hierarchical clustering. The SGD (www.stanford.edu/Saccharomyces) and FunSpec (http://funspec.med.utoronto.ca/) databases were consulted to detect nodes that were enriched in a particular cellular function. Other details related to data processing as well as a query-based website for viewing specific gene fold change data are available online, and hereafter referred to as Supplemental data S1 (http://biopuce.insa-tlse.fr/jmflab/winegenomic).

Determination of fermentation by-products, alcohols and esters

Wine samples (100 ml) were taken throughout the fermentation process to measure acetic acid and ethanol according to the method described in (29). Other by-products, including fusel alcohols, short-chain and medium chain aliphatic esters, terpens and free short and medium aliphatic fatty acids were extracted by liquid/liquid extraction with 200 µl of 1,1,2- Trichlorotrifluoroethane (Fluka) and 0.5 g NaCl using n-decanol (0.2 mg l^{-1}) as standard internal according to the method of Ferreira *et al.* (22). After 2 min agitation, and 2 min centrifugation at 3000 rpm, the organic phase was extracted and 2 µl of this phase was injected in a gas chromatograph HP 6890N (Agilent Technologies, Wilmington, DE, USA) using the automatic injector HP 7683 (Agilent), and mounted with a column TR-WAX (60 m x 0.25 mm x 0.25 µm) equipped with a FID detector. Aromatic volatile compounds were identified and quantified by comparison with standards. Values are the average of two determinations, and except for decanoic acid (18 %), dodecanoic acid (38 %), ethyl octanoate (16 %) and ethyl

decanoate (29 %), the coefficient of variation in all the compounds analyzed was less than 10 %.

Determination of cell fatty acid composition

Yeast cells (5 - 10 mg of dry mass or about 10^8 CFU) were collected at the same stages of fermentation as for microarrays analysis. Fatty acids were extracted from yeast cells and analyzed according to Rozès *et al.* (59). Analytical gas chromatography was performed on a Hewlett-Packard 5890 connected to a HP Vectra computer with the ChemStation software (Agilent Technologies). A 2 µl cellular extract was injected (splitless, 1 min) into an FFAP-HP column (30 m x 0.25 mm x 0.25 µm from Agilent technologies) with an HP 7673 automatic injector (Agilent). The initial temperature was set at 140ºC and increased by 4ºC /min^{-1} to reach 240ºC. Injector and detector temperatures were 250ºC and 280ºC, respectively. The carrier gas was helium at 1.2 ml.min^{-1}. Heptanoic and heptadecanoic acids (1 and 4 mg. ml^{-1}, respectively) were added as internal standards. Relative amounts of fatty acids were calculated from their respective chromatographic peak areas. These values were related to the dry mass of cells and expressed as % of total fatty acids extracted. The mean fatty acids chain length (CL) was calculated as: CL = (P x C) / 100, where P is the percentage of fatty acids and C is the number of carbons.

Determination of sterol composition of the yeast cells

Yeast cells (5-10 mg of dry mass) were collected at the same stages of fermentation as for microarrays analysis, and were analyzed for cell sterol composition as follows. Cells were saponified by incubation at 90°C for 30 min in a methanol solution containing 15 % (w/v) KOH and 5-t -cholestane (1 mg. ml^{-1}) used as an external standard. Sterols were extracted with 300 µl of hexane. Analytical gas chromatography was performed as described above using a SAC-5 column (15 m x 0.25 mm x 0.25 µm from Supelco, Bellefonte, PA, USA). The relative amount of a given sterol component was calculated from its respective chromatographic peak areas. From the same extracts, GC/MS of sterols was conducted on an HP5890 series II coupled to an HP5972 mass selective detector. Electron impact GC/MS (70eV, scanning from 42 to 600 atomic mass units at 1-sec intervals) was performed under the following conditions: SAC-5 column (30 m x 0.25 mm and 0.25 µm film thickness), He as carrier

gas (30 cm sec^{-1}), a detector temperature of 250 ºC, a column temperature of 125-250 ºC (125 ºC for 2 min, 20 ºC min^{-1} to 250 ºC for 60 min), and a injector temperature of 250ºC. All injections were run in a splitless mode.

Lipid extraction and separation of the yeast cells

Prior to lipid extraction, a solution of 100 µl of cold methanol + 10 µl EDTA 0.1 mM were added to yeast cells (5-10 mg dry mass) with 1 g glass beads (0.5 mm, Biospec Products, USA)) in 2.0 ml conical screw cap microtubes (Porex Bio Products, USA), then mixed for 5 min in a minibeadbeater-8 (Biospec Products). Lipid extraction was performed with chloroform: methanol (2 : 1, v/v, two times and 1 : 1 v/v, one time). The inferior organic phase was transferred to a 15 ml glass screw tube in presence of KCl 0.88% (a quarter of the total volume of the extract). After vortexing and being cooled on ice during 15 min, the samples were centrifuged for 10 min at 3000 *rpm*. The organic phase was collected and dried through a Na_2SO_4 column, and then concentrated to dryness with nitrogen. The extract was dissolved in chloroform: methanol (2: 1, v/v).

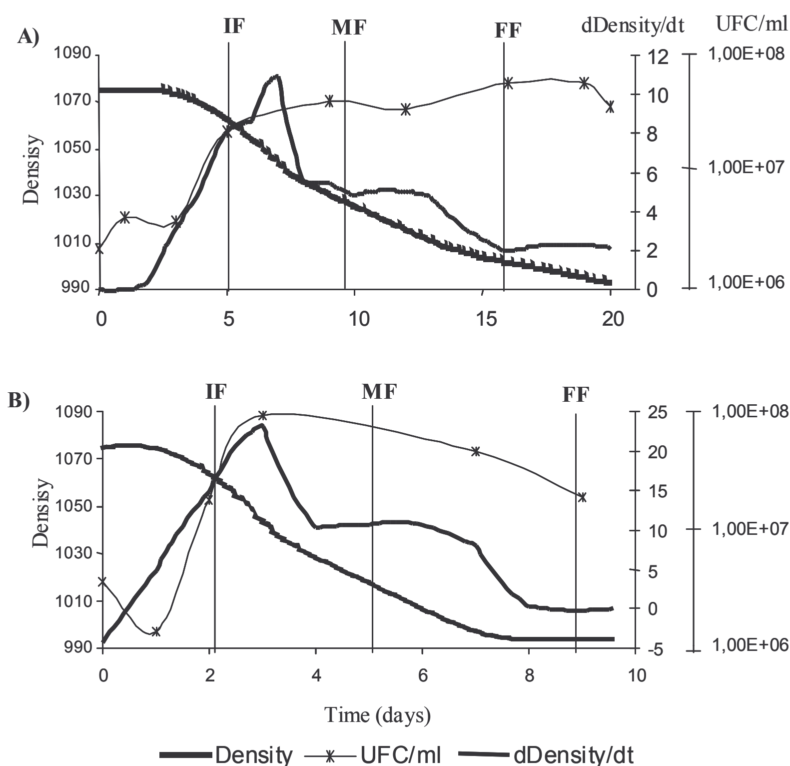
Individual lipid components were separated by thin-layer chromatography (TLC) on silica gel $60F_{254}$ plates (10 x 20 cm, 250 µm, Merck, Germany) with solvent systems as follows: sterol, sterol ester (SE), diacylglycerol (DAG), triacylglycerol (TAG), fatty acid ethyl ester (FAEE) and squalene in hexane: *tert*-butylmethyl ether (MTBE): acetic acid glacial (70: 30: 2, v/v); phosphatidylinositol (PI), phosphatidylserine (PS), phosphatidylcholine (PC), phosphatidylglycerol (PG), phosphatidylethanolamine (PE), cardiolipin (CL) and phosphatidic acid (PA) in chloroform: acetone: methanol: acetic acid glacial: water (50: 15: 10: 10: 5, v/v). The standard lipids lanosterol, ergosterol, squalene, cholesterol oleate (SE), ethyl oleate (EEAG), diolein (DAG), triolein (TAG), PI, PS, PC, PG, PE, CL and PA were obtained from Sigma. Calibration was performed using standard solutions (0.5 - 4 µg/µl). Lipids on TLC plates were detected with 10 % $CuSO_4$ in 8 % H_3PO_4 and heated at 120 ºC for 20 min. The TLC plate showing brown spots was filmed with a Kodak DC290 Zoom digital camera. For lipid quantification, each spot of the image of the TLC plate was processed with the Quantity One software (Biorad, USA).

RESULTS

Macrokinetic analyses of wine fermentation at 25 and 13 °C

Wine fermentation was carried out at 25 and 13 °C in 100-L tanks with fresh rehydrated industrial yeast cells that were inoculated at 2 x 10^6 $CFU.ml^{-1}$ in grape must containing around 200 $g.l^{-1}$ sugars (~ 50 % glucose and ~ 50 % fructose) (Fig.1).

Figure 1: Fermentation kinetic of *S. cerevisiae* strain QA23 on grape musts at 13ºC (A) and 25°C (B). The sampling for RNA extraction and analysis of cell lipid composition is refereed by dotted lines and termed 'IF' (initial fermentation), MF (middle fermentation) and 'FF' (end fermentation).



In the two temperatures fermentation, the duration of the exponential phase was relatively short and was followed by a lengthy non-proliferating phase which lasted for 6 days at 25°C and 14 days at 13°C, during which > 70 % of the sugars initially present in the must were consumed. The fermentation was considered to be finished when the grape must density dropped to below 990 units, *i.e.* when the residual reducing sugars in the medium were ≤ 2 $g.l^{-1}$. At this point, the final ethanol titer reached about 11 % (v/v) in both fermentations. Therefore, the profiles of wine fermentation at 13ºC and 25°C were roughly similar, although as expected, the rate of fermentation was about 2-fold lower at 13ºC than at 25 °C. Interestingly, however, the Colonies Forming Units (CFU ml^{-1}), which estimates the number of viable cells, reached a maximum after 6 and 3

days at 13ºC and 25 °C respectively, remained at this elevated value at 13°C, but decreased gradually at 25°C during the non-proliferating period of fermentation.

The temperature of the fermentation is known to affect the flavor profiles of the wine (12, 24, 72). We therefore measured fermentation by-products and compounds that largely contribute to the sensorial aspect of wines in the broth medium at the end of the process, *i.e.* at day 9 for the fermentation at 25°C and day 20 for that at 13°C. Table 1 shows the contents of floral and fruity aromas (isoamyl acetate and fatty acid esters) obtained from the industrial 13ºC and 25°C -fermentation in which global expression analysis has been carried out. Note that these profiles were qualitatively reproduced in other independent industrial fermentations over years (unpublished data). We can see that isoamyl acetate, hexyl acetate and fatty acid ethyl esters were significantly higher (*i.e*. about 30 - 50 %) at 13ºC than at 25 °C, whereas volatile acidity and fusel alcohols were lower by about 25 %. Also, lowering the wine fermentation temperature increased the production of short-chain fatty acid ethyl esters by 84 %. Taken together, these results show that the fermentation temperature makes an important contribution to the final aroma composition of the wine.

Transcription profiles at 13ºC and 25 °C and comparison of the expression profiles at these temperatures

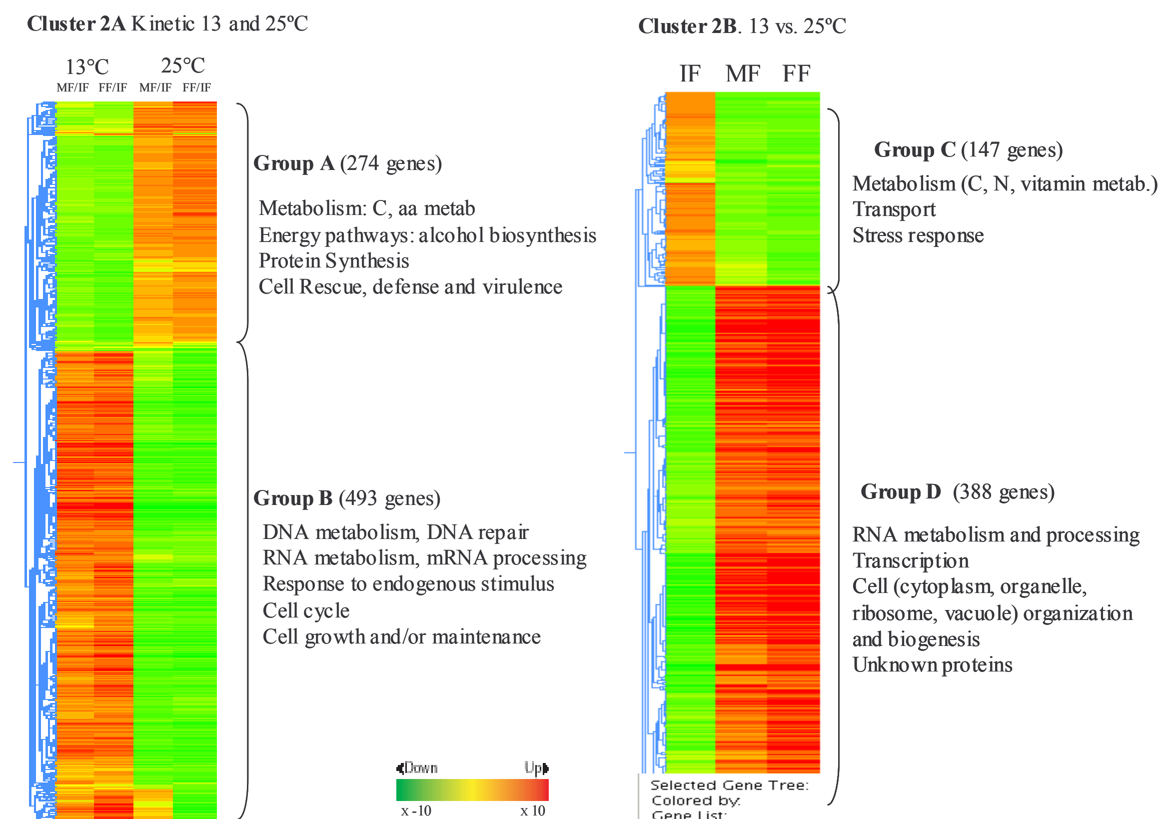
Changes in the global expression of genes during wine fermentation at 13ºC and 25 ºC were analyzed by microarrays containing 70 mers oligonucleotides corresponding to 5915 ORFs from the best annotated genome of the *S. cerevisiae* S288c strain (67). To examine gene expression changes during the wine fermentation at these temperatures, we chose the ‘growing phase’, which we called initial phase (IF) as the reference. With respect to this reference, two other fermentation stages, middle and late stages, were analyzed for global expression changes. One was the middle stage of fermentation (MF), which corresponded to a period in which the cells were at the beginning of the non-proliferating phase, only 50 % of the sugar had been consumed and the content of ethanol was still low. The other was the final stage of fermentation (FF), which corresponded to the arrest of the wine fermentation, and medium contained residual sugar (below 10 $g.l^{-1}$) and a high ethanol concentration (above 10 % (v/v)) in the medium.

Table 1. Effects of temperature fermentation on ethanol content, volatile acidity and aromatic volatile compounds of wines.

Flavors (mg.l^{-1})	13ºC	25ºC	Aroma descriptors
Hexanol	0.40	0.41	Vegetal
Isoamyl alcohols	140	182	Marzipan
Phenethyl alcohol	21.2	35.2	Floral, Rose
∑ Fusel Alcohols	161.6	217.6	
Isobutyl acetate	0.025	0.029	Banana, Pear
Isoamyl acetate	1.5	1.13	Banana
Hexyl acetate	0.13	0.070	Apple
2-Phenethyl acetate	0.14	0.239	Rose
∑ Acetates	1.8	1.5	
Linalool	0.22	0.21	Lemon
α - Terpineol	0.37	0.35	Floral, Lilac
Citronellol	0.022	0.024	Lemon
Nerol	0.011	0.010	Floral
ß - Ionone	0.002	0.001	Violet
∑ Terpens	0.625	0.60	
Ethyl butanoate	0.38	0.21	Floral
Ethyl hexanoate	1.30	0.93	Apple
Ethyl octanoate	1.24	0.96	Pineapple
Ethyl decanoate	0.31	0.36	Floral
∑ Fatty Acid Ethyl Esters	3.23	2.46	
Butyric acid	0.90	0.56	Pungent
Hexanoic acid	2.47	1.21	Sweaty
Octanoic acid	4.56	2.51	Soapy
Decanoic acid	1.27	0.93	Fatty
Dodecanoic acid	0.12	0.05	fatty
∑ Fatty acids	9.32	5.26	
Ethanol (% v/v)	10.9	10.7	
Volatile acidity (g acetic acid l^{-1})	0.36	0.45	Vinegar

The kinetics of the transcription profiles during fermentation at 25ºC and 13°C were therefore made by hybridizing labeled mRNA from stages MF and FF relative to IF. The experiments were performed twice by swapping the fluorescent CY3 and CY5 dyes using two different RNA samples obtained from the same fermentation tank, because it was difficult for technical reasons to perform two independent experiments under this industrial conditions. A total of ~ 5200 ORFs (88 % of the whole genome) were qualitatively detected after filtering procedure (see Material & Methods), from which 1561 genes at 25°C and 1226 at 13°C showed a 2-fold difference for at least one stage of the fermentation. Based on our data quality tests (*i.e.* > 2-fold variation and statistical significance with p value set at <0.05 from the Student's *t*-test), 519 and 379 genes were retained for the fermentations at 25ºC and 13°C, respectively (see supplementary on-line data, Tables S2 & S3).

Figure 2: Global view of differentially expressed genes during fermentation at 13 and 25 °C (A) and between 13 versus 25°C (B) at different time period of the fermentation by hierarchical clustering analysis. Significantly up- and down-regulated genes were analysed by clustering method as described in Material and Methods. This analysis identified the main clusters enriched in genes in specific MIPS, GO functional categories listed on the right (obtained from Funspec analysis, see Material and Methods). The colour scale at the bottom represents the expression ratio x-fold repressed in green and x-fold activated in red with a maximum level of 10 fold.



To obtain an overview of the main differences between the kinetics of wine fermentation at 25ºC and those at 13°C, we clustered the differentially expressed genes into two large groups that were enriched by up-regulated and down-regulated genes (Fig. 2A). Interestingly, genes that showed coordinated up-regulation during fermentation at 25°C were those that were down regulated during the process at 13°C. The main functional categories of this cluster (group A) were genes belonging to the biological processes of carbon and amino acid metabolism, energy, protein synthesis, and cell rescue and defense. Conversely, genes that were down-regulated during fermentation at 25° C were those that were up-regulated during the process at 13°C. The main categories of this group (group B) were genes that encode proteins involved in growth-associated functions (cell growth and maintenance, cell cycle, nucleic acid (DNA and RNA) metabolism, etc.; see also supplementary on-line data in tables S2 & S3). This result is consistent with the fact that the viability of yeast cells remained at a high level during the non-proliferating phase at 13°C, but decreased during this phase at 25°C (Fig. 1).

In a second experiment we compared the gene expressions at each phase of fermentation at 13ºC with the gene expressions at the corresponding phases of fermentation at 25ºC. We decided to perform this direct comparison because the macrokinetics data (Fig. 1) indicated that the three phases were roughly equivalent in terms of global progress of the fermentation at the two temperatures. Labeled transcripts from IF of the fermentation at 13°C were hybridized against the labeled transcripts from IF of the fermentation at 25°C, and so on for MF and FF. From a total of 2275 genes that exhibited a 2-fold change in signal intensity between 13ºC and 25°C, only 535 genes (20 %) were retained based on a second filtering test (Student's *t*-test with p value < 0.05). Following this criterion, differentially expressed genes were organized by hierarchical clustering (18) to yield two main clusters (Fig.2B). One group (group D with 388 genes) contained genes belonging to categories of the RNA metabolism and processing, cell organization and biogenesis and transcription. These transcripts levels were lower during the exponential phase at 13ºC than at 25°C but then became higher in the middle and final phases of the fermentation at the low temperature. The other group (group C) contained a cluster of 147 genes exhibited expression profiles exactly opposite to that of group D. The main functional categories identified in this cluster were genes associated with glucose and amino acids transport, metabolism of vitamin,

carbohydrate and energy reserves and stress response (see supplementary on-line Table S4).

The datasets obtained from the kinetics at 13ºC and 25°C (4 datasets) and from the comparison between fermentation stages at 13ºC and 25°C (3 datasets) were put into an algorithm to estimate the correlation between the expression profiles from these 7 datasets (Table 2). A positive correlation between datasets should indicate similarity in the expression profiles. As Table 2 shows, a correlation close to +1 revealed three subgroups. The first subgroup represented the expression profiles of differentially expressed genes at the middle phase (13MF) and late phase (13FF) during fermentation at 13°C. The second subgroup contained the datasets from 25MF, 25FF and IF (expression changes in the middle and late phases of the fermentation at 25ºC and differential genes expression in the initial phase between 13ºC and 25°C). A third group corresponded to the expression profiles of genes whose expression in the middle and final phases of the fermentation (MF, FF) at 13ºC was different to those in the same phases of the fermentation at 25°C. We concluded from this analysis that the profiles of gene expression at the initial phase of fermentation at 13°C closely resembles that of the middle and late phase of wine fermentation at 25°C. Conversely, the profiles of gene expression at the middle and late phases of fermentation at 13°C resemble those of IF in fermentation at 25°C.

Table 2: Correlation matrix of variables

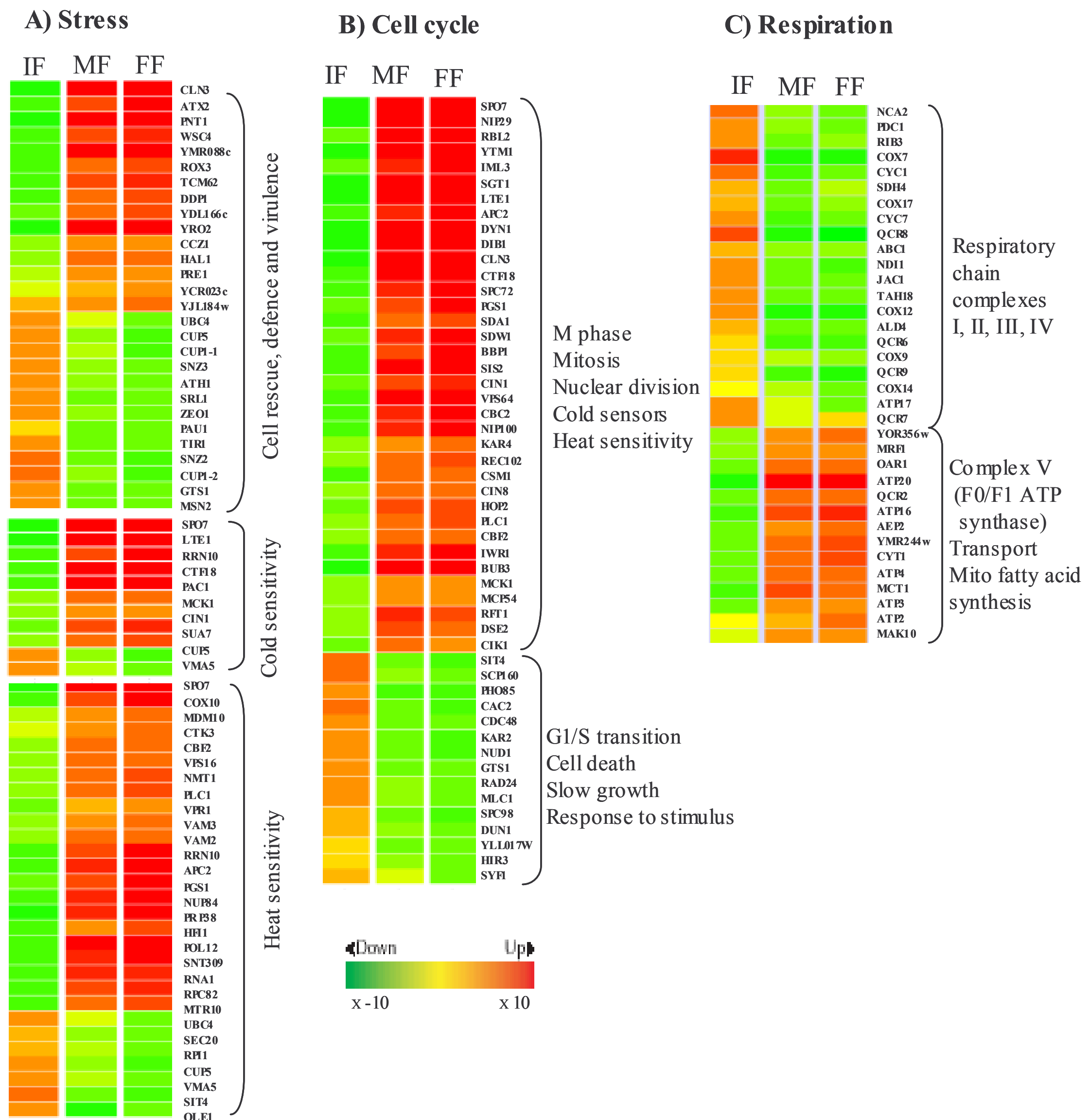
	13MF	13FF	25MF	25FF	IF	MF	FF
13MF	1						
13FF	**+0,930**	1					
25MF	-0,542	-0,532	1				
25FF	-0,474	-0,468	**+0,830**	1			
IF	-0,489	-0,495	**+0,749**	+0,748	1		
MF	+0,632	+0,693	-0,532	-0,531	-0,620	1	
FF	+0,567	+0,649	-0,436	-0,443	-0,512	**+0,917**	1

Cold-stress response, followed by activation of genes involved in cell rescue, viability and cell differentiation characterized 13° C- with respect to 25° C- fermentation

About 10 % of genes in the category of "cell rescue, defense and virulence" were differentially expressed at one of the stages during fermentation at 13ºC with respect to 25°C. Of these, a group of genes including *TIR1*, *PAU1*, *ZEO1* and *SRL1,* showed higher transcript levels at the initial phase of fermentation at 13ºC than at 25ºC, and then showed lower transcript levels at the middle and late phases of fermentation at 13°C than at 25°C. Since the expressions of these genes were also induced during hypoxia and cold shock (39), it is expected that their expression changes may influence cell wall porosity and may be required for the change in membrane fluidity (see below). We also found that the expression of *MSN2,* which encodes the master transcription factor of the general stress response (21, 47), was higher in the initial stage of fermentation at 13°C than at 25°C. This higher expression was accompanied by activation of a set of STRE-responsive genes in this stage of fermentation at 13°C. These results are consistent with the recent study by Schade *et al*. (63), who found that some of the late cold responsive genes are those that belong to the *MSN2/4* dependent stress response. Conversely, transcript levels of a series of genes encoding proteins involved in drug resistance, metal detoxification (*PNT1*, *ATX2, YMR088c*, *DDP1, CCZ1, YCR023c, HAL1*), cell wall stress sensor (*WSC4*) and two genes encoding proteins with chaperone properties (*YRO2* and *TCM62*) reported to be induced very late in response to a cold stress (63), were more abundant in the middle and final phases of the fermentation at 13°C than at the same corresponding phases of fermentation at 25°C. A small cluster called 'cold sensors genes' and comprising 10 differentially expressed genes between 13 and 25°C was also identified. However, apart from *LTE1*, which encodes a GDP/GTP exchange factor required for growth at low temperatures (78) and *MCK1*, which encodes a Ser/ Thr kinase that is involved in cold stress tolerance (34), the other 8 genes were classified as 'cold sensors' because their loss-of-function leads either to very slow growth or unviable cells at 4°C. Some of these genes, namely *SPO7, RRN10*, *PAC1* and *SUA7*, are involved in DNA synthesis and chromosome segregation. The third subgroup of the cell rescue and defense category included a set of genes reported to be 'heat sensitive'. A quick overview of this subgroup indicates that most of those whose expression was higher during late fermentation at 13ºC than at 25°C encode gene products that are associated with cell growth and whose deficiency causes growth defects at temperature above 37°C. Note

that the MIPS classification attributed *OLE1* in the heat sensitive class although it is also known to be induced immediately in response to low temperature (50, 61, 63), as is also seen in the initial growth phase at 13°C (Fig 3).

Figure 3. Expression profiles of differentially expressed genes (2-fold change and p-value < 0.05) in cell rescue, defence and virulence, cell cycle and respiration categories (MIPS function classification) during fermentation at 13 versus 25 °C. Genes with cold and heat sensitivity (MIPS phenotype classification) were also clustered. On the right is shown the nodes of differentially expressed genes classified in cell cycle and respiration (obtained from Funspec analysis, see Material and Methods). Red and green denotes transcripts that are more or less abundant in the low temperature of 13°C versus 25°C.



An interesting cluster containing 51 genes involved in the cell cycle was extracted. The expressions of these genes at the different phases of fermentation at 13ºC were different from those at 25°C (Fig. 3B). This cluster included genes involved in G1 to S transition (*CLN3, BBP1, BUB3*), in chromosomes segregation (*APC2, CTF18,*

CIN1 and *CSM1*), the connection of cellular polarity / spindle position with cell cycle progression (*LTE1, DYN1, DIB1, IML3, CTF18, CIN1, CIN8* and *BUB3*) whose transcript levels became more abundant in the middle and late phase of the fermentation at 13ºC than at 25°C (Fig. 3B). It is interesting to notice the co-regulation of several of these genes is consistent with their genetic interaction as identified by synthetic genetic array (SGA) analysis (71). Another set of genes that showed a similar expression pattern were those that encoded products involved in mating / sporulation (*SPO7, FUS1, KAR4, OPY2, SST2, SPS4*) and in filamentous / invasive growth (*DFG16, DIG1, DFG10*). However, the expression change in these genes was not accompanied by any discernible difference in the morphology of yeast cultures between the two temperatures. Conversely, the most important down-regulated genes of 'the cell cycle cluster' were *PHO85, SIT4* and *GTS1*, which encode, respectively, a cyclin-dependent protein kinase (69), a catalytic subunit of protein phosphatase type 2A (66) and a putative transcription factor involved in the timing of bud emergence and regulation of biological rhythms (49, 79). Taken together, the differential transcription changes of genes related to cell-cycle and growth control seem, at 13ºC, to induce a physiological situation that is comparable to a arrest of growth at G0-G1 that allows a higher protection to stress and keeps cells more viable (5, 30). Consistent with this idea, we recall that the viability of the yeast cells during prolonged cultivation is apparently higher at 13°C than at 25°C (see Fig.1).

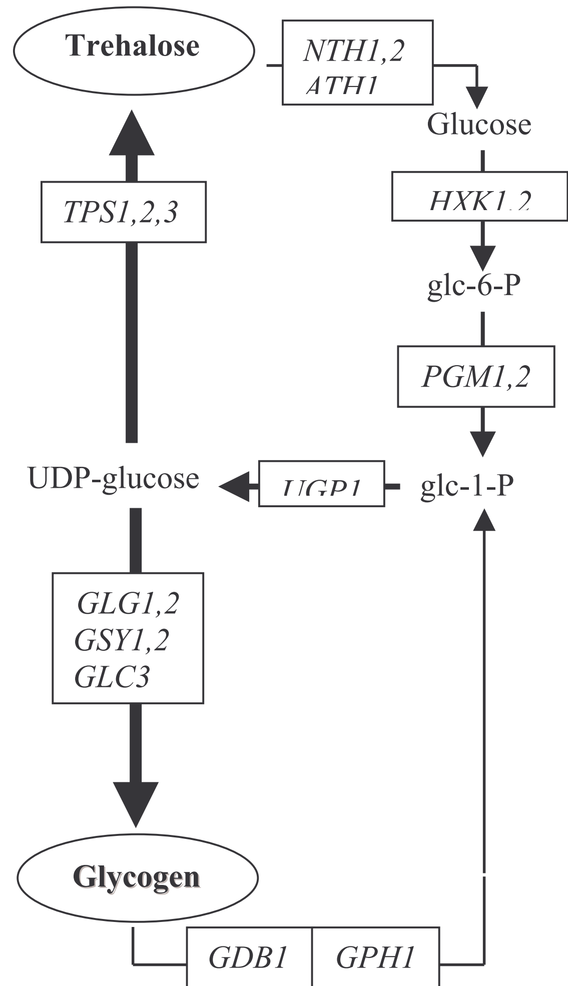
Effect of low temperature on energy production and reserves carbohydrate metabolism

Since enological fermentation occurs in the presence of a high sugar concentration and low oxygen availability, one should expect no major differences between fermentation at 13ºC and fermentation at 25ºC in the expressions of genes involved in respiratory functions. However, genes required for the synthesis of respiratory complexes I to IV were more repressed, whereas genes involved in the synthesis of complex V, which corresponds to the mitochondrial F0/F1 ATPsynthase, were more expressed in the middle and late phases of the fermentation at 13ºC than in the fermentation at 25°C (Fig. 3C). However, careful inspection of the differentially expressed genes from complex V indicated that most of the up-regulated genes at 13°C encode subunits of the F1 unit (*ATP2, 3, 4, 16* and *20*). We also observed that the transcript levels of *MCT1, ETR1* and *OAR1*, which encodes products of type-II

mitochondrial fatty acid synthesis (64), were also more abundant in the middle and late phases of growth at 13ºC than at 25°C.

Table 3: Changes in expression levels of genes that belong to carbohydrate reserves metabolism during the fermentation at 13 versus 25°C.

		(13 *vs*. 25ºC) fold changes		
ORF	Gene	IF	MF	FF
Trehalose metabolism				
YBR126c	*TPS1*	-1,96	2,15	2,89
YDR074	*TPS2*	-2,22	2,57	2,75
YMR261c	*TPS3*	-1,53	1,73	1,74
YDR001c	*NTH1*	1,14	1,05	-1,40
YBR001c	*NTH2*	1,52	-1,61	-1,75
YPR026w	*ATH1*	1,67	-1,92	-2,13
Glycogen metabolism				
YKR058w	*GLG1*	-2,00	2,63	2,61
YJL137c	*GLG2*	-1,29	1,66	1,69
YFR015c	*GSY1*	1,08	1,07	-1,67
YLR258w	*GSY2*	-8,33	6,27	11,86
YEL011w	*GLC3*	1,57	-2,50	-1,79
YPR160w	*GPH1*	1,31	-1,50	-1,67
YPR184w	*GDB1*	-2,56	2,56	3,45
YIL099w	*SGA1*	-1,09	-1,69	-2,27
Others				
YKL127w	*PGM1*	1.19	-1.25	-1.05
YMR105c	*PGM2*	-1.92	1.93	2.36
YFR053c	*HXK1*	1.17	-1.14	-1.45
YGL253w	*HXK2*	1.62	-1.69	-2.22
YKL035w	*UGP1*	1.53	-1.41	-1.72



Our analysis also highlighted that the expressions of genes involved in glycogen and trehalose metabolism in the fermentation at 13ºC were different from those in the fermentation at 25°C (Table 3). Breakdown of trehalose and glycogen was much less efficient at the beginning of fermentation at 13°C than at the beginning of fermentation at 25°C (51), However, our transcriptomic data showed that genes involved in the catabolism (*NTH1, NTH2* and *GPH1*) and those involved in the biosynthesis (*TPS1, TPS2, GLG1, GLG2*, *GSY2*) of these reserve carbohydrates were respectively up and

down-regulated at the beginning of fermentation at 13ºC (Table 3). So, this faster rate of degradation at 25ºC than at 13°C was merely due to a direct effect of temperature on the catalytic activity of the enzymes. As fermentation progressed, this expression profile was reversed, with genes of the catabolic pathways being less expressed and those in the biosynthesis more expressed at 13ºC than at 25ºC. Moreover, this remodeling of the expression level of this genes subset correlated with levels of trehalose and glycogen, which at 13ºC were 15 to 20 % higher in the middle to late phases of fermentation than at 25°C (data not shown).

Differential cellular composition in fatty acid and phospholipid partially correlated with differential expression of their corresponding genes between 13 and 25°C-fermentation.

Previous functional genomic analysis of wine yeast strains under simulated enological conditions showed that there were large variations in the expression of genes involved in fatty acids, phospholipid and sterol metabolism that were mainly a consequence of the low nitrogen availability (3) or the induction of anaerobiosis (58). Expression of this category of genes was also affected in response to a cold shock (61, 63). In the present study, we also found that the expressions of this class of genes were different in the two fermentation processes. Since it is likely that oxygen availability was similar in the two fermentations, this differential gene expression may be due to the effect of temperature. As Table 4 shows, the expression profiles of key genes required for the cytosolic synthesis of long-chain fatty acids (*FAA2, FAA3, OLE1, SUR4, FMR2*) were higher in the early phase and lower in middle and late phases of the fermentation at 13°C than in the corresponding phases of the fermentation at 25°C. On the other hand, the expression profile of genes of the type-II mitochondrial (short-chain) fatty acid synthesis (*OAR1*, *ETR1*, *MCT1*) was opposite to that of genes involved in the cytosolic long-chain fatty acid synthesis. These data prompted us to analyze fatty acids composition of yeast cells at the end the fermentation at 13ºC and 25 °C. As shown in Table 5, cells fermenting at 25°C showed higher levels of saturated fatty acids than cells fermenting at 13°C. On the other hand, the proportion of medium-chain fatty acids (MCFA) remained around 27 % of total fatty acid content throughout the fermentation at 13°C but dropped from 35 % at the initial stage to 11 % at the final stage of the process at 25°C. It is therefore tempting to associate this relative difference in MCFA content between the fermentations at 13 and 25°C to the differential expression of genes

involved in mitochondrial fatty acids synthesis, though the role of this pathway in the biosynthesis of short and medium fatty acid is still a matter of discussion (14, 64).

Table 4: List of genes whose product are implicated in fatty acid, phospholipids and sterol metabolism whose expression is changed at 13 versus 25°C

		Fold change of gene expression between 13 and 25ºC			
ORF	Gene Name	IF	MF	FF	Gene description
Fatty acid metabolism					
YGL055w	*OLE1*	2.08	-5.88	-2.17	stearoyl-CoA desaturase
YOR317w	*FAA1*	1.76	-1.69	-2.38	long-chain-fatty-acid--CoA ligase
YER015w	*FAA2*	2.07	1.18	-1.60	long chain fatty-acid CoA ligase
YIL009w	*FAA3*	2.44	-2.50	-3.57	acyl CoA synthase
YLR372w	*SUR4*	1.80	-1.72	-2.13	sterol isomerase, fatty acid elongase
YCL026c-a	*FMR2*	-4.76	5.70	5.87	involved in fatty acid regulation
YBR026c	*ETR1*	-1.67	1.83	2.19	mitochondrial respiratory function protein
YER061c	*CEM1*	1.49	-1.41	-1.56	beta-keto-acyl-ACP synthase, mitochondrial
YKL055c	*OAR1*	-2.17	2.30	2.52	putative 3-oxoacyl-(acyl carrier protein) reductase
YKL192c	*ACP1*	-1.05	1.02	1.12	mitochondrial acyl-carrier protein
YOR221c	*MCT1*	-4.76	3.38	2.43	malonyl-CoA:ACP transferase
Phospholipid metabolism					
YHL020c	*OPI1*	1.82	-1.75	-2.78	negative regulator of phospholipid biosynthesis
YAL013w	*DEP1*	-2.78	2.83	5.93	regulator of phospholipid metabolism
YCL004w	*PGS1*	-2.63	3.52	6.00	phosphatidylglycerophosphate synthase
YDL142c	*CRD1*	-2.38	2.23	4.63	cardiolipin synthase
YPL268w	*PLC1*	-2.00	2.67	3.19	1-phosphatidylinosiltol-4,5-biphosphate
YDL052c	*SLC1*	-2.50	2.46	3.41	fatty acyltransferase
YBR029c	*CDS1*	-1.13	1.35	1.73	CDP-diacylglycerol synthase
YER026c	*CHO1*	1.60	-1.56	-1.85	CDP-diacylglycerol serine O-phosphatidyltransferase
Sterol metabolism					
YLR056c	*ERG3*	2.62	-2.56	-1.51	sterol C-5 desaturase
YGR060w	*ERG25*	2.47	-2.57	-1.25	sterol C-4 methyoxidase
YGL001c	*ERG26*	2.84	-2.12	-2	sterol C-3 dehydrogenase
YPL117c	*IDI1*	-9.09	11.81	24.18	Isopentenil-diphosphate-isomerase
YPL172c	*COX10*	-4.55	3.64	9.14	farnesyl transferase
YMR202w	*ERG2*	-2	2.66	3.45	sterol C-8 isomerase
YMR208w	*ERG12*	-3.22	3.88	5.81	mevalonate kinase
YGL162w	*SUT1*	-3.03	2.51	2.7	hypoxic protein involved in sterol metabolism

Table 5 also shows that the proportion of unsaturated fatty acids at the initial stage of fermentation at 13°C was higher than at the initial phase of fermentation at 25°C. This may be consistent with higher expression levels of *OLE1* at this stage of fermentation. We also observed comparable amounts of C18:2 in the lipid fractions of

the yeast during the fermentations at 13ºC and 25°C. Since yeast is unable to synthesize polyunsaturated fatty acids (68), this indicates that the temperature did not alter the uptake of this compound from the medium.

Table 5: Fatty acid composition measured by gas chromatography in yeast cells at three stages of the fermentation at 13 and 25°C.

	13°C			25°C		
Fatty acids (%)	IF	MF	FF	IF	MF	FF
Medium Chain Fatty Acids (MCFA)	**25.3**	**26.8**	**27.1**	35.3	26.0	10.7
Saturated Fatty Acids (SFA)	31.8	38.5	**43.6**	33.3	40.8	58.8
Unsaturated Fatty Acids (UFA)	**37.9**	30.7	26.3	26.7	30.0	24.9
Linoleic Acid (C18:2)	5.0	4.0	3.0	4.7	3.2	5.6
Medium Chain Length	14.95	**14.96**	**14.98**	14.39	15.07	16.19

In contrast to genes involved in long-chain fatty acid metabolism, the transcripts of genes of the phospholipid metabolism were more abundant in the middle and late stages of fermentation at 13ºC than in the corresponding stages of fermentation at 25°C. This was particularly the case for mRNA levels of *SLC1*, *CDS1, PGS1*, *CRD1* and also for *OPI1* and *DEP1* (Table 4). These two latter genes encode, respectively, a negative regulator and a positive regulator of phospholipid synthesis pathway. Moreover, it has been reported that *OPI1* was repressed in correlation with increased levels of MFCA (28), which would match our metabolic data (see Table 5).

However, this higher expression of this class of genes at 13ºC than at 25 °C was not accompanied by an increase in cardiolipin, which is the end product of this metabolic pathway (Table 6), but by an increase in phosphatidic acid (PA), the obligate intermediate in the synthesis of several phospholipids, including sphingolipids (14). Another inconsistency was that the expression of *CHO1*, which encodes the phosphatidylserine synthase, was lower at 13°C than at 25°C, while the proportion of phosphatidyl serine (PS) in the middle-to-late phases of fermentation at 13°C was 4-times higher than at 25°C (Table 6). Despite this important difference in genes expression between the two temperatures, which may indicate a reorientation of the metabolic flux in the branches of the phospholipids synthesis, the total content of phospholipids in yeast cultivated at 13°C was twince as low as in yeast cultivated at 25°C (Table 6).

Table 6: Phospholipids composition measured by thin-layer chromatography in yeast cells at three phases of the wine fermentation carried out at 13 and 25°C.

Composition of phospholipids in the cell (% total phospholipids)	13°C			25°C		
	IF	MF	FF	IF	MF	FF
P-Serine (PS)	2.1	**10.1**	**9.6**	7.2	2.7	2.2
P-Ethanolamine (PE)	12.8	**13.0**	**8.2**	13.3	16.8	17.8
P-Choline (PC)	20.2	13.0	13.7	21.1	15.4	14.6
P-Inositol (PI)	11.7	10.1	8.2	10.6	14.1	11.9
P-Acid (PA)	**33.0**	**39.1**	**41.1**	24.9	23.5	29.2
P-Glycerol (PG)	9.6	11.6	15.1	10.0	15.4	13.0
Cardiolipin (CL)	10.6	**2.9**	**4.1**	13.9	12.1	11.4
Total phospholipids (% of cellular lipid content)	9.4	6.9	7.3	18	15	18.5

Table 7. Sterol composition of yeast cells during at three stages of the wine fermentation at 13 and 25 °C.

Composition of sterols in the cell (% total sterols)	13°C			25°C		
	IF	MF	FF	IF	MF	FF
Squalene	48.9	54.2	58.9	38.9	59.3	62.6
Zymosterol	3.8	3.0	2.0	7.8	2.1	2.1
Ergosterol	20.2	16.7	10.8	21.8	11.8	7.7
Stigmasterol	1.4	2.1	-	2.0	0.4	-
ß-Sitosterol	11.0	9.5	12.2	11.5	12.6	13.8
Lanosterol	13.4	13.1	13.8	14.4	13.3	12.4
Total sterol (% of cellular lipid content)	32.4	35.6	36.5	24.4	26.8	29.8

Very few genes involved in sterol synthesis were differentially expressed at the two temperatures of fermentation (Table 4), probably because at both temperatures the wine fermentation was rapid under hypoxic condition. This was also confirmed by the amount of sterol composition in the yeast cell (Table 7). We further observed that cells incorporated equal amounts of phytosterols (mainly β-sitosterol) at both temperatures (Table 7). As a consequence, the significant reduction in total phospholipid in yeast

cells cultivated at 13°C and the lack of significant changes in sterol content led to a ratio sterol/phospholipid that changed from ~ 1.3 in yeast cells cultivated at 25°C to ~ 3.0 in those cultivated at 13°C. This difference is consistent with reduced membrane fluidity and with a higher resistance to ethanol in yeast cultured at temperatures below 15 °C (1).

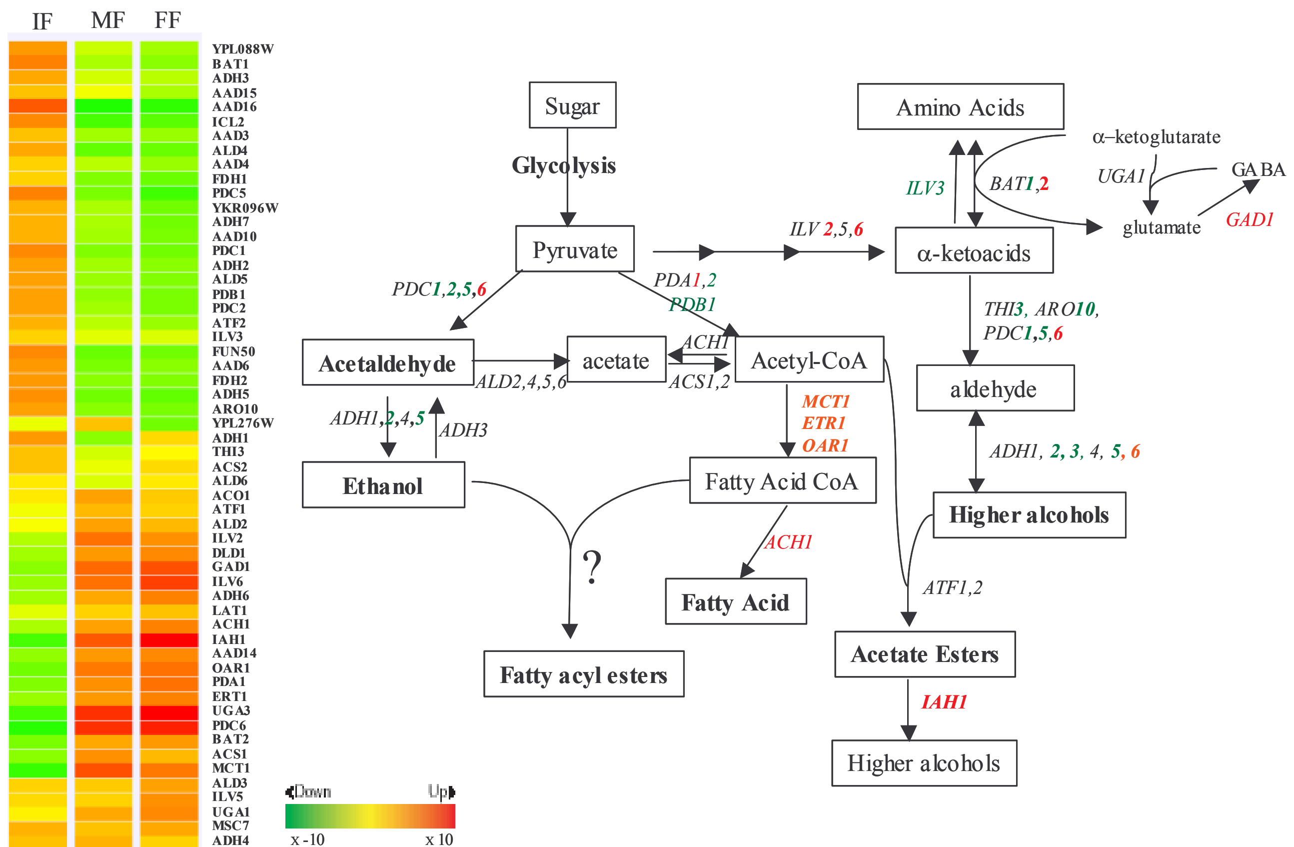
Although content and composition in sterol did not change dramatically in yeast cells between the two temperatures of fermentation, a potential competition at the level of dimethylallyl pyrophosphate (DMPP) (a key intermediate in the sterol biosynthesis) between Erg20p (the farnesyl pyrophosphate synthetase that converts DMPP into FPP) and Mod5p (a tRNA isopentenyltransferase that catalyzes the isopentenylation of adenosine on tRNAs using DMPP (8)) may be more active at 13°C than at 25°C. This is indicated by a down-regulation of *ERG20* and a strong up-regulation of *MOD5* at the end of the fermentation at 13ºC compared to fermentation at 25° C (Table 7).

Enhanced expression of genes implicated in aroma production at low temperature correlates with increased production of volatile and fatty acid ethyl esters.

We reported in Table 1 that levels of some volatile esters (mainly isoamyl acetate), free fatty acids and their corresponding fatty acid ethyl esters was 20, 30 and 85 % higher, respectively, in wine fermentation at 13°C than in wine fermentation at 25°C. This enhanced production of flavors at 13°C may be correlated with a differential expression of some genes involved in their synthesis. As Fig.4 shows, the expressions of genes involved in the catabolism of amino acids by the Erlich pathway such as *BAT2* (which encodes the cytosolic transaminase isoform (35)), *PDC6* (which encodes a minor pyruvate decarboxylase isoform (16, 25)) and *ADH6* (which encodes a NADP-dependent alcohol dehydrogenase (41,62)), and the expressions of genes involved in the degradation of the branched chain amino acids (*ILV2, ILV5, ILV6*) were transcribed higher in the middle and late phases of the fermentation at 13ºC than at 25°C. Also, it is possible that glutamate, 2-ketoglutarate and 4-aminobutyrate (GABA) supplied the Erlich pathway, as is indicated by the enhanced expression of *UGA1*, which encodes GABA transaminase, *GAD1*, which encodes the glutamate decarboxylase and *UGA3*, whose gene product allows the use of GABA (74). While the Erlich pathway appeared to be activated at 13°C, there was actually no major difference in the production of higher alcohols at 13°C and 25°C. However, we noticed a slight but reproducible increase of two volatile esters (isoamyl acetate and hexyl acetate), which are made from

condensation of alcohols with acetyl-CoA. This reaction is catalyzed by an acetylesterase encoded by *ATF1* and *ATF2* (26, 45), whose expression at 13ºC and 25°C is the same. On the other hand, we found from 5 to 10-fold higher expression of *IAH1*, which encodes an esterase that acts antagonistically to the alcohol acetyltransferases (48), at the end of fermentation at 13 than at 25°C.

Figure 4. Expression profiles of differentially expressed genes whose products are implicated in volatile and fatty acid esters pathways during fermentation at 13 versus 25°C. The results obtained from the analysis are shown in the same manner as in Fig.3. On the right is shown a simplified metabolic map of aroma formation from glucose in which are highlighted genes in the cluster whose expression changed significantly at 13°C versus 25°C.



With respect to higher levels of short-chain fatty acid and their corresponding ethyl esters at 13 °C, it is interesting that genes involved in the mitochondrial short-chain fatty acid synthesis were significantly up-regulated at this low temperature (Fig 4). The higher expression of *ACH1* at 13°C than at 25°C may account for the greater production of free fatty acids since the product of this gene, which is an acetyl-CoA hydrolase, is also active on fatty acyl-CoA (43). However, it is unclear how fatty acyl esters are produced, since the enzyme responsible for the esterification reaction has not

been well characterized yet (48). Finally, our comparative transcriptomic analysis also showed that the expression of most of the genes belonging to the aryl alcohol dehydrogenase family (*AAD3, AAD4, AAD6, AAD10, AAD15, YFL057c, YPL088w*) was lower at 13°C than at 25°C. Assuming that a putative function of the products of these genes in yeast is to degrade the complex aromatic notes from grapes into their corresponding unpleasant alcohols (15), their lower expression at 13°C than at 25°C may be in favor of a more fruity quality of wine fermented at 13°C.

DISCUSSION

In this paper, we comprehensively analyzed the expression states of genes in yeast exposed to exact industrial wine fermentations at 25ºC and 13°C, and tentatively correlated the differences in expression patterns between the two fermentation temperatures with major changes in fermentation and metabolic parameters including growth rate, cell viability, flavor production, and lipid composition of the cells. However, difference in the gene expression between 13ºC and 25°C may be due to an arrest of transcription at 25°C, to a transcriptional activation at 13°C or to a higher degradation of the mRNAs at 25°C than at 13°C. Thus, at this stage of the work, we will refer to the relative changes in gene expressions without any meaning about the mechanism of these changes.

From the methodological viewpoint, this study differed from previous genome-wide reports that examined expression profiles in mimicking enological processes (3, 46, 58), since it was carried out under exact industrial wine processing. Also, our genome-wide analysis into the effect of temperature on wine fermentation was different from two previous reports that studied the effects on gene expression of an abrupt drop in temperature from 30ºC to 10°C (61, 63). Nevertheless, our transcriptional investigation of wine fermentation at 25ºC overlapped somewhat with those reported by Rossignol *et al*. (58). This includes increases in stress-responsive genes, genes involved in glycerol and vitamin synthesis and decreases in genes involved in growth-associated function and nucleotide metabolism as the alcoholic fermentation progressed toward the non-proliferating phase of growth. On the other hand, the expression profiles during wine fermentation at 13°C contrasted significantly with those at 25°C. In particular, genes in the cell cycle, cell growth, cell fate and maintenance categories were less expressed at 13ºC during the exponential growth phase than during the stationary phase of the fermentation, whereas genes whose expression was activated during the

exponential phase at 13°C were essentially members of the stress response (30). This indicates, in agreement with previous report (61, 63), that a stress response was induced early during fermentation at 13°C, but activated much latter at 25°C when cells entered the non-proliferating condition. According to the list of genes that were activated at the initial stage of fermentation at 13°C, this stress is comparable to the LCR (Late Cold Response) identified by Schade *et al*. (63). Moreover, this cold-stress response probably prepared the cells to challenge subsequent stresses that may occur later during the growth fermentation, such as ethanol toxicity. A consequence of this cross-protection is that the cell viability is expected to increase (33). This is actually what we can conclude based on a stable CFU measured during the long non-proliferating phase of fermentation at 13°C, while this CFU gradually decreased during this phase at 25°C (see Fig.1). A potential role of *MOD5* in viability can also be inferred from the finding that this gene, which is indirectly involved in protein synthetic machinery via its efficiency to suppress non-sense mutation by tRNAs modification (8), was strongly activated at 13°C. Although whether a *mod5* mutant is sensitive to low temperature has not been tested, other reports stated that a loss of *MOD5* function leads to temperature sensitivity and severe growth defect on YPD after several generations (17, 31). This function of Mod5p should complement other actions mediated by Ded1p and Nsr1p to increase the efficiency of the translation initiation process at low temperature (63). Finally, an additional quantitative illustration of the cold-stress response was that cells at the initial stage of the fermentation at 13°C had higher levels of unsaturated fatty acids than at the same stage of growth at 25°C, which is consistent with a higher expression of *OLE1*, a gene encoding a desaturase know to be induced by cold stress (50, 61, 63).

Another significant difference was that total cellular phospholipid was twice as low at 13ºC than at 25°C. This is partly explained by a lower synthesis of long-chain fatty acids at 13°C than at 25°c, which was correlated with a lower expression of *FAA3*, *FAA1* and *SUR4* (see Table 5). Moreover, a reduction of end products in the phospholipids biosynthesis at 13°C was accompanied by a rise of phosphatidic acid (PA), a general precursor of all glycerolipid (14), which was twice as much at 13 than at 25°C. This suggests that the relative higher expression of genes in the phospholipid synthesis at 13ºC than at 25°C, while phospholipids content was lower, could be a compensatory response that resulted from a reduction in the end products of this pathway, as occurs in response to the depletion of an amino acid (6, 70). Whatever the

exact mechanism, the lower content of phospholipids in yeast cells during growth at 13°C, together with the fact that the levels of sterol were not significantly different in yeast cultivated at 13ºC and yeast cultivated at 25ºC, may explain the modification of membrane fluidity and the higher resistance to ethanol of yeast cultured at low temperature (2, 13, 72, 76, 77).

By comparing expression profiles at different stages of the fermentation between 13ºC and 25°C we obtained additional information that may help to explain how much the cellular metabolic activity may be affected by temperature. In particular, we did not expect to find that genes involved in the synthesis of complexes I, II, III and IV of the respiratory chain were more repressed during the fermentation at 13ºC than during the fermentation at 25°C. These results suggest that the repression of respiratory activity is not only controlled by carbon sources but also by temperature. Moreover, it is possible to establish a link between this apparent reduction in mitochondrial activity and the reduction in cardiolipin in cells cultivated at 13°C, since cardiolipin is especially abundant in fully developed mitochondria (14). On the other hand, the expressions of genes encoding enzymes required for mitochondrial short-chain fatty acid synthesis were up-regulated together with a higher production of short- and medium-chain fatty acid (MCFA; carbon chain < 14). Based on the possibility that short-chain fatty acids (C4 –C8) are mainly produced by the mitochondrial pathway (14, 64), their presence in the medium required energy to be expelled from the mitochondria. Interestingly, genes encoding most of the F1 unit of the F0/F1-ATPsynthase and *AAC3*, which encodes the oxygen-sensitive ADP-ATP translocator (60), were also up-regulated at 13ºC. Thus, one can envisage from these genes activation that the ATP synthase works as an ATPase to generate a protonmotrice force in cooperation with the ADP-ATP translocator that brings glycolytic ATP to the mitochondria (18) to drive fatty acid out of the mitochondria.

A third important result concerns the effect of low temperature on the metabolic pathways that are involved in flavors production. It could be inferred from up-regulation at 13ºC of genes *BAT2*, *PDC6* and *ADH6*, which encode isoenzymes of the transaminase, decarboxylase and dehydrogenase reactions of the Erlich pathway, respectively, that the flux in this pathway was increased at low temperature. However, the lack of significant changes in higher alcohols between the two temperatures may suggest that these genes do not play an important function in this pathway. On the other hand, the lower expression of *ARO10* at 13°C than at 25°C was accompanied by a two-

fold reduction in phenylethanol, which is consistent with a previous study that demonstrated that *ARO10* had a prominent role in the production of this alcohol (75). Similarly, the minor changes in acetate esters production between the two temperatures can be explained by the lack of any transcriptional effect of temperature on *ATF1* and *ATF2*, which encodes the alcohol acetylesterase (48), and whose increased expression was correlated with increased formation of these volatile esters (27, 73). Since *atf1Δatf2Δ* mutant cannot produce isoamyl acetate (73), the slight but reproducible increase of isoamyl acetate at 13°C could then be a consequence of posttranslational effect on Atf1p / Atf2p. Unexpectedly, our overall analysis showed that the expression of *IAH1,* which encodes an esterase described to act antagonistically to alcohol acetylesterase (27), dramatically increased at the end of fermentation at 13°C. Of course, it is likely that this gene activation was not accompanied by an increase in the esterase activity because there was no reduction of these compounds at 13°C. On the other hand, this enzyme could potentially act as an ester synthase to catalyze the formation of ethyl esters from short-chain fatty acyl-CoA and ethanol. This function for Iah1p has already been suggested by Dufour and coworkers (45, 73). This suggestion is supported by the finding that the expression profile of this gene is followed by an increase in the production of ethyl esters during the wine fermentation at 13°C.

To summarize, this genome-wide analysis conducted for the first time under true industrial conditions with a commercial yeast strain reveals major differences in genes expressions both during the course of the wine fermentation and between the two fermentation temperatures. With regard to an industrial output, the advantage of wine fermentation at 13°C is that it induces an early cold stress response that apparently does not penalize the wine fermentation process, but boosts the viability of the cells to sustain a long period of fermentation at low temperature.

ACKNOWLEDGEMENTS

This work was supported by grants from the Spanish government (AGL2000-0205-P4-03) and France-Spain Picasso Integrated Project (HF2001-0015). G. Beltran was granted for one year by the Marie Curie Fellowships (n° HPMT-EC-2000-00135). We thank Torres S.A. for analyzing the volatile compounds of wines. We also thank Kevin Costello of the Languages Services of the Rovira i Virgili University for revising the manuscript.

REFERENCES

1. **Alexandre, H., I. Rousseaux, and C. Charpentier**. 1994. Ethanol adaptation mechanisms in *Saccharomyces cerevisiae*. Biotechnol.Appl.Biochem. **20**:173-183.

2. **Alexandre, H., I. Rousseaux, and C. Charpentier**. 1994. Relationship between ethanol tolerance, lipid composition and plasma membrane fluidity in *Saccharomyces cerevisiae* and Kloeckera apiculata. FEMS Microbiol.Lett. **124**:17-22.

3. **Backhus, L. E., J. DeRisi, P. O. Brown, and L. F. Bisson**. 2001. Functional genomic analysis of a commercial wine strain of *Saccharomyces cerevisiae* under differing nitrogen conditions. FEMS Yeast Res. **1**:111-125.

4. **Barre, P., F. Vézinhet, S. Dequin, and B. Blondin**. 1993. Genetic improvement of wine yeast., p. 421-447. *In* G. H. Fleet (ed.), Wine Microbiology and Biotechnology. Harbor Academic Reading.

5. **Bauer, F. F. and I. S. Pretorius**. 2000. Yeast stress response and fermentation efficiency: how to survive the making of wine - a review. S.Afr.J.Enol.Vitic **21**:27-51.

6. **Beltran, G., M. Novo, N. Rozes, A. Mas, and J. M. Guillamon**. 2004. Nitrogen catabolite repression in *Saccharomyces cerevisiae* during wine fermentations. FEMS Yeast Res. **4**:625-632.

7. **Beltran, G., M. J. Torija, M. Novo, N. Ferrer, M. Poblet, J. M. Guillamon, N. Rozes, and A. Mas**. 2002. Analysis of yeast populations during alcoholic fermentation: a six year follow-up study. Syst.Appl.Microbiol. **25**:287-293.

8. **Benko, A.L., Vaduva, G., Martin, N.C., and A.K. Hopper**. 2000. Competition between a sterol biosynthetic enzyme and tRNA modification in addition to changes in the protein synthesis machinery causes by altered nonsense suppression. Proc.Natl.Acad.Sci., **97** : 61-66.

9. **Bisson, L. F.** 1999. Stuck and sluggish fermentations. American Journal of Enology and Viticulture **50**:107-119.

10. **Boulton, R. B., V. L. Singleton, L. F. Bisson, and R. E. Kunkee**. 1996. Principles and practices of winemaking. Chapman and Hall, New York.

11. **Cavalieri, D., J. P. Townsend, and D. L. Hartl**. 2000. Manifold anomalies in gene expression in a vineyard isolate of *Saccharomyces cerevisiae* revealed by DNA microarray analysis. Proc.Natl.Acad.Sci.U.S.A **97**:12369-12374.

12. **Charoenchai, C., G. H. Fleet, and P. A. Henschke**. 1998. Effects of temperature, pH, and sugar concnetration on the growth rates and cell biomass of wine yeasts. American Journal of Enology and Viticulture **49**:283-288.

13. **Chi, Z. and N. Arneborg**. 1999. Relationship between lipid composition, frequency of ethanol-induced respiratory deficient mutants, and ethanol tolerance in *Saccharomyces cerevisiae*. J Appl.Microbiol. **86**:1047-1052.

14. **Daum, G., N. D. Lees, M. Bard, and R. Dickson**. 1998. Biochemistry, cell biology and molecular biology of lipids of *Saccharomyces cerevisiae*. Yeast **14**:1471-1510.

15. **Delneri, D., D. C. Gardner, and S. G. Oliver**. 1999. Analysis of the seven-member *AAD* gene set demonstrates that genetic redundancy in yeast may be more apparent than real. Genetics **153**:1591-1600.

16. **Dickinson, J. R., S. J. Harrison, J. A. Dickinson, and M. J. Hewlins**. 2000. An investigation of the metabolism of isoleucine to active Amyl alcohol in *Saccharomyces cerevisiae*. J Biol.Chem **275**:10937-10942.

17. **Dunn B, T. Ferea , P. Spellman, J. Schwarz , J. Terraciano, J. Troyanovich, S. Walker , J. Greene, K. Shaw, B. DiDomenico, Q. Wang, M. Kaloper, S. Metzner, E. Chung, C. Bondre, A. Venteicher, D. Botstein and P. Brown.** 2004. Genetic footprinting: A functional analysis of the *S. cerevisiae* genome. Communication to SGD (www.yeastgenome.org).

18. **Dupont, C. H., J. P. Mazat, and B. Guerin**. 1985. The role of adenine nucleotide translocation in the energization of the inner membrane of mitochondria isolated from rho + and rho degree strains of *Saccharomyces cerevisiae*. Biochem.Biophys.Res.Commun. **132**:1116-1123.

19. **Durcos, S., L. Samyn, J. M. Soemen, and B. Médina**. 1989. OENO89: Logiciel d'analyse de données sur Macintosh. Maitrise d'informatique. Université de Bordeaux I

20. **Eisen, M. B., P. T. Spellman, P. O. Brown, and D. Botstein**. 1998. Cluster analysis and display of genome-wide expression patterns. Proc.Natl.Acad.Sci.U.S.A **95**:14863-14868.

21. **Estruch, F.** 2000. Stress-controlled transcription factors, stress-induced genes and stress tolerance in budding yeast. FEMS Microbiol.Rev. **24**:469-486.

22. **Ferreira, V., M. Sharman, J. Cacho, and J. Dennis**. 1996. New and efficient microextraction / solid-phase extraction method for the gas chromatographic analysis of wine volatiles. J.Chromatogr.A **731**:247-259.

23. **Feuillat, M., C. Charpentier, and C. Massoutier**. 1997. Intérêt oenologique des souches de levures *Saccharomyces* cryotolérantes. Revue des Oenologues **85**:18-21.

24. **Fleet, G. H. and G. M. Heard**. 1993. Wine Microbiology and Biotechnology. Harwood Academic Publishers, Switzerland.

25. **Flikweert, M. T., Z. L. Van Der, W. M. Janssen, H. Y. Steensma, J. P. van Dijken, and J. T. Pronk**. 1996. Pyruvate decarboxylase: an indispensable enzyme for growth of *Saccharomyces cerevisiae* on glucose. Yeast **12**:247-257.

26. **Fujii, T., N. Nagasawa, A. Iwamatsu, T. Bogaki, Y. Tamai, and M. Hamachi**. 1994. Molecular cloning, sequence analysis, and expression of the yeast alcohol acetyltransferase gene. Appl.Environ.Microbiol. **60**:2786-2792.

27. **Fukuda, K., N. Yamamoto, Y. Kiyokawa, T. Yanagiuchi, Y. Wakai, K. Kitamoto, Y. Inoue, and A. Kimura**. 1998. Balance of activities of alcohol acetyltransferase and esterase in *Saccharomyces cerevisiae* is important for production of isoamyl acetate. Appl.Environ.Microbiol. **64**:4076-4078.

28. **Furukawa, K., T. Yamada, H. Mizoguchi, and S. Hara**. 2003. Increased ethyl caproate production by inositol limitation in *Saccharomyces cerevisiae*. J.Biosci.Bioeng. **95**:448-454.

29. **García Barceló, J.** 1990. Técnicas analíticas para vinos. GAB, Barcelona.

30. **Gasch, A. P., P. T. Spellman, C. M. Kao, O. Carmel-Harel, M. B. Eisen, G. Storz, D. Botstein, and P. O. Brown**. 2000. Genomic expression programs in the response of yeast cells to environmental changes. Mol.Biol.Cell **11**:4241-4257.

31. **Giaever G, AM. Chu, L. Ni, C. Connelly, L. Riles, S. Veronneau, S. Dow, D. A. Lucau-Danila, K. Anderson, B. Andre, AP. Arkin, A. Astromoff, M. El-Bakkoury, R. Bangham, R. Benito, S. Brachat, S. Campanaro, M. Curtiss, K. Davis, A. Deutschbauer, KD. Entian, P. Flaherty, F. Foury, DJ. Garfinkel, M. Gerstein, D. Gotte, U. Guldener, JH. Hegemann, S. Hempel, Z. Herman, DF. Jaramillo, DE. Kelly, SL. Kelly, P. Kotter, D. LaBonte, DC. Lamb, N. Lan, H. Liang, H. Liao, L. Liu, C. Luo, M. Lussier, R. Mao, P. Menard, SL. Ooi, JL. Revuelta, CJ. Roberts, M. Rose, P. Ross-Macdonald, B. Scherens, G. Schimmack, B. Shafer, DD. Shoemaker, S. Sookhai-Mahadeo, RK. Storms, JN. Strathern, G. Valle, M. Voet, G. Volckaert, CY. Wang, TR. Ward, J. Wilhelmy, EA. Winzeler, Y. Yang, G. Yen, E. Youngman, K. Yu, H. Bussey, JD. Boeke, M. Snyder, P. Philippsen, RW. Davis and M. Johnston** 2002**.** Functional profiling of the Saccharomyces cerevisiae genome. Nature **418** : 387-91

32. **Hauser, N. C., K. Fellenberg, R. Gil, S. Bastuck, J. D. Hoheisel, and J. E. Pérez-Ortín**. 2001. Whole genome analysis of a wine yeast strain. Comp. Func. Genom. **2**:69-79.

33. **Hohmann, S. and W. H. Mager**. 2003. Yeast Stress Response. Springer-Verlag, New York.

34. **Jesus Ferreira, M. C., X. Bao, V. Laize, and S. Hohmann**. 2001. Transposon mutagenesis reveals novel loci affecting tolerance to salt stress and growth at low temperature. Curr.Genet. **40**:27-39.

35. **Kispal, G., H. Steiner, Court DA, B. Rolinski, and R. Lill**. 1996. Mitochondrial and cytosolic branched-chain amino acid transaminases from yeast, homologs of the myc oncogene-regulated Eca39 protein. J Biol.Chem **271**:24458-24464.

36. **Kondo, K. and M. Inouye**. 1991. TIP 1, a cold shock-inducible gene of *Saccharomyces cerevisiae*. J.Biol.Chem. **266**:17537-17544.

37. **Kondo, K., L. R. Kowalski, and M. Inouye**. 1992. Cold shock induction of yeast NSR1 protein and its role in pre-rRNA processing. J.Biol.Chem. **267**:16259-16265.

38. **Kowalski, L. R., K. Kondo, and M. Inouye**. 1995. Cold-shock induction of a family of TIP1-related proteins associated with the membrane in *Saccharomyces cerevisiae.* Mol.Microbiol. **15**:341-353.

39. **Kwast, K. E., L. C. Lai, N. Menda, D. T. James, III, S. Aref, and P. V. Burke**. 2002. Genomic analyses of anaerobically induced genes in *Saccharomyces cerevisiae*: functional roles of Rox1 and other factors in mediating the anoxic response. J.Bacteriol. **184**:250-265.

40. **Lambrechts, M. G. and I. S. Pretorius**. 2000. Yeast and its importance to wine aroma. S.Afr.J.Enol.Vitic **21**:97-129.

41. **Larroy, C., M. R. Fernandez, E. Gonzalez, X. Pares, and J. A. Biosca**. 2002. Characterization of the *Saccharomyces cerevisiae* YMR318C (ADH6) gene product as a broad specificity NADPH-dependent alcohol dehydrogenase: relevance in aldehyde reduction. Biochem.J **361**:163-172.

42. **LeBerre, V., E. Trevisiol, A. Dagkessamanskaia, S. Sokol, A. M. Caminade, J. P. Majoral, B. Meunier, and J. Francois**. 2003. Dendrimeric coating of glass slides for sensitive DNA microarrays analysis. Nucleic Acids Res. **31**:88.

43. **Lee, F. J., L. W. Lin, and J. A. Smith**. 1989. Purification and characterization of an acetyl-CoA hydrolase from *Saccharomyces cerevisiae*. Eur.J Biochem. **184**:21-28.

44. **Llauradó, J. M., N. Rozes, R. Bobet, A. Mas, and M. Constantí**. 2002. Low temperature alcoholic fermentation in high sugar concentration grape must. J.Food Science **67**:268-273.

45. **Malcorps, P. and J. P. Dufour**. 1992. Short-chain and medium-chain aliphatic-ester synthesis in *Saccharomyces cerevisiae*. Eur.J Biochem. **210**:1015-1022.

46. **Marks, V. D., G. K. van der Merwe, and H. J. van Vuuren**. 2003. Transcriptional profiling of wine yeast in fermenting grape juice: regulatory effect of diammonium phosphate. FEMS Yeast Res. **3**:269-287.

47. **Martinez-Pastor, M. T., G. Marchler, C. Schuller, A. Marchler-Bauer, H. Ruis, and F. Estruch**. 1996. The *Saccharomyces cerevisiae* zinc finger proteins Msn2p and Msn4p are required for transcriptional induction through the stress response element (STRE). EMBO J **15**:2227-2235.

48. **Mason, A. B. and J. P. Dufour**. 2000. Alcohol acetyltransferases and the significance of ester synthesis in yeast. Yeast **16**:1287-1298.

49. **Mitsui, K., S. Yaguchi, and K. Tsurugi**. 1994. The GTS1 gene, which contains a Gly-Thr repeat, affects the timing of budding and cell size of the yeast *Saccharomyces cerevisiae*. Mol.Cell Biol. **14**:5569-5578.

50. **Nakagawa, Y., N. Sakumoto, Y. Kaneko, and S. Harashima**. 2002. Mga2p is a putative sensor for low temperature and oxygen to induce OLE1 transcription in Saccharomyces cerevisiae. Biochem.Biophys.Res.Commun. **291**:707-713.

51. **Novo, M. T., G. Beltran, M. J. Torija, M. Poblet, N. Rozes, J. M. Guillamon, and A. Mas**. 2003. Changes in wine yeast storage carbohydrate levels during preadaptation, rehydration and low temperature fermentations. Int.J.Food Microbiol. **86**:153-161.

52. **Phadtare, S., J. Alsina, and M. Inouye**. 1999. Cold-shock response and cold-shock proteins. Curr.Opin.Microbiol. **2**:175-180.

53. **Pretorius, I. S.** 2000. Tailoring wine yeast for the new millennium: novel approaches to the ancient art of winemaking. Yeast **16**:675-729.

54. **Rachidi, N., P. Barre, and B. Blondin**. 2000. Examination of the transcriptional specificity of an enological yeast. A pilot experiment on the chromosome-III right arm. Curr.Genet. **37**:1-11.

55. **Rapp, A.** 1998. Volatile flavour of wine: correlation between instrumental analysis and sensory perception. Nahrung **42**:351-884.

56. **Ribéreau-Gayon, P., D. Dubourdieu, B. Donèche, and A. Lonvaud**. 2000. Handbook of Enology. The Microbiology of wine and vinifications Vol. I. Wiley, West Sussex, England.

57. **Rodriguez-Vargas, S., F. Estruch, and F. Randez-Gil**. 2002. Gene expression analysis of cold and freeze stress in Baker's yeast. Appl.Environ.Microbiol. **68**:3024-3030.

58. **Rossignol, T., L. Dulau, A. Julien, and B. Blondin**. 2003. Genome-wide monitoring of wine yeast gene expression during alcoholic fermentation. Yeast **20**:1369-1385.

59. **Rozès, N., C. García-Jares, F. Larue, and A. Lonvaud-Funel**. 1992. Differentiation between fermenting and spoilage yeasts in wine by total free fatty acid analysis. J.Sci.Food Agric. **59**:351-357.

60. **Sabova, L., I. Zeman, F. Supek, and J. Kolarov**. 1993. Transcriptional control of AAC3 gene encoding mitochondrial ADP/ATP translocator in *Saccharomyces cerevisiae* by oxygen, heme and ROX1 factor. Eur.J Biochem. **213**:547-553.

61. **Sahara, T., T. Goda, and S. Ohgiya**. 2002. Comprehensive expression analysis of time-dependent genetic responses in yeast cells to low temperature. J.Biol.Chem. **277**:50015-50021.

62. **Saint-Prix, F., L. Bonquist, and S. Dequin**. 2004. Functional analysis of the ALD gene family of *Saccharomyces cerevisiae* during anaerobic growth on glucose: the NADP+-dependent Ald6p and Ald5p isoforms play a major role in acetate formation. Microbiology **150**:2209-2220.

63. **Schade, B., G. Jansen, M. Whiteway, K. D. Entian, and D. Y. Thomas**. 2004. Cold Adaptation in Budding Yeast. Mol.Biol.Cell.

64. **Schneider, R., B. Brors, F. Burger, S. Camrath, and H. Weiss**. 1997. Two genes of the putative mitochondrial fatty acid synthase in the genome of *Saccharomyces cerevisiae*. Curr.Genet. **32**:384-388.

65. **Schreier, P.** 1979. Flavour composition of wines: a review. CRC Crit.Rev.Food Sci.Nutr. **12**:59-111.

66. **Sutton, A., D. Immanuel, and K. T. Arndt**. 1991. The SIT4 protein phosphatase functions in late G1 for progression into S phase. Mol.Cell Biol. **11**:2133-2148.

67. **Talla, E., F. Tekaia, L. Brino, and B. Dujon**. 2003. A novel design of whole-genome microarray probes for *Saccharomyces cerevisiae* which minimizes cross-hybridization. BMC.Genomics **4**:38.

68. **Taylor, G. T., P. Thurston, and B. Kirsop**. 1979. The influence of lipids derived from a malt spent grains on yeast metabolism and fermentation. J.Inst.Brew. **85**:219.

69. **Tennyson, C. N., J. Lee, and B. J. Andrews**. 1998. A role for the Pcl9-Pho85 cyclin-cdk complex at the M/G1 boundary in *Saccharomyces cerevisiae*. Mol.Microbiol. **28**:69-79.

70. **ter Schure, E. G., N. A. van Riel, and C. T. Verrips**. 2000. The role of ammonia metabolism in nitrogen catabolite repression in *Saccharomyces cerevisiae*. FEMS Microbiol.Rev. **24**:67-83.

71. **Tong, A.H., G. Lesage, GD. Beader, H. Ding, H. XU, X. Xin, J. Young, G.F. Berriz, R.L. Brost, M. Chang et al**. 2004. Global mapping of the yeast genetic interaction network. Science, **303**: 808 –813.

72. **Torija, M. J., G. Beltran, M. Novo, M. Poblet, J. M. Guillamon, A. Mas, and N. Rozes**. 2003. Effects of fermentation temperature and *Saccharomyces* species on the cell

fatty acid composition and presence of volatile compounds in wine. Int.J.Food Microbiol. **85**:127-136.

73. **Verstrepen, K. J., S. D. Van Laere, B. M. Vanderhaegen, G. Derdelinckx, J. P. Dufour, I. S. Pretorius, J. Winderickx, J. M. Thevelein, and F. R. Delvaux**. 2003. Expression levels of the yeast alcohol acetyltransferase genes *ATF1*, *Lg-ATF1*, and *ATF2* control the formation of a broad range of volatile esters. Appl.Environ.Microbiol. **69**:5228-5237.

74. **Vissers, S., B. Andre, F. Muyldermans, and M. Grenson**. 1989. Positive and negative regulatory elements control the expression of the *UGA4* gene coding for the inducible 4-aminobutyric-acid-specific permease in *Saccharomyces cerevisiae*. Eur.J Biochem. **181**:357-361.

75. **Vuralhan, Z., M. A. Morais, S. L. Tai, M. D. Piper, and J. T. Pronk**. 2003. Identification and characterization of phenylpyruvate decarboxylase genes in *Saccharomyces cerevisiae*. Appl.Environ.Microbiol. **69**:4534-4541.

76. **Walker, M. A.** 1998. Yeast Physiology and biotechnology. Wiley & Sons, West Sussex, England.

77. **Watson, K.** 1987. Temperature relations, p. 41-71. *In* A. H. Rose and J. S. Harrison (ed.), The yeast. Yeast and Environment, vol. 2. Academic Press, London.

78. **Wickner, R. B., T. J. Koh, J. C. Crowley, J. O'Neil, and D. B. Kaback**. 1987. Molecular cloning of chromosome I DNA from *Saccharomyces cerevisiae*: isolation of the MAK16 gene and analysis of an adjacent gene essential for growth at low temperatures. Yeast **3**:51-57.

79. **Yaguchi, S., K. Mitsui, K. Kawabata, Z. Xu, and K. Tsurugi**. 1996. The pleiotropic effect of the *GTS1* gene product on heat tolerance, sporulation and the life span of *Saccharomyces cerevisiae.* Biochem.Biophys.Res.Commun. **218**:234-237.

CHAPTER 3

Nitrogen Catabolite Repression in *Saccharomyces cerevisiae* during wine fermentations

Gemma Beltran, Maite Novo, Nicolas Rozès, Albert Mas and José M. Guillamón

Unitat d'Enologia del Centre de Referència de Tecnologia d'Aliments. Dept. Bioquímica i Biotecnologia. Facultat d'Enologia de Tarragona. Universitat Rovira i Virgili. Marcel·lí Domingo s/n 43007 Tarragona, Spain

FEMS Yeast Research, *4*, 625-632 (2004)

ABSTRACT

We carried out fermentations with several nitrogen sources in different concentrations and studied nitrogen regulation by following the transcriptional profile of the general amino acid permease (*GAP1*) and the ammonium permeases (*MEP1*, *MEP2*, *MEP3*). In wine fermentations the cells evolve from a nitrogen-repressed situation at the beginning of the process to a nitrogen-derepressed situation as the nitrogen is consumed. These nitrogen-repressed/derepressed conditions determined the different patterns of ammonium and amino acid consumption. Arginine and alanine were hardly used under the repressed conditions, while the uptake of branched-chain and aromatic amino acids increased.

Keywords: wine yeast, alcoholic fermentation, nitrogen consumption, ammonium, amino acids, *GAP1*, *MEP* genes

INTRODUCTION

A wide variety of nitrogen-containing compounds are present in grape juice. These depend on the variety of grape and the time of harvest. The two main sources of yeast assimilable nitrogen compounds are amino acids and ammonium ions. Ammonium ions make up a large percentage of total assimilable nitrogen (up to 40%) while proline and arginine are the most common nitrogenous compounds in grape juice (30-65% of total amino acid content) [1]. *S. cerevisiae* is able to use different nitrogen sources for growth but not all nitrogen sources support growth equally well. *S. cerevisiae* selects nitrogen sources that enable the best growth by a mechanism called Nitrogen Catabolite Repression (NCR) [2-3]. Good nitrogen sources such as glutamine, asparagine or ammonium decrease the level of enzymes required for utilisation of poorer nitrogen sources [4].

Amino acids are transported into the cell by general and specific transport systems. The general high-capacity permeases like *GAP1* and *AGP1* or the specific proline permease *PUT4* are nitrogen-regulated and become down-regulated at the transcriptional as well as the post-translational level, in response to high-quality nitrogen sources like ammonium [5]. However, specific permeases like the histidine permease (*HIP1*), the lysine permease (*LYP1*) and the basic amino acid permease *CAN1* are expressed constitutively [4]. Up to now, only three permeases (Mep1p, Mep2p and Mep3p) have been related with the ammonium uptake [6]. Mep2p displays the highest affinity for NH_4^+ (Km 1.4 to 2.1 μM), followed closely by Mep1p (Km 5 to 10 μM) and finally by Mep3p, whose affinity is much lower (Km 1.4 to 2.1 mM) [6]. The *MEP* genes are also subject to nitrogen control. These genes are expressed when low ammonium concentrations are present in the growth medium but, at high concentration of a good nitrogen source (including ammonium) all three *MEP* genes are repressed. With a poor nitrogen source, *MEP2* expression is much higher than *MEP1* and *MEP3* expression [6].

Despite major advances in characterising the genome of the yeast *S. cerevisiae* and numerous reports on the transcriptional regulation of individual genes in laboratory strains of this yeast, we have a limited understanding of the expression of genes in yeast during industrial fermentations [7]. Specifically, few data are available about the metabolism of nitrogen and its regulation in winemaking conditions, which are characterised by a high concentration of sugar and ethanol, a low content of assimilable nitrogen, a shortage of oxygen and a low pH. Our main objective in this study is an

initial approach to study nitrogen regulation under these conditions. We monitored NCR during fermentations in synthetic grape juice with several nitrogen sources added in different concentrations. We also studied how NCR affects nitrogen uptake and fermentation kinetics.

MATERIALS AND METHODS

Strain, fermentations and sampling

The commercial wine strain *Saccharomyces cerevisiae* QA23 (Lallemand S.A., Canada) was used in this study. Fermentations were carried out in a synthetic grape must (pH 3,3) as described by Riou *et al.* [8], but with 200 g l^{-1} of reduced sugars (100 g l^{-1} Glucose and 100 g l^{-1} Fructose) and without anaerobic factors. Only the nitrogen content changed in the different fermentations. The Yeast Assimilable Nitrogen (YAN) content in the control synthetic grape must (CNC) was 300 mg N l^{-1}: ammoniacal nitrogen (NH_4Cl) 120 mg N l^{-1} and amino acids 180 mg N l^{-1} (Table 1).

Table1. Content of amino acids and ammonium expressed as mg l^{-1} and mg N l^{-1} (YAN) in control synthetic grape must (CNC).

Amino acid	mg/l	mg N/l
Asp	44,20	4,65
Glu	119,60	11,39
Ser	78,00	10,40
Gln	499,20	47,87
His	33,80	3,05
Gly	18,20	3,40
Thr	75,40	8,87
Arg	367,90	29,60
Ala	145,60	22,90
Tyr	19,50	1,51
Cis	20,80	2,41
Val	44,20	5,29
Met	31,20	2,93
Trp	174,20	11,95
Phe	37,70	3,20
Ile	32,50	3,47
Leu	48,10	5,14
Lys	16,90	1,62
Pro	599,30	-
Total aas	1807,00	179,66
Amonia	154,28	120,00
Total YAN		299,66

The High Nitrogen Content (HNC) and Low Nitrogen Content (LNC) conditions contained 4-fold (1200 mg l^{-1}) and 1/5-fold (60 mg l^{-1}) the YAN of the control must, respectively. The proportions of the different amino acids and ammonium were maintained in the HNC and LNC synthetic musts. Two extra fermentations with low nitrogen content (60 mg N l^{-1}) were also carried out. In these fermentations, the nitrogen content was either amino acids (LNC-aas) or ammonium (LNC-NH4).

Fermentations were done at room temperature (22-28ºC) in laboratory-scale fermenters: 2-litre bottles filled with 1.8 l medium and fitted with closures that enabled the carbon dioxide to escape and the samples to be removed. Fermentations were in semi anaerobic conditions, since limited aeration was necessary in order to harvest samples for subsequent analysis. The final population inoculated in every flask was $2x10^7$ cell ml^{-1} from dry yeast rehydrated in water at 37ºC.

Fermentations were monitored by the media density. Residual sugars was also determined by enzymatic kits (Roche Applied Science, Germany). Yeast cell biomass was determined by absorbance at 600 nm. Yeasts cells were harvested at different points of the fermentation for analysing mRNA and determining arginase activity. Yeast cells were also analysed before their inoculation in the fermentation media (time 0). The supernatant of these samples was stored at –20 ºC for analysis of their nitrogen content.

Analysis of nitrogen content

YAN was analysed by the formol index method [9], and the ammonium content was quantified using an enzymatic method (Roche Applied Science, Germany); both determinations were expressed as mg nitrogen ml^{-1}. Analysis of individual amino and imino acids was determined by OPA and FMOC derivatizations, respectively, using the Agilent 1100 Series HPLC. The sample (2 µl) was injected into a 4.6 x 250 mm x 5 µm Hypersil ODS column (Agilent Technologies, Germany). The concentration of each amino acid was calculated using external and internal standards and expressed as mg l^{-1}.

Determination of arginase activity

Arginine degradation is first catalyzed by arginase yielding ornithine and urea. Arginase activity can be determinated by measuring an increase in the concentration of ornithine by the reaction with ninhydrin, known as the Chinard reaction [10]. This method has been widely used in animal tissues and has been developed in yeast by Carrasco *et al*. [11].

Real-time quantitative PCR

Total RNA was isolated from yeast samples as described by Sierkstra *et al.* [12] and resuspended in 50 µl of DEPC-treated water. cDNA was synthesised from total RNA using Superscript™ II RNase H⁻ Reverse Transcriptase (Invitrogen, USA) in a GenAmp PCR System 2700 (Applied Biosystem, USA). The protocol provided by the manufacturer was used.

The PCR primers used in this study are listed in Table 2. The Real-Time Quantitative PCR reaction was performed using SYBR® Green I PCR (Applied Biosystems, USA). For each gene, a standard curve was made with serial 10-fold dilution of yeast genomic DNA, ranging from 4 to 4 x 10^{-5} ng/µl. The starting quantity of the studied gene was calculated from the standard curve by interpolation, and normalized with Actin gene, as housekeeping gene.

Table 2. Primers used in this study (supplied by PE Applied Biosystems).

Primer	Nucletide sequence (5' to 3')
ACT-F	TGGATTCCGGTGATGGTGTT
ACT-R	CGGCCAAATCGATTCTCAA
GAP1-F	CTGTGGATGCTGCTGCTTCA
GAP1-R	CAACACTTGGCAAACCCTTGA
MEP1-F	CCTGAGCTCGCGTATGCA
MEP1-R	GGCGCCAGCGATAATACTTAA
MEP2-F	GGTATCATCGCTGGCCTAGTG
MEP2-R	ACAACGGCTGACCAGATTGG
MEP3-F	GCCGGTGTGGTGGGATT
MEP3-R	TTGTGCCGTCCATTCCAAT

In the PCR reaction, final concentration of primers was 300 nM, together with 1 µl of cDNA (or 5 µl of each DNA serial dilution for standard tubes). All PCR reactions were mixed in 96-well optical plates and cycled in a GeneAmp 5700 Sequence Detection System (Applied biosystems, USA) under the following conditions: 50ºC for 2 min, 95ºC for 10 min, and 40 cycles at 95ºC for 15 sec and at 60ºC for 60 sec.

The threshold was positioned to intersect the exponential part of the amplification curve of positive reactions, as recommended by Applied Biosystems. The C_t value is inversely proportional to the log amount of template in the PCR reaction. All samples were

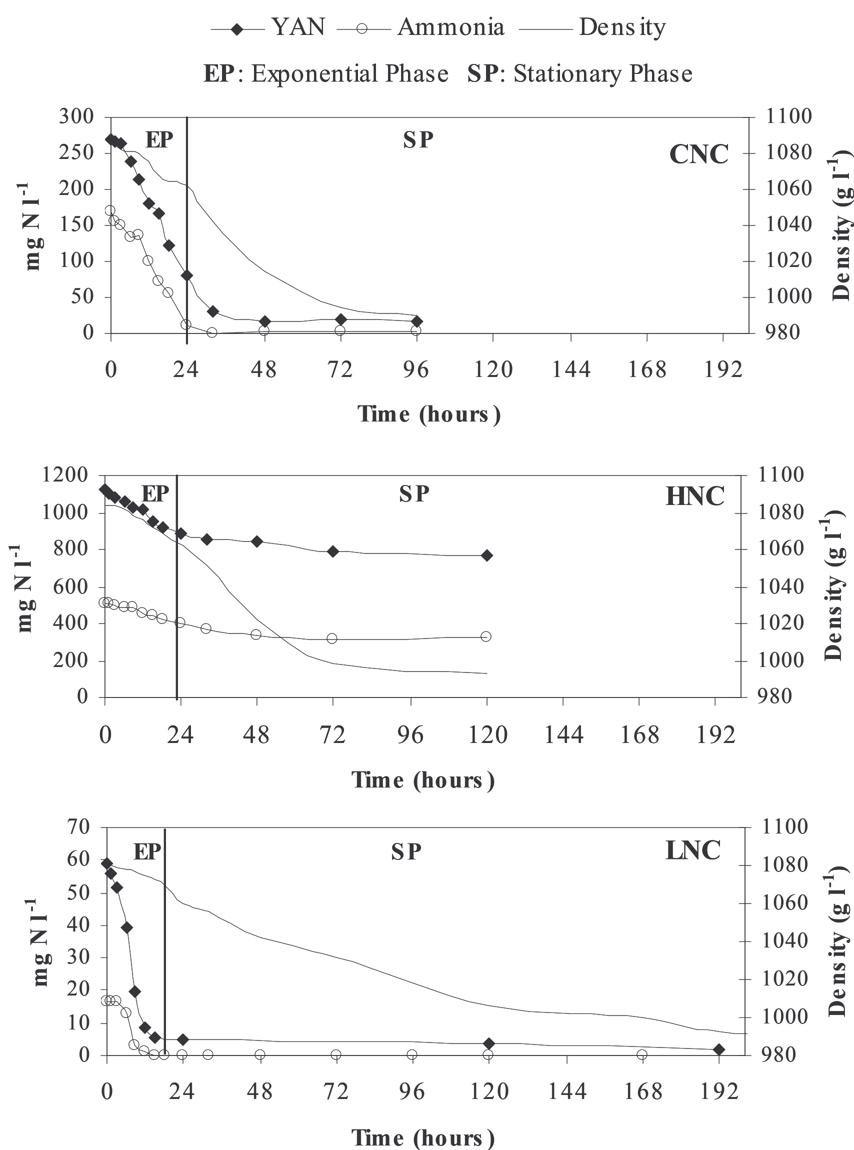
analysed in duplicate and the expression values were averaged by the analysis software (Applied Biosystems, USA). The coeficient of variation in all samples analysed was less than 10 %.

RESULTS

Fermentation kinetics and nitrogen consumption

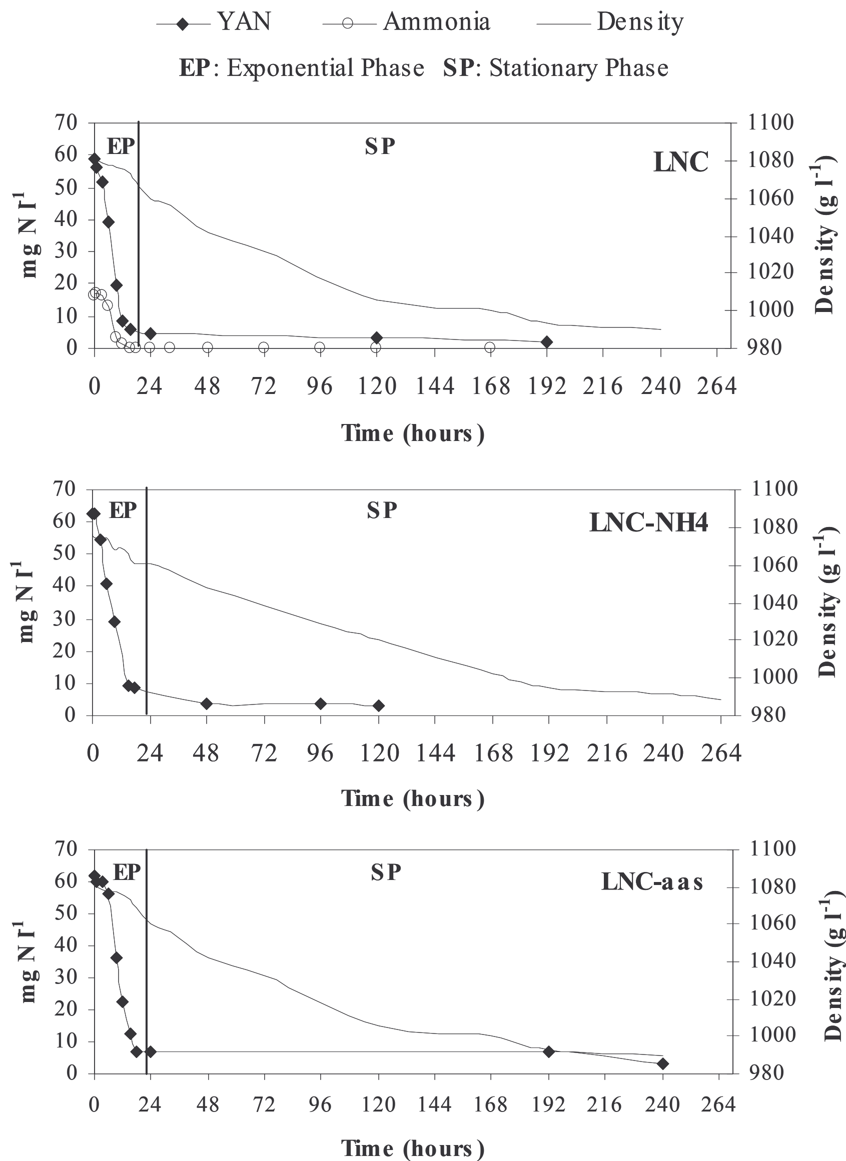
Fermentations with a low (60 mg/l; LNC) and a high nitrogen content (1200 mg/l; HNC) were carried out with the commercial *S. cerevisiae* strain QA23. As a control, we used the same synthetic media with usual nitrogen content in natural grape musts (300 mg/l; CNC). Media density and consumption of yeast assimilable nitrogen (YAN) and ammonium were monitored throughout these fermentations (Figure 1).

Figure 1. Must density evolution and nitrogen consumption during control (CNC), high (HNC) and low (LNC) nitrogen content fermentations.



Fermentations were completed after 96 hours, 120 hours and 240 hours for the CNC, HNC and LNC fermentations, respectively. In the HNC fermentation, yeasts only consumed approximately 30% of the total YAN and 40% of the initial ammonium. Most of the assimilable nitrogen was consumed in the first 24 hours of the three fermentations. Biomass production did not show any differences between the HNC and the CNC fermentations (maximum OD_{600} = 4.37 and 4.31, respectively) but it was significantly lower in the LNC fermentation (maximum OD_{600} = 3.85).

Figure 2. Must density evolution and nitrogen consumption during low nitrogen content fermentations: mixture of ammonia and amino acids (LNC), only ammonia (LNC-NH4) and only amino acids (LNC-aas).



To check the importance of the nitrogen source, we complemented these results with two new fermentations. The synthetic grape must of these fermentations contained the same amount of YAN as in the LNC fermentation (60 mg/l), but the mixture of ammonium and amino acids was replaced by only ammonium (LNC-NH4) or only amino acids (LNC-aas) (Figure 2).

The extracellular amino acids were also determined at different points for all the fermentations. Table 3 shows the consumption of the amino acids and ammonium at the end of the CNC and HNC fermentations. The excess nitrogen in HNC fermentation decreased the proportion of uptake of some amino acids (represented by group A) and increased the consumption of others (represented by group C). Group B represented the amino acids that were taken up in similar proportions in both fermentations.

Table 3. Consumption (expressed as mg l^{-1} and percentage of the total YAN consumed) of ammonia and amino acids at the end of the Control Nitrogen Content (CNC) and High Nitrogen Content (HNC) fermentations. Ratio between HNC and CNC consumption (%) is also indicated. Nitrogen sources are grouped as: A: Ratio < 1; B: Ratio 1-2; C: Ratio > 2.

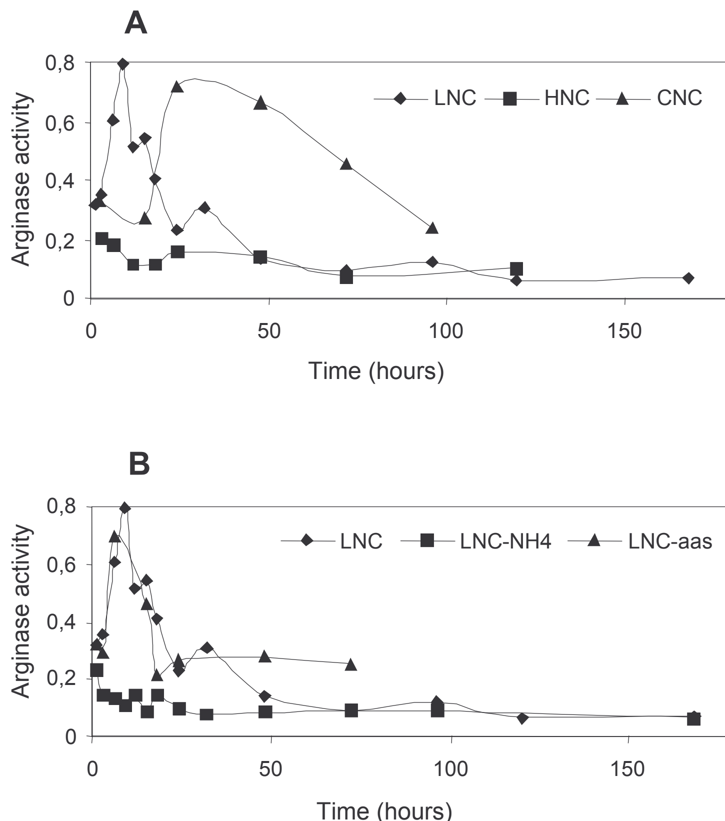
	Consumption (mg/l)		**Consumption (%)**			**Consumption**
	CNC	**HNC**	**CNC**	**HNC**	**Ratio**	**group**
Amino acid						
Asp	27,12	27,43	1,01	0,74	0,73	**A**
Glu	94,64	18,64	3,54	0,50	0,14	**A**
Ser	68,60	103,29	2,56	2,80	1,09	**B**
Gln	505,34	526,46	18,89	14,25	0,75	**A**
His	32,65	84,57	1,22	2,29	1,88	**B**
Gly	3,02	11,12	0,11	0,30	2,67	**C**
Thr	80,74	122,88	3,02	3,33	1,10	**B**
Arg	328,71	11,20	12,29	0,30	0,02	**A**
Ala	103,97	0,10	3,89	0,00	0,00	**A**
Tyr	9,69	38,38	0,36	1,04	2,87	**C**
Cis	3,01	55,34	0,11	1,50	13,31	**C**
Val	31,52	99,97	1,18	2,71	2,30	**C**
Met	24,10	85,08	0,90	2,30	2,56	**C**
Trp	87,84	184,88	3,28	5,00	1,52	**B**
Phe	31,91	149,92	1,19	4,06	3,40	**C**
Ile	23,83	120,88	0,89	3,27	3,67	**C**
Leu	43,11	87,24	1,61	2,36	1,47	**B**
Lys	14,81	61,77	0,55	1,67	3,02	**C**
Total	1514,61	1784,36	56,63	48,30	0,85	
Amonia N	116,00	191,00	43,37	51,70	1,19	**B**
Total YAN	267,46	369,44	100,00	100,00	1,00	

Arginase activity

We determined the arginase activity of the yeast cells collected during the various fermentations (Figure 3). As far as the nitrogen concentration is concerned (Figure 3a), the excess nitrogen in the HNC fermentation prevented the activation of this enzyme (below 0.2 nmol x μg protein^{-1} x min^{-1} throughout the process). On the other hand, in the CNC and LNC fermentations, we detected the activation of this enzyme at the time that the ammonium was completely consumed. This fermentation point also matched with the beginning of the arginine uptake (data not shown).

As far as the nitrogen source is concerned (Figure 3b), the activity of arginase increased in the LNC-aas condition during the first few hours of fermentation. On the other hand, the lack of amino acids in the LNC-NH4 condition kept arginase activity low throughout the fermentation.

Figure 3. Arginase activity throughout (A) CNC, HNC and LNC fermentations and (B) LNC, LNC-NH4 and LNC-aas fermentations. Arginase activity was expressed as: nmol ornithine x μg protein^{-1} x min^{-1}.



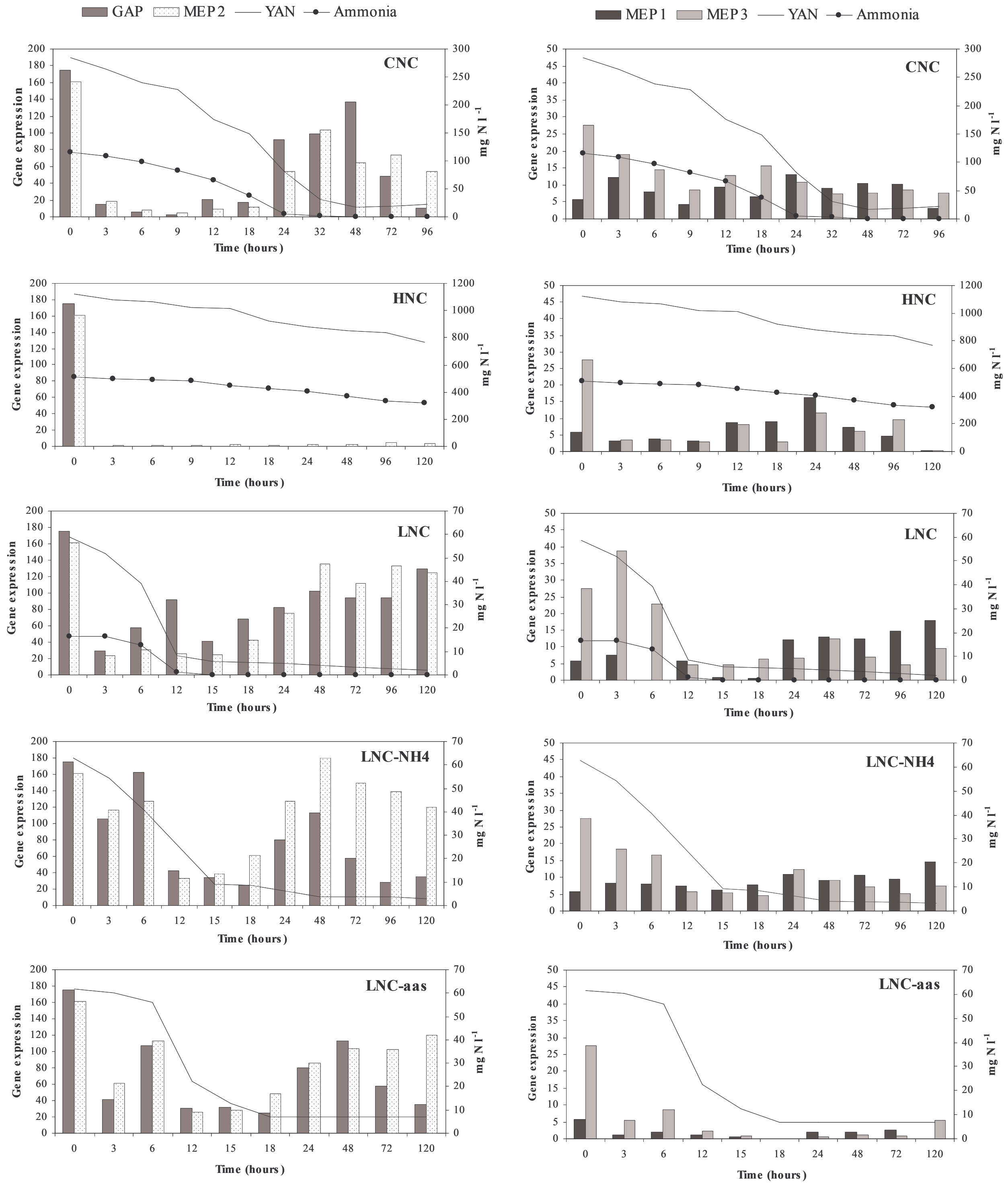
***GAP1* and *MEP*s gene expression**

We analysed the transcriptional activity of *GAP1, MEP1*, *MEP2* and *MEP3* genes during the alcoholic fermentations under several nitrogen concentrations (Figure 4). We grouped the results of *GAP1* and *MEP2* because the level of expression and type of response were very similar and much higher than for *MEP1* and *MEP3*.

In the CNC and HNC fermentations, *GAP1* and *MEP2* were repressed in the first hours after the inoculation in the must-like media. On the other hand, both genes were activated/derepressed in the CNC fermentation when ammonia was depleted, even though the YAN concentration in the media was still high (81,2 mg l^{-1}). In the fermentations with low nitrogen content (LNC), this repression after the inoculation was also observed but it was slower and not so stronger as this one detected in the HNC and CNC fermentations. The lowest values of gene expression were detected in the three LNC fermentations from 12 to 18 hours after inoculation. Afterwards, the expression level of these genes increased when nitrogen was depleted.

As mentioned above, *MEP1* and *MEP3* showed a lower level of gene expression than *MEP2* (Figure 4). *MEP3* was repressed after the inoculation and this repression was quicker and stronger in the medium with high nitrogen content. The gene expression profile of *MEP1* throughout the fermentation registered few changes. However, the highest repression in both genes was detected in the medium with low nitrogen content but without ammonium (LNC-aas).

Figure 4. Relative gene expression of ammonia permeases (*MEP1*, *MEP2*, *MEP3*) and general amino acid permease (*GAP1*) at time zero (before inoculation) and at several fermentation points in all nitrogen conditions studied. The data were quantified by calculating the ratio between the concentration of the studied genes normalized with the concentration of the housekeeping gene (Actine), and expressed as percentage (the quantity ratio 1 was set as 100%). YAN and ammonia consumption along the fermentations are also indicated.



DISCUSSION

A commercial wine yeast has been inoculated in a synthetic grape must at several concentrations and sources of nitrogen. CNC fermentation could not be considered as nitrogen-limited because biomass and fermentation rate was very similar to the HNC fermentation. LNC fermentations were clearly nitrogen-limited, with a lower biomass production and a slower fermentation rate than the CNC fermentation. During the CNC and LNC fermentations, the cells evolved from a nitrogen-repressed situation at the beginning of the process to a nitrogen-derepressed situation as the nitrogen is consumed. Accordingly, the high concentration of nitrogen in the HNC fermentation maintained the nitrogen repression throughout the process. These nitrogen-repressed/derepressed conditions determined the different patterns of amino acid consumption. An NCR condition (as in the HNC fermentation) inhibited the arginine and alanine uptake and led to a lower consumption of glutamic acid, aspartic acid and glutamine. Arginine and alanine must be mainly transported by the general amino acid permease (Gap1p) or by other specific permeases also subjected to NCR. On the other hand, the consumption of branched-chain and aromatic amino acids was higher in the HNC fermentation, which might be explained by a stimulation of their specific permeases. The presence of some amino acids in the medium induced the transcription activity in the genes encoding the branched-chain amino acid permease Bap2p or the tyrosine and tryptophan permease Tat1p in ammonium-grown cells [5, 13]. Branched-chain amino acids are the principal precursors of higher alcohols during wine fermentation, significant for the sensory properties of the wine. Therefore, the degree of uptake of these amino acids at the different nitrogen conditions may influence the production of fusel alcohols in the wine [7].

The ammonium uptake was higher in the HNC fermentation than in the CNC fermentation. This higher consumption of ammonium might also influence the production glycerol [14], which is also an important metabolite in the composition of the wine. Curiously, the repression of the three *MEP* genes was stronger in the HNC fermentation than in the CNC fermentation. Marini et al. [6] tried to explain this paradox by two possible hypothesis: either the yeast might posses additional $NH4^+$ transport systems unrelated to the Mep proteins or NH_4^+ at a high concentration might be taken up into cells by simple diffusion. Regarding the transcription profile of these genes, our results confirmed the highest transcription activity in *MEP2* and its regulation by NCR [6]. The transcription profiles of *MEP1* and *MEP3* during

fermentation did not respond to NCR as clearly as the transcription profile of *MEP2* did. The absence of ammonium in the medium (LNC-aas fermentation) caused the strongest repression in *MEP1* and *MEP3* throughout the fermentation. Therefore, the extracellular ammonium may act as an inducer of the expression of these genes.

Recently, Carrasco *et al.* [11] suggested that the argininase activity was a useful marker for the intracellular nitrogen shortage and a good indicator of the availability of nitrogen during wine fermentations. Our results showed that activation coincided with the beginning of arginine uptake. As already mentioned, this time point also coincided with ammonium depletion and *GAP1* activation/derepression. That is, arginase activity could be considered as a good marker of the shift from a repressed to a derepressed nitrogen condition during alcoholic fermentation. However, this is only true when this amino acid is present in the medium.

The objective of this study was to apply the basic knowledge about nitrogen regulation to a wine fermentation conditions by using a commercial wine yeast strain. The repression of *GAP1* and *MEP2* genes in the cells, low arginase activity or inhibition of arginine uptake could be considered as a good NCR markers. Winemakers systematically supplement grape musts with diammonium phosphate to prevent nitrogen-related fermentation problems. Greater knowledge of this system should improve the control of nitrogen availability and addition during wine fermentations. A nitrogen-repressed condition throughout fermentation modifies the uptake of ammonium and amino acid and this different uptake may determine the production of important secondary metabolites. Moreover a wine with an excess of nitrogen could be detrimental for the microbiology stability during ageing, storage or bottling.

ACKNOWLEDGEMENTS

This work was supported by grant AGL2000-0205-P4-03 from the *Comisión Interministerial de Ciencia y Tecnología*, Spain. The authors wish to thank the language service of the Rovira i Virgili University for revising the manuscript.

REFERENCES

[1] Henschke, P.A. and Jiranek, V. (1991) Hydrogen sulfide formation during fermentation: Effect of nitrogen composition in model grape must. In: Proceedings of the International Symposium on Nitrogen in Grapes and Wine (Rantz JM, Eds.), Am. Soc. Enol. Vitic. Davis, CA. 172-184.

[2] Wiame, J.M., Grenson, M. and Arst, HNJR. (1985) Nitrogen catabolite repression in yeast and filamentous fungi. Adv. Microb. Physiol. 26, 1-88.

[3] Magasanik, B. (1992) Regulation of nitrogen utilization. In: The molecular and cellular biology of the yeast *Saccharomyces*: Gene expression. (Jones, E.W., Pringle and J.R., Broach, J.R., Eds.), pp. 283-317. Cold Spring Harbor Laboratory Press, Cold Spring Harbor: NY, USA.

[4] Ter Schure, E.G., van Riel, N.A.W. and Verrips, C.T. (2000). The role of ammonia metabolism in nitrogen catabolite repression in *Saccharomyces cerevisiae*. FEMS Microbiol. Rev. 24, 67-83.

[5] Forsberg, H. and Ljungdahl, O. (2001) Sensors of extracellular nutrients in *Saccharomyces cerevisiae*. Curr. Genet. 40, 91-109.

[6] Marini, A.M., Soussi-Boudekou, S., Vissers, S. and Andre, B. 1997. A Family of Ammonium Transporters in *Saccharomyces cerevisiae*. Mol. Cell. Biol. 17, 4282-4293.

[7] Marks, V.D., van der Merwe, G.K. and van Vuuren, H.J.J. (2003) Transcriptional profiling of wine yeast in fermenting grape juice: regulatory effect of diammonium phosphate. FEMS Yeast Res. 3, 269-287.

[8] Riou, C., Nicaud, J.M., Barre, P. and Gaillardin, C. (1997) Stationary-phase gene expression in *Saccharomyces cerevisiae* during wine fermentation. Yeast 13, 903-915.

[9] Aerny, J. (1996) Composés azotés des moûts et vins. Revue suisse Vitic. Arboric. Hortic. 28, 161-165.

[10] Chinard, F.P. (1952) Photometric estimation of proline and ornithine. J. Biol. Chem. 199, 91-95.

[11] Carrasco, M.P., Pérez-Ortín, J.E. and del Olmo, M. (2003) Arginase activity is a useful marker of nitrogen limitation during alcoholic fermentation. Syst. Appl. Microbiol. 26, 471-479.

[12] Sierkstra, L.N., Verbakel, J.M.A. and Verrips, C,T. (1992) Analysis of transcription and translation of glycolytic enzymes in glucose-limited continuous cultures of *Saccharomyces cerevisiae*. J. Gen. Microbiol. 138, 2559-2566.

[13] Regenberg, B., During-Olsen, L., Kielland-Brandt, M.C. and Holmberg, S. (1999) Substrate specificity and gene expression of the amino-acid permeases in Saccharomyces cerevisiae. Curr. Genet. 36, 317-28.

[14] Albers, E., Larson, C., Lidén, G., Niklasson, C. and Gustafsson, L. (1996) Influence of the nitrogen source on *Saccharomyces cerevisiae* anaerobic growth and product formation. Appl. Environ. Microbiol. 62, 3187-3195.

CHAPTER 4

Influence of the timing of nitrogen additions during synthetic grape must fermentations on fermentation kinetics and nitrogen consumption

Gemma Beltran[1], Braulio Esteve-Zarzoso[2], Nicolas Rozès[1], Albert Mas[1] and José M. Guillamón[1]

[1]Unitat d'Enologia del Centre de Referència de Tecnologia d'Aliments. Dept. Bioquímica i Biotecnologia. Facultat d'Enologia de Tarragona. Universitat Rovira i Virgili. Marcel·lí Domingo, s/n 43007 Tarragona, Spain

[2]Bodegas Miguel Torres. Miquel Torres i Carbó 6, 08720 Vilafranca del Penedès, Barcelona, Spain

Journal of Agricultural and Food Chemistry

(*In press*)

ABSTRACT

Nitrogen deficiencies in grape musts are one of the main causes of stuck or sluggish wine fermentations. In the present study, we have supplemented nitrogen-deficient fermentations with a mixture of ammonium and amino acids at various stages throughout the alcoholic fermentation. The timing of the nitrogen additions influenced the biomass yield, the fermentation performance, the patterns of ammonium and amino acid consumption, and the production of secondary metabolites. These nitrogen additions induced a nitrogen-repressed situation in the cells, and this situation determined which nitrogen sources were selected. Glutamine and tryptophan were the main amino acids consumed in all the fermentations. Ammonium is the preferred nitrogen source for biomass production but was hardly consumed when it was added in the final stages of the fermentation. The higher ammonium consumption in some fermentations correlated with a greater synthesis of glycerol, acetate and acetaldehyde but with a lower synthesis of higher alcohols.

Keywords: *Saccharomyces cerevisiae*, Alcoholic Fermentation, Amino acids, Ammonium, *GAP1, MEP2*.

INTRODUCTION

The nitrogen composition of grape musts affects the growth and metabolism of yeast, the fermentation rate and the completion of fermentation (*11*). Nitrogen deficiencies are one of the main causes of stuck or sluggish fermentations. One way of avoiding these problems is to add nutritional supplements, usually inorganic forms of nitrogen such as ammonium salts, to grape must prior to fermentation (*9;16;26*). These additions are generally made empirically in wine cellars, and the initial nitrogen concentration in the must or the nitrogen requirements of the usual yeast strain used in the cellar are not determined. Yeasts respond metabolically to differences in nitrogen availability so this lack of control of nitrogen leads to differences in wine composition.

Nitrogen affects yeast cells in two ways: it increases biomass production and stimulates the rate of sugar utilization. Nitrogen additions during the period of cell growth have resulted in maximum cell populations. Later additions during the stationary phase have had no effect on the cell population, but have increased the specific fermentation rate, thus reducing the length of the fermentation (*9;10;24*).

Nitrogen supplementation affects the pattern of nitrogen uptake. Ammonium is a preferred yeast nitrogen source and, when plentiful, it represses the expression of catabolic pathways by degrading other nitrogenous compounds (*12;37*). This mechanism, called Nitrogen Catabolite Repression (NCR), has recently been studied during wine fermentations (*8*). It inhibits the uptake of arginine and alanine, and stimulates the consumption of branched-chain and aromatic amino acids. Changes in the nitrogen uptake patterns influence the production of aroma and spoilage compounds (particularly hydrogen sulfide), and the amount of urea, the major precursor of the carcinogen ethyl carbamate (*18;23;27;31*).

The volatiles identified in wines are usually dominated by fermentation products. Organic acids, higher alcohols and esters are the main group of flavor compounds coming from yeast metabolism (*31*). Higher alcohols can be produced either by the catabolic conversion of the branched chain amino acids (via Ehrlich) or by the anabolic formation of these amino acids *de novo* from a sugar substrate (*6*). An excess of higher alcohols (above 400 mg L^{-1}) can be regarded as a negative influence on the quality of wine but at the concentrations generally found in wines (below 300 mg L^{-1}), they usually contribute to the desirable complexity of wine. Furthermore, these alcohols, together with the acids in wine, are substrates for ester formation. Most esters, with the

exception of ethyl acetate, impart a pleasant smell of fruits and flower notes in the wine (*19*).

As mentioned, in winemaking most of the nitrogen additions are made empirically and do not take into account the different nitrogen needs of the cell during wine fermentation, the proper timing of these additions or the nitrogen source added. In this study, we supplemented nitrogen-deficient fermentations with a mixture of ammonium and amino acids at different stages of the alcoholic fermentation. We then studied the effect of these additions on the fermentation kinetics, the consumption of organic and inorganic nitrogen throughout the fermentation and the influence of this consumption on the organoleptic profile of the wines. We also monitored the effect of the nitrogen supplementations on the NCR system and the effect of the nitrogen-repressed situation on nitrogen uptake.

MATERIALS AND METHODS

Strain, fermentations and sampling

A commercial *Saccharomyces cerevisiae var. bayanus* wine strain QA23 (Lallemand S.A., Toulouse, France) was used in this study. Fermentations were carried out in a synthetic grape must (pH 3.3) as described by Riou et al. (*33*) but with 200 g L^{-1} of reducing sugars (100 g L^{-1} Glucose and 100 g L^{-1} Fructose) and without anaerobic factors. Only the nitrogen content changed in the different fermentations.

The Yeast Assimilable Nitrogen (YAN) content in the control synthetic grape must was 300 mg N L^{-1}, ammoniacal nitrogen (NH_4Cl) 120 mg N L^{-1}, and amino acids 180 mg N L^{-1} (Table 2). This medium also contained 426 mg L^{-1} of Proline but it should not be considered as assimilable nitrogen (*34*). The proportions of the different amino acids and ammonium were maintained in all the fermentations. Nitrogen-limited fermentations were carried out with 60 mg L^{-1} of YAN (24 mg L^{-1} of ammoniacal nitrogen and 36 mg L^{-1} of amino acid nitrogen), and 240 mg L^{-1} of YAN nitrogen was added at different fermentation points (96 mg L^{-1} of ammoniacal nitrogen and 144 mg L^{-1} of amino acid nitrogen).

The supplementation points were chosen by monitoring the decrease in density of the media. Density was measured throughout the fermentation by weighing 5 mL, and nitrogen was added when the density of the must was: 1060 g L^{-1} (30 hours after inoculation), 1040 g L^{-1} (72 hours), 1020 g L^{-1} (144 hours) and 1000 g L^{-1} (240 hours).

Fermentations took place at room temperature (22-28ºC) in laboratory-scale fermenters: 2 L bottles filled with 1.8 L of medium and fitted with closures that enabled the carbon dioxide to escape and the samples to be removed. Fermentations were in semi-anaerobic conditions, since limited aeration was necessary in order to harvest samples for the subsequent analysis. The population inoculated in every flask was $2x10^6$ cell mL^{-1} from dry yeast rehydrated in water at 37ºC.

In the latter stages of the fermentation, the sugar consumption was assayed by enzymatic kits (Roche Applied Science, Germany). Fermentation was considered to be complete when the residual sugars were below 2 g L^{-1}. Cell growth was determined by absorbance at 600 nm. Absorbance values were corrected for the initial absorbance reading obtained for juice.

Cells were harvested at different points during the fermentation so that mRNA could be analysed. Flasks were magnetically stirred to resuspend settled biomass, transferred to centrifuge tubes and centrifuged at 5000 rpm for 5 min at room temperature to prevent temperature shock. Cell pellets were transferred to 1.5 mL Eppendorf tubes and frozen immediately in liquid nitrogen. They were kept at –80 ºC until they were analysed. The supernatant of these samples was stored at –20 ºC for extracellular metabolites and nitrogen content analysis.

Nitrogen content analysis

YAN was analysed by the formol index method (*1*), and the ammonium content was quantified using an enzymatic method (Roche Applied Science, Germany). The individual amino and imino acids were analysed by OPA and FMOC derivatizations, respectively, using the Agilent 1100 Series HPLC equipped with a low pressure gradient quaternary pump, a thermostatted autosampler, a DAD ultraviolet detector and a fluorescence detector (Agilent Technologies, Germany). The sample (2 μL) was injected into a 4.6 x 250 mm x 5 μm Hypersil ODS column (Agilent Technologies, Germany). The gradient solvent system was: solvent A (16 mM sodium acetate and 0.022 % triethylamine, adjusted to pH 7.2 with 1-2 % acetic acid, and 0.6 % tetrahydrofuran) from 100 % at time 0 to 0 % at time 18 min, and solvent B (20 % of 66 mM sodium acetate, adjusted to pH 7.2 with 1-2 % acetic acid, 40 % acetonitrile and 40 % methanol) from 0 % at time 0 to 100 % at time 18 min. The analysis temperature was 40 ºC, and the flow rate was 1.5 mL min^{-1}. Several dilutions of each sample were analysed and averaged using the analysis software. The concentration of each amino

acid was calculated using external and internal standards, and expressed as mg L^{-1}. The software used was Agilent ChemStation Plus (Agilent Technologies, Germany).

Ethanol, glycerol and organic acid analysis

Ethanol, glycerol and organic acids were analysed in all the samples at the end of the fermentation process. Analytical HPLC was carried out on a Hewlett-Packard HP 1050 connected to a Hewlett-Packhard Integrator 3395 equipped with an HP 1047 RI detector (Agilent Technologies, Wilmington, DE, USA) (*38*). The wine sample (450 μL) was mixed with 50 μl of formic acid (internal standard), and 25 μl was injected into a 300 x 7.8 mm AMINEX HPX-87H column (BioRad, Hercules, CA, USA). The solvent used was sulfuric acid 2.5 mM at 0.5 mL min^{-1}. The analysis temperature was 60ºC. The concentration of each metabolite was calculated using external and internal standards.

Fatty acid analysis

Fatty acids were extracted using the method published by López *et al.* (*20*). Analytical GC was carried out on a Hewlett Packard 6890N connected to a computer with the ChemStation software (Agilent Technologies, Wilmington, DE, USA). The extract (2 μL) was injected (splitless, 0.75 minutes) into a Tracer TR column of 60 m x 250 μm and 0.25 μm phase thickness with an HP automatic injector (Agilent). The temperature program was 40ºC for 5 min followed by 2ºC min^{-1} to 240 ºC (15 min.). Injector and detector temperatures were 220 and 240ºC, respectively. The carrier gas was hydrogen at 60 mL min^{-1}. 2-ethylphenol (0.2 mg L^{-1}) was added as internal standard. Internal patterns were used to estimate the quantity of the different compounds.

Analysis of higher alcohols and esters

Higher alcohols and esters were extracted by liquid/liquid extraction (wine 10 ml, 200 μL 1,1,2-Trichlorotrifluoroethane, 0.5 g NaCl), with n-decanol (0.2 mg L^{-1}) as internal standard (*13*). After agitation for 2 min and centrifugation, the organic phase was extracted and 2 μL was injected. The chromatographic program used is the same as that used for the fatty acid analysis. Quantification was conducted by comparison with known quantities of different products in a hydroalcoholic solution.

RNA extraction and cDNA synthesis

Total RNA was isolated from yeast samples as described by Sierkstra *et al.* (*36*) and resuspended in 50 μL of DEPC-treated water. Total RNA suspensions were purified using the High Pure Isolation kit (Roche Applied Science, Germany) following the protocol provided by the manufacturer. RNA concentrations were determined using a GenQuant spectrophotometer (Pharmacia, Canada) and the quality of RNA was verified electrophoretically on 0.8% agarose gels. Solutions and equipment were treated so that they were RNase free, as outlined in Sambrook *et al.(35)*.

Total RNA was reverse-transcripted with Superscript^{TM} II RNase H^{-} Reverse Transcriptase (Invitrogen, USA) in a GenAmp PCR System 2700 (Applied Biosystem, USA). 0.5 μg of Oligo $(dT)_{12\text{-}18}$ Primer (Invitrogen, USA) was used with 0.8 μg of total RNA as template in a reaction volume of 20 μl. Following the protocol provided by the manufacturer, after denaturation at 70ºC for 10 min, cDNA was synthesized at 42ºC for 50 min. Finally, the reaction was inactivated at 70ºC for 15 min.

Real-time quantitative PCR

The PCR primers used in this study are: *ACT-F*: TGGATTCCGGTGATGGTGTT and *ACT-R*: CGGCCAAATCGATTCTCAA (*ACT*, for actine gene); *GAP1-F*: CTGTGGATGCTGCTGCTTCA and *GAP1-R*: CAACACTTGGCAAACCCTTGA (*GAP1*, for general amino acid permease gene); *MEP2-F*: GGTATCATCGCTGGCCTAGTG and *MEP2-R*: ACAACGGCTGACCAGATTGG (*MEP2*, for ammonium permease gene) (*8*). They were all designed with the available GenBank sequence data and the Primer Express software (Applied Biosystems, USA) in accordance with the Applied Biosystems guidelines for designing PCR primers for quantitative PCR. All amplicons were short, which ensured maximal PCR efficiency and, therefore, the most precise quantification.

For each gene, a standard curve was made with yeast genomic DNA. DNA extraction was performed as described by Querol *et al.* (*29*), digested by RNase and isolated by two-fold phenol-chloroform extractions and ethanol precipitation. Concentration was determined using a GeneQuant spectrophotometer (Pharmacia, Canada). Serial 10-fold dilutions of DNA were carried out to yield DNA concentrations from 4 to 4 x 10^{-5} ng μL^{-1}. These dilution series were amplified (in duplicate) by SYBR PCR for each gene to obtain standard curves (see above). The standard curve displays the Ct value *vs.* $\log_{10}$ of each standard's starting quantity. The starting quantity of the unknown samples was

calculated against the standard curve by interpolation, and gene expression levels are shown as the concentration of the studied gene normalized with the concentration of the housekeeping *ACT* gene.

The Real-Time Quantitative PCR reaction was performed using SYBR® Green I PCR (Applied Biosystems, USA). In SYBR PCR, amplification is monitored by the gain in fluorescence of the double-strand specific DNA-binding dye SYBR green. The 25 μL SYBR PCR reactions contained 300 nM of each PCR primer, together with 1 μL cDNA (or 5 μL of each DNA serial dilution for standard tubes) and one time SYBR master mix (Applied Biosystems, USA).

All PCR reactions were mixed in 96-well optical plates (Applied Biosystems, USA) and cycled in a PE Applied Biosystems 5700 thermal cycler under the following conditions: 50ºC for 2 min, 95ºC for 10 min, and 40 cycles at 95ºC for 15 sec and at 60ºC for 60 sec.

The PE5700 cycler provided cycle-by-cycle measurement of the fluorescence emission from each PCR reaction. Analysis resulted in the assignation of a threshold cycle (C_t) value to each PCR reaction. The C_t value is the cycle number at which an increase in reporter fluorescence above a baseline signal can first be detected. The threshold was positioned to intersect the exponential part of the amplification curve of positive reactions, as recommended by Applied Biosystems. The C_t value is inversely proportional to the log of the amount of template in the PCR reaction; the lower the C_t value, the higher the concentration of template in the PCR reaction. Assuming a 100 % effective PCR amplification, a difference of one C_t value corresponds to a 2^1 = 2-fold difference in the amount of template. All samples were analysed in duplicate and the expression values were averaged by the analysis software (Applied Biosystems, USA). The coefficient of variation in all samples analysed was less than 10 %.

RESULTS

Effect of nitrogen addition on fermentation kinetics and nitrogen consumption

Five fermentations started with a nitrogen content of 60 mg L^{-1}, which is low enough for a fermentation to be sluggish but high enough for it to finish. Four of these nitrogen-deficient fermentations were supplemented at different points with 240 mg L^{-1} of YAN, the first one at a density of 1060 g L^{-1} and the second, third and fourth at 1040, 1020 and 1000 g L^{-1} respectively. The remaining fermentation was not supplemented, but

subjected to nitrogen deficiency throughout the process. As a fermentation control, we used the same medium with a non-deficient amount of nitrogen (300 mgN L^{-1}) (*8*).

Figure 1 shows the effect of nitrogen additions on O.D. measures throughout the fermentations studied. The nitrogen-deficient fermentations had lower O.D. values than the control fermentation. When nitrogen was added in the first half of the fermentations (density of 1060 and 1040), these effects were almost overcome, and the O.D. values were similar to those of the control fermentation. Additions at densities of 1020 and 1000, however, had minimal effects on O.D. measures.

Figure 1. Effect of nitrogen additions on O.D. measures (λ = 600 nm) throughout synthetic grape must fermentations. The arrows indicate the time of addition.

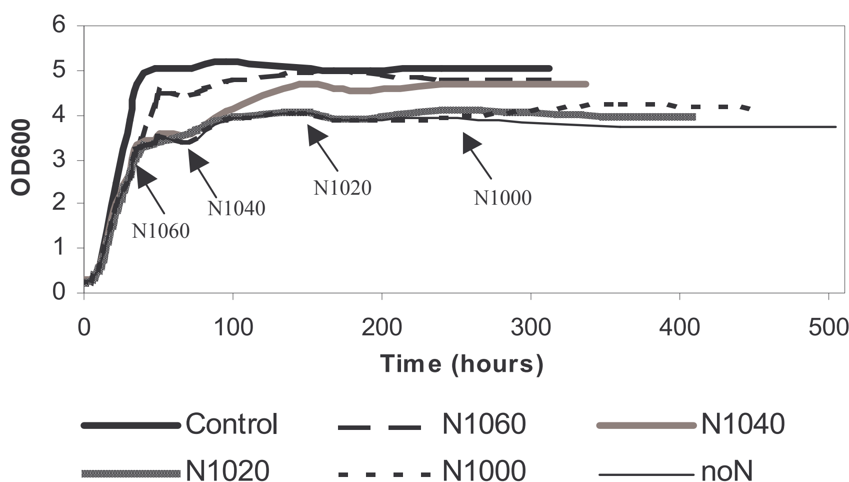


Table 1 summarizes the evolution of the fermentation and nitrogen consumption, measured as ammonium and amino acid nitrogen. Unlike their effect on the O.D. values, the nitrogen additions clearly stimulated the fermentation regardless of when they were made. In the nitrogen-deficient fermentation, yeast consumed the total YAN after the first day (data not shown). However, nitrogen was not completely consumed in the control fermentation. The nitrogen additions were all carried out when the initial YAN had already been depleted, and the later the nitrogen was added, the lower the amount of YAN was consumed (Table 2).

The ammonium consumed was 54% of the total YAN consumed in the control fermentation (Table 2) but this proportion decreased when nitrogen was added later in the fermentation. Ammonium was proportionally preferred as the nitrogen source when the additions were made in the first half of the fermentations (N1060 and N1040). In later additions (N1020 and N1000), the small amount of nitrogen consumed was mostly from amino acids.

Table 1. Determination of Yeast Assimilable Nitrogen (YAN) in the fermentation media, represented by the amino acid fraction (YAN aas) and by the ammonium fraction (YAN NH_4^+).

density (ρ)	control fermentation time (h)	YAN NH_4^+ (mg N L^{-1})	YAN aas (mg N L^{-1})	N addition at $\rho = 1060$ time (h)	YAN NH_4^+ (mg N L^{-1})	YAN aas (mg N L^{-1})	N addition at $\rho = 1040$ time (h)	YAN NH_4^{2+} (mg N L^{-1})	YAN aas (mg N L^{-1})
1080	0	120	168	0	25	41	0	25	40
1060	30	70	98	36	0.5 (90[a])	4 (145[a])	36	0.1	4
1040	56	52	95	62	50	105	78	0.1 (92[a])	4 (136[a])
1020	96	36	98	96	45	104	120	68	104
1000	168	35	98	168	42	108	192	65	110
990 (end[b])	312	41	102	312	50	110	336	66	114

density (ρ)	N addition at $\rho = 1020$ time (h)	YAN NH_4^+ (mg N L^{-1})	YAN aas (mg N L^{-1})	N addition at $\rho = 1000$ time (h)	YAN NH_4^+ (mg N L^{-1})	YAN aas (mg N L^{-1})	no N addition time (h)	YAN NH_4^+ (mg N L^{-1})	YAN aas (mg N L^{-1})
1080	0	25	41	0	26	40	0	26	40
1060	36	0	7	36	0	7	36	0	5
1040	78	0	7	78	0	4	78	0	2
1020	150	0 (92[a])	4 (143[a])	150	0	4	150	0	1
1000	240	81	109	264	0 (99[a])	2 (147[a])	264	1	1
990 (end[b])	408	88	118	456	100	130	504	1	1

[a] NH_4^+ and aas YAN content just after the nitrogen addition. [b] End of fermentation.

Table 2. Total consumption of amino acids and ammonia at the end of each fermentation expressed as mg N L^{-1} [a]

amino acids	full N media content	control consumption total	N1060 consumption total	N1060 post-add	N1040 consumption total	N1040 post-add	N1020 consumption total	N1020 post-add	N1000 consumption total	N1000 post-add	no N consumption total
Gln	47.4	25.5	34.9	22.1	24.5	11.8	29.0	16.2	23.1	10.3	12.8
Trp	10.3	6.6	6.8	4.2	7.7	4.1	8.1	4.5	7.8	4.1	3.6
Thr	9.9	9.4	3.6	1.4	2.6	0.7	2.0	0.2	1.9	0.1	1.8
His	3.8	3.7	1.5	1.5	1.2	1.2	0.8	0.8	0.4	0.4	–
Leu	4.7	4.2	2.6	1.1	1.8	0.8	1.4	0.4	1.2	0.2	1.0
Ile	3.8	3.2	1.8	0.6	1.4	0.7	0.8	0.2	0.7	0.1	0.6
Phe	2.8	2.4	1.2	0.4	1.3	0.5	1.2	0.4	0.9	0.1	0.8
Val	5.1	3.0	1.6	0.4	1.1	0.1	1.0	–	1.1	–	1.0
Ser	7.5	2.8	2.9	0.8	1.7	–	1.7	–	1.9	0.1	1.8
Met	1.8	1.8	1.1	0.5	1.0	0.6	0.6	0.3	0.5	0.2	0.3
Lys	1.9	1.1	0.7	–	1.0	0.9	1.0	0.9	0.1	–	0.2
Arg	47.9	0.6	5.9	–	9.0	0.9	8.8	0.7	8.2	–	8.2
Tyr	1.4	0.4	0.2	–	0.3	–	0.3	–	0.3	–	0.3
Glu	11.8	0.5	2.3	–	2.0	–	2.7	0.2	2.5	–	2.5
Gly	3.1	0.2	–	–	–	–	–	–	0.3	–	0.4
Ala	12.1	–	1.2	–	1.7	–	2.7	–	3.1	–	3.2
Asp	4.2	–	–	–	–	–	–	–	–	–	0.1
YAN aas	179.5	65.5	68.4	34.5	58.2	22.2	62.1	24.6	53.9	15.7	38.6
YAN NH_4^+	120.0	79.2	64.9	39.5	51.4	26.0	29.5	4.1	25.4	–	25.4

[a] In the fermentations with nitrogen additions, post-add represents the nitrogen taken up after this addition.

The consumption of amino acids was monitored at different points during the fermentations. The yeast's pattern of amino acid utilization changes with the time of YAN supplementation (Table 2). The amino acids can be grouped in different sets according to the preference of the cell in the different conditions. The amino acids that are most consumed are glutamine and tryptophan. Together they represented 32% of the total assimilable amino acids of the synthetic grape must (Table 2) and, regardless of the fermentation conditions, their consumption accounted for 50% to 65% of the total amino acids consumed. On the other hand, the consumption of arginine, glutamate, glycine, alanine and aspartate, which were approximately 44% of the total assimilable amino acids in the medium (Table 2), was together hardly 2% of the total amino acids

consumed in the control fermentation. The consumption of these amino acids was much higher in the fermentations supplemented with nitrogen. However, yeast cells only consumed these amino acids before the nitrogen addition: that is, when the fermentations were nitrogen-deficient. Lastly, there is one other set of amino acids, consisting of threonine, histidine, leucine, isoleucine, phenylalanine, valine and methionine, which was consumed proportionally more in the control fermentation than in the supplemented fermentations.

GAP1 and *MEP2* gene expression

The expression of the nitrogen transporters *GAP1* and *MEP2* was analysed and quantified relative to the expression of the housekeeping actine gene. Time zero was the expression of yeast before inoculation (and after rehydration). Both genes were repressed in the first hours after inoculation in the must-like medium (Figure 2).

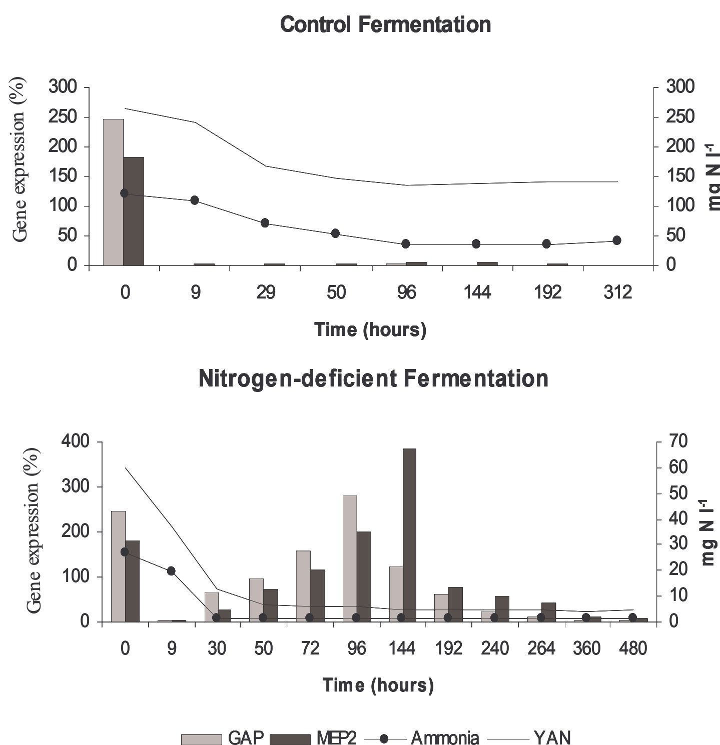


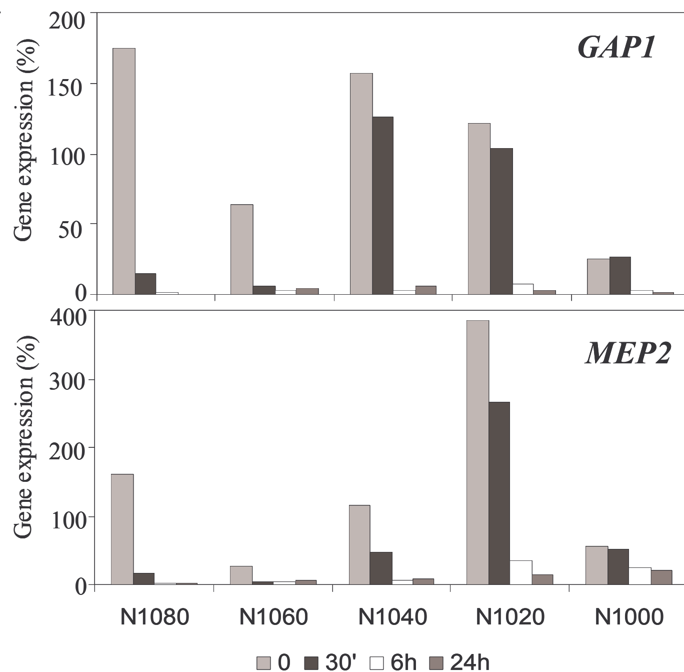
Figure 2. Gene expression of ammonium permease (*MEP2)* and general amino acid permease (*GAP1)* at time zero (before inoculation) and at different points during the control fermentation and the nitrogen-deficient fermentation (without nitrogen addition).
The data were quantified by calculating the ratio between the concentration of the studied genes normalized with the concentration of the housekeeping *ACT* gene, and expressed as a percentage (the quantity ratio 1 was set as 100%). YAN and ammonia consumption throughout the fermentations are also indicated.

In the nitrogen-deficient fermentation, these genes started to be activated/derepressed after 30 hours, when nitrogen was almost depleted. The expression of both genes increased continuously during the first days of fermentation and peaked after four and six days for *GAP1* and *MEP2*, respectively. The expression of the genes decreased in

the last days of fermentation, after several days without a nitrogen source. On the other hand, the presence of residual nitrogen in the control fermentation repressed these genes throughout.

Figure 3 shows the gene expression of *GAP1* and *MEP2* in the first twenty-four hours after the nitrogen addition. They were both repressed in all the fermentations. However, the later the addition took place in the fermentation process, the longer it took for the genes to be repressed. When nitrogen was added at the end of the fermentation (density 1000), the effect was negligible because of the low expression at this point.

Figure 3. Relative gene expression of *GAP1* and *MEP2* at different points in the first twenty-four hours after the nitrogen addition. Time zero represents the point just before this addition. The data were calculated as in Figure 2.



Analytical profile

We analysed the residual sugars, ethanol, glycerol and acids in the wines obtained from the different fermentations and such flavour compounds as higher alcohols, volatile fatty acids and esters, which arose from yeast metabolism (Table 3). The later the nitrogen addition was, the lower the concentration of glycerol, acetic acid and acetaldehyde was. The higher alcohol content was lower when excess nitrogen was available at the beginning of the fermentation (control fermentation and N1060). These different concentrations were accounted for by the increase in isoamyl alcohol and 2-phenyl ethanol. The concentration of these compounds increased considerably in the fermentations with nitrogen additions in the later phases (or no addition), and were approximately 2- and 5-times higher than in the control fermentation.

Table 3. Secondary metabolites produced by yeasts during the different fermentations [(*)].

	Control Ferm.	N1060	N1040	N1020	N1000	No N Addition
Alcohols and Acids ($g L^{-1}$)						
Ethanol	98.7	97.2	98.0	98.0	98.7	101.1
Glycerol	6.56	6.57	6.32	6.11	5.78	6.12
Acetate	1.17	1.22	0.98	0.80	0.89	0.81
Acetaldehyde	0.33	0.28	0.26	0.25	0.24	0.22
Citrate	0.41	0.41	0.38	0.41	0.39	0.41
Succinate	0.13	0.21	0.27	0.26	0.23	0.23
Lactate	0.04	0.05	0.04	0.03	0.02	0.02
Higher alcohols ($mg L^{-1}$)						
N-propanol	37	33	28	20	13	12
Isobutanol	11	13	16	16	16	16
Isoamylic alcohol	50	48	81	97	94	94
Phenyl-2-ethanol	11	21	42	46	53	43
∑	109	115	167	179	176	165
Fatty acids ($mg L^{-1}$)						
Isobutyric acid	0.39	0.40	0.43	0.39	0.50	0.41
Butyric acid	0.63	0.73	0.67	0.72	0.59	0.60
Isovaleric acid	0.63	0.37	0.30	0.44	0.80	0.60
Valeric acid	0.12	0.09	0.09	0.10	0.18	0.15
Hexanoic acid	2.06	1.58	1.40	1.53	1.90	1.85
Octanoic acid	2.30	1.95	1.84	2.34	2.43	2.49
Decanoic acid	0.39	0.37	0.21	0.19	0.14	0.46
Dodecanoic acid	0.16	0.14	0.09	0.16	0.32	0.28
∑	6.70	5.63	5.02	5.87	6.86	6.83
Acetate Esters ($mg L^{-1}$)						
Ethyl acetate	35	35	32	25	19	28
Isobutyl acetate	0.023	0.024	0.012	0.016	0.011	0.011
Isoamyl acetate	0.49	0.46	0.39	0.69	0.39	0.29
Hexyl acetate	0.006	0.005	-	-	-	-
Phenyl-2-ethanol acetate	0.21	0.37	0.41	0.47	0.41	0.29
∑	35.73	35.86	32.81	26.18	19.81	28.59
Fatty acid Esters ($mg L^{-1}$)						
Ethyl butyrate	0.224	0.220	0.163	0.232	0.164	0.124
Ethyl Isobutyrate	0.006	0.005	0.006	0.005	0.004	0.007
Ethyl hexanoate	0.089	0.071	0.23	0.31	0.23	0.085
Ethyl Octanoate	0.022	0.022	0.060	0.081	0.059	0.020
Ethyl Decanoate	0.002	0.004	0.019	0.024	0.019	0.004
∑	0.343	0.322	0.478	0.652	0.446	0.240

* Values are the average of two determinations and the coefficient of variation in all the compounds analysed was less than 10% with the exception of decanoic acid (18%), dodecanoic acid (38%), ethyl octanoate (16%) and ethyl decanoate (29%).
(-) Undetectable values

The increase in isoamyl alcohol did not lead to a corresponding clear increase in its ester (isoamyl acetate) in these fermentations and the phenyl-2-ethanol acetate ester only increased slightly. In fact, the differences in the concentration of the total acetate esters between the fermentations were due to the concentration of ethyl acetate, which was more than 95% of the total acetate esters. Its concentration was higher in the control fermentation and the N1060 and 1040 fermentations, which correlated with a higher acetate concentration. The differences in the concentration of fatty acids and their esters were smaller in the final products of the fermentations.

DISCUSSION

The addition of nitrogen to grape musts, especially in the form of ammoniacal nitrogen, is a common winemaking practice that prevents nitrogen-related fermentation problems. Several studies, in which grape musts were supplemented with diammonium phosphate, have proved that nitrogen supplements can optimise fermentation performance (*9;16;26*). In the present study, we supplemented a nitrogen-deficient synthetic must with a mixture of ammonium and amino acids at different stages of the alcoholic fermentation. Then we studied the effect of these additions on the fermentation kinetics, the consumption of organic and inorganic nitrogen throughout the fermentation and the influence of this consumption on the aroma compound profile of the wines.

We observed a reduction in the fermentation length regardless of the time of addition and, consequently, a reduction in the total fermentation time. However, the fermentation length decreased even further when nitrogen was added during the exponential phase and yeast cells probably used this nitrogen for biomass production. These results largely agree with those previously reported (*9;16;26*). However, the yeast strain QA23 used in this study seems to have low nitrogen requirements. It used only 147 mg NL^{-1} in the control fermentation and finished it with only 60 mg NL^{-1}. Agenbach (*2*) established that fermentations require a minimal amount of 140 mg NL^{-1} to avoid getting stuck. In fact, nitrogen demands and preferences are strain dependent (*17;21;28*) and, therefore, it should be taken into account that we only used one strain.

Like previous experimental studies (*9;24*), we observed that nitrogen additions during the period of cell growth resulted in an increase in cell biomass. During the cell growth phase of the fermentation, most carbon- and nitrogen-containing compounds are diverted to biomass production. When growth stops, however, only small amounts of these nutrients are required, primarily for cell maintenance (*3*).

The metabolism of nitrogen depends heavily on its uptake through the different nitrogen transporters. In this study, we monitored the activity of the genes encoding two important permeases in the transport of amino acids (*GAP1*) and ammonium (*MEP2*) throughout fermentation. In a previous study (*5*), we observed that both permeases were repressed in a nitrogen-rich medium by the mechanism called nitrogen catabolite repression (NCR). The present study confirms this repression, because in the control fermentation their expressions were almost negligible and in the limiting nitrogen condition their expressions dropped sharply at the beginning, when nitrogen was still available, and increased continuously when it was not. The NCR of both transporters was fast and effective, as seen with the nitrogen additions, although the cell response to the excess of nitrogen in the medium was quicker when the nitrogen addition was in the first half of fermentation. During the last stages of fermentation, ethanol content is high and it is well established that the first target of ethanol toxicity is the plasma membrane (*4;5*), which can be impaired for a long period of anaerobic growth. Therefore, the sensing system of the cell, mainly located in the plasma membrane, may be affected by both effects (*15*).
The moment of the fermentation process at which the NCR was established (by the nitrogen addition) determined the pattern of amino acid consumption. As previously reported (*8*), arginine, alanine, aspartate, glutamate and glycine were the amino acids that were most affected by the NCR because they were hardly consumed when there was an excess of nitrogen. In fact, they were not taken up until the medium was depleted of good nitrogen sources. These amino acids must be transported mainly by the general amino acid permease (Gap1p) or by other specific permeases also subjected to NCR. Similar uptake pattern for these amino acids was previously reported in both synthetic and natural grape juices (*1; 26*). On the other hand, in brewing conditions (*32*) arginine and glutamine were rapidly consumed whereas ammonium uptake was delayed. Branched-chain and aromatic amino acids behaved in a completely different way. Except for tryptophan, they were mostly consumed in the first stages of the control fermentation; that is, when the cells were subjected to NCR from the beginning of the fermentation process. A common feature of the genes that encode the permeases of the branched-chain amino acids (*BAP1* and *BAP2*) and aromatic amino acids (*TAT1* and *TAT2*) is that they are induced in a nitrogen-rich medium (*14;32*).

Regardless of the time of addition, glutamine and tryptophan were the main amino acids consumed after the nitrogen additions and, therefore, they may be very important for the yeast cell metabolism throughout the process.

Ammonium accounted for 40% of the total YAN of the fermentation media. However, its consumption depended on the timing of the addition. Ammonium is the preferred nitrogen source for biomass production but was hardly consumed when it was added in the final stages of the fermentation. These differences in ammonium uptake are difficult to explain in terms of permease regulation. In the present study and our previous one (*8*), we detected that the more nitrogen there was in the fermentation media, the more repressed the three MEP genes were. Marini et al. (*22*) have proposed two possible hypotheses to explain this paradox: either the yeast possesses additional ammonium transport systems unrelated to the Mep proteins, or highly concentrated ammonium is taken up into the cells by simple diffusion.

The timing of the nitrogen additions directly determined the likely aroma characteristics of the wines. Glycerol increased in the fermentations with higher biomass production and higher ammonium consumption. The relationship between biomass formation and glycerol synthesis has already been reported (*7;30;39*). Likewise, a higher glycerol yield was also observed on a synthetic glucose-rich medium when ammonium was used as the sole nitrogen source instead of a mixture of ammonium and amino acids (*3*). Michnick *et al.* (*25*) also related the production of glycerol to the accumulation of acetate and acetaldehyde.

Higher alcohols were also affected by the changes in nitrogen utilization. These compounds can be produced either by the catabolic conversion of the branched-chain amino acids (via Ehrlich) or by the anabolic formation of these amino acids *de novo* from a sugar substrate (*6;19*). Our results show that the anabolic route is of greater importance because the increase in isoamyl alcohol and 2-phenyl ethanol was inversely proportional to the consumption of leucine and phenylalanine, respectively. Furthermore, the closer the nitrogen concentration is to the growth-limiting level, the higher the yield of fusel alcohols is. There is also an inverse correlation between ammonium consumption and the production of fusel alcohols (*31*). A greater concentration of higher alcohols did not seem to determine an increase in esters. On the contrary, the acetate concentration seemed to determine a greater concentration of acetate esters, especially ethyl acetate.

In conclusion, our study shows the quantity and quality of the nitrogen demands of the wine strain QA23. Although further studies should be carried out with other wine strains, our data show that cell growth and fermentation have different preferred nitrogen sources. Nitrogen additions always improved fermentation performance but had a minimal effect on biomass production when added in the second half of the fermentation. These nitrogen additions subjected the cells to NCR and changed the profile of nitrogen consumption. The differences in the pattern of nitrogen consumption were related to different aroma compound composition in the wines. In our opinion, this study is a starting point for further investigation into using an ammonium/amino acid mixture as nitrogen supplementation in the wine industry and the effect that these additions have on yeast physiology, fermentation performance and wine quality.

ACKNOWLEDGEMENTS

This work was supported by grant AGL2000-0205-P4-03 from the *Comisión Interministerial de Ciencia y Tecnología*, Spain. The authors wish to thank the Language Service of the Rovira i Virgili University for revising the manuscript.

REFERENCES

1. Aerny,J. Composés azotés des moûts et vins. *Revue suisse Vitic.Arboric.Hortic.* **1996**, *28*, 161-165.

2. Agenbach,W.A. A study of must nitrogen content in relation to incomplete fermentations, yeast production and fermentation. *Proceedings of the South African Society for Enology and Viticulture* **1977**, 87.

3. Albers,E.; Larsson,C.; Liden,G.; Niklasson,C.; Gustafsson,L. Influence of the nitrogen source on Saccharomyces cerevisiae anaerobic growth and product formation. *Appl.Environ.Microbiol.* **1996**, *62*, 3187-3195.

4. Alexandre,H.; Rousseaux,I.; Charpentier,C. Ethanol adaptation mechanisms in Saccharomyces cerevisiae. *Biotechnol.Appl.Biochem.* **1994**, *20*, 173-183.

5. Alexandre,H.; Rousseaux,I.; Charpentier,C. Relationship between ethanol tolerance, lipid composition and plasma membrane fluidity in Saccharomyces cerevisiae and Kloeckera apiculata. *FEMS Microbiol.Lett.* **1994**, *124*, 17-22.

6. Äyräpää,T. Biosynthetic formation of higher alcohols by yeast. Dependence on the nitrogenous nutrient level of the medium. *J.Inst.Brew.* **1971**, *77*, 276.

7. Bakker,B.M.; Overkamp,K.M.; van Maris,A.J.; Kotter,P.; Luttik,M.A.; van Dijken,J.P.; Pronk,J.T. Stoichiometry and compartmentation of NADH metabolism in Saccharomyces cerevisiae. *FEMS Microbiol.Rev.* **2001**, *25*, 15-37.

8. Beltran,G.; Novo,M.; Rozes,N.; Mas,A.; Guillamon,J.M. Nitrogen catabolite repression in Saccharomyces cerevisiae during wine fermentations. *FEMS Yeast Res.* **2004**, *4*, 625-632.

9. Bely,M.; Sablayrolles,J.M.; Barre,P. Automatic detection of assimilable nitrogen deficiencies during alcoholic fermentation in oenological conditions. *J.Ferment.Bioeng.* **1990**, *70*, 246-252.

10. Bely,M.; Sablayrolles,J.M.; Barre,P. Description of alcoholic fermentation kinetics: its variability and significance. *American Journal of Enology and Viticulture* **1990**, *41*, 319-324.

11. Bisson,L.F. Influence of nitrogen on yeast and fermentation of grapes. In *Proceedings of the International Sumposium on Nitrogen in Grapes and Wine*; Ranz,J.M., Ed.; Am. Soc. Enol. Vitic.: Davis, CA, 1991.

12. Cooper,T.G. Nitrogen metabolism in *Saccharomyces cerevisiae*. In *The molecular Biology of the Yeast Saccharomyces*; Stratherm,J.N.; Jones,E.W.; Broach,J.R., Eds.; Cold Spring Harbor Laboratory, Cold Spring Harbor: NY, USA, 1982.

13. Ferreira,V.; Sharman,M.; Cacho,J.; Dennis,J. New and efficient microextraction / solid-phase extraction method for the gas chromatographic analysis of wine volatiles. *J.Chromatogr.A* **1996**, *731*, 247-259.

14. Forsberg,H.; Ljungdahl,P.O. Sensors of extracellular nutrients in Saccharomyces cerevisiae. *Curr.Genet.* **2001**, *40*, 91-109.

15. Gagiano,M.; Bauer,F.F.; Pretorius,I.S. The sensing of nutritional status and the relationship to filamentous growth in Saccharomyces cerevisiae. *FEMS Yeast Res.* **2002**, *2*, 433-470.

16. Jiranek,V.; Langridge,P.; Henschke,P.A. Yeast nitrogen demand: selection criterion for wine yeasts for fermenting low nitrogen musts. In *Proceedings of the International Symposium on Nitrogen in Grapes and Wine*; Ranz,J.M., Ed.; Am.Soc.Enol.Vitic.: Davis, CA, 1991.

17. Jiranek,V.; Langridge,P.; Henschke,P.A. Amino acid and ammonium utilization by Saccharomyces cerevisiae wine yeasts from a chemical defined medium. *American Journal of Enology and Viticulture* **1995**, *46*, 75-83.

18. Jiranek,V.; Langridge,P.; Henschke,P.A. Regulation of hydrogen sulfide liberation in wine-producing Saccharomyces cerevisiae strains by assimilable nitrogen. *Appl.Environ.Microbiol.* **1995**, *61*, 461-467.

19. Lambrechts,M.G.; Pretorius,I.S. Yeast and its importance to wine aroma. *S.Afr.J.Enol.Vitic* **2000**, *21*, 97-129.

20. Lopez,R.; Aznar,M.; Cacho,J.; Ferreira,V. Determination of minor and trace volatile compounds in wine by solid-phase extraction and gas chromatography with mass spectrometric detection. *J.Chromatogr.A* **2002**, *966*, 167-177.

21. Manginot,C.; Roustan,J.L.; Sablayrolles,J.M. Nitrogen demand of different yeast strains during alcoholic fermentation. Importance of the stationary phase. *Enzyme Microb.Technol.* **1998**, *23*, 511-517.

22. Marini,A.M.; Soussi-Boudekou,S.; Vissers,S.; Andre,B. A family of ammonium transporters in Saccharomyces cerevisiae. *Mol.Cell Biol.* **1997**, *17*, 4282-4293.

23. Marks,V.D.; van der Merwe,G.K.; van Vuuren,H.J. Transcriptional profiling of wine yeast in fermenting grape juice: regulatory effect of diammonium phosphate. *FEMS Yeast Res.* **2003**, *3*, 269-287.

24. Mendes-Ferreira,A.; Mendes-Faia,A.; Leao,C. Growth and fermentation patterns of Saccharomyces cerevisiae under different ammonium concentrations and its implications in winemaking industry. *J.Appl.Microbiol.* **2004**, *97*, 540-545.

25. Michnick,S.; Roustan,J.L.; Remize,F.; Barre,P.; Dequin,S. Modulation of glycerol and ethanol yields during alcoholic fermentation in Saccharomyces cerevisiae strains overexpressed or disrupted for GPD1 encoding glycerol 3-phosphate dehydrogenase. *Yeast* **1997**, *13*, 783-793.

26. Monteiro,F.F.; Bisson,L.F. Nitrogen supplementation of grape juice. I. Effect on amino acid utilization during fermentation. *Am.J.Enol.Vitic.* **1992**, *43*, 1-10.

27. Ough,C.S. Influence of nitrogen compounds in grapes in ethyl carbamate formation in wines. In *Proceedings of the International Sumposium on Nitrogen in Grapes and Wine*; Ranz,J.M., Ed.; Am.Soc.Enol.Vitic.: Davis, CA, 1991.

28. Ough,C.S.; Huang,D.A.; Stevens,D. Amino acid uptake by four commercial yeasts at two different temperatures of growth and fermentation: Effects on urea excretion and re-adsorption. *American Journal of Enology and Viticulture* **1991**, *41*, 26-40.

29. Querol,A.; Barrio,E.; Ramon,D. A comparative study of different methods of yeast strain characterization. *Syst.Appl.Microbiol.* **1992**, *21*, 315-323.

30. Radler,F.; Schütz,H. Glycerol production of various strains of *Saccharomyces*. *American Journal of Enology and Viticulture* **1982**, *33*, 36-40.

31. Rapp,A.; Versini,G. Influence of nitrogen compounds in grapes on aroma compounds of wines. In *Proceedings of the International Symposium on Nitrogen in Grapes and Wine*; Ranz,J.M., Ed.; Am. Soc. Enol. Vitic. Davis, CA, 1991.

32. Regenberg,B.; During-Olsen,L.; Kielland-Brandt,M.C.; Holmberg,S. Substrate specificity and gene expression of the amino-acid permeases in Saccharomyces cerevisiae. *Curr.Genet.* **1999**, *36*, 317-328.

33. Riou,C.; Nicaud,J.M.; Barre,P.; Gaillardin,C. Stationary-phase gene expression in Saccharomyces cerevisiae during wine fermentation. *Yeast* **1997**, *13*, 903-915.

34. Salmon,J.M.; Barre,P. Improvement of nitrogen assimilation and fermentation kinetics under enological conditions by derepression of alternative nitrogen-assimilatory pathways in an industrial Saccharomyces cerevisiae strain. *Appl.Environ.Microbiol.* **1998**, *64*, 3831-3837.

35. Sambrook,J.; Fritsch,E.F.; Maniatis,T. *Methods in Yeast Genetics: A Laboratory Manual*, 2 ed.; Cold Spring Harbor: NY, USA, 1989.

36. Sierkstra,L.N.; Verbakel,J.M.; Verrips,C.T. Analysis of transcription and translation of glycolytic enzymes in glucose-limited continuous cultures of Saccharomyces cerevisiae. *J.Gen.Microbiol.* **1992**, *138 (Pt 12)*, 2559-2566.

37. ter Schure,E.G.; van Riel,N.A.; Verrips,C.T. The role of ammonia metabolism in nitrogen catabolite repression in Saccharomyces cerevisiae. *FEMS Microbiol.Rev.* **2000**, *24*, 67-83.

38. Torija,M.J.; Beltran,G.; Novo,M.; Poblet,M.; Rozes,N.; Mas,A.; Guillamon,J.M. Effect of organic acids and nitrogen source on alcoholic fermentation: study of their buffering capacity. *J.Agric.Food Chem.* **2003**, *51*, 916-922.

39. Watanabe,M.; Uehara,M.; Shinohara,T. Effect of cell number on the formation of glycerol and some volatile components by yeast. *J.Brew.Soc.Jpn* **1982**, *77*, 346.

CHAPTER 5

Effect of low temperature fermentation on yeast nitrogen metabolism

Gemma Beltran, Nicolas Rozès, Albert Mas and José M. Guillamón

Unitat d'Enologia del Centre de Referència de Tecnologia d'Aliments. Dept. Bioquímica i Biotecnologia. Facultat d'Enologia de Tarragona. Universitat Rovira i Virgili. Marcel·lí Domingo s/n 43007 Tarragona, Spain

(In preparation)

INTRODUCTION

Low temperatures (10-15ºC) can be used in wine fermentations to enhance production and retain flavour volatiles. In this way, white and rosé wines of greater aromatic complexity can be produced at low temperatures (Lambrechts and Pretorius, 2000; Llauradó *et al.*, 2002; Beltran *et al.*, 2002; Torija *et al.*, 2003; Novo *et al.*, 2003a; Novo *et al.*, 2003b). However, temperature affects the rate of fermentation, with lower temperatures giving slower fermentation rates and longer fermentations. Changes in fermentation may also modify the yeast and bacteria ecology, and impair the nutrient uptake, ethanol sensitivity and yeast metabolism (Fleet and Heard, 1993).

Yeast metabolism firstly depends on the nutrient uptake capacity of the cell by the different permeases. The membrane permeases are highly temperature-dependent, because changes in temperature provoke conformational changes in these molecules (Entian and Barnett, 1992). Thus, several studies of the effect of ethanol and temperature on yeast growth and fermentation have shown that, for temperatures of 15ºC to 35ºC, glucose transport and glycolytic flux increase steadily with temperature (Sa-Correia and van Uden, 1983; Leao and van Uden, 1985).

Nitrogen assimilation has been shown to depend on fermentation temperature. Fermentation temperature is an important factor determining utilization of nitrogen sources during fermentation of grape juice (Lopez *et al.*, 1996). Moreover, changes in plasma-membrane composition, especially the increase in unsaturated fatty-acids at low temperatures, result in a decrease solute transport (Watson, 1987; Walker, 1998). Low temperatures result in a lower rate of amino acid assimilation (Ough *et al.*, 1991; Lopez *et al.*, 1996; Llauradó *et al.*, 2002), consistent with lower rates of fermentation and yeast growth.

In this study, we have analysed the patterns of assimilable nitrogen uptake (amino acid and ammonia) by yeasts in order to determine how fermentation temperature affects to nitrogen regulation. The uptake of nitrogen by the cells is regulated by the mechanism known as Nitrogen Catabolite Repression (NCR). When good nitrogen sources are present in the medium, the transcription of some genes involved in the uptake and utilization of the poorer nitrogen sources is repressed, and their corresponding products are inactivated and degraded (ter Schure *et al.*, 2000; Magasanik and Kaiser, 2002). *GAP1* and *MEP2*, which encoded two important permeases in the transport of amino acids and ammonium respectively, are a good example of genes down-regulated in a nitrogen-rich medium by the NCR mechanism (Beltran *et al.*, 2004; Beltran *et al.*,

2005a). We have monitored the transcriptional activity of these genes to determine the nitrogen repressed or derepressed situation of the cells throughout fermentations at different temperatures.

MATERIALS AND METHODS

Strain, fermentations and sampling

A commercial *Saccharomyces cerevisiae var. bayanus* wine strain QA23 (Lallemand S.A., Toulouse, France) was used in this study. Fermentations were carried out in a synthetic grape must (pH 3.3) but with 200 g L^{-1} of reducing sugars (100 g L^{-1} Glucose and 100 g L^{-1} Fructose) and without anaerobic factors (Beltran *et al.*, 2004). The Yeast Assimilable Nitrogen (YAN) content was 300 mg N L^{-1}, ammoniacal nitrogen (NH_4Cl) 120 mg N L^{-1}, and amino acids 180 mg N L^{-1}. Fermentations took place at 13ºC and 25ºC in laboratory-scale fermentors: 2 L bottles filled with 1.8 L of medium and fitted with closures that enabled the carbon dioxide to escape and the samples to be removed. Fermentations were in semi-anaerobic conditions, since limited aeration was necessary in order to harvest samples for the subsequent analysis. The population inoculated in every flask was $2x10^6$ cell mL^{-1} from dry yeast rehydrated in water at 37ºC.

The growth of yeast was calculated by counting the viable yeast after plating them on YPD agar at an adequate dilution for 2 days at 28ºC.

Every day the density was measured by weighing 5 ml of medium. In the latter stages of fermentation the sugar consumption was assayed by enzymatic kits (Roche Applied Science, Germany). Fermentation was considered to be complete when the residual sugars were below 2 g L^{-1}.

Nitrogen content analysis

YAN was analysed by the formol index method (Aerny, 1996), and the ammonium content was quantified using an enzymatic method (Roche Applied Science, Germany). The individual amino and imino acids were analysed by HPLC as described by Beltran *et al.* (2004).

RNA extraction, cDNA synthesis and Real-time quantitative PCR

Total RNA was isolated from yeast samples as described by Sierkstra *et al.* (1992), and purified and quantified as described as Beltran *et al.* (2004).

Total RNA was reverse-transcripted with Superscript^{TM} II RNase H^{-} Reverse Transcriptase (Invitrogen, USA) in a GenAmp PCR System 2700 (Applied Biosystem, USA). 0.5 µg of Oligo $(dT)_{12-18}$ Primer (Invitrogen, USA) was used with 0.8 µg of total RNA as template in a reaction volume of 20 µl. Following the protocol provided by the manufacturer, after denaturation at 70ºC for 10 min, cDNA was synthesized at 42ºC for 50 min. Finally, the reaction was inactivated at 70ºC for 15 min.

The PCR primers used in this study are: *ACT-F*: TGGATTCCGGTGATGGTGTT and *ACT-R*: CGGCCAAATCGATTCTCAA (*ACT*, for actine gene); *GAP1-F*: CTGTGGATGCTGCTGCTTCA and *GAP1-R*: CAACACTTGGCAAACCCTTGA (*GAP1*, for general amino acid permease gene); *MEP2-F*: GGTATCATCGCTGGCCTAGTG and *MEP2-R*: ACAACGGCTGACCAGATTGG (*MEP2*, for ammonium permease gene).

For each gene, a standard curve was made with yeast genomic DNA. The Real-Time Quantitative PCR reaction was performed using SYBR® Green I PCR (Applied Biosystems, USA). The 25 µL SYBR PCR reactions contained 300 nM of each PCR primer, together with 1 µL cDNA (or 5 µL of each DNA serial dilution for standard tubes) and one time SYBR master mix (Applied Biosystems, USA).

All PCR reactions were cycled in a PE Applied Biosystems 5700 thermal cycler under the following conditions: 50ºC for 2 min, 95ºC for 10 min, and 40 cycles at 95ºC for 15 sec and at 60ºC for 60 sec. All samples were analysed in duplicate and the expression values were averaged by the analysis software (Applied Biosystems, USA). The coefficient of variation in all samples analysed was less than 10 %.

RESULTS

Wine fermentations were carried out at 25ºC and 13ºC using synthetic media in order to estimate the effect of temperature on nitrogen consumption (ammonia and amino acid uptake), and on the Nitrogen Catabolite Repression (NCR) system (expression of permeases Gap1p and Mep2p).

Effect of temperature on yeast growth and nitrogen consumption

Figure 1 shows the fermentation kinetic and cell growth at both fermentation temperatures. As expected, low temperatures decreased the fermentation rate and increased the length of fermentation. Yeast growth rate was also slower at 13ºC than at

25ºC and maximum population was higher at 25ºC than at 13ºC. However the viability of yeast cells fermenting at 13ºC remained constant up to the end of fermentation (29 days in total).

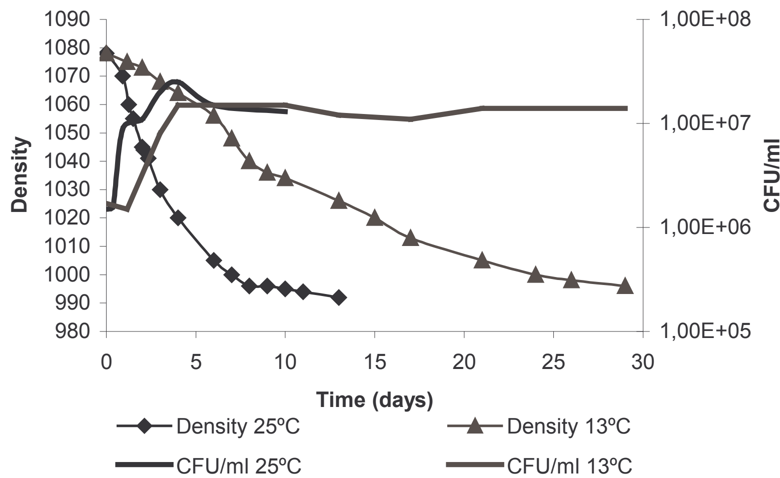


Figure 1. Fermentation kinetics and cell growth throughout 13ºC and 25ºC fermentations.

We observed different nitrogen requirements in yeasts fermenting at both temperatures (Table 1). Correlating with a higher biomass production, the final nitrogen consumption by yeasts was higher at 25ºC than at 13ºC. This difference between both temperatures was more significant in the consumption of ammonium (79 mg/l at 25ºC, 59 mg/l at 13ºC) than in the consumption of amino acids (67 mg/l at 25ºC, 59 mg/l at 13ºC).

By analysing this consumption in the different fermentation stages, cells at 13ºC consumed most of the nitrogen in the first stages (density of 1065), which overlapped the biomass production during exponential phase (Figure 1). For this biomass production, cells at 13ºC preferred amino acids to ammonium. Conversely cells at 25ºC used nitrogen throughout and ammonium as preferred source.

Table1. Consumption of Yeast Assimilable Nitrogen (YAN), expressed as ammonia (YAN NH4) and amino acid (YAN aas) nitrogen, at different phases of 13ºC and 25ºC fermentation. FF: Final Fermentation

	Density		
	1065	**1020**	**990 (FF)**
25ºC			
YAN aas	38,3	50,6	66,8
YAN NH4	45,7	83,6	79,2
Total YAN	84,1	134,1	146,0
13ºC			
YAN aas	44,11	53,5	59,4
YAN NH4	37,88	61,4	59,0
Total YAN	81,98	114,8	118,4

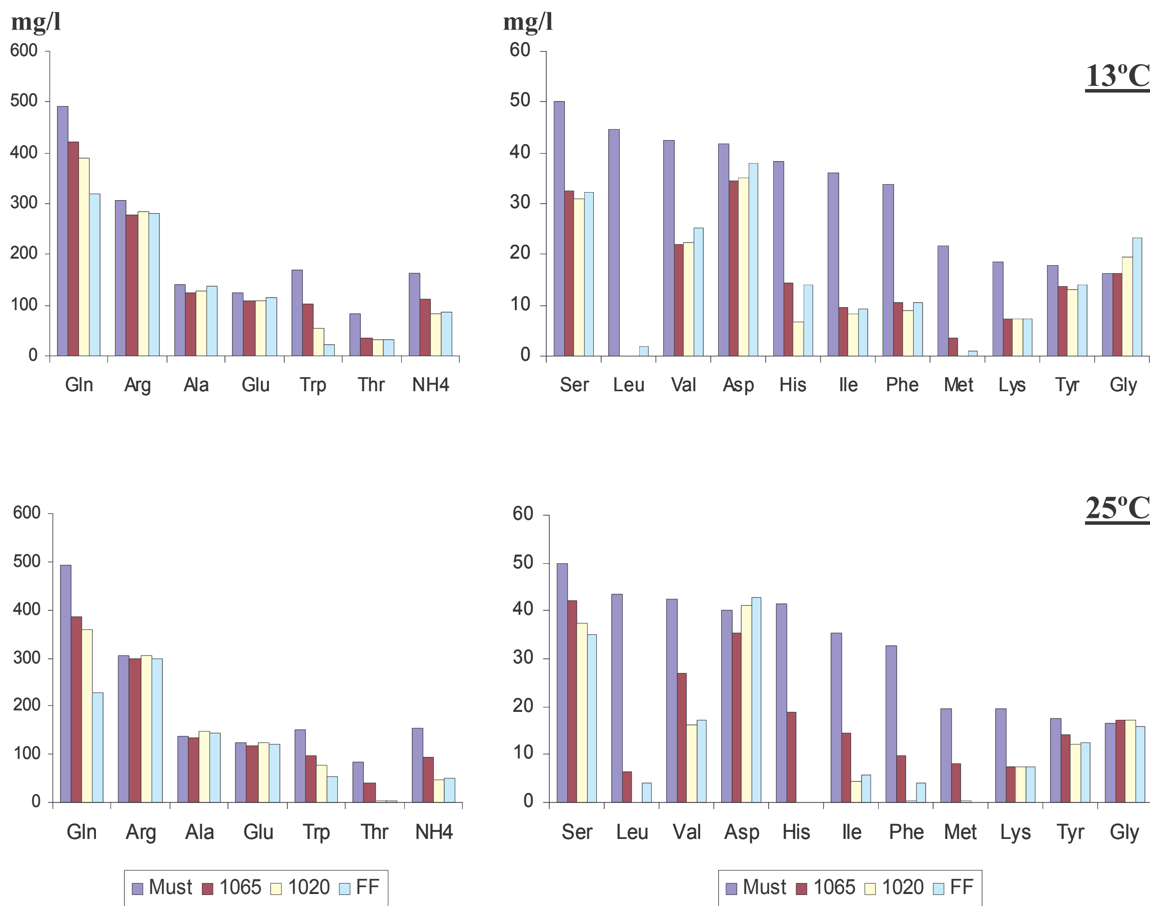
Amino acid preferences were also different at both temperatures (Table 2). Interesting enough was the higher uptake of tryptophan by the cells growing at 13ºC and of glutamine at 25ºC. Threonine, histidine, valine and serine were proportionally more consumed at 25ºC. On the other hand, there is a set of amino acids (arginine, glutamate, alanine and aspartate) that were hardly consumed at 25ºC but proportionally much more consumed at 13ºC. In previous studies (Beltran *et al.*, 2004; 2005b) we already reported the minimal consumption of these amino acids in a rich-nitrogen medium.

Table 2. Final consumption of amino acids (mg l^{-1}) by yeast cells fermenting at 13ºC and 25ºC. As the ratio between total amino acids consumed at 25ºC and 13ºC was 1,1, amino acids with ratio > 1,1 were more consumed at 25ºC and amino acids with ratio < 1,1 were more consumed at 13ºC.

	25ºC	**13ºC**	*Ratio*
Gln	266,28	175,34	1,5
Trp	96,71	144,94	**0,7**
Thr	79,79	51,60	1,5
Leu	39,43	42,71	0,9
His	41,38	24,21	1,7
Ile	29,58	26,86	1,1
Phe	28,70	23,10	1,2
Val	25,43	17,40	1,5
Met	19,24	20,70	0,9
Ser	21,03	12,82	1,6
Lys	11,92	10,92	1,1
Arg	7,78	28,48	**0,3**
Tyr	5,11	3,80	1,3
Glu	4,83	11,07	**0,4**
Gly	0,86	0,00	
Ala	0,00	2,98	
Asp	0,00	4,09	
Suma	668,42	593,80	1,1

Figure 2 shows the pattern of amino acids and ammonia consumption in different phases of fermentation. The major bulk of amino acids were consumed at the initial stages of fermentation (density 1065), during the growth phase. Only glutamine and tryptophan were consumed in the latter phases of 13ºC fermentation, whereas at 25ºC yeast cells were able to consume a more variety of amino acids throughout the fermentation. At the end of fermentation, levels of several amino acids in the wine had increased because the cell autolysis.

Figure 2. Amino acid and ammonium concentration in the synthetic grape-must media at different stages of the fermentations at 13ºC and 25ºC.



Effect of low temperature fermentation on *GAP1* and *MEP2* expression

The expression of the nitrogen transporters *GAP1* and *MEP2* was analysed and quantified relative to the expression of the housekeeping actine gene. Time zero was the expression of yeast before inoculation (and after rehydration).

Both genes were repressed in the first hours after inoculation in the must-like medium (Figure 3) due to the presence of good nitrogen sources and, therefore, to the effect of NCR (ter Schure *et al.* 2000; Beltran *et al.*, 2004, 2005a). As nitrogen was not completely consumed, its presence in the media repressed these genes throughout the fermentation. However this nitrogen catabolite repression of the permeases seemed to be less efficient at 13ºC, where we obtained higher values of gene expression, overall of *MEP2* expression at the final stages of fermentation.

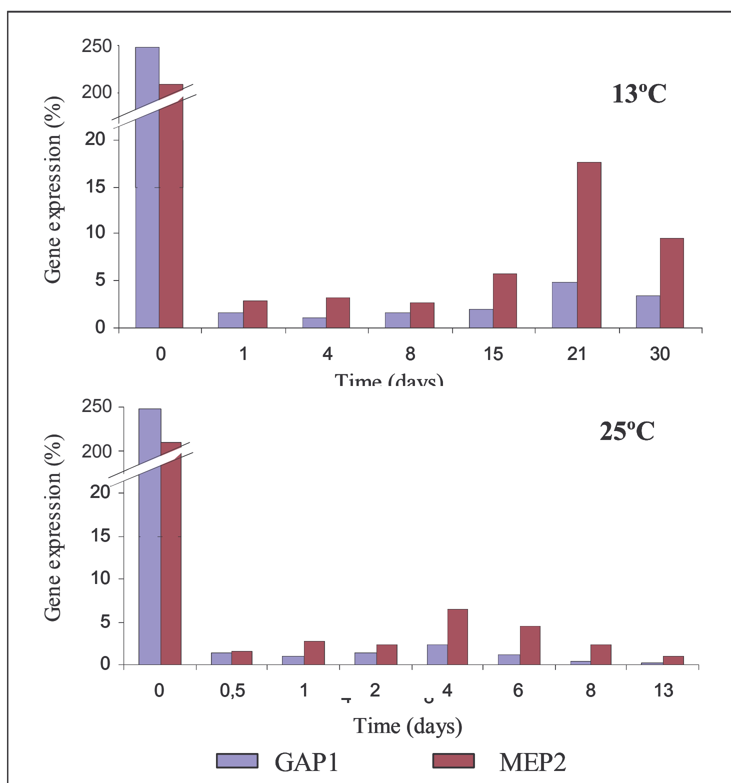


Figure 3. Gene expression of ammonium permease (*MEP2)* and general amino acid permease (*GAP1)* at time zero (before inoculation) and at different points during the fermentations at 13ºC and 25ºC.
The data were quantified by calculating the ratio between the concentration of the studied genes normalized with the concentration of the housekeeping *ACT* gene, and expressed as a percentage (the quantity ratio 1 was set as 100%).

DISCUSSION

Temperature of fermentation is an important factor in winemaking due to its influence in fermentation performance and in final product characteristics. Previously, we have observed that fermentations carried out at low temperatures (13ºC) improved certain characteristics of taste and aroma, not only related to primary aroma retention (Beltran *et al.*, 2005b;Llauradó *et al.*, 2002;Torija *et al.*, 2003). In addition fermentation temperature also affects the growth yeast, with a better viability at 13ºC than at 25ºC, and the lipid composition of the cell, with changes in the membrane fluidity (Beltran *et al.*, 2005b). Nitrogen assimilation has also been shown to depend on fermentation temperature (Ough *et al.* 1991, Lopez *et al.*, 1996).

We recently studied (Beltran *et al.*, 2004) nitrogen regulation by using a commercial wine *S. cerevisiae* strain and a synthetic medium which mimics grape-must. In the present study our aim is to analyse the influence of low temperature on this nitrogen regulation and, therefore, on nitrogen consumption. Our results pointed to a different nitrogen regulation at low temperature, which can affect to the growth rate and nitrogen preferences. This is not unexpected since, in a global transcriptomic analysis of *S. cerevisiae*, genes associated with nitrogen transport and amino acid metabolism were

importantly up-regulated by shifting growing cultures from 30ºC to 10ºC (Schade *et al.*, 2004).

Somehow low temperature produces similar metabolic effects to these obtained in nitrogen-deficient cultures. Low temperature and nitrogen-limited growth decreased biomass yield and relaxed the nitrogen catabolite repression. As a consequence, ammonium and glutamine, which are the preferred source for biomass production (Beltran et al., 2005), were less consumed at low temperature. Likewise amino acids which are only taken up under derepressed conditions were more consumed at low temperature. Another amino acid whose uptake increased at low temperature is tryptophan. 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 endowed to the cell with a good growth capacity at low temperature (Abe and Horikoshi, 2000).

In wine fermentations, most of the transformation of sugars into ethanol and CO_2 happens during the non-proliferating or stationary phase. During this stationary phase most of the nitrogen compounds are devoted to cell maintenance. Comparing the data of amino acid uptake, yeast cells in fermentation at 25ºC needs much more nitrogen than these ones fermenting at 13ºC. Nevertheless cell viability was kept very high during the 29 days of low temperature fermentation (no decline phase was detected). Therefore fermentations at low temperature seems to be more homeostatic than fermentations at high temperature. This could be related with a higher tolerance to ethanol at low temperature.

In conclusion, low temperature is a restrictive condition for yeast growth (biomass yield) but not for the maintenance of this growth (high viability). Although further studies should be done to assure this hypothesis, low temperature influences the quantity and the quality of nitrogen requirement. Nitrogen-deficient grape musts and low temperature are two main prevalent causes of sluggish fermentations and, therefore, implications between both growth conditions on yeast metabolism are of capital interest for wine making.

ACKNOWLEDGEMENTS.

This work was supported by grant AGL2000-0205-P4-03 from the *Comisión Interministerial de Ciencia y Tecnología*, Spain.

REFERENCES

1. Abe,F.; Horikoshi,K. Tryptophan permease gene TAT2 confers high-pressure growth in Saccharomyces cerevisiae. *Mol.Cell Biol.* **2000**, *20*, 8093-8102.

2. Aerny,J. Composés azotés des moûts et vins. *Revue suisse Vitic.Arboric.Hortic.* **1996**, *28*, 161-165.

3. Beltran,G.; Esteve-Zarzoso,B.; Rozes,N.; Mas,A.; Guillamon,J.M. Influence of the timing of nitrogen additions during synthetic grape must fermentations on fermentation kinetics and nitrogen consumption. *J Agric.Food Chem* **2005a**, *on web*.

4. Beltran,G.; Novo,M.; Guillamon,J.M.; Mas,A.; Rozes,N. Effect of fermentation temperature and culture media on the lipid composition and volatile compounds in wine. *In preparation* **2005b**.

5. Beltran,G.; Novo,M.; Rozes,N.; Mas,A.; Guillamon,J.M. Nitrogen catabolite repression in Saccharomyces cerevisiae during wine fermentations. *FEMS Yeast Res.* **2004**, *4*, 625-632.

6. Beltran,G.; Novo,M.; Torija,M.J.; Poblet,M.; Rozes,N.; Guillamon,J.M.; Mas,A. Fermentaciones a bajas temperaturas. *Tecnología del vino* **2002**, *1*, 73-76.

7. Entian,K.D.; Barnett,J. Regulation of sugar utilization by Saccharomyces cerevisiae. *Trends Biochem.Sci.* **1992**, *17*, 506-510.

8. Fleet,G.H.; Heard,G.M. Yeast growth during fermentation. In *Wine Microbiology and Biotechnology*; Fleet,G.H., Ed.; Harwood Academic Publishers: Switzerland, 1993.

9. Lambrechts,M.G.; Pretorius,I.S. Yeast and its importance to wine aroma. *S.Afr.J.Enol.Vitic* **2000**, *21*, 97-129.

10. Leao,C.; van Uden,N. Effect of ethanol and other alkanols on the temperature of glucose transport and fermentation in *Saccharomyces cerevisiae*. *Appl Microbiol Biotechnol.* **1985**, *22*, 359-363.

11. Llauradó,J.M.; Rozes,N.; Bobet,R.; Mas,A.; Constantí,M. Low temperature alcoholic fermentation in high sugar concentration grape must. *J.Food Science* **2002**, *67*, 268-273.

12. Lopez,R.; Santamaria,P.; Gutierrez,A.; Iñiguez,M. Changes on amino acids during the alcoholic fermentation of grape juice at different temperatures. *Sci.Aliments* **1996**, *16*, 529-535.

13. Magasanik,B.; Kaiser,C.A. Nitrogen regulation in Saccharomyces cerevisiae. *Gene* **2002**, *290*, 1-18.

14. Novo,M.; Beltran,G.; Torija,M.J.; Poblet,M.; Guillamon,J.M.; Mas,A.; Rozes,N. Fermentaciones a bajas temperatras: análisis químico y sensorial. *Tecnología del vino* **2003a**, *2*, 51-55.

15. Novo,M.; Torija,M.J.; Beltran,G.; Rozes,N.; Mas,A.; Guillamon,J.M. Revisión sobre las fermentaciones vínicas a baja temperatura. Perspectivas de futuro. *Tecnología del vino* **2003b**, *3*, 38-43.

16. Ough,C.S.; Huang,D.A.; Stevens,D. Amino acid uptake by four commercial yeasts at two different temperatures of growth and fermentation: Effects on urea excretion and re-adsorption. *American Journal of Enology and Viticulture* **1991**, *41*, 26-40.

17. Riou,C.; Nicaud,J.M.; Barre,P.; Gaillardin,C. Stationary-phase gene expression in Saccharomyces cerevisiae during wine fermentation. *Yeast* **1997**, *13*, 903-915.

18. Sa-Correia,I.; van Uden,N. Temperature profiles of ethanol tolerance: effects of ethanol on the minimum and the maximum temperatures for growth of the yeasts *Saccharomyces cerevisiae* and *Kluyveromyces fragilis*. *Biotechnol.Bioeng.* **1983**, *25*, 1665-1667.

19. Sambrook,J.; Fritsch,E.F.; Maniatis,T. *Methods in Yeast Genetics: A Laboratory Manual*, 2 ed.; Cold Spring Harbor: NY, USA, 1989.

20. Schade,B.; Jansen,G.; Whiteway,M.; Entian,K.D.; Thomas,D.Y. Cold Adaptation in Budding Yeast. *Mol.Biol.Cell* **2004**.

21. Sierkstra,L.N.; Verbakel,J.M.; Verrips,C.T. Analysis of transcription and translation of glycolytic enzymes in glucose-limited continuous cultures of Saccharomyces cerevisiae. *J.Gen.Microbiol.* **1992**, *138 (Pt 12)*, 2559-2566.

22. ter Schure,E.G.; van Riel,N.A.; Verrips,C.T. The role of ammonia metabolism in nitrogen catabolite repression in Saccharomyces cerevisiae. *FEMS Microbiol.Rev.* **2000**, *24*, 67-83.

23. Tokai,M.; Kawasaki,H.; Kikuchi,Y.; Ouchi,K. Cloning and characterization of the CSF1 gene of Saccharomyces cerevisiae, which is required for nutrient uptake at low temperature. *J Bacteriol.* **2000**, *182*, 2865-2868.

24. Torija,M.J.; Beltran,G.; Novo,M.; Poblet,M.; Guillamon,J.M.; Mas,A.; Rozes,N. Effects of fermentation temperature and Saccharomyces species on the cell fatty acid composition and presence of volatile compounds in wine. *Int.J.Food Microbiol.* **2003**, *85*, 127-136.

25. Walker,M.A. *Yeast Physiology and biotechnology*, Wiley & Sons: West Sussex, England, 1998.

26. Watson,K. Temperature relations. In *The yeast. Yeast and Environment*; Rose,A.H.; Harrison,J.S., Eds.; Academic Press: London, 1987.

CONCLUSIONS

&

PERSPECTIVES

The temperature of fermentation is important in winemaking because it affects the performance of fermentation and the characteristics of the final product. The temperature used depends on the type and style of wine involved: red wines are produced between 25-30ºC because this improves maceration and colour extraction, and white and rosé wines are produced at lower temperatures (~18ºC) because this improves the production and retention of aromatic compounds. Previous studies by our research group (Llauradó, 2002; Torija, 2002) found that fermentations carried out at lower temperatures (13ºC) improved certain characteristics of taste and aroma that were not only related to primary aroma retention. However, low temperature fermentations also have several disadvantages, including a longer process and a greater risk of stuck or sluggish fermentations.

Despite the major advances in characterizing the genome of the yeast *S.cerevisiae* in laboratory strains of this yeast, we have limited understanding of the expression of genes in yeast during industrial fermentations. In this thesis we used DNA microarray technology and Real-Time quantitative PCR for the analysis of gene expression of yeasts throughout the fermentation. The different transcriptional data were complemented with different metabolic and phenotypic characteristics such as cell's viability, fermentation by-products and flavours, cellular lipid composition or nitrogen consumption during the wine process. The global aim was to compare transcriptomic and metabolomic data in order to understand the degree of connection between both systems and, therefore, improve our knowledge about the global response of the cell at low temperature fermentations.

A low fermentation temperature affects the growth of the yeast, the lipid composition of cells and the aromatic intensity of wines and leads to:

- a slow growth rate but a higher viability of yeast cells throughout the fermentation,
- changes in the lipid composition of the yeast membrane i.e. a higher degree of unsaturation at the beginning of fermentation and a lower chain length and higher ergosterol proportion at the end than with the optimal temperature fermentation, and

- an improvement in the aromatic composition of wine i.e. an increase in flavor-active compounds (acetate and fatty acid ethyl esters) and a decrease in unpleasant ones such as acetic acid and fusel alcohols.

Most of these changes are observed in both natural and synthetic must fermentations, but we also observed that the complexity of grape must allows yeast cells to reach higher values of viability that seem to be related, among others, to the incorporation of the linoleic acid and ß-sitosterol present in the grape must. It also produces fruitier and more aromatic wines with less volatile acidity contents than those produced in the laboratory. More studies are needed into the complexity of grape musts and how their components (e.g. lipids, sterols, nitrogen compounds, vitamins, etc) affect yeast metabolism and the quality of the final product.

The analysis of the transcriptomic profile of *S. cerevisiae* at low temperature fermentation confirmed some of the differences observed in the lipid constitution of the cells. A higher expression of the fatty acid desaturase gene (*OLE1*) and fatty acid synthesis genes at the beginning of fermentation, and an increase in mitochondrial MCFA synthesis genes at the later stages of fermentation may be related with a higher synthesis of unsaturated and short-chain fatty acids. These short- and medium-chain fatty acids produced at higher concentrations at lower temperature could be exported into the medium directly or converted into ethyl esters to improve the aromatic quality of the wine. In our fermentations we found that these compounds, and acetate esters, increased in wines produced at low temperatures. The up-regulation of the genes *ATF1* and *ATF2*, which encodes the alcohol acetylesterases (Mason and Dufour, 2000), has been correlated with the increased formation of volatile esters (Fukuda *et al.*, 1998; Lilly *et al.*, 2000). Curiously we observed a large increase in the esterase *IAH1* gene at low temperature, but we detected no transcriptional difference in *ATF1* and *ATF2* between the two fermentation temperatures. Further accurate studies into the metabolism of aromatic compounds during this phase of fermentation should be done in order to better understand this consistent improvement in aroma profile. The higher production of aromatic compounds at low temperature may be also due to a higher viability and therefore, higher activity of yeast cells in the later stages of low temperature fermentation.

The greater viability of yeast cells at low temperatures also correlated with a higher expression of the genes involved in cell growth and maintenance at the later stages of fermentation. In fact, it could be related to an early stress response and a higher tolerance to ethanol. We found that most known responses of yeast to ethanol toxicity i.e. a decrease in membrane-saturated fatty acids such as palmitic acid, an increase in membrane-unsaturated long-chain fatty acids such as oleic acid and phosphatidylinositol biosynthesis (Alexandre *et al.*, 1994a; 1994b; Walker, 1998) were also triggered at low temperature. The adaptation response therefore seems to be similar in both kinds of stresses.

In wine fermentation, the general stress response (determined by the function of the two transcription factors, Msn2p and Msn4p) is primarily associated with the stationary phase (Gasch *et al.*, 2000; Rossignol *et al.*, 2003). However, this response was detected much earlier at 13ºC than a 25ºC (See Annex2, Figure12). This early stress response might arrange the cells for better resistance to further stresses such as ethanol toxicity. A consequence of this cross-protection (Beney *et al.*, 2000; Gasch, 2003) is that the cell viability is expected to increase. During the lag and exponential phase, yeast cells may enhance the cell protection mechanism to survive under low temperature conditions, as previously observed (Sahara *et al.*, 2002; Schade *et al.*, 2004). It would be very interesting, therefore, to better understand the first phase, from the rehydration phase to the first days of fermentation. These mechanisms of cell adaptation at low temperature should be confirmed with further experimental work because they would be of great industrial interest. The ability of wine yeast to adapt to the hostile environment and stress conditions in grape juice fermentation is vitally important for fermentation performance. Yeast cells could be pre-adapted at low temperature to endure ethanol toxicity during alcoholic fermentation, thus increasing their viability throughout and viceversa, to pre-adapt yeast cells to ethanol toxicity for a better performance of the low temperature fermentations.

The DNA chip technology is one of the promising tools for the analysis of cell physiology. This global studies of the cell as a whole can help us to understand many of the technical problems facing winemaking. Some of this knowledge could be also used in the future perspectives of genetic improvement of wine yeasts strains (Perez-Ortin *et al.*, 2002; Pretorius and Bauer, 2002). Curiously, the expression of several genes (e.g. genes involved in trehalose and glycogen metabolism, stress response, sterol formation

and esterases, etc.) identified as targets for the genetic improvement of wine-yeast strains, fermentation performance, wine quality and other sensory qualities (Pretorius and Bauer, 2002), is modified at low-temperature fermentations.

Another important factor for avoiding problematic fermentations and improving fermentation performance is the control of nutritional requirements, especially nitrogen deficiencies. Moreover, nitrogen uptake patterns also influence the production of several aromatic compounds.

In wine fermentations, cells evolve from a nitrogen-repressed situation at the beginning of the process to a nitrogen-derepressed situation as the nitrogen is consumed. The transcriptional activity of the general amino acid and ammonium permeases, *GAP1* and *MEP2* genes, is repressed during the first hours after the inoculation in the media by the Nitrogen Catabolite Repression (NCR), and both genes are activated/derepressed when ammonia is depleted. The transcription profiles of *MEP1* and *MEP3* during fermentation, with lower levels of gene expression, did not respond to NCR as clearly as the transcription profile of *MEP2* did.

These nitrogen-repressed/derepressed conditions determined the different patterns of ammonium and amino acid consumption. Glutamine and tryptophan were the main amino acids consumed throughout the fermentations. Arginine, alanine, aspartate, glutamate and glycine were the amino acids that were most affected by the NCR because they were hardly consumed when there was an excess of nitrogen. In fact, they were not taken up until the medium was depleted of good nitrogen sources. On other hand, the uptake of branched-chain and aromatic amino acids increased in NCR situation.

The transcriptional activity of *GAP1* and *MEP2* genes, the arginase activity or the uptake of some amino acids as arginine could be used as markers to check the state of nitrogen repression or derepression in the cell.

Winemakers systematically supplement grape musts with diammonium phosphate to prevent nitrogen-related fermentation problems. Better knowledge of this system should improve the control of nitrogen availability and addition during wine fermentations. Nitrogen-repressed conditions throughout fermentation modify the uptake of ammonium and amino acid and this different uptake influence the production of aroma

and spoilage compounds (particularly hydrogen sulfide), and the amount of urea, the major precursor of the carcinogen ethyl carbamate (Rapp and Versini, 1991; Ough, 1991; Jiranek *et al.*, 1995). Moreover, a wine with an excess of nitrogen could have a negative effect on microbiological stability during ageing, storage or bottling.

The timing of the nitrogen additions influenced the biomass yield, the fermentation performance, the patterns of ammonium and amino acid consumption, and the production of secondary metabolites. Ammonium was the preferred nitrogen source for biomass production but it was hardly consumed when added at the final stages of fermentation. The higher ammonium consumption in some fermentations correlated with a greater synthesis of glycerol, acetate and acetaldehyde but also with a lower synthesis of higher alcohols.

Although further studies with other wine strains are needed, our data showed that the preferred nitrogen sources of cell growth and sugar fermentation are different. Nitrogen additions always improved fermentation performance but had a minimal effect on biomass production when added in the second half of fermentation. These nitrogen additions induced the NCR and changed the profile of nitrogen consumption. The differences in the pattern of nitrogen consumption were related to different aroma compound compositions in the final wines. In our opinion, this study is a starting point for further research into the use of an ammonium/amino acid mixture as a nitrogen supplement in the wine industry and the effects of these additions on the physiology of the yeast, the performance of the fermentation and the quality of the wine.

Nitrogen assimilation also depends on fermentation temperature. Fermentation temperature is an important factor determining utilization of nitrogen sources during fermentation of grape juice, and influences the quantity and the quality of nitrogen requirement. Ammonium and glutamine, the preferred source for biomass production, are less consumed at low temperature. Likewise amino acids that are only taken up under derepressed conditions (arginine, alanine, asparagine, etc.) are more consumed at low temperature. The uptake of tryptophan also increased at low temperature. In fact, low temperature seems to relax the nitrogen catabolite repression. Although nitrogen needs are strain dependent, we could conclude that low temperature fermentation decrease nitrogen requirements.

The information provided by this thesis represents a starting point for deciphering the regulatory circuits during wine fermentation, overall at low temperature, and should help us to understand the properties of wine yeasts.

General Conclusions

- The low temperature fermentation affects the kinetics and the yeast growth resulting in a slow growth and fermentation rate but a higher viability of yeast cells throughout the process.

- The low temperature fermentation affects the lipid composition of the yeast membrane resulting in:
  - a higher degree of unsaturation at the beginning of fermentation,
  - a decrease in chain length at the end of the process (higher MCFA),
  - a higher ergosterol proportion at the end of the fermentation at 13ºC than at 25ºC.

- The low fermentation temperature affects the wine aromatic composition, resulting in:
  - an increase in flavour-active compounds, such as fusel alcohol acetates and fatty acid ethyl esters,
  - a decrease in unpleasant ones such as acetic acid and fusel alcohols.

  These effects are observed in both natural and synthetic media fermentations.

- Most of the effects of low temperature in yeast metabolism are observed in both natural and synthetic must fermentations, which confirms that this synthetic medium can be used in subsequent studies of yeast metabolism in which natural must fermentations are mimicked.

- Fermentation of natural grape must permits yeast cells to reach higher values of viability, which seems to be related, among others, to the incorporation of linoleic acid and ß-sitosterol, present in the grape media.

- Fermentation of natural grape must permits to obtain fruitier and more aromatic wines, with low volatile acidity content than those of laboratory medium. The low temperature emphasized this feature.

- At low temperatures two evident phases of fermentation are observed in relation to yeast gene response, which coincided with the exponential and stationary phases, respectively. In fact, gene expression of lag phase was not analysed in this study.

    - During exponential phase at 13ºC yeasts seem to enter in a phase adaptation to the hostile environment and stress conditions (as it was an extension of the lag phase). Cells respond to this stressful low temperature condition with up-regulation of genes involved in cold-shock, but also genes of general stress response. Genes involved in metabolism (carbon and nitrogen), energy and transport are also up-regulated in this phase at low temperature fermentation, but down-regulated as it progresses.

    - In the stationary phase of 13ºC fermentation genes belonging to cell fate, growth control, and maintenance are up-regulated at 13ºC with respect to 25ºC, which correlated with a higher cell's viability at low temperature. In this phase, genes involved in RNA metabolism and processing, transcription and trehalose and glycogen accumulation are also up-regulated.

- The adaptation response of yeast to low temperature seems to be correlated with the ethanol resistance.

- The analysis of the transcriptomic profile of *S. cerevisiae* at low temperature fermentation confirmed some of the differences observed in the lipid constitution of the cells.

- The increase of aroma-related compounds at low temperatures may be due both to a modification of yeast metabolism by temperature, and to the higher activity of yeasts in the latter stages of fermentation.

- In wine fermentations the cells evolve from a nitrogen-repressed situation at the beginning of the process to a nitrogen-derepressed situation as the nitrogen is consumed. These nitrogen-repressed/derepressed conditions determined the different patterns of ammonium and amino acid consumption.

- *GAP1* and *MEP2* are repressed during the first hours after the inoculation in the media, and both genes are activated/derepressed when ammonia is depleted.

- The transcriptional activity of two important permeases of amino acids and ammonium as *GAP1* and *MEP2* genes, the arginase activity or the uptake of some amino acids as arginine could be used as markers to check the state of nitrogen repression or derepression in the cell.

- Nitrogen additions always improved the fermentation performance but had a minimal effect on biomass production when they were added in the second half of the fermentation.

- The timing of nitrogen additions influenced the biomass yield, the fermentation performance, the patterns of ammonium and amino acid consumption, and the production of secondary metabolites.

- Ammonium is the preferred nitrogen source for biomass production but was hardly consumed when it was added in the final stages of the fermentation.

- Amino acids could be classified into three categories regarding the timing of consumption:

    - the amino acids mostly consumed all along the fermentation: glutamine and tryptophan

    - the amino acids whose consumption is not NCR-dependent and that were mainly consumed during the growing phase: branched-chain amino acids

    - the amino acids whose uptake is repressed in the presence of ammonia in the media, such as arginine, alanine, asparagine, glycine, tyrosine or glutamate.

- The higher ammonium consumption correlates with a greater synthesis of glycerol, acetate and acetaldehyde but with a lower synthesis of higher alcohols.

- Low temperature fermentation produces similar metabolic effects to these obtained in nitrogen-deficient cultures. Low temperature and nitrogen-limited growth decreased biomass yield and relaxed the nitrogen catabolite repression.

- Low temperature fermentation decrease the nitrogen consumption, overall ammonia uptake.

- Low temperature influences the quantity and the quality of nitrogen requirement. Ammonium and glutamine, the preferred source for biomass production, are less consumed at low temperature. Likewise amino acids that are only taken up under derepressed conditions (arginine, alanine, asparagine, etc.) are more consumed at low temperature. The uptake of tryptophan also increases at low temperature.

Perspectives

This thesis has focused on the effects and responses of low temperatures and nitrogen compounds on wine yeast metabolism. In my opinion, our results open up a lot of interesting perspectives that will further our knowledge of wine yeast metabolism during wine fermentations. Some of them could be the study of:

- The higher aroma production at low temperature: expression of related genes and enzymatic activities.
- Metabolism of short- and medium-chain fatty acids and their corresponding ethyl esters.
- Early cold-shock and general stress response at low temperature.
- Higher ethanol tolerance at low temperature?
- Trehalose and glycogen metabolism at low temperature.
- Yeast pre-adaptation to increase the fermentation performance at low temperature: pre-adaptations with ethanol or low temperatures.
- Screening of other *S. cerevisiae* strains comparing the good-fermenting strains with strains the weak fermenting strains at low temperatures to find which are the main responses needed to survive and perform in this condition.
- Nitrogen Catabolite Repression and nitrogen requirements at low temperature.

REFERENCES

Alexandre,H., Rousseaux,I., Charpentier,C. (1994a). Ethanol adaptation mechanisms in Saccharomyces cerevisiae. Biotechnol.Appl.Biochem. *20*, 173-183.

Alexandre,H., Rousseaux,I., Charpentier,C. (1994b). Relationship between ethanol tolerance, lipid composition and plasma membrane fluidity in Saccharomyces cerevisiae and Kloeckera apiculata. FEMS Microbiol.Lett. *124*, 17-22.

Beney,L., Martinez,d.M., I, Marechal,P.A., Gervais,P. (2000). Influence of thermal and osmotic stresses on the viability of the yeast Saccharomyces cerevisiae. Int.J Food Microbiol. *55*, 275-279.

Fukuda,K., Yamamoto,N., Kiyokawa,Y., Yanagiuchi,T., Wakai,Y., Kitamoto,K., Inoue,Y., Kimura,A. (1998). Balance of activities of alcohol acetyltransferase and esterase in Saccharomyces cerevisiae is important for production of isoamyl acetate. Appl.Environ.Microbiol. *64*, 4076-4078.

Gasch,A.P. (2003). The Environmental Stress Response: a Common Yeast Response to Diverse Environmental Stresses. In: Yeast Stress Responses, ed. S.Hohmann, W.H.MagerBerlin Heidelberg: Springer-Verlag, 11-70.

Gasch,A.P., Spellman,P.T., Kao,C.M., Carmel-Harel,O., Eisen,M.B., Storz,G., Botstein,D., Brown,P.O. (2000). Genomic expression programs in the response of yeast cells to environmental changes. Mol.Biol.Cell *11*, 4241-4257.

Jiranek,V., Langridge,P., Henschke,P.A. (1995). Regulation of hydrogen sulfide liberation in wine-producing Saccharomyces cerevisiae strains by assimilable nitrogen. Appl.Environ.Microbiol. *61*, 461-467.

Lilly,M., Lambrechts,M.G., Pretorius,I.S. (2000). Effect of increased yeast alcohol acetyltransferase activity on flavor profiles of wine and distillates. Appl.Environ.Microbiol. *66*, 744-753.

Llauradó, J. M. Avaluació dels condicionants del most en el desenvolupament de la fermentació alcohòlica a baixes temperatures. 2002. PhD thesis Universitat Rovira i Virgili, Tarragona (Spain).

Mason,A.B., Dufour,J.P. (2000). Alcohol acetyltransferases and the significance of ester synthesis in yeast. Yeast *16*, 1287-1298.

Ough,C.S. (1991). Influence of Nitrogen Compounds in Grapes in Ethyl Carbamate Formation in Wines. In: Proceedings of the International Sumposium on Nitrogen in Grapes and Wine, ed. J.M.RanzDavis, CA: Am.Soc.Enol.Vitic., 165-171.

Perez-Ortin,J.E., Garcia-Martinez,J., Alberola,T.M. (2002). DNA chips for yeast biotechnology. The case of wine yeasts. J.Biotechnol. *98*, 227-241.

Pretorius,I.S., Bauer,F.F. (2002). Meeting the consumer challenge through genetically customized wine-yeast strains. Trends Biotechnol. *20*, 426-432.

Rapp,A., Versini,G. (1991). Influence of Nitrogen Compounds in Grapes on Aroma Compounds of Wines. In: Proceedings of the International Symposium on Nitrogen in Grapes and Wine, ed. J.M.RanzDavis, CA: Am. Soc. Enol. Vitic. 156-164.

Rossignol,T., Dulau,L., Julien,A., Blondin,B. (2003). Genome-wide monitoring of wine yeast gene expression during alcoholic fermentation. Yeast *20*, 1369-1385.

Sahara,T., Goda,T., Ohgiya,S. (2002). Comprehensive expression analysis of time-dependent genetic responses in yeast cells to low temperature. J.Biol.Chem. *277*, 50015-50021.

Schade,B., Jansen,G., Whiteway,M., Entian,K.D., Thomas,D.Y. (2004). Cold Adaptation in Budding Yeast. Mol.Biol.Cell.

Torija, M. J. Ecología de levaduras: selección y adaptación a fermentaciones vínicas. 2002. PhD Thesis. Universitat Rovira i Virgili, Tarragona (Spain).

Walker,M.A. (1998). Yeast Physiology and Biotechnology. West Sussex, England: Wiley & Sons.

ANNEX 1

Materials & Methods

Índex

1. Medis de llevats 192
1.1. YEPD agar 192
1.2. YEPD líquid 192
1.3. Medi Lisina 192
2. Medis de fermentació i fermentacions 192
2.1. Medi de rehidratació 192
2.2. Fermentacions industrials: Most natural 192
2.3. Fermentacions de laboratori: Most sintètic (MS) 193
3. Seguiment de les fermentacions i les poblacions de llevat 195
3.1. Mesura de la densitat del most 195
3.2. Recompte de llevats totals 195
3.3. Recompte de llevats viables 195
3.4. Mesura de la densitat òptica 195
3.5. Mesura del pes sec 195
4. Mètodes de Biologia Molecular 196
4.1. Determinació de l'expressió gènica per RT-PCRq 196
4.2. Determinació de l'expressió gènica per Northern-blot 200
4.3. Comparació dels resultats de RT-PCRq i Northerns 202
4.4. Determinació de l'expressió gènica per chips de DNA o Microarrays 203
4.4. Identificació de llevats a nivell de soca: Anàlisis dels perfils de restricció (RFLPs) del mtDNA 207
4.5. Identificació de llevats a nivell d'espècie: Anàlisis dels perfils de restricció (RFLPs) del rDNA 208
5. Analítiques bàsiques en mostos i vins 209
5.1. Determinació de sucres reductors (mètode GAB) 209
5.2. Determinació de la Glucosa i Fructosa (mètode enzimàtic) 209
5.3. Determinació de l'acidesa total 209
5.4. Determinació de l'acidesa volàtil (mètode García-Tena) 210
5.5. Determinació del grau alcohòlic per ebullimetria 210
5.6. Determinació del sulfurós total i lliure 210
5.7. Determinació dels àcids orgànics, sucres, glicerol i etanol per HPLC 211
6. Analítiques de compostos secundaris 211
6.1. Determinació dels àcids grassos del vi 211
7. Analítiques de compostos nitrogenats 212
7.1. Determinació dels aminoàcids (AA) per HPLC 212
7.2. Determinació del nitrogen assimilable pels llevats (YAN total) 213
7.3. Determinació del amoni per kit enzimàtic 213
8. Anàlisis dels lípids del llevat 213
8.1. Determinació dels àcids grassos de membrana 213
8.2. Determinació dels esterols 214
8.3. Determinació dels lípids neutres i fosfolípids 215
9. Altres: 216
9.1. Determinació de la Trehalosa i Glicogen intracelular 216
9.2. Activitat arginasa 217
Pàgines web utilitzades: 219
REFERENCES 220

1. Medis de llevats

1.1. YEPD (Yeast Extracte Peptone Dextrose) agar

Glucosa		20 g
Peptona (Cultimed)		20 g
Extracte de llevat (Cultimed)		10 g
Agar (Tipus E, Cultimed)		20 g
Aigua destil·lada	c.s.p.	1000 ml

La mescla s'autoclava a 120ºC, 15 min, i es dispensa a les plaques Petri a una temperatura convenient (uns 50ºC).

1.2. YEPD líquid

La mateixa composició que el medi anterior, però sense agar.

1.3. Medi Lisina (Morris and Eddy 1957)

Aquest medi s'utilitza per distingir les soques *Saccharomyces* de la resta d'espècies víniques. *Saccharomyces* no pot créixer en un medi que contingui lisina com a única font de nitrogen, en canvi els altres llevats si que es desenvolupen.

- Preparar una solució de lactat potàssic: 18 ml àcid làctic al 85 %
  14 g KOH
- En un litre de aigua destil·lada se li afegeixen 4 ml d'aquesta solució.
- Addicionar 66 g de pols de medi Lisina (ADSA micro).
- Escalfar la mescla fins dissoldre totalment el medi, agitant contínuament per evitar el sobreescalfament.
- Es refreda fins uns 50ºC en un bany i s'afegeix 1 ml d'àcid làctic al 10 % per ajustar el pH aproximadament a 5.0.
- Distribuir en plaques.

2. Medis de fermentació i fermentacions

2.1. Medi de rehidratació

El llevat utilitzat en les fermentacions realitzades és un llevat sec actiu (LSA) comercial *Saccharomyces cerevisiae var. bayanus* QA23 (Lallemand). Seguint la metodologia recomanada per la casa comercial, el llevat es rehidrata en aigua tèbia (uns 37ºC) durant 30 minuts (1 g de LSA per cada 10 ml d'aigua: 10^9 cells/ml). Un cop rehidratat el llevat s'inocula en els fermentadors a una població inicial aproximada de $2\ 10^6$ cells /ml.

2.2. Fermentacions industrials: Most natural

Les fermentacions industrials es duen a terme a la finca experimental Mas dels Frares, localitzada a 4 km de Tarragona, i propietat de la Facultat d'Enologia de la URV. Per les proves de fermentacions industrials a diferents temperatures s'utilitza most de la varietat de raïm Muscat. El most es deixa clarificar durant 24 hores, s'hi afegeix sulfurós (50 mg l^{-1}) i es reparteix en tines d'acer inoxidable de 100 litres, plenes amb uns 80 litres de most. S'inocula amb el llevat comercial QA23 tal com s'indica en l'apartat 2.1. Les fermentacions control tenen lloc a temperatura ambient (~25ºC) mentre que la temperatura es controla a 13ºC en les fermentacions a baixes temperatures.

2.3. Fermentacions de laboratori: Most sintètic (MS)

Les fermentacions de laboratori o microfermentacions es duen a terme utilitzant un most sintètic que presenta les característiques mitjanes d'un most de raïm (Bely *et al.* 1990). Aquest medi s'utilitza per modelitzar les condicions enològiques, i es caracteritza per tenir una forta concentració en sucres (200g/l), un contingut relativament feble en nitrogen (300 mg/l) i un pH àcid.
Les microfermentacions es realitzen a temperatura controlada (25ºC o 13ºC) en fermentadors de 2 litres, emplenats amb 1.8 litres de medi, i amb taps que permeten la sortida de CO_2 i la presa de mostra. Les fermentacions s'inoculen amb el llevat QA23, i s'agiten periòdicament per prendre la mostra (fermentacions semi-anaeròbiques).

<u>Composició d'un litre de MS</u>

Glucosa	100 g
Fructosa	100 g
Àcid cítric	5 g
Àcid màlic	0.5 g
Àcid tartàric	3 g
KH_2PO_4	0.750 g
K_2SO_4	0.500 g
$MgSO_2$ 7 H_2O	0.250 g
$CaCl_2$ 2 H_2O	0.155 g
NaCl	0.200 g
Nitrogen (300mg N assimilable /l)*	
NH_4Cl (120 mgN/l)	0.460 g
Solució mare d'aminoàcids (180 mgN/l)	13.09 ml
Solució mare d'oligo-elements	1 ml
Solució mare de vitamines	10 ml
Facors d'anaerobiosis (calentar 70ºC)	1 ml
H_2O destil·lada	Csp 1 L

El medi s'esterilitza al autoclau 20 min. a 120ºC abans d'afegir-hi les solucions mare.
El pH s'ajusta a 3.3 amb una solució de NaOH 10M

*Contingut en Nitrogen Assimilable (YAN) de les fermentacions control (CNC). Pels estudis del metabolisme nitrogenat variem aquesta concentració:
HNC: 1200 mg YAN/l (480 mgN/l en forma d'amoni, 720 mgN/l en forma d'amino àcids)
LNC: 60 mg YAN/l (sols amoni, sols amino àcids i amoni + amino àcids)
Per l'estudi d'addicions de nitrogen les fermentacions comencen amb 60 mg YAN/l i s'hi afegeix 240 mg YAN/l en diferents punts de la fermentació, mantenint les proporcions d'amoni i amino àcids.

<u>Solució mare d'oligo-elements (per 1 litre)</u>

$MnSO_4$, H_2O	4 g
$ZnSO_4$, 7 H_2O	4 g
$CuSO_4$, 5 H_2O	1 g
KI	1 g
$CoCl_2$, 6 H_2O	0.4 g
H_3BO_3	1 g
$(NH_4)_6Mo_7O_{24}$	1 g
H_2O destil·lada	csp 1L

Aquesta solució és esterilitzada per filtració i guardada a 4ºC.

Solució mare de factors d'anaerobiosis (per 100 ml)

Ergosterol	1.5 g
Oleci Acid	0.5 ml
Tween 80	50 ml
Etanol pur	cps 100 ml

Calentar a 70 ºC per dissoldre.
Aquesta solució és aliquotada i conservada a 4ºC.

*En els experiments duts a terme no hem afegit aquest factors d'anaerobiosi degut a que treballem en condicions semi-anaeròbiques, i que en alguns casos es pretenia analitzar la producció de lípids per part del llevat, resultat que es podia veure modificat per l'addició d'aquests compostos.

Solució mare d'amino àcids (per 1 litre de solució tampó Na_2CO_3 2%)

Tirosina (Tyr)	1,5 g	calentar a 100ºC
Triptòfan (Trp)	13,4 g	70ºC
Isoleucina (Ile)	2,5 g	70ºC
Àcid Aspàrtic (Asp)	3,4 g	desgasificar de CO_2
Àcid Glutàmic (Glu)	9,2 g	desgasificar de CO_2
Arginina (Arg)	28,3 g	
Leucina (Leu)	3,7 g	aumentar la Tª
Treonina (Thr)	5,8 g	
Glicina (Gly)	1,4 g	
Glutamina (Gln)	38,4 g	
Alanina (Ala)	11,2 g	
Valina (Val)	3,4 g	
Metionina (Met)	2,4 g	
Fenilalanina (Phe)	2,9 g	
Serina (Ser)	6,0 g	
Histidina (His)	2,6 g	
Lisina (Lys)	1,3 g	
Cisteïna (Cys)	1,5 g	
Proline(Pro)	46,1 g	
H_2O destil·lada	cps 1 L	
Total	**138g**	(correspon a 13,75 g N alfa-amino/l)

Aquesta solució és esterilitzada per filtració i conservada a –20ºC en alíquotes.

Solució mare de vitamines (per 1 litre)

Myo-inositol	2 g
Pantothenate calcium*	0.15 g
Thiamine, hydrocloride	0.025 g
Nicotinic acid	0.2 g
Pyridoxine*	0.025 g
Biotine*	3 ml**
H_2O destil·lada	csp 1L

* Vitamines conservades en fred.
* *Solució mare de biotina a 100 mg/l, conservada al congelador.
Aquesta solució és esterilitzada per filtració i conservada a –20ºC en alíquotes.

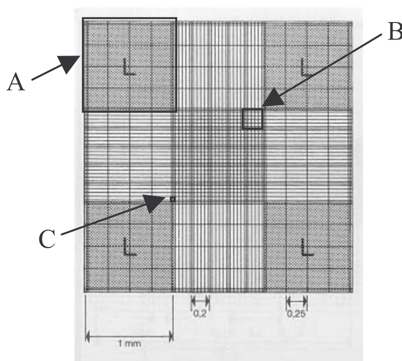
3. Seguiment de les fermentacions i les poblacions de llevat

3.1. Mesura de la densitat del most

Un mètode àmpliament utilitzat en bodega pel seguiment de la fermentació és la mesura de la densitat del most, la qual està relacionada en un 99 % amb la concentració de sucres del most (Ribéreau-Gayon *et al.* 2000). La corba de disminució de la densitat al llarg de la fermentació és directament proporcional a la corba de consum de sucres. En les ultimes fases de fermentació el seguiment es fa per mesura dels sucres residuals de la mostra (Apartats 5.1 i 5.2).
En les fermentacions industrials la densitat del most es mesura mitjançant un densímetre de Baumé, que dóna directament la densitat del most. En les fermentacions de laboratori la mesura de la densitat es realitza per pesada de 5 ml de medi (prèviament centrifugat i desgasificat), en una balança de precisió. Aquesta pesada es fa per triplicat, i el valor que s'obté s'utilitza per calcular-ne la densitat del medi en mg/l.

3.2. Recompte de llevats totals

El nombre de llevats totals durant la fermentació es determina per recompte al microscopi òptic, utilitzant la cambra de Neubauer.



$A = 16B \qquad B = 25C$

Volum C = $0.05 \times 0.05 \times 0.1\text{mm} = 2.5\ 10^{-4}\ \text{mm}^3 = 2.5\ 10^{-7}$ ml
Volum B = $0.25 \times 0.25 \times 0.1 \times 10^{-3} = 6.25\ 10^{-6}$ ml
Volum A = $1 \times 1 \times 0.1 \times 10^{-3} = 10^{-4}$ ml

$$\frac{\text{Nº cèl·lules x dilució}}{\text{Nº quadres N x Volum N}} = \text{nº cells / ml}$$

Profunditat = 0.1 mm

3.3. Recompte de llevats viables

Per la determinació de llevats viables al llarg de la fermentació es sembren diverses dilucions de la mostra en medi sòlid YPD, i s'incuben a 28ºC durant 2 dies. El nombre de colònies crescudes per la dilució realitzada correspon a nombre unitats formadores de colònies per ml (UFC/ml).

3.4. Mesura de la densitat òptica

La mesura d'absorbància a 600nm ens dóna un valor directament proporcional a la biomassa del llevat. Durant la fermentació, el medi correctament diluit ($DO_{600} < 0.5$), es mesura al espectofotèmetre a 600nm, utilitzant com a blanc el propi medi inicial.

3.5. Mesura del pes sec

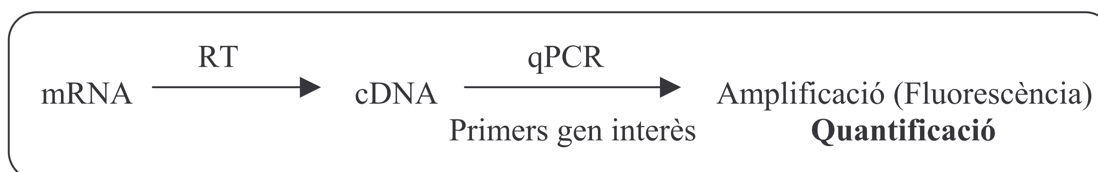
Una mesura més directa de la biomassa del llevat és el pes sec. Un volum conegut de medi es centrifuga (10000g 2min) i les cèl·lules de llevat es passen a un tub Eppendorf prèviament assecat (100ºC 24 hores) i pesat (W0). Un cop centrifugat i eliminat el sobrenedant, aquest tub es posa de nou a 100ºC durant 24h, es deixa refredar en un dessecador unes hores, i es pesa de nou (W1).

Pes Sec (mg) = W1 – W0 $\qquad$ mg Pes Sec/ml = (W1-W0)/Volum

4. Mètodes de Biologia Molecular

4.1. Determinació de l'expressió gènica per RT-PCRq

La tècnica de RT-PCR consisteix en aïllar el RNA missatger, i fer-ne una retrotranscricció (RT) per obtenir-ne el DNA complementari (cDNA) a la seqüència de RNA. La quantitat de cDNA d'un gen dependrà del seu nivell d'expressió, és a dir del nombre de molècules de RNA que la cèl·lula hagi sintetitzat en les condicions d'estudi. Posteriorment, la PCR específica amplificarà selectivament el cDNA corresponent al gen d'interès. La PCR quantitativa a temps real permet el seguiment a cada cicle del producte de PCR format, obtenint unes corbes d'amplificació a partir de les quals es pot extrapolar la concentració inicial de cDNA del gen d'interès.



A. Extracció de RNA (Sierkstra *et al.* 1992)

- Rentar les cèl·lules (provenint de 5-10 ml de medi de fermentació) amb 1 ml d'**Extraction Buffer** (100mM Tris-HCl pH7.4, 100mM LiCl, 0.1mM EDTA).
- Centrifugar 5 min. a 10000 rpm a 4ºC i eliminar el sobrenendant.
- Resuspendre el *pellet* en 0.5 ml de **Vortex Buffer** (100mM LiCl, 10mM EDTA, 0.5% LitiumDodecylSulfat, pH 7.4). Transferir la suspensió en Eppendorfs de 2ml que continguin 1g de perles de vidre.

A partir d'aquest moment treballar en condicions lliures de RNAses.

- Trencar les cèl·lules en intervals de 30 segons vórtex / 30 segons en gel (8-10 cops).
- Afegir 500 µl de **fenol/cloroform/alcohol isoamílic** (25:24:1) (v/v) i agitar 2 seg al vórtex..
- Centrifugar 5 min a 10000 rpm 4ºC.
- Extreure la fase aquosa (superior) amb **fenol/cloroform/alcohol isoamílic** (25:24:1) (v/v). (Repetir aquest pas un parell de cops o fins no s'observi interfase).
- Centrifugar i extreure la fase aquosa (superior) amb **cloroform** (v/v).
- Centrifugar i afegir a la fase aquosa (superior) 1/10 del volum de **NaAc 3M** (pH 5.6) i 2.5 del volum de **Etanol absolut.** Precipitar 15 min a -80ºC.
- Centrifugar 30 min a 4ºC i rentar el *pellet* amb 500 µl de **Etanol 70%**. Centrifugar 5 min. a 10000 rpm.
- Assecar el RNA a l'aire i resuspendre en 50 µl d'aigua-DEPC durant una nit.

B. Purificació del RNA

Per eliminar bé el DNA que pugui quedar en la solució i que ens podria interferir en el resultat de la RT-PCR, el RNA es digereix amb DNAsa. Per fer-ho s'utilitza un kit comercial de purificació: High Pure Isolation kit (Roche Applied Science, Germany), seguint el protocol subministrat per la casa comercial.

C. Visualització i Quantificació del RNA

La concentració de RNA es determina mitjançant lectura al espectofotòmetre (GenQuant spectrophotometer, Pharmacia, Canada). Es realitza una lectura entre 200 i 300, mesurant les absorbàncies a 230, 260 i 280 nm.

RNA (μg/ml) = Abs 260 x 2 (Cubeta 0,5mm) x 40 μg/ml x dilució mostra
$Abs260/Abs235 \geq Abs260/Abs280 \geq 1,8$

La qualitat del RNA es verifica també electroforèticament mitjançant gels d'agarosa del 0.8% (p/v), utilitzant com a marcador de pes una mescla de 50% (v/v) del marcador DNA Molecular Weight MarkerII i 50 % (v/v) de DNA Molecular Weight MarkerIII (0.12-21.1 kbp, ADN del fag λ digerit amb EcoRI I HindIII) de Boehringer Mannheim.

D. Síntesis del cDNA (Retrotranscripció o RT)

La reacció es du a terme en un termociclador Perkin Elmer GeneAmp PCR System 2400.

- Diluir les mostres de RNA total fins obtenir una concentració d'uns 0.320 μg/μl.
- Afegir a cada tub:

A (RT)	**B** (control noRT)*
2.5 μl mostra (0.8 μg RNA)	2.5 μl mostra (0.8 μg RNA)
1 μl Oligo-dT 12-18 Primer (Invitrogen) (0.1μg)	9.5 μl H_2O-DEPC
8.5 μl H_2O-DEPC	

- Incubar en el termociclador 5 min. 70ºC
- Deixar-ho 1 min en gel
- Afegir a cada tub: 4 μl 5xPCR Buffer (Invitrogen)
  1 μl dNTPs 10mM
  2 μl DTT 0,1M
- Incubar 5 min. 42ºC.
- Afegir als tubs: **A** 1μl Superscript II Rnase H^- Reverse Transcriptase (Invitrogen).
  B 1 μl H_2O-DEPC
  Volum final 20μl amb 40 ng RNA /μl
- Incubar 50 min. 42ºC.
- Per aturar la reacció s'incuba 15 min. a 70ºC.

*Per comprovar les possibles restes de contaminació per DNA que puguin haver quedat en la mostra es fa un control noRT, en el que s'afegeixen tots els reactius excepte l'enzim transcriptasa inversa i el OligodT.

E. PCR quantitativa a temps real (Real Time Quantitative PCR)

Termocilcador Applied Biosystems GeneAmp 5700 Sequence Detection System.

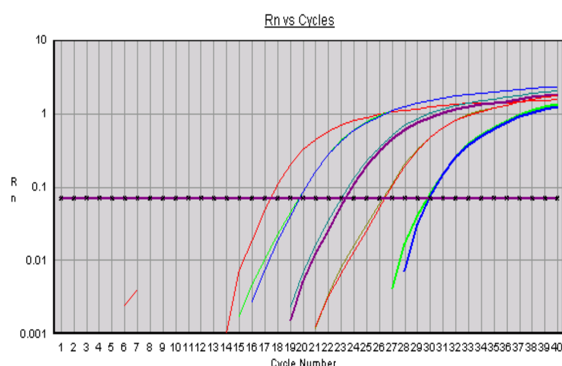
El fonament de la tècnica de PCR Quantitativa a temps real és el mateix que el de la PCR, però en aquest cas es fa un seguiment de la reacció a temps real mitjançant la

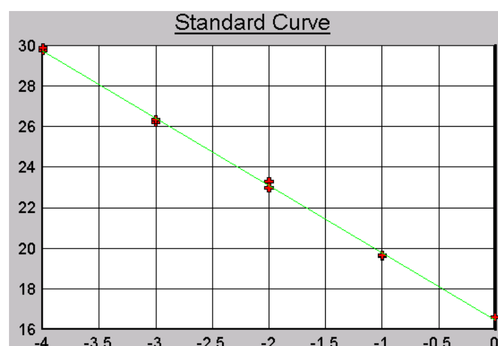
monitorització del senyal de fluorescència que emet un fluorocrom present en la reacció (SYBR Green I, Applied Biosystems). Aquest compost s'intercala específicament al DNA de doble cadena format i en aquestes condicions emet fluorescència. Per tant el senyal emès és proporcional a la quantitat de molècules de doble cadena formades en el cicle de PCR.

Encebadors o Primers utilitzats: El disseny de primers s'ha realitzat a partir de les seqüències dels gens obtingudes en GenBank i el software Primer Express (Applied Biosystems, USA), seguint les instruccions d'Applied Biosystems pel disseny de primers per PCR quantitativa.

Primer	Nucletide sequence (5' to 3')
ACT-F	TGGATTCCGGTGATGGTGTT
ACT-R	CGGCCAAATCGATTCTCAA
GAP1-F	CTGTGGATGCTGCTGCTTCA
GAP1-R	CAACACTTGGCAAACCCTTGA
MEP1-F	CCTGAGCTCGCGTATGCA
MEP1-R	GGCGCCAGCGATAATACTTAA
MEP2-F	GGTATCATCGCTGGCCTAGTG
MEP2-R	ACAACGGCTGACCAGATTGG
MEP3-F	GCCGGTGTGGTGGGATT
MEP3-R	TTGTGCCGTCCATTCCAAT

Rectes patró per cada gen:
Per a cada gen d'estudi es construeix una recta patró. Es realitza la PCR quantitativa utilitzant com a mostra DNA genòmic de concentració coneguda, en un rang de 40 a 4 x 10^{-4} ng/µl (que en el tub de PCR representa entre 200 ng i 2 x 10^{-3} ng de DNA genòmic). Per a cada concentració de DNA s'obté una Ct corresponent, a partir de les quals es pot construir una recta patró diferent per a cada parella de primers utilitzats.





Rectes patró:

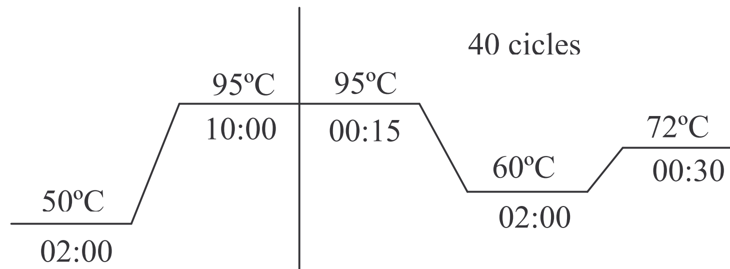
ACT:	**Ct=**	**-3,144**	**log(x) +**	**18,061**	**R=0,9928**
GAP:	**Ct=**	**-3,44**	**log(x) +**	**19,469**	**R=0,9974**
Mep1:	**Ct=**	**-3,414**	**log(x) +**	**19,802**	**R=0,9992**
Mep2:	**Ct=**	**-3,252**	**log(x) +**	**19,326**	**R=0,9988**
Mep3:	**Ct=**	**-3,232**	**log(x) +**	**18,840**	**R=0,9982**

Realització de la PCR:

Preparar una mescla amb : 12.5 µl Mix SYBR Green 2X
1.5 µl Primer Forward (5 µM)
1.5 µl Primer Reverse (5 µM)
4.5 µl aigua

Afegir-hi 5.0 µl cDNA (diluir 1:5 la RT)

La concentració final dels primers en el tub de PCR és de 0.3 µM.
Les condicions de PCR utilitzades són:



El pas de 10 minuts a 95ºC és per activar la Taq polimerasa Gold, que és l'enzim subministrat per Applied en la mescla SYBR Green 2X per fer la reacció.
El pas d'extensió a 72ºC no és necessari si l'amplicó és petit (50-150 pb), ja que durant el pas de 60 a 95ºC s'arriba a 72ºC i la polimerasa té temps suficient per realitzar la extensió.

Càlculs: Per a determinar l'expressió relativa, s'interpola en les rectes de calibratge per determinar a partir de les Ct obtingudes en la PCR, la concentració de cDNA del gen d'interes així com la concentració del gen de referència. Es calcula el quocient entre la concentració de cDNA del gen d'interès i del gen de referència, i aquest valor ens indica els nivells d'expressió relativa del gen d'interès.

Gene expression = [Gene d'interès] / [Gen referència]

Valor de 1 significa que la mostra analitzada conté la mateixa concentració d'actina que del gen d'estudi. Aquest valors els podem expressar també en % multiplicant el valor obtingut per 100.

4.2. Determinació de l'expressió gènica per Northern-blot

Aïllament, purificació i quantificació del RNA

La extracció, purificació i quantificació del RNA es du a terme seguint el mateix protocol que el descrit en l'apartat 4.1 per la RT-PCR.

Electroforesi de RNA en gels desnaturalitzants

Les mostres de RNA es preparen seguint el protocol descrit a (Sambrook *et al.* 1989).

Preparació del gel desnaturalitzant:

Preparar un gel d'agarosa al 1-2 % amb tampó MOPS 1X (MOPS 20mM, acetat sòdic 8 mM, EDTA 1mM, pH7) i formaldèhid 2.2 M.

Preparació de les mostres:

- Afegir en un tub: 2.5 µl RNA (~ 10µg RNA)
  - 0.5 µl MOPS 10x
  - 1.5 µl formaldèhid 35-40% (v/v)
  - 5 µl formamida desionitzada
  - H_2O fins volum final 10 µl

  (0.5 µl MOPS 10x, 1.5 µl formaldèhid, 5 µl formamida i H_2O: dissolvent de mostres)

  (1 volum de RNA per 3 volums de dissolvent de mostres)
- Incubar les mostres a 65ºC durant 10 minuts.
- Deixar les mostres en gel uns 5 minuts.
- Afegir 2 µl tampó de càrrega.
- Aplicar les mostres en el gel d'agarosa desnaturalitzant.

L'electroforesi es realitza amb tampó MOPS 1x a 50 mA unes 3-4 hores. Els gels es tenyeixen amb una solució de bromur d'etidi (1µg/ml) durant 10 minuts, i es destenyeix amb aigua destil·lada durant uns 30 minuts. Un cop tenyit es realitza una fotografia en llum ultravioleta per comprovar la integritat del RNA ribosòmic i la quantificació realitzada per absorbància a 260 nm.

Transferència del àcids nucleics a filtres

La transferència del RNA del gel d'electroforesi a les membranes de niló es realitza seguint el protocol de Northern descrit a Sambrook et al. (1989). S'utilitzen membranes de niló comercialitzades com Hybond-N (Amersham Pharmacia Biotech). El tampó de transferència és SSC 6x (NaCl 3M, citrat trisódic 0.3M, pH 7). La transferència es realitza almenys durant una nit.

La fixació del RNA a la membrana es realitza mitjançant exposició a llum ultravioleta a 120mJ 3 minuts en un forn Bio-link BLX-254 de Ecogen (es realitza per les dos cares de la membrana).

Marcatge radiactiu de les sondes de DNA

Els fragments de DNA utilitzats com a sondes es marquen radioactivament mitjançant la tècnica de marcatge aleatori utilitzant com a nucleòtid marcat [α-^{32}P]-dCTP.

Pel marcatge s'utilitza un kit comercial Redipime^{TM} II random prime labelling system (Amersham Biosciences), i es segueix el protocol subministrat per la casa comercial.

- Posar en un tub 50-100 ng sonda en un volum final de 45 µl TE.
- Bullir 5 minuts per desnaturalitzar el DNA.
- Posar 5 minuts en gel.

A partir d'aquí es treballa en la zona de radioactivitat

- Posar els 45 µl en l'Eppendorf del kit que ja conté liofilitzats tots els nucelòtids (excepte el dCTP que serà radioactiu) i la resta de reactius.
- Agitar bé utilitzant el vórtex.
- Afegir a cada tub 3-5µl de [α-^{32}P]-dCTP.
- Incubar-ho a 37ºC (temperatura a la que actuarà el fragment de Klenow incorporant el nucleòtid marcat) un mínim de 30 minuts.
- Aturar la reacció afegint EDTA a una concentració final de 50mM.

Hibridació dels àcids nucleics

SSPE 20X: (pH 7.4)		Denhart 20X:	
	NaCl		BSA 0.4 % (p/v)
	NaH_2PO_4		Ficoll 0.4 % (p/v)
	EDTA		PVP 0.4 % (p/v)

Solució pre-hibridació:		Solució hibridació:	
SSPE	5X	SSPE	5X
Denhart	5X	Denhart	5X
SDS	0.5%	SDS	0.5 % (p/v)
DNA salmó	200 µg/ml	Sulfat de dextrà	10 % (p/v)
		Formamida desionitzada	50% (v/v)
		DNA salmó (afegit a últim moment)	100 µg/ml

- Pre-incubar les membranes a 42ºC durant 2 hores amb la solució de pre-hibridació (el DNA de salmó bloquejarà la superfície de la membrana on no hi hagi RNA).
- Canviar-ho per la solució de hibridació que conté:
  20 ml Solució hibridació
  200 µl DNA salmó 10 mg/ml (100µg /ml)
  55 µl sonda marcada desnaturalitzada (bullir 5 min/ en gel 5 min)
  - Aquesta solució d'hibridació es pot utilitzar més d'un cop, per hibridar altres membranes amb la mateixa sonda. Guardar-la a 4ºC.
  - Podem afegir més d'una sonda al mateix temps si tenen llocs d'hibridació suficientment distanciats, com és el cas de GAP1 (1806 pb) i MEP3 (1470 pb).

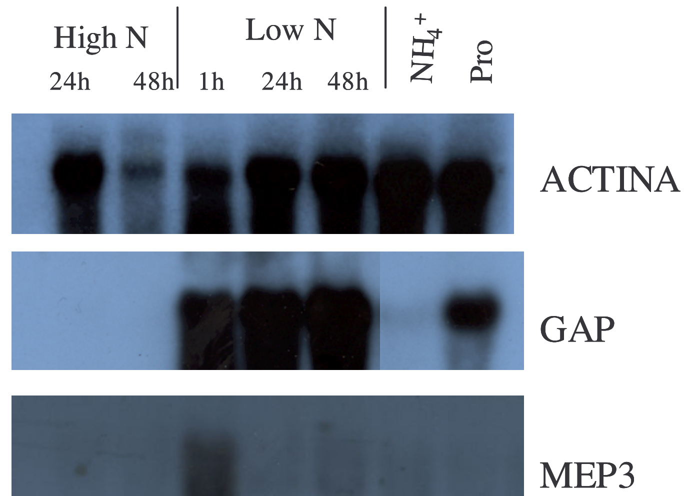
- La hibridació té lloc a 42ºC durant 16-24 hores.
- Rentats de les membranes: desprès de recuperar la solució d'hibridació rentem la membrana 2 x 10 minuts a temperatura ambient amb SSPE 2X i SDS 0.1% i després 5-10 minuts a 65ºC amb SSPE 0.1X i SDS 0.1 % (per eliminar hibridacions inespecífiques de la membrana).

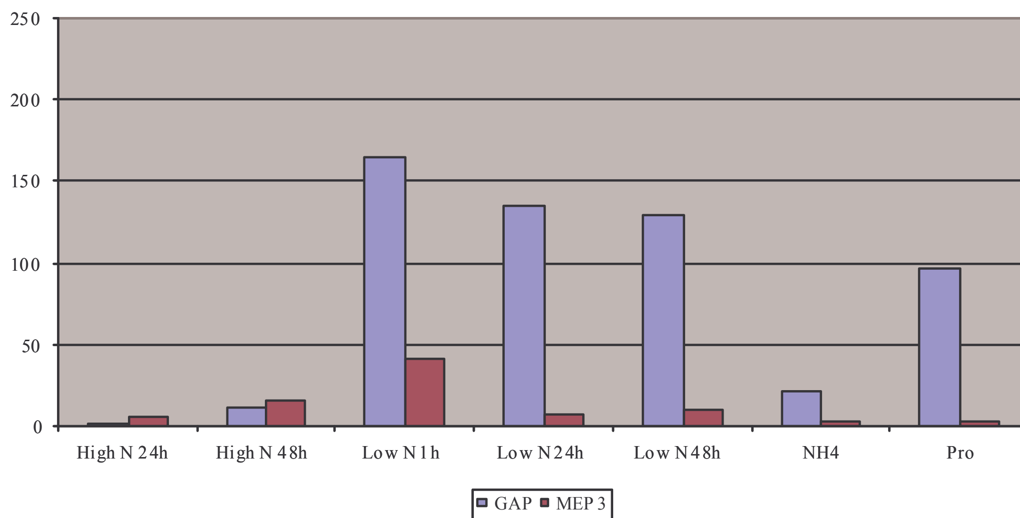
Autoradiografia

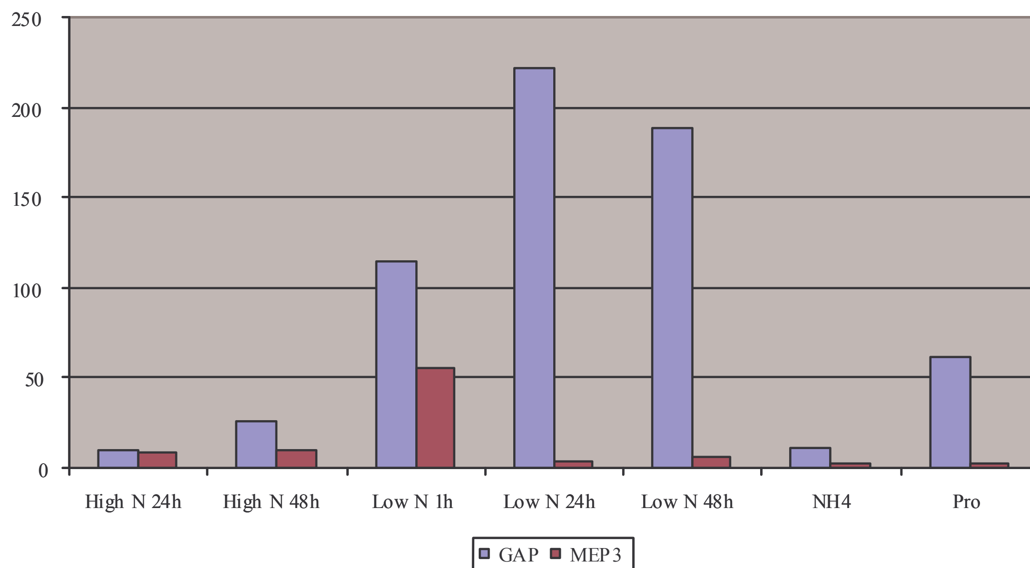
Per l'autoradiografia dels filtres hibridats amb sondes radioactives s'utilitzen pel·lícules de 100 NIF (MXB Film, Kodak) i cassets d'exposició (Amersham Pharmacia Biotech) equipats de pantalles intensificadores Kodak X-Omatic. La exposició es realitza a –80ºC. Les pel·lícules es revelen amb una màquina reveladora automàtica Curix 60 (Agfa). La quantificació de la intensitat de les bandes es realitza amb el software Quantity One software (Biorad, USA).

Controls: rRNA 18S (6333 pb) i 28S (2366 pb).

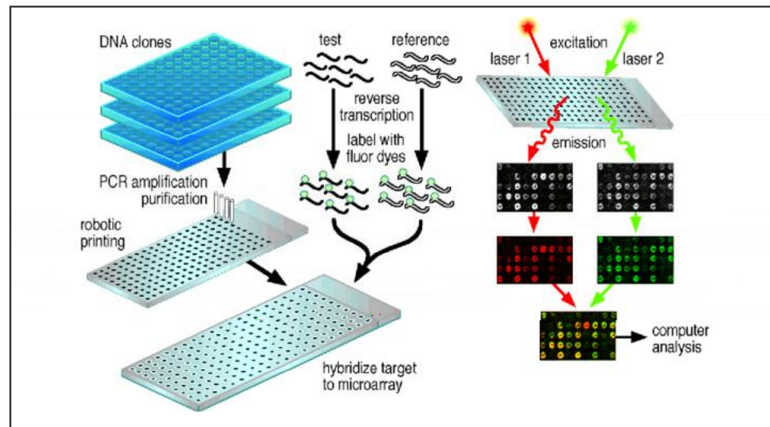
4.3. Comparació dels resultats de RT-PCRq i Northerns







4.4. Determinació de l'expressió gènica per chips de DNA o Microarrays
La realització dels chips de DNA de llevat es va dur a terme als laboratoris de Jean Marie François al departament DGBA (INSA, Toulouse) i a la Transcriptome-Biochips plateforme de la Genopole Midi-Pyrénees, Toulouse. (http://biopuce.insa-tlse.fr/).



A. Trencament de les cèl·lules de llevat

- Es centrifuga un volum adequat de cèl·lules en fermentació a 5000rpm 3 min.
- Es recuperen les cèl·lules amb una pipeta Pasteur i es congelen en petites gotes en nitrogen líquid. Les gotes de cèl·lules en guarden congelades a –80ºC.
- Pre-refredar el material que s'utilitzarà per trencar les cèl·lules en nitrogen líquid (recipient de Teflon de 5 ml, bola de Tungstè de 7mm, espàtula).
- Afegir en el recipient de Teflon la bola de 7 mm de Tungstè i les cèl·lules congelades (2 o 3 gotes equivalents a unes 15 unitats de DO).
- Tancar el flascó i posar-lo en el microDismembrator (Braun, Melsungen) a 2600 rpm durant 2 minuts.

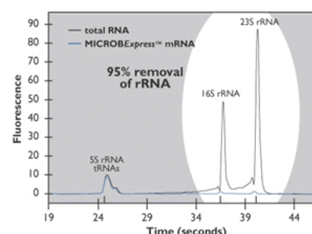
B. Extracció de RNA amb RNeasy midi kit (QIAgen)

- Transferir les cèl·lules trencades a un tub de 15 ml amb 1.9 ml de tampó RTL del kit i 19 μl de β-mercaptoetanol.
- Centrifugar 5 min a 4000 rpm a temperatura ambient.
- Seguir el protocol del kit QIAgen a partir del pas nº6.
- Després del pas d'elució, recuperar el RNA amb la pipeta i transferir-ho a un Epperdorf (RNAse free).

Concentrar el RNA:

- Als 500 μl de la solució RNA afegir-hi 50 μl d'acetat de sodi (3M, pH 5.2) i 500 μl d'isopropanol. Deixar-ho a –20ºC un mínim de 2 hores perquè precipiti el RNA.
- Centrifugar a 12000 rpm durant 30 min, a 4ºC.
- Rentar el *pellet* amb 250 μl d'etanol al 70%. Centrifugar 5 min 12000 rpm.
- Assecar el RNA al aire uns 30 min.
- Dissoldre el *pellet* en 50 μl de aigua-DEPC.

C. Control qualitatiu i quantitatiu del RNA



Comprovar la qualitat i quantitat de RNA per electroforesi micro-capil·lar utilitzant el Bioanalyzer Agilent 2100 (Agilent Technologies), seguint el protocol subministrat per la casa comercial. Ratio 28S/18S ≥ 1.7

D. Síntesis i marcatge del cDNA amb Cy3 i Cy5

CyScribe First-Strand cDNA Labelling kit (Amersham Pahrmacia Biotech)

- Afegir en un Eppendorf: x µl RNA (20-30 µg RNA, màxim 9 µl)
  1 µl random monamers
  1 µl oligo(dT)
  H_2O fins Volum total 11 µl
- Mesclar amb la pipeta i incubar a 70ºC 5 min.
- Deixar-ho 10 min. a temperatura ambient.
- Centrifugar 30 seg. per recuperar els reactius al fons del tub.
- Posar el tub en gel i afegir-hi els components de marcatge en el següent ordre:
  4 µl 5X CyScript buffer
  2 µl DTT 0.1M
  1 µl dCTP nucletide mix
  1 µl dCTP CyDye-labelled nucleotids (Cy3-dCTP o Cy5-dCTP)*
  1 µl CyScript Reverse Trancriptase
  Volum total 20 µl
- Agitar la mescla amb el vórtex i fer un pulse de 30 segons en una microcentrifuga.
- Incubar la reacció a 42ºC durant 1.5 hores.
- Guardar el cDNA marcat en gel o a –20ºC fins la seva purificació. Protegir-ho de la llum.

*Cada mostra a comparar es marca amb un fluorocrom diferent, Cy3-dCTP o Cy5-dCTP
25IF, 13MF, 13FF amb Cy5-dCTP (color blau, senyal al escàner vermella)
13IF, 25MF, 25FF amb Cy3-dCTP (color rosa, senyal al escàner verda)
I en la repetició de la mostra es marca al invers (swapping)

E. Purificació del cDNA marcat

És necessari eliminar el mRNA restant del cDNA abans de la hibridació amb les sondes immobilitzades en la làmina de vidre, així com els nucleòtids-CyDye marcats no incorporats, per eliminar soroll de fons i millorar la sensitivitat de detecció. El mRNA es degrada en petits oligòmers amb tractament alcalí. Després els petits oligòmers i els nucleòtids no marcats s'eliminen mitjançant una columna cromatogràfica.

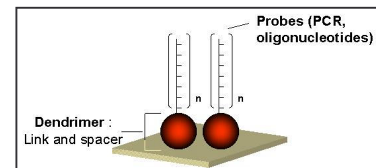
Degradació del mRNA

- Afegir 2 µl de NaOH 2.5M en cada un dels tubs que contenen el cDNA marcat.
- Mesclar els tubs amb el vórtex i centrifugar 30 segons.
- Incubar les mostres a 37ºC durant 15 minuts.
- Afegir 10 µl de HEPES free acid 2M a cada tub de reacció.
- Mesclar amb el vórtex i fer un pulse de 30 segons.

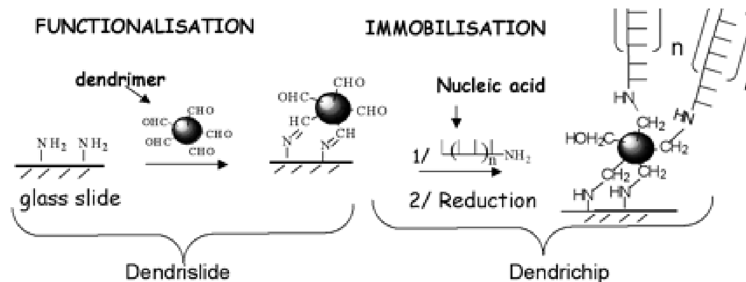
Eliminació dels oligomers i nucleòtids

Per la purificació s'utilitza un kit comercial, CyScribe GFX purification kit (Amersham Pharmacia), seguint el protocol subministrat per la casa comercial.

F. Obtenció de les làmines o chips de DNA



Les làmines utilitzades pels microarrays són fabricades a la plate-forme de Biochips, Genopole-Toulouse, i consisteixen en dedri-làmines (dendrimer-activated glass slides) (Le Berre et al. 2003).



Les dendri-làmines augmenten la sensibilitat i fiabilitat dels chips de DNA. Els dendrimers són estructures nanomèriques de llongitud de diàmetre controlat. La seva unió covalent a la superfície de la làmina de vidre, permet una unió covalent i estable de les sondes de DNA. Això permet tenir spots uniformes i homogenis, reduir la quantitat de sonda a immobilitzar, així com disminuir el límit de detecció dels cDNA hibridats.

Les sondes de DNA són oligonucleòtids de 70 mers, representant el 99% del genoma de llevat obtingut de Operon Inc, i són immobilitzades mecànicament en la superfície de la làmina mitjançant un robot automàtic (Eurogridder from Virtek/Bio-Rad). Cada spot conté uns 2 nl de DNA, i un diàmetre d'uns ~80 µm.

G. Hibridació

Les hibridacions de les làmines es duen a terme en una cambra d'hibridació automàtica (Discovery™, Ventana).

Pre-hibridació de la làmina

- Pre-hibridar la làmina (microarray) amb 3 ml d'una solució que conté: 2xSSC, 0.2% SDS i 1% BSA.
- Deixar-ho en la cambra d'hibridació (Discovery™, Ventana) a 42ºC durant 30 minuts.

Preparació de la mostra i hibridació

- Mesclar en un Eppendorf: 10 µl cDNA marcat Cy3
10 µl cDNA marcat Cy5
180 µl RiboHybe™ (Ventana)
- Fer un pulse per recuperar bé tot el volum
- Dipositar la mescla sobre la làmina (microarray).
- Hibridar a 42ºC en la cambra d'hibridació tota una nit (10-14 hores).

Rentats de post-hibridació

- Submergir la làmina 5 minuts en 2x SSC, 0.1% SDS, a temperatura ambient.
- Rentar 4 vegades en tampó 0.1x SSC durant 2 minuts a temperatura ambient.
- Assecar suaument la làmina utilitzant N_2 comprimit.

H. Escaner de les làmines i tractament de les dades

Les làmines hibridades s'escanegen utilitzant GenPix4000B laser Scanner (AxonInstruments), i les imatges adquirides són analitzades i quantificades per l'aplicació GenPix Pro v.3.01. Les dades exportades d'aquest programa s'incorporen en la base de dades de la plate-forme Biopuce i són tractades per les aplicacions Bioplot, BioClust (programes desenvolupats per Biopuce) i GeneSpring (Silicon Genetics).
Els experiments (des de la extracció de RNA fins l'anàlisi de les imatges) van ser realitzats en duplicat, invertint el marcatge Cy3 i Cy5 (swapping) entre els dos experiments per reduir la variabilitat deguda al efecte marcador.

Normalització de les dades

Correcció per background: la intensitat mitjana de cada spot ha estat corregida per la subtracció de la mitjana del background (soroll de fons) sobre una corona al voltant del spot.
Log transformation: les intensitats corregides han estat transformades a log10.
Normalizació: la intensitat en log de cada spot va ser normalitzada per la subtracció de la mitjana de les intensitats-log. Donat que els microarrays contenen el genoma complet, aquest tipus de normalització sembla justificada, doncs un feble percentatge de gens canvia d'expressió d'una condició per respecte a una altra.
Log-Ratio: es calculen els log-ratios de les log-intensitats (log-int-normCy3 vs. log-int-normCy5 o a l'inversa).
Mitjana dels replicats sobre la mateixa làmina: es calcula la mitjana aritmètica dels log-ratios entre els replicats de cada spot. D'aquesta manera cada gen té un log-ratio per làmina.
Mitjana, error entre làmines: s'ha calculat la mitjana aritmètica així com l'error dels log-ratios per cada gen sobre la parella de làmines corresponent a les repeticions de canvi de marcatge (swapping).
Filtratge per test Student i ratio: la mitjana dels log-ratios i els errors calculats per cada gen han estat utilitzats per fer el test de Student i retenir els gens que tenen un p-value inferior a un 5% i un $|\text{log-ratio}| > \log10(2)$ (ratio $\leq 0{,}5$ pels down-regulated i ≥ 2 pels up-regulated).

4.4. Identificació de llevats a nivell de soca: Anàlisis dels perfils de restricció (RFLPs) del mtDNA (Querol *et al.* 1992)

Extracció de DNA

- Fer créixer les cells en **1,5 ml de YEPD** a 26ºC (durant 12 hores o el temps suficient per tenir un bon creixement).
- Centrifugar 2 minuts a 10 000 rpm. Eliminar el sobrenedant.
- Rentar amb 1 ml d'aigua destil·lada estèril.
- Centrifugar 2 minuts a 10 000 rpm. Eliminar el sobrenedant.
- Resuspendre el *pellet* amb 500 μl de **Tampó 1** (T1: sorbitol 0.9M, EDTA 0.1M pH 7.5).
- Afegir 30 μl d'una solució de **Zymoliasa 60 000** (1,5mg en 1300μl T1). Incubar la suspensió final a 37ºC durant 20 minuts.
- Centrifugar 2 minuts a 10 000 rpm i eliminar el sobrenedant.
- Resuspendre les cells en 500 μl de **Tampó 2** (T2: Tris 50mM pH 7,4, EDTA 20mM).
- Afegir 13 μl de **SDS 10 %**, agitar i incubar a 65ºC durant 5 minuts.
- Afegir 200 μl de **Acetat de potasi 5M** i agitat bé. Incubar en gel 5 minuts.
- Centrifugar en fred (4ºC) 10 min a 12 000 rpm.
- Transferir el sobrenedant a un altre Eppendorf, afegir 700 μl de **Isopropanol** i incubar a T^a ambient 5 min. (Tb es pot guardar a -20ºC).
- Centrifugar 10 min a 12 000 i eliminar el sobrenedant.
- Afegir 500 μl d'**etanol 70 %** (v/v), centrifugar 5 min a 12 000 rpm i eliminar el sobrenedant.
- Eixugar el *pellet* al buit. Resuspendre el DNA en 15 μl de **TE** (Tris 10mM pH 7.4, EDTA1mM, pH 8.0).

Digestió del DNA total

- Preparar la mescla de digestió en un volum final de 25 μl

  10-12 μl de DNA

  2.5 μl de Tampó H (Boehringer Mannheim)

  1 μl d'enzim de restricció *Hinf*I (Boehringer Mannheim) (quantitat?)

  H_2O mili-Q fins 25 μl

- Incubar a 37ºC un mínim de 3 hores (es sol deixar tota una nit)

Electroforesi

- Preparar un gel d'agarosa al 0.8% (p/v) en tampó TBE (Tris base 89 mM, Boric acid 89 mM, EDTA 2mM, pH 8). Afegir-hi Bromur d'etidi, 1 μl per cada 25 ml de solució TBE (conc. final: 0,4 μg/ml).
- Preparar la mostra: als 25 μl de la mostra digerida s'hi afegeix 4 μl de tampó de càrrega (solució 10X: 0.25 % (p/v) blau de bromofenol, 1% (p/v) SDS, 20% (v/v) glicerol, 0,1M Na_2EDTA, pH8).
- L'electroforesi es realitza 25-30 V en TBE1X durant un mínim de 8 hores.

  La visualització es porta a terme en un transil·luminador de llum UV. El marcador consisteix en una mescla de 50% (v/v) del marcador DNA Molecular Weight MarkerII i 50 % (v/v) de DNA Molecular Weight MarkerIII (0.12-21.1 kbp, ADN del fag λ digerit amb EcoRI I HindIII) de Boehringer Mannheim.

4.5. Identificació de llevats a nivell d'espècie: Anàlisis dels perfils de restricció (RFLPs) del rDNA (Guillamon *et al.* 1998)

Reacció en cadena de la polimerasa (PCR)

- La mescla de reacció de PCR conté per un volum final de 50 µl:
  1 µl de cada un dels primers (10pm de cada un)
  ITS1 (5' TCCGTACGTGAACCTGCGG 3')
  ITS4 (5' TCCTCCGCTTATTGATATGC 3')
  4 µl dNTPs d'una mescla que conté 1 µl de cada un (10mM)
  3 µl $MgCl_2$ (d'una solució 100 mM, Ecotaq)
  5 µl Tampó Taq 10X, sense Mg (Ecotaq)
  33 µl H_2O mili-Q estèril
  3 µl ADN extret
  0.5 µl Taq-Polimerasa (Ecotaq)

- Iniciar el programa de PCR amb les següents condicions:
  5 min 95ºC (Desnaturalització)
  30 seg. 95ºC (desnaturalització) } 35 cicles
  1 min. 52ºC (hibridació) } 35 cicles
  1 min. 72ºC (extensió) } 35 cicles
  7 min a 72ºC (extensió final)

Gel per visualitzar l'amplificat

- Preparar un gel al 1,2 % (p/v) amb agarosa multipurpose (Boehringer Mannheim) en tampó TBE (Tris base 89 mM, Boric acid 89 mM, EDTA 2mM, pH 8). Afegir-hi Bromur d'etidi, 1 µl per cada 25 ml de solució TBE (conc. final: 0,4 µg/ml).
- Preparar la mostra: a 5 µl del DNA amplificat se li afegeix 1 µl de **tampó de càrrega** (solució 10X: 0.25 % (p/v) blau de bromofenol, 1% (p/v) SDS, 20% (v/v) glicerol, 0,1M Na_2EDTA, pH8).
- L'electroforesi es porta a terme entre 50-100 V durant un mínim de dues hores. La visualització es porta a terme en un transil·luminador de llum UV. Les mides de les bandes s'estimen per comparació amb un marcador de pes molecular: 100 bp DNA Ladder (Gibco BRL).

Digestió del amplificat amb endonucleases

- Preparar la mescla de digestió (un tub per enzim) en un volum final de 25 µl
  8 µl del DNA amplificat (entre 0,5-1,0 µg d'ADN)
  2 µl del tampó corresponent segons el enzim
  5 unitats d'enzim (CfoI, HaeIII o HinfI (Boeringer Manheim)
  H_2O mili-Q fins 25 µl
- Incubar a 37ºC un mínim de 3 hores (es sol deixar tota una nit).

Gel per visualitzar la digestió de l'amplificat

- Preparar un gel d'agarosa multipurpose (Boeringer Manheim) al 2% (p/v) en TBE 1X i bromur d'etidi 0.4µg/ml.
- Als 25 µl de la mostra digerida s'hi afegeix 4 µl de tampó de càrrega.
- L'electroforesi es porta a terme entre 25-30 volts durant un mínim de 8 hores. Les mides de les bandes s'estimen per comparació amb un marcador de pes molecular: 100bp DNA Ladder (Gibco BRL).

5. Analítiques bàsiques en mostos i vins

5.1. Determinació de sucres reductors (mètode GAB) (García Barceló 1990)

Es mesuren les quantitats d'hexoses en el most i en mostres d'estadis finals de fermentació per determinar l'acabament de la fermentació. És un mètode utilitzat freqüentment en bodega, i es basa en la reducció del coure per part dels sucres i la valoració del coure residual. S'utilitza el mètode modificat de Rebelein (García-Barceló, 1990). Els reactius i bureta provenen de GAB (Moja-Olèrdola, Barcelona).

Procediment

- Afegir en un Erlenmeyer de 250 ml: 10 ml exactes de solució de coure (mesurats amb una pipeta de doble enràs), 5 ml de solució alcalina, 2 ml de mostra (diluïda corresponentment) i pedra pómez per homogenitzarla ebullició.
- Tapar el matrau amb un vidre de rellotge i escalfar en un calefactor elèctric prèviament calent. Esperar la ebullició i comptar a partir de llavors un minut i mig exactament. Refredar el matrau amb aigua corrent.
- Afegir 10 ml de solució de iodur, 10 ml de solució de midó i 10 ml d'àcid sulfúric. La mostra agafa un color negre.
- Valorar (en la bureta GAB o una bureta de 30 ml)amb tiosulfat de sodi fins a viratge de color a crema/blanc lletós.

Càlcul

La graduació de la bureta GAB ja dóna directament els grams de sucres reductors per litre. Si s'ha utilitzat una bureta de 30 ml normal, el volum de tiosulfat de sodi no gastat correspon als g/l de sucres reductors. Si la motra s'ha diluit el resultat s'haurà de multiplicar pel factor de dilució.

5.2. Determinació de la Glucosa i Fructosa (mètode enzimàtic)

La glucosa i fructosa residual dels vins es calculen de manera més precisa mitjançant un kit enzimàtic (Boeringher Mannheim). Primer es valora la quantitat de glucosa més fructosa. Després e quantifica la glucosa de la mostra. La diferència entre ambdós valors ens dóna la concentració de fructosa de la mostra.

5.3. Determinació de l'acidesa total (Ough and Amerine 1988)

Procediment

- Desgasificar els vins.
- Colocar 10 ml de mostra en un Erlenmeyer, uns 50 ml d'aigua i afegir 2-3 gotes de fenoftaleïna (1%).
- Valorar amb NaOH 0,1 N fins al canvi de color.

Càlcul:

$$N_{mostra} = \text{(ml de NaOH gastats x 0,1 N) / 10 ml mostra}$$

$$\text{Acidesa total (g/l de àc Tartàric)} = N_{mostra}\ (eq/l) \times \frac{\text{1 eq. àc.Tart}}{\text{2 eq NaOH}} \times \frac{\text{150 g àc. Tart}}{\text{1 eq. àc.Tart}}$$

$$\text{Ac. Total} = \text{ml NaOH} \times 0.75$$

5.4. Determinació de l'acidesa volàtil (mètode García-Tena) (García Barceló 1990)

Es basa en una destil·lació parcial dels àcids grassos del vi i valoració amb NaOH. El primer volum es recull en una proveta de 5.1 ml i el segon en una proveta de 3.2 ml. L'equip utilitzat es el GAB, que consisteix en una placa calefactora elèctrica i un destil·lador.

Procediment

- Encendre l'aparell uns 15 minuts abans del primer anàlisis perquè el calefactor arribi a la temperatura adequada.
- Mesurar 10 ml de la mostra desgasificada i colocar-la en el matrau de destil·lació. Afegir pedra pómez.
- Colocar el matrau al destil·lador i la proveta de 5.1 ml a la sortida del refrigerant.
- Recollir exactament 5.1 ml en la proveta, i sustituir-la ràpidament per la proveta de 3.2 ml.
- El líquid recollit en la proveta de 3.2 ml es valora amb NaOH 0,0204 M utilitzant fenoftaleïna (1%) com indicador.

Càlcul

La acidesa volàtil es calcula a partir del volum de NaOH gastats en la valoració, consultant les taules subministrades pel mètode GAB o per la fórmula.

$$\text{g/l àcid acètic} = \frac{N \times 0.0204 \times 60 \times 100}{V \times 33.3}$$

V= volum de mostra (10ml)
N= ml de NaOH 0.0204 consumits

5.5. Determinació del grau alcohòlic per ebullimetria

Es basa en la disminució del punt d'ebullició que experimenten les solucions hidroalcohòliques a mesura que augmenta el percentatge d'alcohol (llei de Raoult). S'utilitza l'ebullometre de la firma GAB (Moja-Olèrdola, Barcelona).

Procediment

- Engegar la toma d'aigua corrent pel refrigerador
- Omplir amb aigua destil·lada el recipient de l'aparell i engegar l'escalfador.
- Prendre nota de la temperatura d'ebullició de l'aigua.
- Passar un volum de mostra de vi pel recipient i omplir el recipient amb mostra fins la ratlla indicativa (uns 50 ml).
- Anotar la temperatura d'ebullició de la mostra quan s'hagi estabilitzat.

Càlcul

Ajustar la regleta subministrada per la casa comercial GAB amb la temperatura d'ebullició de l'aigua, i interpolar grau alcohòlic amb la temperatura d'ebullició del vi.

5.6. Determinació del sulfurós total i lliure

Per la valoració es pot utilitzar l'aparell Toning de la firma GAB (Moja-Olèrdola, Barcelona) que detecta el viratge en les mostres de mostos i vins rosats i negres.

Sulfurós lliure:

- Afegir en un Erlenmeyer (o en el tub de vidre del Toning): 25 ml de mostra (mesurats amb pipeta), 2-3 ml indicador (solució de midó al 1%) i 5 ml d'àcid sulfúric al 33%.

- Valorar amb iodat potàssic. (Fins que l'aparell ho indica amb la senyal acústica)
Per calcular la quantitat de sulfurós lliure en la mostra es multiplica el volum gastat per 10 (1ml equival a 10 mg SO_2/l).

Sulfurós total:
- Afegir en un Erlenmeyer (o en el tub de vidre del Toning): 25 ml mostra i 10 ml NaOH al 4 %.
- Tapar, agitar i deixar en repòs 10-15 minuts.
- Afegir-hi 2-3 ml d'indicador (solució de midó al 1%) i 5 ml d'àcid sulfúric al 33%.
- Valorar amb iodat potàssic. (Fins que l'aparell ho indica amb la senyal acústica)
Per calcular la quantitat de sulfurós total en la mostra es multiplica el volum gastat per 10.

5.7. Determinació dels àcids orgànics, sucres, glicerol i etanol per HPLC

Els àcids organics, sucres, etanol i glicerol van ser analitzats al final de la fermentació per HPLC. L'equip utilitzat va ser un Hewlett-Packard HP 1050 connectat a un integrador Hewlett-Packard 3395A equipat amb un detector HP 1047 RI (Agilent Technologies, Wilmington, DE, USA). La mostra de vi (450 μl) es barreja amb 50 μl d'àcid fòrmic (1 g l^{-1}), i 25 μl s'injecten a la columna AMINEX HPX-87H de 300 x 7,8 mm (BioRad, Hercules, CA, USA). El solvent utilitzat és H_2SO_4 2,5 mM, prèviament desgasificat, a un flux constant de 0,5 ml/min. La temperatura d'anàlisis és de 60ºC. La concentració de cada compost es calcula mitjançant rectes patró de cada compost i el patró intern.

6. Analítiques de compostos secundaris

Aquests anàlisis han estat realitzats per la empresa Miguel Torres S.A (Vilafranca del Penedés, Barcelona).

6.1. Determinació dels alcohols superiors, esters i àcids grassos del vi (Ferreira *et al.* 1996)

Es realitza l'extracció dels alcohols superiors i esters del vi per extracció liquid/liquid: 10 ml vi, 200 μL 1,1,2-Trichlorotrifluoroetà i 0.5 g NaCl, utilitzant n-decanol (0.2 mg/l) com a patró intern. Després d'agitar durant 2 minuts i centrifugar, la fase orgànica es recupera i s'injecta en el cromatògraf de gasos. L'equip utilitzat és un Hewlett-Packard 6890N connectat a un ordenador que conté el software ChemStation (Agilent Technologies). L'extracte (2 μl) s'injecta en una columna Tracer TR (60 m x 250 μm i 0.25 μm de gruix) mitjançant un injector automàtic HP (Agilent Technologies). La temperatura del programa és de 40ºC 5 min., 2ºC/min fins 240ºC (15 min). Les temperatures del injector i detector són 220 i 240ºC, respectivament. El gas vector és heli, a 60 ml/min. Com a patró intern s'afegeix 2-etilfenol (0.2 mg/l). La quantificació dels compostos té lloc per comparació amb solucions patró de concentracions conegudes en solució hidroalcohòlica.

7. Analítiques de compostos nitrogenats

7.1. Determinació dels aminoàcids (AA) per HPLC

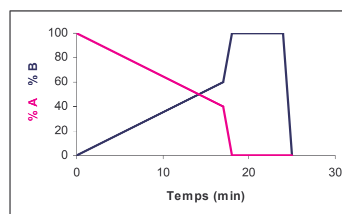
Els aminoàcids i iminoàcids individuals es determinen mitjançant derivatització amb OPA (o-ftalaldèhid) i FMOC (9-fluorenilmetil cloroformat), respectivament. L'equip utilitzat és un HPLC Agilent 1100 Series, equipat amb una bomba quaternària, un detector d'ultravioleta i un detector de fluorescència (Agilent Technologies). La mostra, prèviament diluïda i filtrada (0.22µm, Millipore) s'injecta automàticament a una columna Hypersil ODS 4.6 x 250 mm x 5 µm (Agilent Technologies). La temperatura d'anàlisi és de 40ºC, i el flux de 1.5 ml/min. Els solvents utilitzats, prèviament desgasificats en un bany d'ultrasons, són:

Solvent A: Acetat sòdic 16 mM, trietilamina 0.022 % (ajustat a pH 7.2 amb 1-2 % àcid acètic) i tetrahidrofurà 0.6 %.

Solvent B: 20 % d'acetat sòdic 66 mM (ajustat a pH 7.2 amb 1-2 % àcid acètic), 40 % acetonitril i 40 % metanol.

El gradient dels solvents utilitzats és:

0-100% B (0-18 min)
100%B (18-24 min)
100-0% B (24-25 min)



Programa de punxada: (automatitzat)

5 µl Tampó borat
1 µl OPA
2 µl mostra
1 µl Patrons Interns
1 µl FMOC

} Mesclar i injectar en la columna

Detecció:

UV: 338 nm (OPA-aminoàcids), 262 nm (FMOC-iminoàcids)

Fluorescència:

Temps	λ Excitació (nm)	λ Emissió (nm)
0	340	450
15	266	305
25	340	450

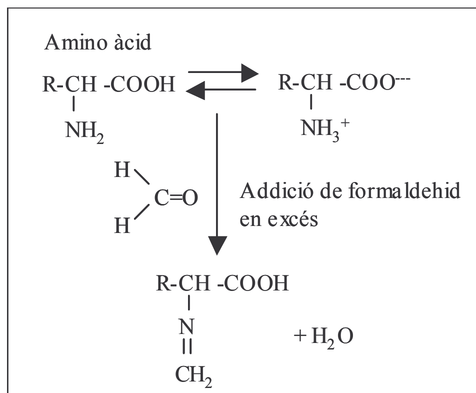
- Es van utilitzar com a patrons interns (PI) solucions 250 µM (en HCl 0.1N) de Norvalina (per als aminoàcids) i de t-Prolina (per als iminoàcids: Pro, OH-Pro)
- Per cada aminoàcid analitzat es va efectuar una corba patró. Aquests patrons externs, per ordre d'elució, són: Asp, Glu, Ser, Gln, His, Gly, Thr, Ala, Arg, Tyr, Cys, Cys-Cys, Val, Met, (N-Val), Trp, Phe, Ile, Leu, Lys, Pro, (t-Pro). El rang de cada corba patró és de 0-500 µM (en HCl 0.1N).

S'analitzen diverses dilucions de cada mostra (en HCl 0.1N). La identificació s'efectua a partir del temps de retenció, per comparació amb els patrons externs. La concentració de cada AA es calcula a partir de l'àrea respectiva dels pics cromatogràfics i dels patrons interns i externs, de concentració coneguda. El software utilitzat és el Agilent ChemStation Plus (Agilent Technologies).

Recta patró:
$$\frac{\text{Àrea AA}_{ref}}{\text{Àrea PI}_{ref}} = a + b\,\frac{[AA]_{ref}}{[PI]_{ref}}$$

$$[AA]_{mostra}\,(mg/l) = \frac{\frac{\text{Àrea AA}_{mostra}}{\text{Àrea PI}_{mostra}} - a}{b} \times [PI]_{mostra} \times \text{dilució} \times \text{Pes molecular}$$

7.2. Determinació del nitrogen assimilable pels llevats o índex de formol (YAN total) (Aerny 1996)



Permet conèixer la quantitat de nitrogen disponible pels llevats (nitrogen amoniacal i nitrogen d'amino acids) en mostos i vins. Es basa en una valoració amb formaldèhid. El formaldèhid, prèviament ajustat a pH 8.1, bloqueja el grup amino dels aminoàcids i l'amoni, alliberant-se un protó. El protó alliberat es valora amb sosa.
L'índex de formol és el nombre de ml de NaOH 1M necessaris per neutralitzar 1L de mostra. Un índex de 1 correspon a 14 mg/l de nitrogen assimilable.

Procediment:
- Ajustar la solució de formaldèhid a pH 8,1 amb NaOH 0,25 M.
- Posar 25 ml de mostra en un vas de precipitats. Si la mostra conté anhídrid sulfurós, afegir unes gotes d'aigua oxigenada al 30%.
- Ajustar la mostra a pH 8,1 amb la solució de NaOH 0,25 M.
- Afegir 10 ml de formaldèhid (pH 8,1), mesclar bé i deixar-ho reposar durant 1,5 min.
- Valorar amb NaOH 0,25M per portar la solució de nou a pH 8,1.

Si la valoració necessita més de 20 ml de solució de NaOH 0,25M, diluir la mostra, si necessita menys de 2 ml de solució de NaOH 0,25M repetir el procés amb NaOH 0,025M.

Càlculs:
Índex de formol (no té unitats) = volum (ml) de NaOH 0,25M gastats x 10
Nitrogen total assimilable (mg N / l) = índex de formol x 14

7.3. Determinació del amoni per kit enzimàtic

Per la determinació del amoni en mostos i vins s'utilitza un mètode enzimàtic (Boehringer Mannheim). En presència de l'enzim glutamat-deshidrogenasa i de NADH, l'amoni reacciona amb 2-cetoglutarat a L-glutamat, i el NADH s'oxida a NAD^+. La quantitat de NAD^+ és proporcional a la quantitat d'amoni. NADH es determina per l'absorbància de llum de 365 nm mitjançant un espectofotòmetre.

8. Anàlisis dels lípids del llevat

8.1. Determinació dels àcids grassos de membrana (Rozès *et al.* 1992)

Obtenció del extracte lipídic:
- Centrifugar les cèl·lules de llevat (aprox. 1 x 10^8 cells/ml, 5-10 mg Pes Sec)
- Afegir: 1ml NaOH 5% en Metanol/H_2O (v/v)
  10 µl dels patrons interns (C7:0, 1mg/ml; C17:0, 4 mg/ml)
- Agitar i incubar 30 minuts a 100ºC.
- Deixar refredar i afegir 500 µl de HCl 5N. Agitar bé.
- Extracció amb 400 µl de Hexà / MTBE (v/v).
  6 intervals de: 30 segons agitació en vórtex
  30 segons de repòs
- Recuperar la fase orgànica i passar-la a un vial
- Repetir la extracció amb la fase aquosa per recuperar el màxim de fase orgànica.
- Tancar el vial.

Anàlisi del extracte per Cromatografia de Gasos:
L'equip utilitzat és un Hewlett-Packard 5890 connectat a un ordenador HP Vectra amb el software ChemStation (Agilent Technologies). La columna utilitzada és una FFAP-HP 30 m x 0.25 mm i 0.25 mm de gruix (Agilent). La mostra (2μl) és injectada per un injector automàtic HP 7673 (Agilent).
El programa de temperatura és: 140ºC x 4ºC/min fins 240ºC (13 minuts). La temperatura del injector i detector són de 250 i 280ºC respectivament.
El gas vector utilitzat és el Heli a un flux de 1.2 ml/min.
Els patrons interns són el àcid heptanoic (mg/ml) i heptadecanoic (4 mg/ml). El àcid heptanoic serveix com a patró intern pels àcids C6:0, C8:0, C10:0, C12:0, C14:0 i C14:1, mentre que el àcid heptadecanoic s'utilitza pels àcids C16:0, C16:1, C18:1, C18:2, C18:3 i C20:0.
Els àcids grassos s'identifiquen a partir dels temps de retenció obtinguts al injectar una solució de referència o patró que conté una concentració coneguda de tots els àcids grassos que es volen determinar. La solució de referència va ser tractada igual que si fos una mostra.
Els càlculs realitzats són:

$$K_{ref} = [A.G.]_{ref.} \times \frac{\text{Àrea P.I.}_{ref.}}{\text{Àrea A.G.}_{ref}} \qquad [A.G.]_{mostra} = K_{ref.} \times \frac{\text{Àrea A.G.}_{mostra}}{\text{Àrea P.I.}_{mostra}}$$

8.2. Determinació dels esterols

Obtenció del extracte lipídic:

- Centrifugar les cèl·lules de llevat (aprox. 1 x 10^8 cells/ml, 5-10 mg Pes Sec)
- Afegir: 1ml KOH 15% en Metanol/H_2O (v/v)

  10 μl dels patrons interns (5-α-cholestane, 10μg μl^{-1})
- Agitar i incubar 30 minuts a 90ºC.
- Extracció amb 300 μl d'Hexà.
  6 intervals de: 30 segons agitació en vórtex
  30 segons de repòs
- Recuperar la fase orgànica i passar-la a un vial
- Repetir la extracció amb la fase aquosa per recuperar el màxim de fase orgànica.
- Tancar el vial.

Anàlisi del extracte per Cromatografia de Gasos (GC):
L'equip utilitzat és un Hewlett-Packard 5890 connectat a un ordenador HP Vectra amb el software ChemStation (Agilent Technologies). La columna utilitzada és una SAC-5 15 m x 0.25 mm i 0.25 mm de gruix (Supelco). La mostra (2μl), és injectada per un injector automàtic HP 7673 (Agilent).
El programa de temperatura és: 140ºC x 4ºC/min fins 240ºC (13 minuts). La temperatura del injector i detector són de 250 i 280ºC respectivament. El gas vector utilitzat és el Heli a un flux de 1.2 ml/min.
La quantitat relativa dels esterols es calcula a partir de l'àrea respectiva dels pics cromatogràfics i a partir dels temps de retenció obtinguts al injectar una solució de referència que conté una concentració coneguda de tots els esterols que es volen determinar.

Anàlisi del extracte per Cromatografia de Gasos i Espectometria de Masses (GC/MS):
Els mateixos extractes s'analitzen també per GC/MS. L'equip utilitzat és un HP5890 sèrie II acoblat a un HP5972 detector selectiu de masses. L'impacte dels electrons en GC/MS (70eV, escanejant de 42 a 600 unitats de massa atòmica a intervals de 1 segon) té lloc en les següents condicions: columna SAC-5 (30 m x 0.25 mm i 0.25 mm de gruix), heli com a gas vector (30 cm/segon), temperatura de detecció 250 º©, temperatura de la columna 125-250º© (125º© 2 minuts, 20ºC/min fins 250º© 60 minuts), temperatura d'injecció 250ºC.
Els esterols majoritaris (esqualè, lanosterol, ergosterol, stigmasterol i ß-sitosterol) s'identifiquen a partir dels pics cromatogràfics i dels temps de retenció respectius, en relació als patrons d'esterols (Supelco). La confirmació de l'assimilació de fitosterols es determina per comparació dels temps de retenció del GC i del espectre de masses.

8.3. Determinació dels lípids neutres i fosfolípids

Extracció lipídica amb pre-trencament de les cèl·lules

- Centrifugar les cèl·lules de llevat (5-10 mg pes sec) en tubs de rosca de 2 ml.
- Afegir-hi: 20µl EDTA 0.1 M
100 µl Metanol
perles de vidre
- Agitar durant 5 minuts en un minibeadbeater-8 (Biospec Products).
- Extracció amb cloroform: metanol (2:1, v/v, dos cops i 1: 1, un cop).
  Les fases orgàniques es transfereixen a un tub de vidre de 15 ml en presència de KCl 0.88% (1/4 del volum total del extracte).
- Agitar amb el vórtex i refredar en gel en intervals de 30 segons durant 15 min.
- Centrifugar les mostres 10 minuts a 2000 rpm durant 2 min.
- Recuperar la fase orgànica i assecar-la mitjançant una columna de Na_2SO_4 anhidra.
- Concentrar fins a assecar amb nitrogen comprimit.
- Disoldre l'extracte amb Cloroform:metanol (2:1, v/v).

Anàlisi de l'extracte per cromatografia de capa fina (TLC)
Separar els lípids individuals per cromatografia de capa fina (TLC) en plaques de silica gel 60F254 (10 x 20cm, 250µm, Merck, Germany), amb els següents sistemes de solvent:

- Lípids neutres (esterols, esters d'esterols (SE), diacilglicèrids (DAG), triacilglicèrids (TAG), àcids grassos lliures (AG), esters d'àcids grassos (EEAG) i esqualè): Primer solvent, fins la meitat de la placa: **hexà : tert-butilmetil eter (MTBE) : àcid acètic glacial** (70:30:2, v/v). Segon solvent fins a dalt de la placa: **hexà**.

- Fosfolípids (fosfatidil-inositol (PI), fosfatidil-serina (PS), fosfatidil-colina (PC), fosfatidil-glicerol (PG), fosfatidil-etanolamina (PE), cardiolipina (CL) i àcid fosfatídic (PA)): **cloroform : acetona : metanol : acid acètic glacial : aigua** (50:15:10:10:5, v/v).

La calibració es realitza utilitzant solucions patró (0.5-4 µg/µl) de lanosterol, ergosterol, esqualè, colesterol oleate (SE), oleate (AG), etil oleate (EEAG), diolein (DAG), triolein (TAG), PI, PS, PC, PG, PE, CL and PA (Sigma).

El revelat i detecció dels lípids en les plaques es realitza amb $CuSO_4$ 10% en H_3PO_4 8%, esclafant a 120ºC 20 minuts. La placa es filma amb una camera digital Kodak DC290 i la quantificació de les taques corresponents als lípids neutres i fosfolípids es realitza mitjançant el programa Quantity One software (Biorad, USA).

Determinació de la composició en àcids grassos dels fosfolípids per GC

Els fosfolípids (PL) separats dels lípids neutres en TLC són recuperats de la placa després de la visualització amb vapors de iode.

- Dissoldre els fosfolípids en 100 µl de MTBE.
- Afegir 200 µl de 0.2N metilat de sodi en metanol durant 2 minuts a T^a ambient.
- Afegir 60 µl de H_2SO_4 4N i 400 µl d'aigua, vórtex durant 1 minut.
- Centrifugar 2 minuts 3000 rpm per recuperar els esters d'àcids grassos metilats.
- Afegir-hi el patró intern: ester metil-heptanoat (1 µg µ-1).

Injectar l'extracte (3 µl) en una columna FFAP-HP 30 m x 0.25 mm i 0.25 mm de gruix (Agilent),mitjançant un injector automàtic HP7673 (Agilent). La temperatura del programa és 100ºC x 3ºC/min a 240ºC (13 min.). Les temperatures del injector i detector són 250 i 280ºC, respectivament. El gas vector és heli 1.2 ml/min.
Els FAME són identificats a partir dels seus temps de retenció relatius als patrons corresponents (Supelco) i quantificats a partir de les àrees dels seus pics.

9. Altres:

9.1. Determinació de la Trehalosa i Glicogen intracelular (Parrou and Francois 1997)

A. Extracció i digestió de la trehalosa i glicogen intracel·lulars

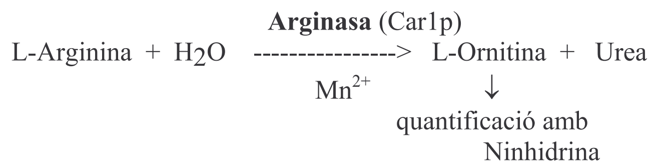
- Fer créixer les cèl·lules en 5 ml de YEPD 12 hores a 28 C (pes sec 4-10 mg, DO_{600} 10-15)
- Centrifugar 3 minuts a 5000g (0-4 C).
- Resuspendre les cèl·lules amb 0.25 ml de **Na_2CO_3 0.25M***.
- Incubar durant **2-4 hores a 95ºC** (permeabilitza les cèl·lules a l'entrada de enzims i sortida de glucosa).
- Afegir 0.15 ml de **Àcid acètic 1M** i 0.6 ml de **Acetat sòdic 0.2M** a pH=5,2 i separar-ho en dos meitats.
- Incubar una meitat de la suspensió amb **Trehalasa** 0.05 U/ml (Sigma Cat.No T-8778) durant **una nit a 37ºC** per la determinació de trehalosa.
- Incubar l'altra meitat amb **Amyloglucosidasa** 1.2 U/ml de *A.niger* (Boehringer Cat.No. 208469) **una nit a 57ºC** amb agitació constant (cambra d'hibridació) per la determinació de glicogen*.
- Centrifugar 3 minuts a 5000g.

*Preparar-ho en Eppendorfs de 2 ml de rosca per evitar fuites.

B. Determinació de la glucosa formada:

- A 20 µl de mostra (sobrenedant adequadament diluït amb aigua) afegir-hi 200 µl de **glucosa oxidasa** (Sigma Cat.No. 510-A)
- Llegir l'absorbància de la mostra a **420 nm**.
- Fer el càlcul a partir d'una solució estàndard de glucosa, i expressar el resultat en mg glc / mg pes sec

9.2. Activitat arginasa (Carrasco *et al.* 2003)



A. Obtenció de l'extracte proteic:

- Homogeneïtzar 200 mg de cèl·lules en 500µl en Tampó Tris i 1g de perles de vidre.
- Agitar al vórtex 4-8 cops en intervals de 1 min vórtex i 1 min gel.
- Centrifugar a 5000g 5 minuts en fred.
- Transferir el sobrenedant (extracte proteic) a un Eppendorf.

B. Quantificació de proteïnes per Bradford (µassaig: 0-25µg/ml):

- Fer una recta patró amb Albúmina (Sigma, A-4503): 0, 5, 10, 20, 40 µg/ml
  (a partir d'una solució de 400 µg BSA/ml)
- Diluir les mostres corresponentment.
- Mesclar en la cubeta 800µl de mostra i 200µl de reactiu de Bradford (casa comercial).
- Esperar 5 minuts i llegir l'absorbància a 595 nm.
- Calcular la concentració de proteïna a partir de la recta patró

C. Assaig de l'activitat enzimàtica:

*preparar-ho en un Epp de 2mlde rosca perquè al bullir durant 30min la mostra no s'obri

- *Mesclar 25µl µl de l'extracte proteic (2-40 µg prot total) i 25 µl de $MnCl_2$ incubant 20 min a 55ºC. (tants tubs de cada mostra com punts a aturar la reacció)
- Deixar a temperatura ambient 4 minuts.
- Afegir 150 µl de tampó carbonat 0,1M pH 9,5 atemperat a 37ºC.
- Començar la reacció afegint 50µl del substrat (arginina 0,1M).
- Incubar a 37ºC.
- Aturar la reacció afegint 750 µl d'àcid acètic glacial a diferents temps: 0, 3, 6,... minuts (un tub per cada temps).

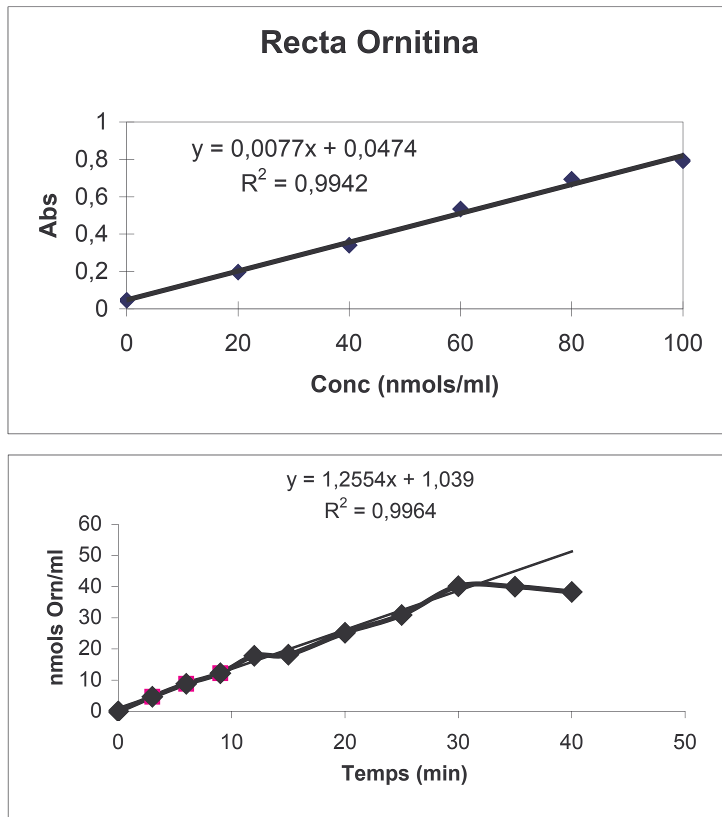
D. Determinació amb ninhidrina:

- Afegir 250 µl del reactiu de ninhidrina (140 mM ninhidrina en 60% àc. acètic, 40% àc. fosfòric 6M en Metanol).
- Bullir la mostra a 100ºC durant 30 min.
- Mesurar a 515 nm i calcular l'ornitina a partir d'una recta patró de la mateixa (afegir tots els reactius però enlloc de l'extracte proteic diferents concentracions d'Ornitina: de 0 a 100 nM).
- Fer també un blanc sense extracte proteic i un sense arginina per cada mostra.

Càlculs: a partir de la recta patró es calcula la concentració d'ornitina cada punt de reacció. La pendent de la recta ens donarà les unitats, nmols Orn ml^{-1} min^{-1}., que dividint-ho per la concentració de proteïna ens donarà l'activitat

$$\text{Activitat (nmols Orn min}^{-1}\ \mu\text{g prot}^{-1}) = \frac{\text{Unitats x dilució}}{\text{[prot] x volum (0,025 ml)}}$$

Prova realitzada amb el llevat sec actiu QA23 (Lallemand) rehidratat 30 minuts a 37ºC



Unitats: 1,255 nmols Orn ml^{-1} min^{-1}
Concentració proteïna: 8647 µg prot/ml (dil 1:100)
Volum: 0,025 ml
Activitat: (1,255 x 100) / (8647 x 0,025) = **0,58 nmols Orn min^{-1} µg prot $^{-1}$**

Pàgines web utilitzades:

Biopuce de Toulouse
http://bio71.gba.insa-tlse.fr/

Saccharomyces Genome Database (SGD)
http://www.yeastgenome.org/

Yeast Project at MIPS: Comprehensive Yeast Genome Database (CYGD)
http://mips.gsf.de/genre/proj/yeast/index.jsp

MIPS Funcional Catalogue
http://mips.gsf.de/proj/funcatDB/search_main_frame.html

AmiGO: your friend in Gene Oncology
http://www.godatabase.org/cgi-bin/amigo/go.cgi

Yeast Proteome Database (YPD)
https://www.incyte.com/login.html

Funspec: A Web-Based Cluster Interpreter for Yeast
http://funspec.med.utoronto.ca/

FatiGO: data mining with Gene Oncology
http://fatigo.bioinfo.cnio.es/

EPCLUST: clustering, visualization and analysis
http://ep.ebi.ac.uk/EP/EPCLUST/

Yeast Microarray Global Viewer (yMGB)
http://www.transcriptome.ens.fr/ymgv/

The Yeast Transport Protein Database (YTPdb)
http://mips.gsf.de/proj/eurofan/eurofan_1/n6/

KEGG Pathway Database
http://www.genome.jp/kegg/pathway.html

REFERENCES

Aerny,J. (1996) Composés azotés des moûts et vins. *Revue suisse Vitic. Arboric. Hortic.* **28**, 161-165.

Bely,M., Sablayrolles,J.M. and Barre,P. (1990) Description of alcoholic fermentation kinetics: its variability and significance. *American Journal of Enology and Viticulture* **41**, 319-324.

Carrasco,P., Perez-Ortin,J.E. and del Olmo,M. (2003) Arginase activity is a useful marker of nitrogen limitation during alcoholic fermentations. *Syst. Appl. Microbiol.* **26**, 471-479.

Ferreira,V., Sharman,M., Cacho,J. and Dennis,J. (1996) New and efficient microextraction / solid-phase extraction method for the gas chromatographic analysis of wine volatiles. *J. Chromatogr. A* **731**, 247-259.

García Barceló, J. (1990) *Técnicas analíticas para vinos*. Barcelona: GAB.

Guillamon,J.M., Sabate,J., Barrio,E., Cano,J. and Querol,A. (1998) Rapid identification of wine yeast species based on RFLP analysis of the ribosomal internal transcribed spacer (ITS) region. *Arch. Microbiol.* **169**, 387-392.

Lopez,R., Aznar,M., Cacho,J. and Ferreira,V. (2002) Determination of minor and trace volatile compounds in wine by solid-phase extraction and gas chromatography with mass spectrometric detection. *J. Chromatogr. A* **966**, 167-177.

Morris,E.O. and Eddy,A.A. (1957) *J. Inst. Brew.* **63**, 34.

Ough, C. S. and Amerine, M. A. (1988) *Methods for analysis of must and wines*. California: Wiley-Interscience Publication.

Parrou,J.L. and Francois,J. (1997) A simplified procedure for a rapid and reliable assay of both glycogen and trehalose in whole yeast cells. *Anal. Biochem.* **248**, 186-188.

Querol,A., Barrio,E. and Ramon,D. (1992) A comparative study of different methods of yeast strain characterization. *Syst. Appl. Microbiol.* **21**, 315-323.

Ribéreau-Gayon, P., Dubourdieu, D., Donèche, B. and Lonvaud, A. (2000) *Handbook of Enology. The Microbiology of wine and vinifications Vol. I*. West Sussex, England: Wiley.

Rozès,N., García-Jares,C., Larue,F. and Lonvaud-Funel,A. (1992) Differentiation between fermenting and spoilage yeasts in wine by total free fatty acid analysis. *J. Sci. Food Agric.* **59**, 351-357.

Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) *Methods in Yeast Genetics: A Laboratory Manual*. NY, USA: Cold Spring Harbor.

Sierkstra,L.N., Verbakel,J.M. and Verrips,C.T. (1992) Analysis of transcription and translation of glycolytic enzymes in glucose-limited continuous cultures of Saccharomyces cerevisiae. *J. Gen. Microbiol.* **138**, 2559-2566.

ANNEX 2

Supplementary data of functional genomics at low temperature fermentation

13ºC *vs.* 25ºC

Table 1. Number and ratios of genes up or down-regulated (>2-fold variation) obtained at each phase of fermentation (initial, mid and final). Ratios were obtained by dividing the low temperature (13ºC) by the control temperature (25ºC) intensities for the up-regulated genes (positive values) and by dividing the 25ºC by the 13ºC intensities for the down-regulated genes (negative values) at the same stages of fermentation (13ºC *vs.*25ºC).

	IF		MF		FF	
	Up	**Down**	**Up**	**Down**	**Up**	**Down**
All genome						
Nº gens	**285**	**907**	**1154**	**461**	**1327**	**867**
% genome	4.70	14.95	19.02	7.60	21.87	14.29
Maximal/minimal ratio	7.39	-25	22.93	-7.69	62.04	-14.29
Average Ratio	2.42	-3.99	4.04	-2.52	5.41	-2.59
t-Student & Ratio						
Nº gens	**10**	**25**	**128**	**27**	**313**	**138**
Maximal/minimal ratio	3.04	-20	21.07	-2.63	40.54	-4.35
Average Ratio	2.85	-3.63	5.58	-2.22	6.96	-2.47

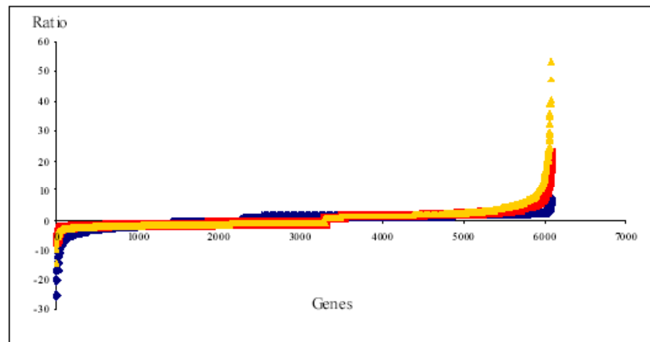


Table 2. Number of genes significantly up or down-regulated (*i.e.* >2-fold variation and Student's *t*-test with p value < 0.05) in each functional category (MIPS) comparing both temperatures at three phases of fermentation (initial, mid and final)

		Number of Genes					
		IF		MF		FF	
Functional Category		Down(1)	Up (2)	Down(1)	Up (2)	Down(1)	Up (2)
Transport Facilitation	(313)	1	-	-	3	16	15
Subcellular localization	(2258)	8	5	13	49	51	145
Control of cellular organization	(209)	-	-	1	6	4	9
Cell Fate	(427)	3	-	3	6	11	24
Regulation of cell Envinonment	(199)	-	-	-	3	4	10
Cell Rescue, Defense and Virulence	(278)	-	1	-	3	13	11
Cellular comunication/signal Transduction	(59)	-	-	-	1	2	2
Cellular Transport, Tranport Mechanism	(495)	1	1	1	17	14	32
Protein Fate	(595)	3	1	3	16	15	30
Protein Synthesis	(359)	3	1	4	5	8	17
Transcription	(771)	-	-	6	17	12	51
Cell Cycle and DNA processing	(628)	1	1	4	21	13	46
Energy	(252)	-	-	2	-	6	4
Metabolism	(1066)	2	3	12	9	36	48
Classification not yet clear-cut	(115)	-	-	1	2	4	6
Unkown proteins	(2399)	14	3	7	58	47	111
Total number of genes		**25**	**10**	**27**	**128**	**138**	**313**

(1) ≤ 2-fold down-regulated genes at 13ºC condition (2) ≥ 2-fold up-regulated genes at 13ºC condition

Kinetic 13ºC & Kinetic 25ºC

Table 3. Number and ratios of genes up or down-regulated (>2-fold variation) obtained at the middle and final phases of 13ºC and 25ºC fermentations comparing with expression levels of this yeast strain at the initial phases of fermentation. (BioPlot software)

	13MF/IF		13FF/IF		25MF/IF		25FF/IF	
	Up	Down	Up	Down	Up	Down	Up	Down
All genome								
Nº gens	563	245	762	462	347	788	480	991
Maximal/minimal ratio	23.17	-7.69	33.08	-8.33	7.91	-20	18.49	-25
Average Ratio	3.12	-2.40	3.29	-2.54	2.41	-3.26	2.47	-3.56
t-Student & Ratio								
Nº gens	**128**	**90**	**117**	**125**	**20**	**233**	**112**	**317**
Maximal/minimal ratio	14.66	-6.67	20.82	-6.69	7.91	-20	5.35	-20
Average Ratio	3.39	-2.39	3.78	-2.55	3.01	-3.80	2.49	-4.40

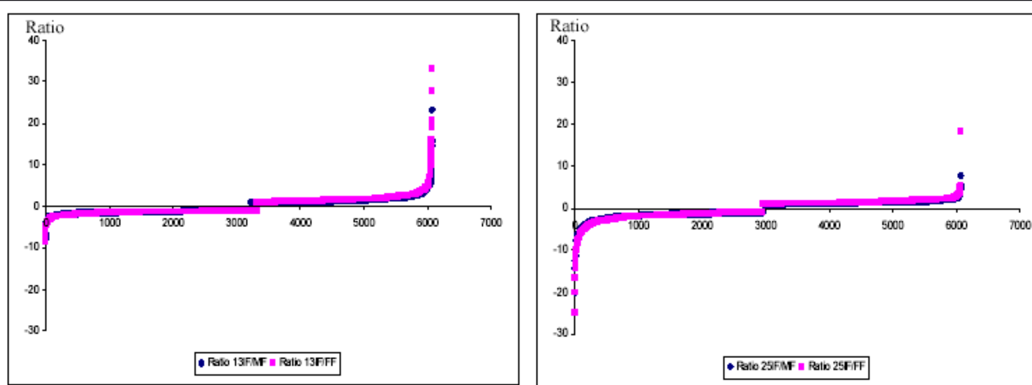


Table 4. Number of genes significantly up or down-regulated (*i.e.*>2-fold variation and Student's *t*-test with p value < 0.05) in each functional category (MIPS).

		Kinetic 13ºC				Kinetic 25ºC			
		13 MF/IF		13 FF/IF		25 MF/IF		25 FF/IF	
Functional Category		Down(1)	Up (2)	Down(1)	Up (2)	Down(1)	Up (2)	Down(1)	Up (2)
Transport Facilitation	(313)	6	8	11	8	8	1	9	9
Subcellular localization	(2258)	66	40	76	38	87	12	115	62
Control of cellular organization	(209)	5	6	2	2	7	1	3	7
Cell Fate	(427)	8	13	10	5	18	2	26	8
Regulation of cell Envinonment	(199)	3	5	4	6	8	1	5	1
Cell Rescue, Defense and Virulence	(278)	9	4	10	-	9	-	7	13
Cellular comunication/signal Transduction	(59)	1	1	-	-	2	-	2	-
Cellular Transport, Tranport Mechanism	(495)	15	15	15	8	18	4	22	17
Protein Fate	(595)	19	8	13	10	25	5	25	16
Protein Synthesis	(359)	21	4	27	4	11	1	20	8
Transcription	(771)	8	14	20	18	31	1	42	18
Cell Cycle and DNA processing	(628)	7	18	8	15	32	1	42	18
Energy	(252)	8	1	5	5	6	4	5	8
Metabolism	(1066)	33	10	33	16	20	1	27	30
Classification not yet clear-cut	(115)	-	5	2	1	3	-	6	-
Unkown proteins	(2399)	19	63	31	58	101	8	116	27
Total number of genes		**90**	**128**	**125**	**117**	**233**	**20**	**317**	**112**

(1) $\leq$ 2-fold down-regulated genes at 13ºC condition (2) $\geq$ 2-fold up-regulated genes at 13ºC condition

Figure 1. Distribution of the Ratios and logRatios of all genes showing the correlation between the different samples analysed: **A)** comparing the gene expression at 13ºC and 25ºC fermentation at different points of fermentation, and **B)** throughout the fermentation process at 25ºC and 13ºC. The ratios of genes retained based on our filtering tests (*i.e.* > 2-fold ratio variation and Student's *t*-test p-value <0.05) are represented in pink.

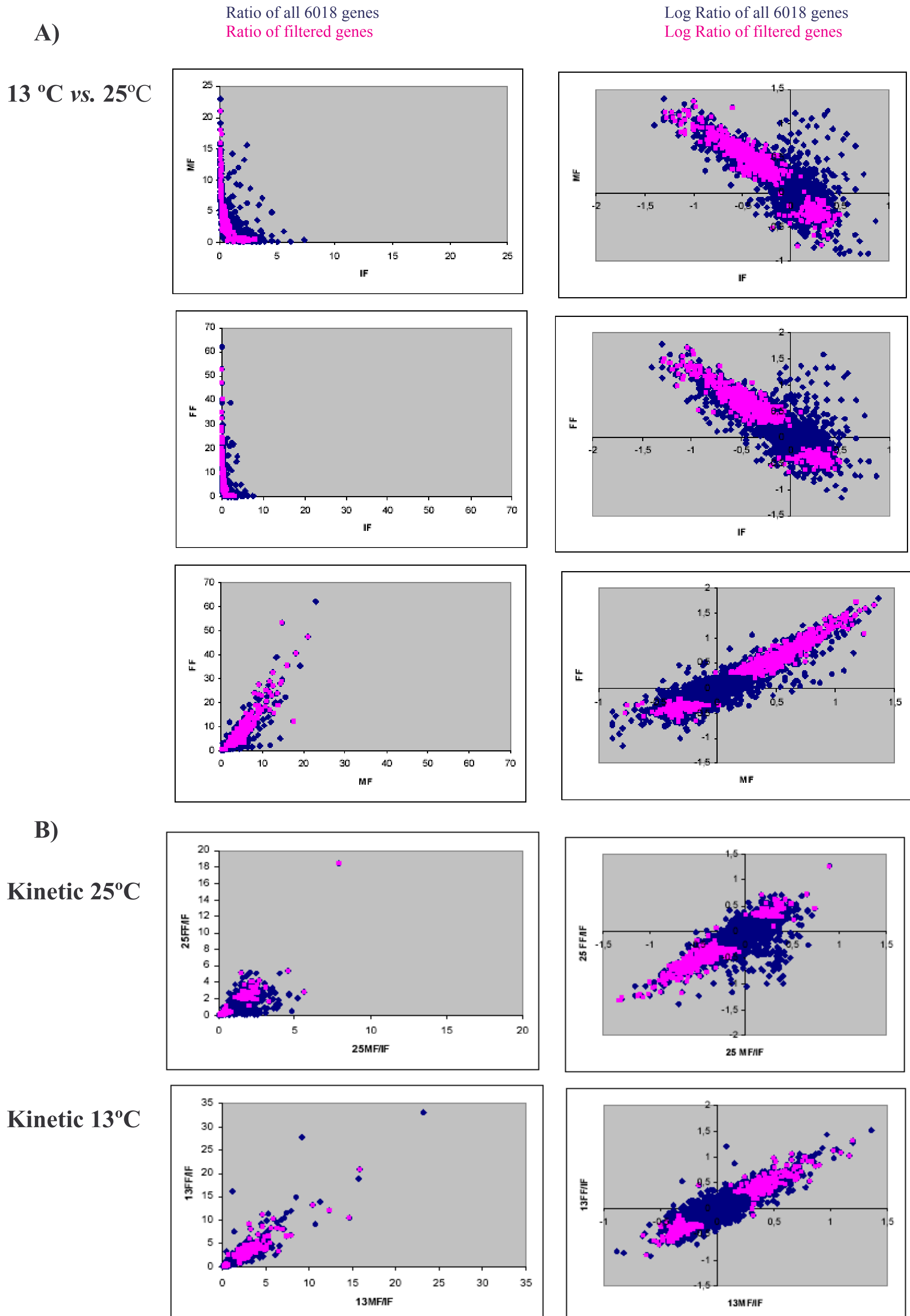


Table 5. Total number of genes up- and down-regulated at 13ºC *vs*. 25ºC in each phase of fermentation, and distributed in each chromosome.

Chromosome		IF				MF				FF			
	Nº genes	Down	Up	Total	%	Down	Up	Total	%	Down	Up	Total	%
I	114	36	2	38	33,3	1	41	42	36,8	9	41	50	43,9
II	431	88	9	97	22,5	23	116	139	32,3	54	120	174	40,4
III	173	34	7	41	23,7	9	55	64	37,0	18	61	79	45,7
IV	816	79	32	111	13,6	66	103	169	20,7	116	130	246	30,1
V	308	17	21	38	12,3	25	25	50	16,2	48	33	81	26,3
VI	136	5	9	14	10,3	16	14	30	22,1	24	12	36	26,5
VII	565	45	21	66	11,7	37	52	89	15,8	71	70	141	25,0
VIII	297	24	16	40	13,5	18	38	56	18,9	34	43	77	25,9
IX	239	12	16	28	11,7	28	17	45	18,8	49	20	69	28,9
X	388	30	29	59	15,2	45	41	86	22,2	65	53	118	30,4
XI	337	22	14	36	10,7	25	30	55	16,3	42	42	84	24,9
XII	541	83	36	119	22,0	47	117	164	30,3	70	139	209	38,6
XIII	486	57	12	69	14,2	27	89	116	23,9	57	108	165	34,0
XIV	420	39	20	59	14,0	29	47	76	18,1	58	58	116	27,6
XV	572	159	19	178	31,1	34	177	211	36,9	92	189	281	49,1
XVI	494	177	22	199	40,3	31	192	223	45,1	60	208	268	54,3
Total	6317	907	285	1192	18,9	461	1154	1615	25,6	867	1327	2194	34,7

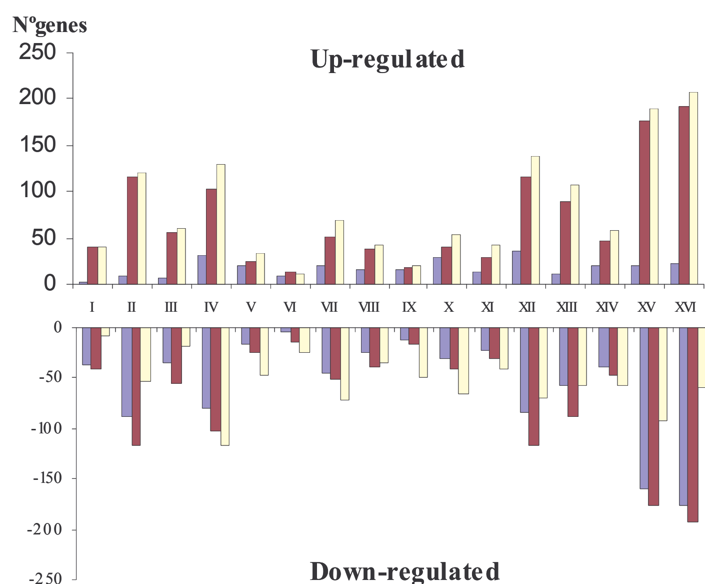


Table 6. Number of genes retained based on our filtering tests (Bioclust software): *i.e.* > 2-fold ratio variation and /or statistical significance with Student's *t*-test p-value <0.05, at least in one point of study.

	13*vs*.25	Kinetic13ºC	Kinetic25ºC	Kinet.13+25ºC
Ratio (<0.5, >2)	2275	1226	1561	1952
t-Student (<0.05)	1361	1349	1342	2288
t-Student & Ratio	536	381	520	767

Figure 3. Hierarchical clustering of significant up- and down- regulated genes (statistically significant variation of at least twofold in at least on of the experiments) during fermentation at 13ºC and 25°C and comparing 13ºC and 25°C at different times of the fermentation (Genespring version 4.2 software, Silicon genetics Inc, USA). Ratios of the changes obtained by dividing the experimental by the reference samples are represented with a green-to-red color-scale. Down regulated are green and up-regulated are red.

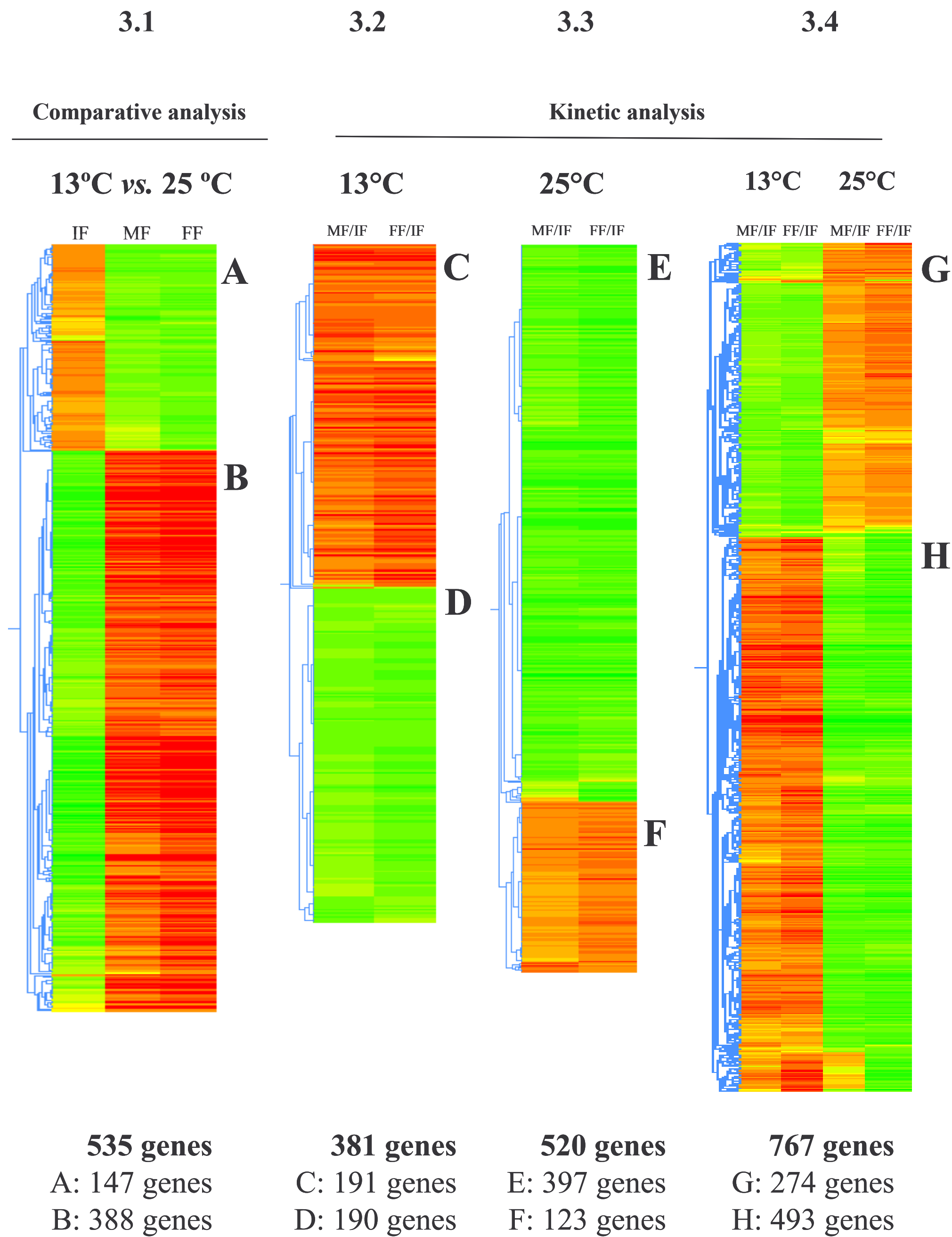
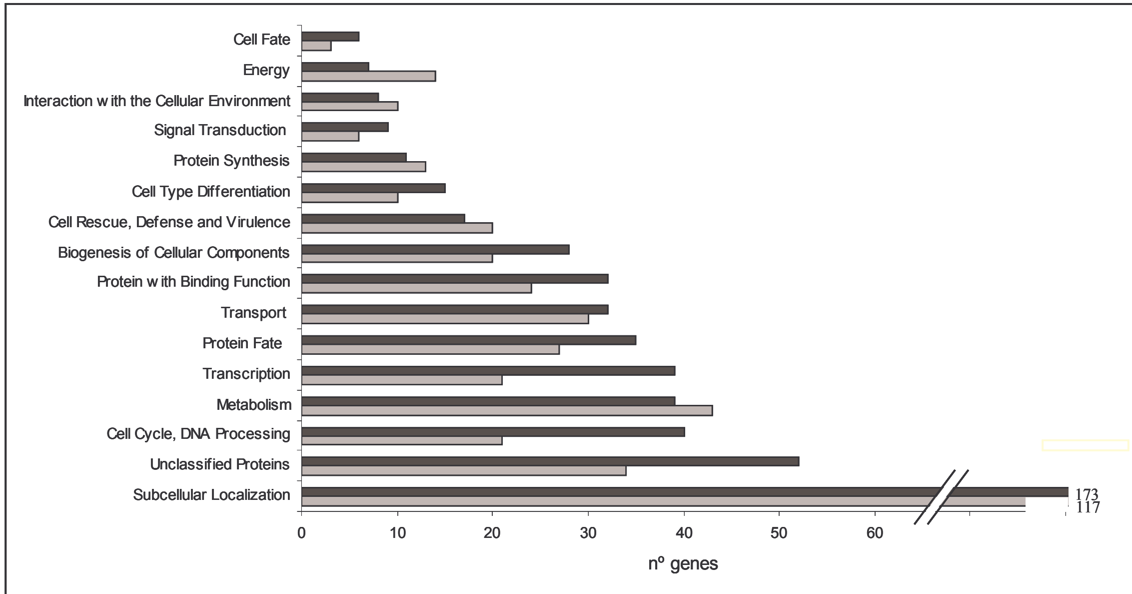
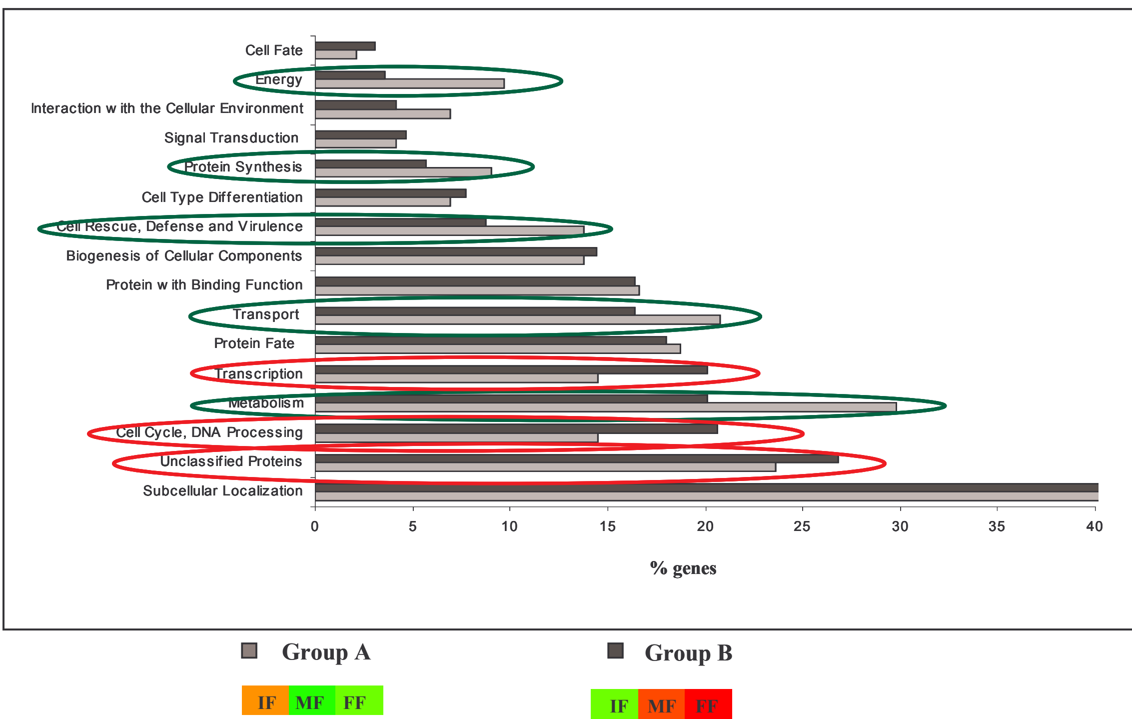


Figure 4. Genes with similar expression patterns obtained in the cluster of Figure 3.1 (13 *vs* 25ºC, GroupA and Group B) are represented showing the distribution of the most representative MIPS functional categories. Group A (147 genes) are the genes up-regulated at 13ºC IF, but down-regulated at 13ºC MF and FF. Group B (388 genes) are the genes down-regulated at 13ºC IF, but up-regulated at 13ºC MF and FF. The results are expressed as number of genes in each category, but also as % of genes of each group in each category.

13 vs. 25ºC



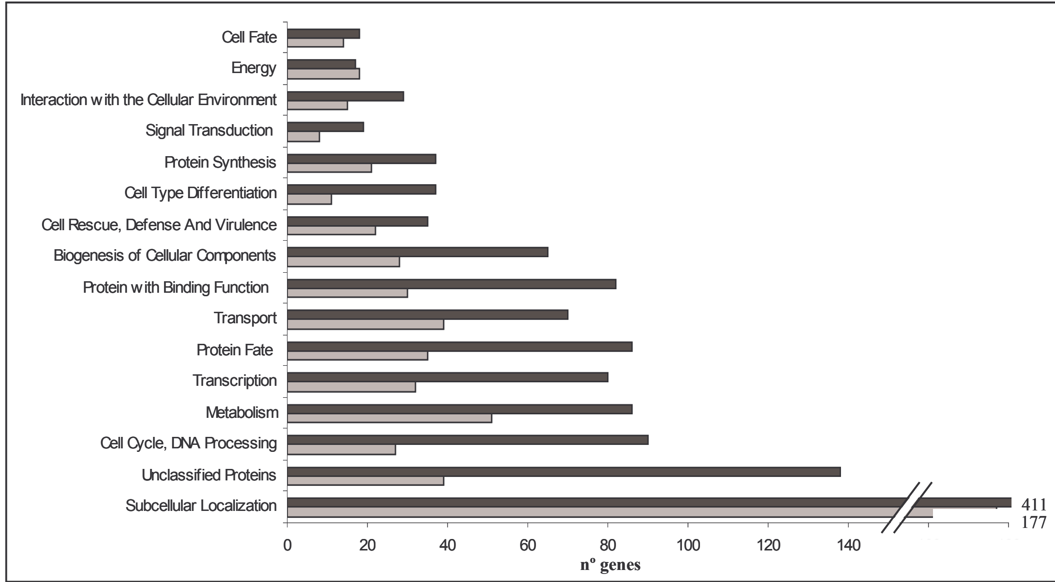


Group A IF MF FF

Group B IF MF FF

Figure 5. Genes with similar expression patterns obtained in the cluster of Figure 3.4 (Kinetic 13ºC and Kinetic 25ºC, GroupG and GroupH) are represented showing the distribution of the most representative functional categories. The results are expressed as number of genes in each category, but also as % of genes of each group in each category. Group G (274 genes) Group H (493 genes)

Kinetic 13ºC and Kinetic 25ºC



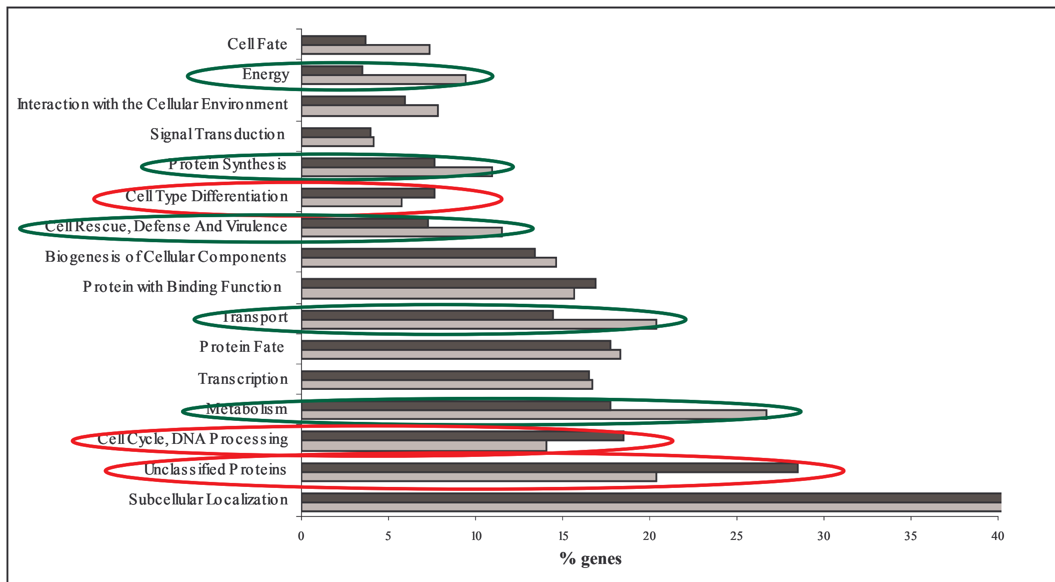


Figure 6. The datasets obtained from the kinetics at 13ºC and 25°C (4 datasets) and from the comparison between fermentation stages at 13ºC and 25°C (3 datasets) were filtered and clustered as indicated in figure 1 to estimate the correlation between the expression profiles from these 7 datasets.

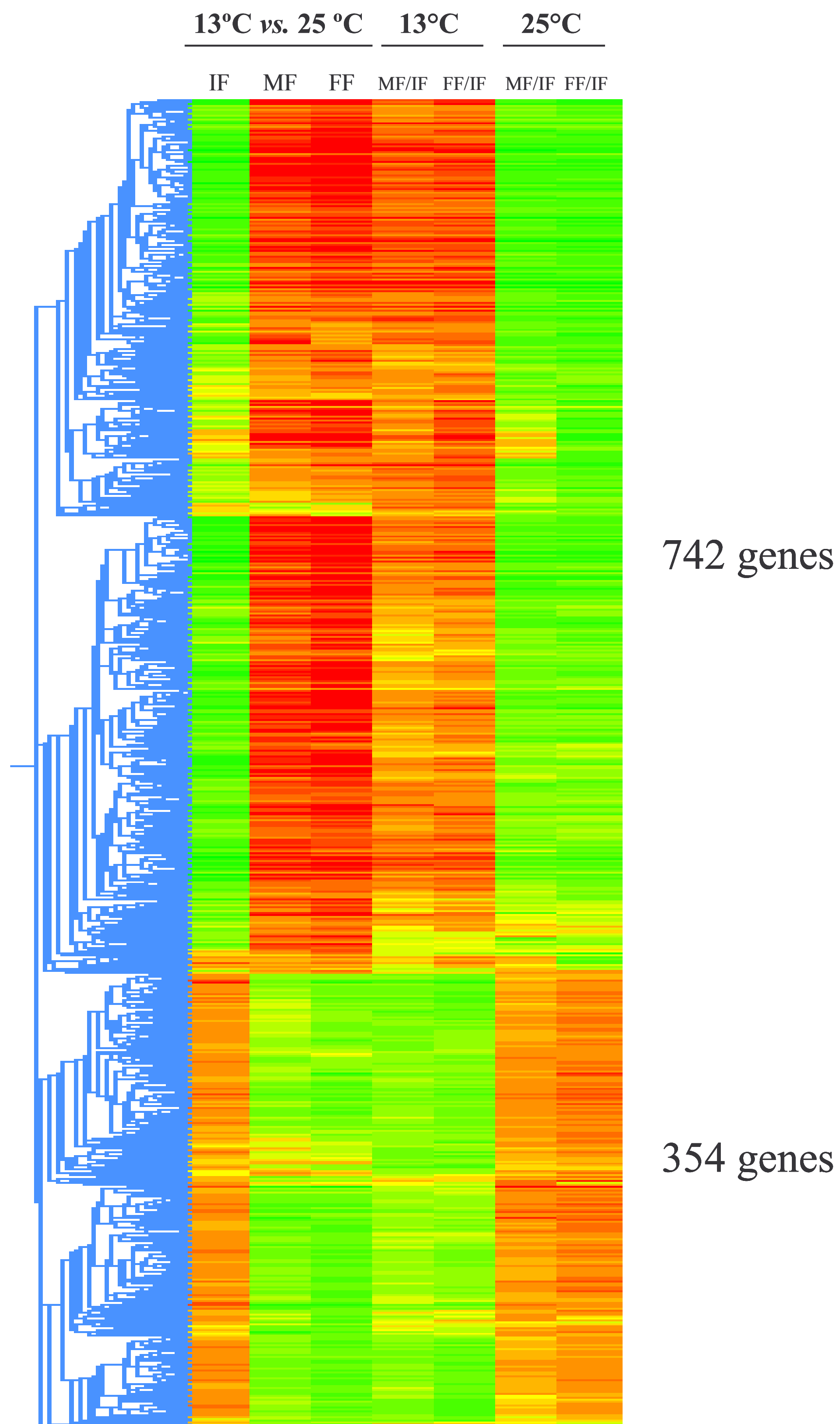
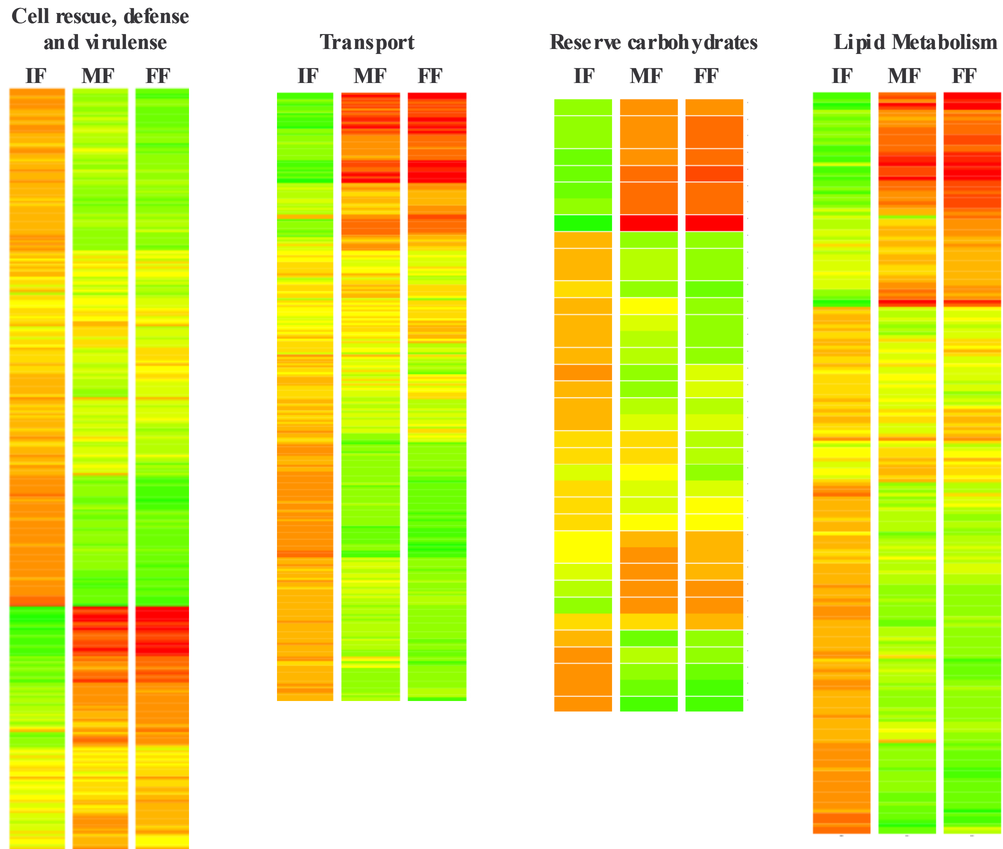


Figure 7. Hierarchical clustering of all genes involved on Cell rescue, defence and virulence; Transport; Reserve carbohydrates and Lipid Metabolism (MIPS functional classification) comparing gene expression of yeast fermenting at 13ºC and 25ºC at three points of fermentation (initial, mid and final). Ratios of the changes obtained by dividing the experimental by the reference samples are represented with a green-to-red color-scale. Down regulated are green and up-regulated are red. (Genespring version 4.2 software, Silicon genetics Inc, USA).



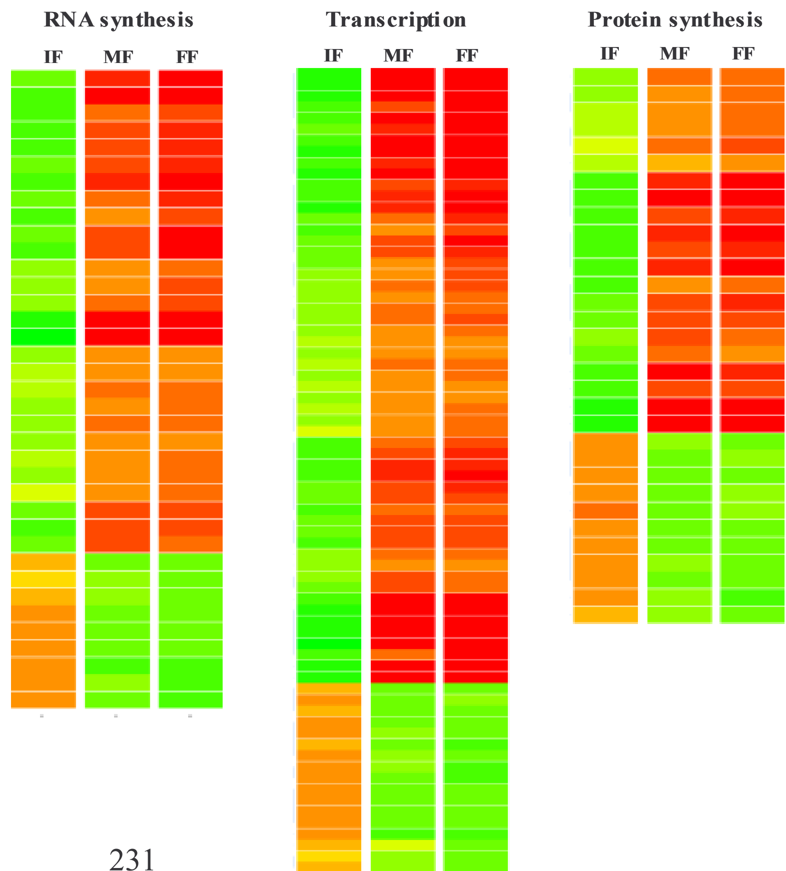


Figure 8. Hierarchical clustering of up- and down-regulated (2-fold changes) genes involved on RNA synthesis, Transcrition and Protein synthesis (MIPS functional classification) comparing gene expression of yeast fermenting at 13ºC and 25ºC at three points of fermentation (initial, mid and final).

Figure 9. Hierarchical clustering of all genes involved on Fatty acid, Sterol, Phospholipid and Sphingolipid Metabolism (Gene Ontology classification) comparing gene expression of yeast fermenting at 13ºC and 25ºC at three points of fermentation (initial, mid and final). Ratios of the changes obtained by dividing the experimental by the reference samples are represented with a green-to-red color-scale. Down regulated are green and up-regulated are red. (Genespring version 4.2 software, Silicon genetics Inc, USA).

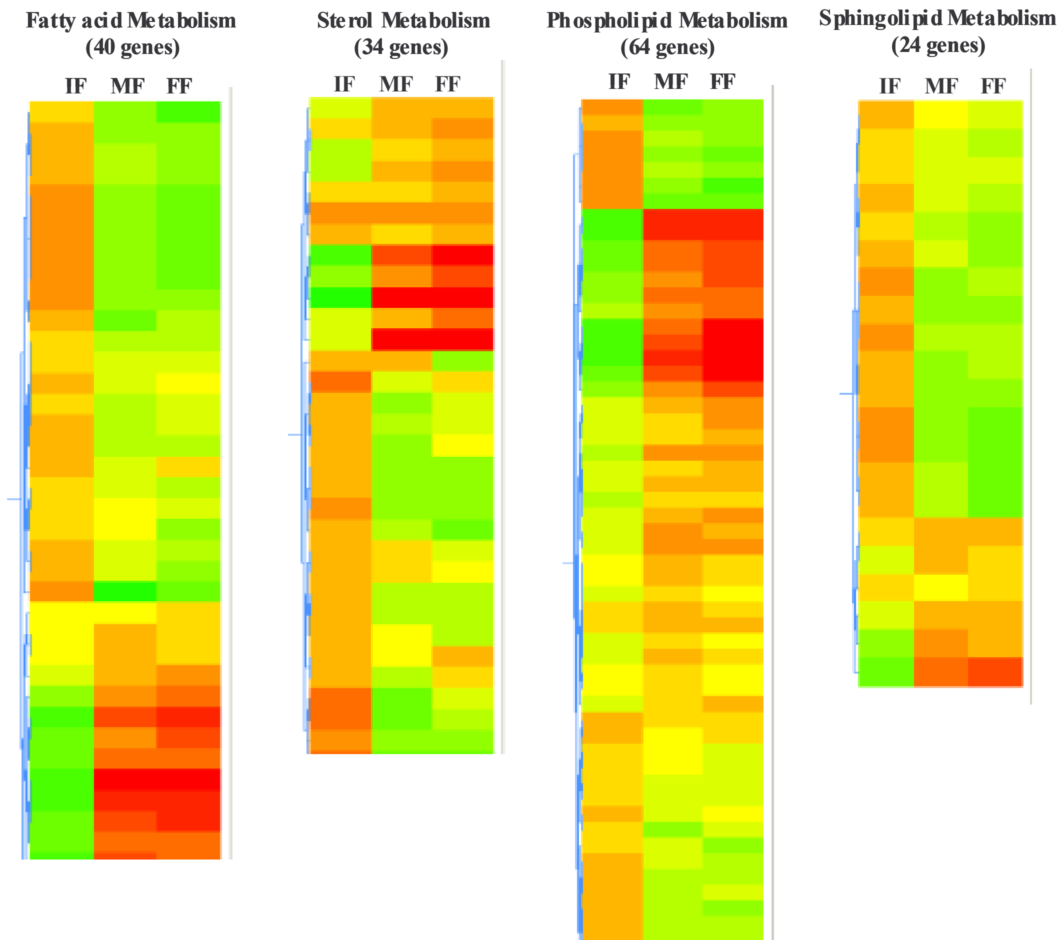


Figure 10. Phospholipid biosynthetic pathway in *S.cerevisiae* with the expression of genes represented with a green-to-red color-scale. Down regulated are green and up-regulated are red.

CL: Cardiolipine; DAG: Diacylglicerid; FA: Fatty acid; G-3-P: Glycerol 3P; PA: Phosphatidic acid; PC: Phosphatidylcholine; PE: Phosphatidylethanolamine; PG: Phosphatidylglycerol; PI: Phosphatilylinositol; PS: Phosphatidylserine; TAG: Triacylglicerid

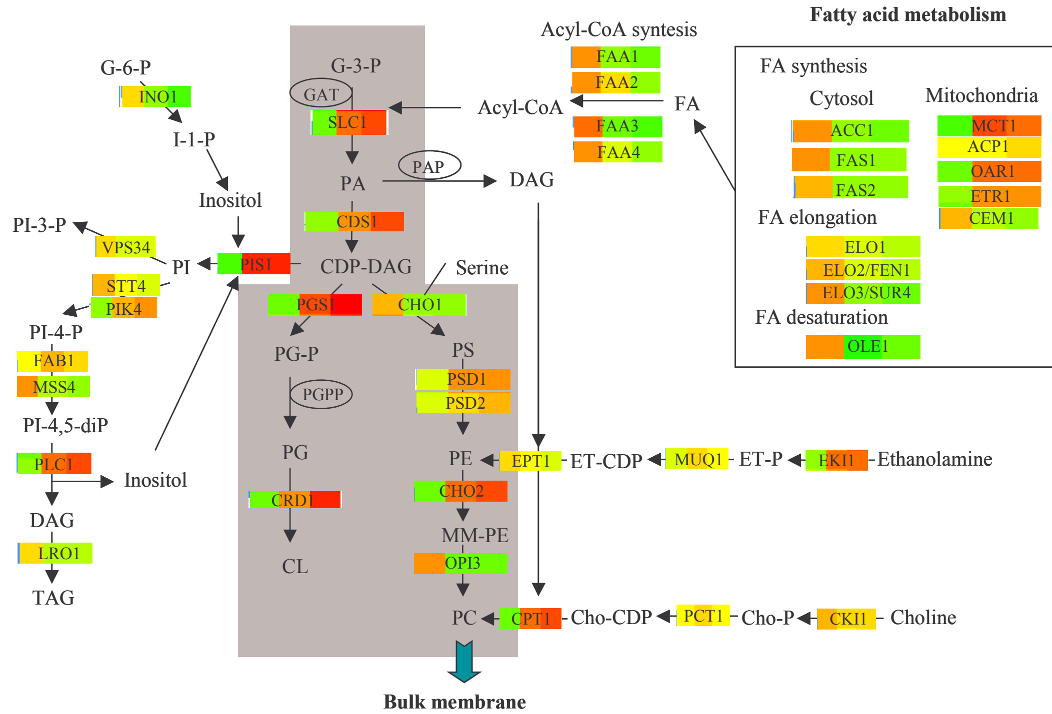


Figure 11. Sterol (A) and sphingolipid (B) biosynthetic pathways in *S.cerevisiae* with the expression of genes represented with a green-to-red color-scale. Down regulated are green and up-regulated are red.

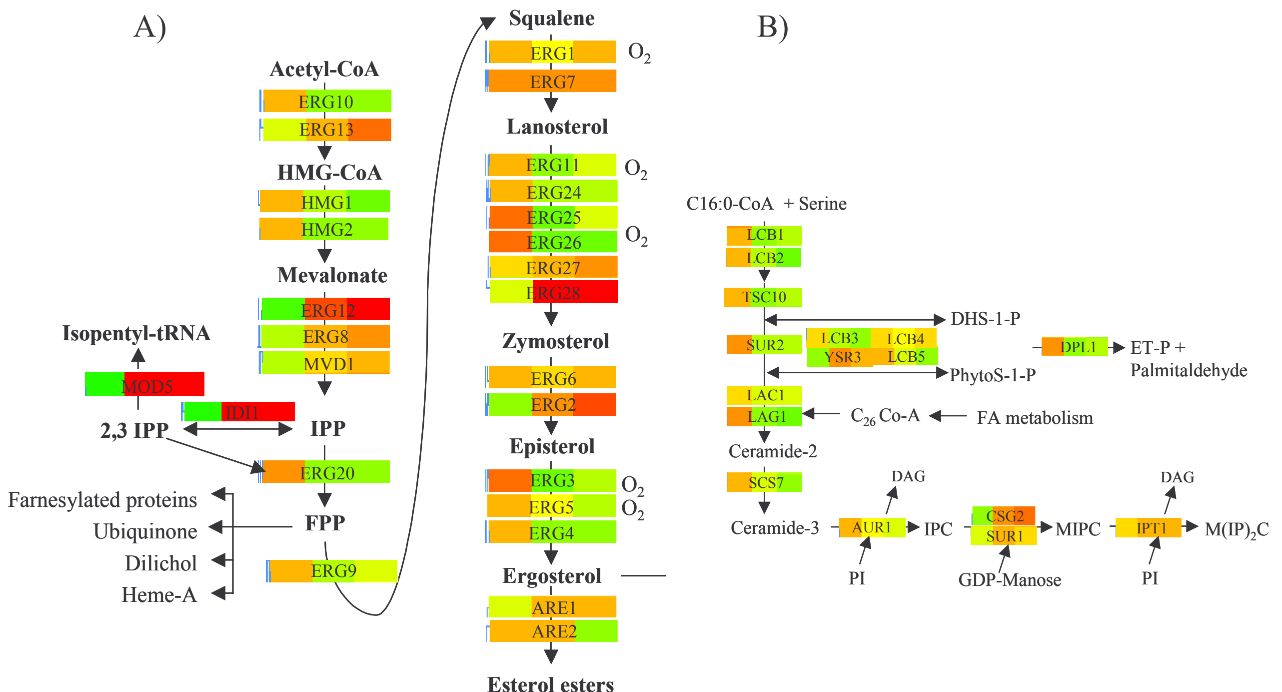
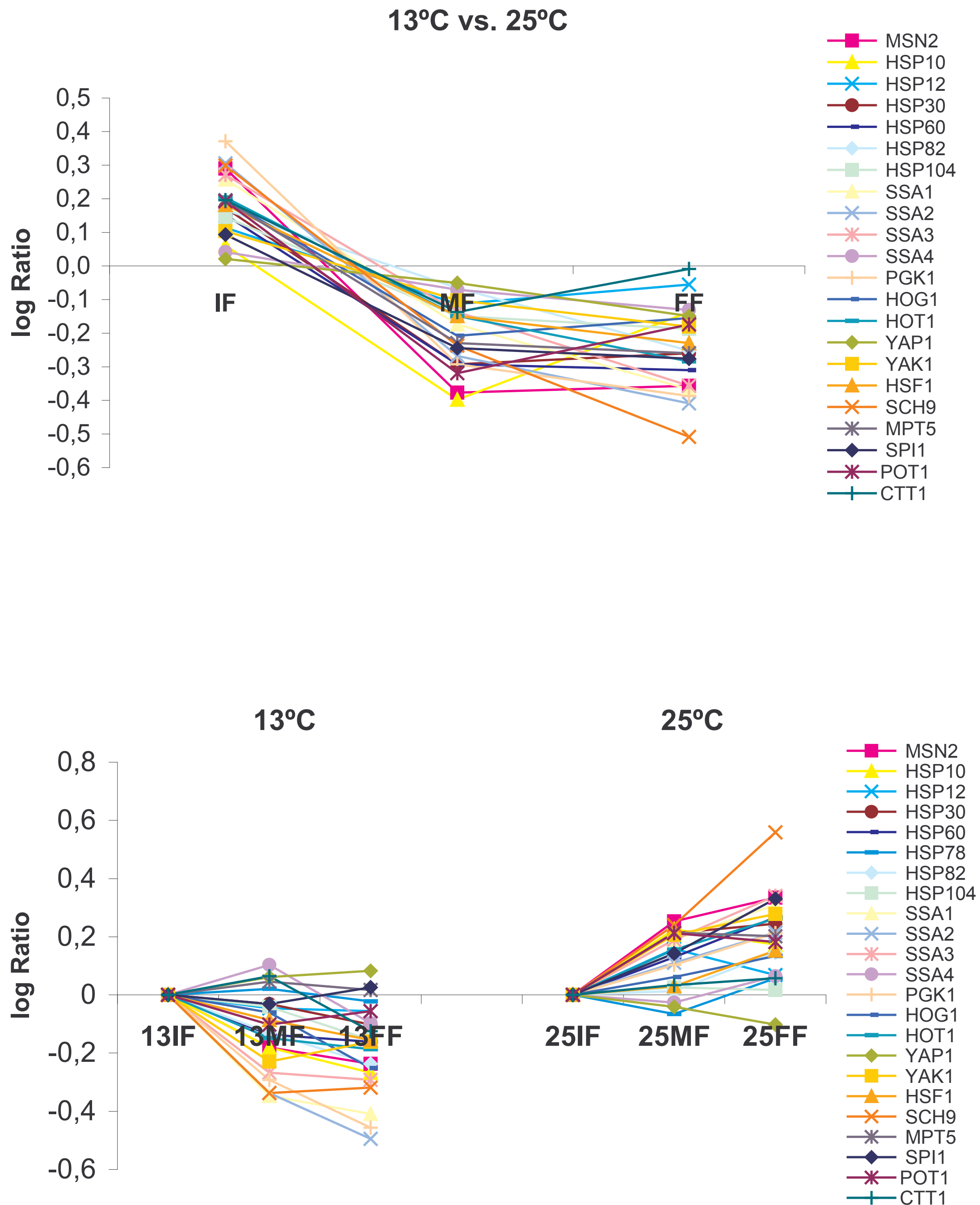


Figure 12. Expression profiles of genes whose products are involved in the stress response of yeast throughout wine fermentation.



ANNEX 3

Fermentaciones a bajas temperaturas

Gemma Beltran, Maite Novo, Mª Jesús Torija, Montse Poblet, Nicolas Rozès,

José M. Guillamón y Albert Mas

Unitat d'Enologia del Centre de Referència de Tecnologia d'Aliments. Dept. Bioquímica i Biotecnologia. Facultat d'Enologia de Tarragona. Universitat Rovira i Virgili. Marcel·lí Domingo s/n. 43007 Tarragona, Spain

Tecnología del Vino, 2, 73-76 (2001)

Fermentaciones a bajas temperaturas

G. Beltrán, M. Novo, M. J. Torija, M. Poblet, N. Rozès, J. M. Guillamón y A. Mas
Unitat d'nologia del Centre de Referència en Tecnologia dels Aliments
Facultad Enologia de Tarragona. Universitat Rovira i Virgili

La fermentación a baja temperatura realizada para la obtención de vinos de calidad, aún cuando puede presentar algunas desventajas, ofrece a cambio la conservación de otros efectos notablemente positivos, principalmente lo relacionado con el aroma ya que contribuye a la fermentación de las sustancias volátiles. Este trabajo aporta un mayor conocimiento, en detalle, sobre el condicionamiento que supone la aplicación del frío sobre la fermentación alcohólica.

1. Introducción

Las fermentaciones a bajas temperaturas (13°C o inferiores) tienen un gran interés para la producción de vinos blancos y rosados, especialmente los de gran potencial aromático. Estos aromas, al ser volátiles a temperatura ambiente, ven mermada su concentración durante la fermentación alcohólica. Así mismo, las fermentaciones a bajas temperaturas condicionan notablemente el desarrollo de las bacterias acéticas y lácticas, lo que permite su mejor control. A pesar de todas estas ventajas, las bajas temperaturas dificultan la realización de la fermentación alcohólica, llegando incluso al punto de comprometer su viabilidad. Los efectos más notables son:

- Disminución de la velocidad del consumo de azúcares.

- Fermentaciones más largas y mayor riesgo de paradas de fermentación.

- Modificación de las poblaciones de microorganismos.

- Cambio de la actividad metabólica de los microorganismos: modificaciones en la producción de metabolitos secundarios como consecuencia de las necesidades adaptativas.

Una vez que se ha conseguido un control efectivo de las temperaturas de fermentación por parte de la industria enológica, un aspecto de creciente interés ha sido la realización de fermentaciones a muy bajas temperaturas (13°C o inferiores, [13]). Dicha práctica tiene una gran aplicación en vinificaciones en blanco o rosado al retenerse de esta forma gran parte de los aromas, tanto primarios como secundarios [6, 13 y 7], que al ser volátiles a temperatura ambiente, ven mermada su concentración durante la fermentación alcohólica. Actualmente se llevan a cabo vinificaciones a dichas temperaturas en diversas bodegas, especialmente en los países denominados nuevos productores (Chile, Australia, Sudáfrica, Canadá y California).

No obstante, a pesar de que las fermentaciones a bajas temperaturas influyen positivamente sobre la calidad de determinados vinos, éstas son problemáticas y pueden llegar a comprometer la viabilidad de la propia fermentación [11]. La tasa de crecimiento de las levaduras y la fermentación alcohólica aumentan en paralelo con la temperatura, con tasas máximas entre 25-28°C. Las fermentaciones por debajo de 18°C se caracterizan porque se retrasa varios días el inicio (fases de latencia largas) y el desarrollo de las mismas es muy lento [12 y 10].

2. Efecto de la cepa de levadura

En la figura 1 se puede observar la influencia de la temperatura de fermentación sobre el desarrollo de las cinéticas fermentativas de tres cepas de levadura distintas. Cabe destacar que las fermentaciones se desarrollaron en el laboratorio sobre

© Tecnología del Vino
www.alcion.es

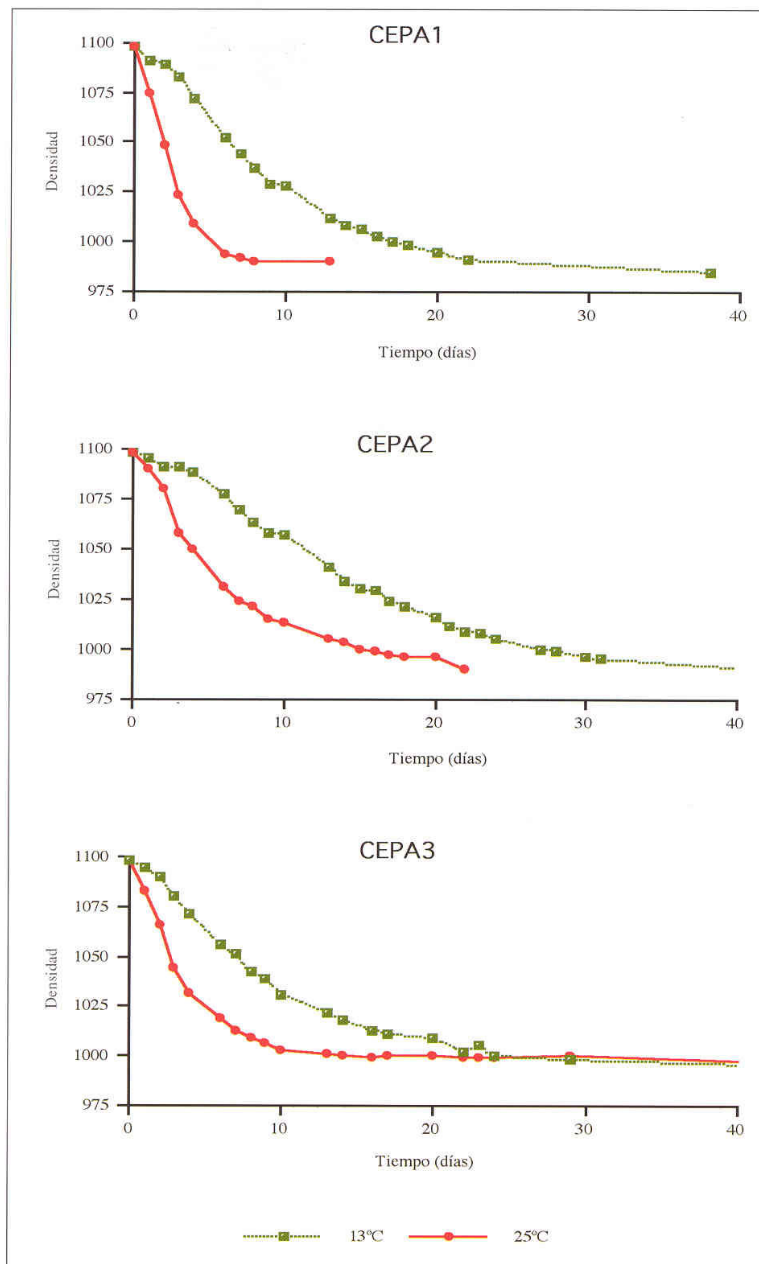


Figura 1. Cinéticas de fermentación a escala de laboratorio con distintas levaduras a 13° y 25°C.

mostos nutricionalmente pobres y con grado alcohólico probable elevado (contenido de azúcares de 220 g/l, mediante diluciones de Mosto Concentrado). De las tres cepas de levadura ensayadas, la primera se utiliza en fermentaciones de todo tipo de mostos, siendo una de las más utilizadas también frecuentemente en el tiraje de vinos espumosos. La segunda cepa es una levadura recomendada exclusivamente para vinificaciones en tinto a temperaturas que oscilan entre los 20 y 30°C. La tercera cepa se considera criotolerante, aunque para mostos de baja densidad. A pesar de que las cepas dos y tres tuvieron fermentaciones muy largas, ésta última fue la única en la que se observó una parada en la fermentación a 13°C.

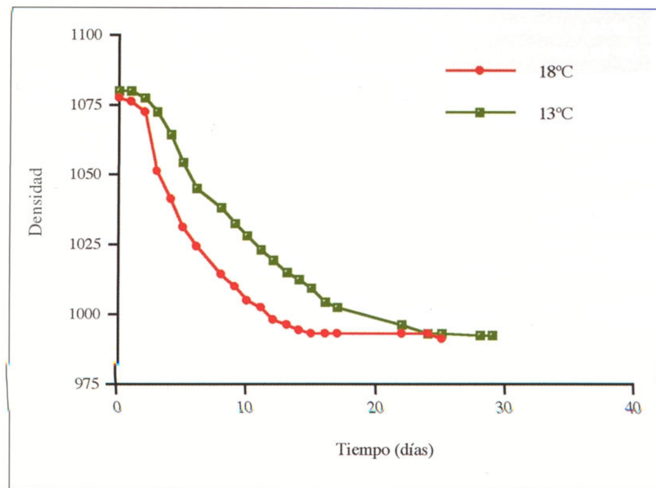
Independientemente de la cepa de levadura, en todas las fermentaciones se produjo un retraso considerable en el inicio, así como una cinética de fermentación más lenta, lo que condujo a un tiempo final de fermentación muy largo. Esto supone un problema tecnológico (fermentadores ocupados durante un largo período) y un problema microbiológico porque en ocasiones estas fermentaciones pueden llegar a pararse sin haber consumido todos los azúcares. Como consecuencia de ello, otros microorganismos del vino (bacterias lácticas y acéticas) pueden desarrollarse aprovechando estos niveles elevados de azúcares residuales y mermar las características organolépticas del producto final. No obstante, es poco probable su desarrollo durante la fermentación ya que las bacterias son sensibles a las bajas temperaturas. Tanto las bacterias lácticas como acéticas ven disminuída su multiplicación y actividad celular. Las bacterias lácticas presentan un rango óptimo de 25 a 30°C, y el crecimiento se ralentiza al disminuir la temperatura y resulta casi imposible entre 14 y 15°C. En el caso de las bacterias acéticas, a más temperatura, mayor actividad: la formación de ácido acético es diez veces más rápida a 28°C que a 23°C. Hay que señalar que no hay un método eficaz para eliminar estas bacterias, solamente las temperaturas inferiores a 15°C pueden limitar el problema.

3. Efecto sobre la imposición de inóculos

A pesar de este aspecto positivo con respecto al desarrollo bacteriano, la situación no es comparable con respecto a las otras levaduras. Se ha demostrado que las bajas temperaturas de fermentación afectan al desarrollo de las diferentes especies de levaduras que nos encontramos

a lo largo del proceso. *Saccharomyces cerevisiae* es la principal especie fermentativa, siendo mayoritaria a los pocos días del inicio de la fermentación. Sin embargo, en fermentaciones a bajas temperaturas, Heard y Fleet [4] demostraron que *Kloeckera apiculata* dominaba la fermentación durante un período de tiempo superior cuando se llevaba a cabo a bajas temperaturas (por debajo de 20°C). Estos autores sugirieron que esta especie está más adaptada a desarrollarse a bajas temperaturas que *S. cerevisiae*, dificultándose la imposición de esta última en el proceso. La supervivencia excesiva de especies de no-*Saccharomyces* durante la fermentación incide en la producción de determinadas sustancias volátiles indeseables, como ácido acético o acetato de etilo [5 y 14].

Figura 2. Evolución de la densidad en fermentaciones industriales a 13° y 18°C.



En nuestro caso, dicha competencia se analizó en una fermentación en condiciones industriales (depósitos de 100 Hl), comparando la temperatura usual de fermentación en blanco (18°C) con una temperatura inferior (13°C). Se utilizó la cepa 1 de levadura que fue la que presentó mejor comportamiento en el laboratorio (Figura 2). A pesar de que al principio de la fermentación se encontraban levaduras no-*Saccharomyces* salvajes, la imposición de la levadura inoculada fue rápida y completa, analizado mediante técnicas moleculares de análisis del DNA mitocondrial (Fig. 3).

4. Efecto sobre el metabolismo y los productos finales

Es evidente que la temperatura de fermentación afecta a las actividades bioquímicas de las levaduras, y este afectará al producto final, el vino y sus componentes. El efecto más notable observado en la adaptación de levaduras a bajas temperaturas es el aumento del grado de insaturación de los ácidos grasos [12] y la reducción de la síntesis de esteroles. Ambos cambios condicionan notablemente la fluidez de las membranas celulares y reducen el paso de nutrientes [15], resultando dicha disminución de la permeabilidad de las membranas en una inhibición de la actividad fermentativa de *Saccharomyces* [8]. También se han observado modificaciones en la producción de metabolitos secundarios, de gran impacto en la composición organoléptica del vino [1, 9 y 14].

En este aspecto, cabe destacar las diferencias observadas en algunos de los metabolitos más relevantes analizados en las fermentaciones a 13 y 25°C, de gran relevancia en la calidad del vino final (Tabla I). El rendimiento en etanol a bajas temperaturas (incluyendo hasta 20°C) es significativamente mayor que a temperaturas superiores. No obstante, y aunque es previsible una cierta pérdida por evaporación, la diferencia fundamental se debe a un cambio significativo del metabolismo de las levaduras, ya que se pueden observar diferencias significativas tanto en el rendimiento en etanol como en otros productos secundarios. Así, la producción de glicerol y ácido acético aumentan progresivamente con la temperatura, y hay que recordar que en este caso, al tratarse de fermentaciones de laboratorio, sólo las levaduras estaban presentes. Aunque aumenten los metabolitos secundarios, no se puede considerar que esta producción pueda compensar la disminución en etanol, ya que al tener en cuenta todos los metabolitos con mayor presencia cuantitativa durante la fermentación del mosto (etanol, dióxido de carbono, glicerol, acetaldehido, ácidos acético y succínico) sigue habiendo una disminución significativa. Esta disminución se puede deber a diversos motivos aunque probablemente lo más razonable implique una diferencia en la producción de biomasa. Así, a bajas temperaturas se alcanza progresivamente la población máxima, y se mantiene durante casi toda la fermentación. En cambio, a temperaturas más elevadas la población máxima se alcanza muy rápidamente, produciéndose una mayor mortandad celular durante la fermentación. Esta diferencia, que se refleja en el corto período de latencia a temperaturas superiores a

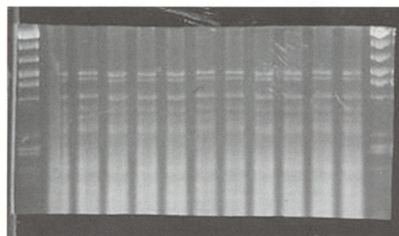


Figura 3. Perfiles electroforéticos del DNA mitocondrial digerido con enzimas de restricción. Carreras 1 y 13: Marcadores moleculares. Carreras del 2 al 12: Muestra de colonias aisladas en la fermentación industrial. Todos los perfiles son idénticos lo que implica una imposición total de la cepa inoculada.

Tabla I. Análisis de los productos de fermentación a 13°C y 25°C

	13°C	25°C
Etanol (%, v/v)	11,70	11,25
Glicerol (g/l)	6,05	6,91
Acido acético (g/l)	0,08	0,14
Suma de productos de fermentación (g/l)	190,05	183,94

20°C, también puede producir un mayor consumo de nutrientes para el crecimiento de las levaduras, lo que repercutirá en un menor rendimiento alcohólico.

La aplicación de las fermentaciones a bajas temperaturas se ve limitada, pues, por los condicionantes anteriormente expresados, a pesar de las ventajas que se obtienen en algunas variedades con alto potencial aromático. Una posible solución biotecnológica será la selección de levaduras (*S. cerevisiae*) con capacidad para desarrollarse a bajas temperaturas. Los sistemas de selección clonal de levaduras se han centrado en características tales como elevada capacidad fermentativa, baja producción de acidez volátil, resistencia al alcohol, etc. [2]. La capacidad para la fermentación a bajas temperaturas aún no ha sido un criterio de selección, y sólo un número limitado de criolevaduras se encuentran disponibles en el mercado. La mayoría han sido seleccionadas en países más septentrionales que España, y no siempre funcionan en nuestros mostos que se caracterizan por un mayor contenido en azúcares (sirva como ejemplo la cepa de levadura tres del apartado dos). Es evidente que es necesario un trabajo de selección para posibilitar dichas fermentaciones con nuestros mostos y aumentar el control de las fermentaciones a bajas temperaturas.

5. Bibliografía

[1] Castellari, L., Ferruzzi, M., Magrini, A., Giudici, P., Passarelli, P, y Zambonelli, C. "Unbalanced wine fermentation by cryotolerant vs non-cryotolerant Saccharomyces strains". Vitis, 33, 49-52 (1994).

[2] Degre, R. "Selection and commercial cultivation of wine yeast inoculation techniques by use of a "marked" yeast strain". Austalian New Zealand Wine Ind. J., 3, 53-56 (1993).

[3] Feuillat, M., Charpentier, C. y Massoutier, C. "Interes enológico de las cepas de levaduras Saccharomyces criotolerantes". Nueva Tecnología del tratamiento del mosto. Ed. Fidia, pág.: 7-15 (1997).

[4] Heard, G. M. y Fleet, G. H. "The effects of temperature and pH on the growth of the yeast species during the fermentation grape juice". J. Appl. Bacteriol., 65, 23-28 (1988).

[5] Herraiz, T., Reglero, G., Herraiz, M., Martin-Alvarez, P. J. y Cabezudo, M. D. "The influence of yeast and type of culture on the volatile composition of wines fermented without sulfur dioxide". Am. J. Enol. Vitic., 41, 313-318 (1990).

[6] Killian, R. E. y Ough, C. S. "Fermentation esters - formation and retention as affected by fermentation temperature". Am. J. Enol. Vitic., 30, 301-305 (1979).

[7] Kunkee, R. E. "Selection and modification of yeasts and lactic acid bacteria for wine fermentation". Food Microbiol., 1, 315-322 (1984).

[8] Larue, F., Lafon-Lafourcade, S. y Ribereau-Gayon, P. "Relationship between the sterol content of yeast cells and their fermentation activity in grape must". Appl. Environm. Microbiol., 29, 808-811 (1980).

[9] Lema, C., Garcia-Jares, C., Orriols, I. y Angulo, L. "Contribution of Saccharomyces and Non-Saccharomyces populations to the production of some components of Albariño wine aroma". Am. J. Enol. Vitic., 47, 206-216 (1996).

[10] Llaurado, J., Constantí, M., Rozés, N. y Mas, A. "Fermentaciones a bajas temperaturas (13ºC)". ALIMENTACIÓN, EQUIPOS Y TECNOLOGÍA, 2, 87-94 (2000).

[11] Meurgues, O. "La valorisation des arômes du Chardonnay en Bourgogne par les techniques de vinification". " Les arômes du vin, Caractérisation et genèse". E. Lallemand, Toulouse, pág.: 43-45 (1996).

[12] Rozès, N., Larue, F. y Ribéreau-Gayon, P. "Effect of a variation of grape must temperature on the fermentative ability and the neutral lipid content". Biotechnol. Lett., 10, 821-824 (1988).

[13] Soufleros, E. y Bertrand, A. "Incidence de l'action conjuguée de la temperature de fermentation et de l'acidité du milieu sur les teneurs en substances volatiles formées par les levures". Conn. Vigne Vin, 14, 97-109 (1980).

[14] Suarez Lepe, J. A. "Levaduras vínicas". Mundi-Prensa (1997).

[15] Zamora, F., Fort, F., Fuguet, J., Bordons, A. y Arola, L. "Influence de certain facteurs lors de la fermentation alcoolique sur le transport de glucose dans les levures". "Œnologie-95" Ed. Lavoisier, pág.: 167-171 (1995).

Agradecimientos

El presente trabajo forma parte del proyecto 2FD97-1854 financiado por fondos FEDER y el Ministerio de Ciencia y Tecnología.