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Maite Novo Molinero
ISBN: 978-84-690-7597-5 / DL: T.1409-2007

Universitat Rovira i Virgili

Departament de Bioquímica i Biotecnologia

Facultat d'Enologia

Biochemistry and physiology of rehydration and adaptation of active dry wine yeast for winemaking

Memoria presentada per

MAITE NOVO MOLINERO

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

sota la direcció del Dr. Albert Mas i el Dr. Nicolas Rozès

Tarragona, 2006

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Els sotasignants, Dr. Albert Mas Barón, Catedràtic 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 **"Biochemistry and physiology of rehydration and adaptation of active dry wine yeast for winemaking"** que presenta la Srta. **Maite Novo Molinero** 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. Albert Mas Barón

Dr. Nicolas Rozès

Tarragona, 5 de juny de 2006

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A mis padres y a Ana

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Agradecimientos

Esta etapa termina. Es momento de mirar atrás antes de poner el punto final, momento de agradeceros todo vuestro apoyo y toda vuestra ayuda. Sin vosotros, simplemente, esta tesis no hubiera existido.

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I entre micropipetes i bates blanques vaig trobar un munt de gent que mereix molt més que un simple «gràcies». Doctorands desconeguts que han esdevingut amics imprescindibles. Tots vosaltres ja sabeu qui sou. Miriam (mami) i Ramon (tan filosòfiques les nostres converses!), Roser, Carmen i Mireia, Ton (tot i que vas estar poc temps, t'has quedat per sempre), Josep Valls (què dir-te! mil gràcies per tots els moments compartits i…pels teus CD's sempre tan encertats!), Ángel (de trato 'encantador', soy yo la encantada de haberte conocido), Núria (la tortosina sempre disposada a tot, tant al labo com a les pistes de ski!), Rosana (Rose, que no te voy a

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comprar una mascota! jeje Mil gracias por tu paciencia en todo este tiempo compartiendo piso y vida) i Zoel (Gràcies pel teu recolçament de 10, bacione). Las nuevas incorporaciones al grupo han resultado ser ideales, Estitxu (mil muxus, preciosa! Gracias por tu entusiasmo y cariño), Braulio (gracias por tus buenos consejos y tus risas). Y a todos los que estáis empezando la tesis, muchos ánimos y mucha suerte.

Durante los momentos más difíciles de la tesis encontré gente nueva que me abrieron las puertas de "su tren de largo recorrido": Sandra, Juan y Santi, unidos ya para siempre. Merci beaucoup à tous les amis toulousiens, merci Vincent (pour nos marathons au bord du canal de Midi), Pierre-Alain, Babeth, Ingrid et Tony (pour m'avoir ouvert toutes les portes de votre amitié). Jean-Roch, merci énormement pour avoir dévenu ma boussole au milieu de la tempête, merci pour ton soutien, même à la distance.

Gracias a mis Apechusques (Javi, Almu, Emma y Carme), que no han dejado de alentarme y apoyarme en todo este tiempo. I com no, a Sumpta (pels bons moments…i pels que vindran!), Xavi (per la portada i per estar sempre disposat a compartir cada instant), Jordi (saben aquell que diu...4-10 de la Reina). I a elles… les meves "Àngeles" (Chus y Gemma, Gemma i Chus)… Chicas, esto no lo hubiera conseguido sin vosotras. Nunca. Habéis sido mi punto de apoyo, mi fuerza, lo habéis sido todo. Amb el vostre entusiasme, la vostra comprensió i el vostre amor he pogut superar dificultats que semblaven impossibles. Todo esto, va por ustedes!

Y ahora: mi familia. A todos, desde los abuelos y tíos que ya se fueron a los que acaban de llegar (Paula y Gerard), os quiero dedicar el fruto de mi esfuerzo. Gracias por vuestro apoyo incondicional y amor infinito. Lo sois todo. Papá, mamá, Ana os quiero.

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OBJECTIVES &

OUTLINE OF THESIS

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OBJECTIVES

The present PhD thesis has been carried out within the Oenological Biotechnology research group (Department of Biochemistry and Biotechnology, Faculty of Oenology) at the University Rovira i Virgili (Tarragona, Spain). This research group deals with all the microorganisms that could be involved in winemaking, that is yeasts, acetic acid and lactic acid bacteria. Due to my background in Oenology, I was ascribed to work in yeasts and alcoholic fermentation. The research is focused on the oenological industry, and specifically on yeast metabolism during winemaking fermentations (previous PhD Thesis of this group are those of J.M. Llauradó, M.J. Torija and G. Beltran). Since I had joined the group, the main target of study has been the molecular characterization of wine yeast metabolism at low temperature fermentations, financially supported by different projects (FEDER 2FD97-1875, AGL2000-0205-P4-0, France-Spain Integrated Project-Acciones Integradas HF2001-0015) and in tightly collaboration with oenological industry: wine cellars (Caves Gramona S.A, Torres S.A and Señorío de Sarria S.A., all of Spain) and yeast producers (Lallemand S.A., Toulouse, France).

The wine industry, in its attempt to face the continuous challenge of a demanding global market, has managed to control fermentation temperature effectively. Wines produced at low temperatures (10–15ºC) develop certain characteristics of taste and aroma that improve the quality and reproducibility of white and *rosé* wines. These enhanced sensorial properties are due to a major retention of volatile substances and a major metabolic yeast production of aromatic compounds at low temperatures. However, optimal growth temperature for *Saccharomyces cerevisiae* is 25ºC and low temperatures are restrictive and increase the risks of stuck or sluggish fermentations. Low temperatures increase the length of alcoholic fermentation, decrease the rate of yeast growth and modify the ecology of wine fermentation. Thus, although low temperature has interesting applications in the oenological industry, it also has an adverse effect on cell metabolism, increasing the yeast stress during wine production.

During winemaking, in addition to low temperatures, yeast cells are subjected to several environmental factors which also have an adverse effect on growth (high sugar content, high ethanol concentration, nitrogen starvation, oxygen limitation, pH, SO_2 treatments).

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Thus, stress response is essential for survival, adaptation and growth in both, natural environments and industrial applications. Trehalose and Glycogen are the main storage carbohydrates in yeast cells and they are key factors for the viability, vitality and physiological activity of yeasts. Trehalose protects cells by preserving the integrity of biological membranes and stabilising proteins in their native state. Glycogen provides a readily usable carbon and energy source while the yeasts adapt to a new growth medium. One of the main characteristics for optimal performance of alcoholic fermentation is the set of physiological and metabolic changes occurring immediately after must inoculation. Is in this early phase when the yeast must rapidly change its metabolism to take maximum advantage into the new environment. Within the last 20 years, wine technology also allows the inoculation of selected wine yeast strains to ensure and control better the alcoholic fermentation. These selected yeasts are used in the form of "Active Wine Dry Yeast" that needs to be rehydratated before being inoculated into the musts in the fermentation tanks. Thus, yeast rehydratation is the first step to ensuring healthy cells and a good fermentation.

Thus, it is rather evident that a good control of the yeast stress response will improve the must fermentation and that there is a relative lack of knowledge of some of the processes involved. The working hypothesis is that the early metabolic responses of the yeast allow their success in performing the alcoholic fermentation. Furthermore, the knowledge of those responses at the biochemical and physiologic level will allow to develop sensible parameters to foresee the fermentation development and act to control it. This hypothesis will be tested through the following objectives:

✓ The analysis of Trehalose and Glycogen metabolism in a commercial wine yeast strain (QA23, Lallemand S.A.) in response to stress: We studied the metabolism under winemaking conditions, considering as variables the fermentation temperatures (low, 13ºC, and control, 25ºC), nitrogen content (low and high) and the rehydratation and preadaptation of active wine dry yeast.

✓ The determination of the very early transcriptional responses after inoculation of the grape must: It was analysed considering the presence of glucose and related fermentable carbon sources, the osmotic shock and the yeast metabolic activation, including the presence of the drug cycloheximide.

✓ The characterisation, at different levels (transcriptional, morphological, etc), of the rehydratation process of active wine dry yeast.

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OUTLINE OF THE THESIS

Changes in wine yeast storage carbohydrate levels during preadaptation, rehydration and low temperature fermentations

In order to characterise the low temperature fermentations, we focused in the yeast response to stress. Changes in trehalose and/or glycogen concentrations may be an early indication of cell metabolic activity. The metabolism of glycogen and trehalose was analysed in a wine yeast strain fermenting at 25 and 13 ºC, in both, laboratory and industrial winemaking conditions. Fermentation kinetics, yeast growth and total Yeast Assimilable Nitrogen (YAN) and Ammonia uptake were also determined.

Additionally, various preadaptation media were tested so that their influence on trehalose and glycogen degradation was determined. We tested the presence of fermentable carbon sources, such as glucose or fructose, and the effect of increasing of the osmotic pressure by a non-available carbon source such as sorbitol.

Results are reported and discussed in Chapter 1.

Effect of nitrogen limitation and surplus upon trehalose metabolism in wine yeast

Nitrogen is known to be one of the limiting factors in alcoholic fermentation. For a better understanding of trehalose metabolism and its genetic control in our commercial wine yeast strain we analysed the changes during alcoholic fermentation at 25ºC with different nitrogen availability. Additionally, we considered also important to know the possible links between Trehalose and Nitrogen metabolisms in order to determine the importance of nitrogen in regulating trehalose metabolism.

So, trehalose patterns of degradation and accumulation and gene expression of *TPS1*, *NTH1* and *NTH2* were determined all along alcoholic fermentations of three musts with different nitrogen content: Control (300 mg/l YAN), High (1200 mg/l YAN) and Low (60 mg/l YAN). As in the previous study, Fermentation kinetics, yeast growth and total Yeast Assimilable Nitrogen (YAN) and Ammonia uptake were also determined. This work was done in parallel with the analysis of nitrogen metabolism and uptake, which were a part of another PhD Thesis of the group (Gemma Beltran, 2005)

Results are reported and discussed in Chapter 2.

Early transcriptional response of wine yeast after rehydration: osmotic shock and metabolic activation

To know the main features of yeast metabolism as early response to the presence of a fermentable medium, we decided to use the powerful DNA microarray technique (http://biopuce.insa-toulouse.fr). We drew the global transcriptomic landscape of yeast after rehydratation and permanence for one hour in the followed media: Water, Sorbitol (200 g/l), Glucose/Fructose (200g/l, 50:50) and Synthetic Must (SM). We also tested the presence of the drug cycloheximide in water and SM. Transcriptomic results were verified by Real-Time quantitative RT-PCR, determining the expression of some critical genes. Additionally, vitality and viability of yeast cells after permanence in these media were also determined.

Results are reported and discussed in Chapter 3.

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INTRODUCTION

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1. YEAST AND WINEMAKING

The history of winemaking parallels that of civilization: historians believe that wine was being made in the Caucasus and Mesopotamia as early as 6000 BC. It was in the 19th century that several scientists, among them Louis Pasteur, proved that yeasts from the surface of grape berries are responsible for spontaneous fermentation. *Saccharomyces cerevisiae* is invariably the species isolated when fermentation is over and it has become known as "the wine yeast" (Pretorius 2000, Ribéreau-Gayon 2004).

1.1. Yeast ecology in the winemaking process

The microflora of grapes depends on numerous factors: grape variety, climatic influences, soil and viticultural practices, physical damage to the grapes, harvesting equipment, etc. (Pretorius *et al.,* 1999). Damage to grapes increases the yeast population (above 10^6 cfu/ml), particularly that of the *Hanseniaspora/Kloeckera*, *Metschnikowia* and *Candida* species, and also *Saccharomyces* and *Zygosaccharomyces* (Fleet *et al.,* 2002). Yeast species whose fermentative activity is lower than that of *S. 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 (Querol *et al.,* 1992, Torija *et al.,* 2001). However, *Hanseniaspora* and *Candida* species, when the fermentation is performed at temperatures lower than 15-20ºC, decrease their sensitivity to ethanol, making a significant contribution to wine flavour (Erten 2002). The *Saccharomyces sensu stricto* group of wine yeasts, which are more ethanol-tolerant, become predominant (10^7-10^8 cfu/ml) and complete the fermentation. The *Saccharomyces sensu stricto* group comprises *S. cerevisiae, S.paradoxus, S. bayanus* and *S. pastorianus*, which are associated with wine fermentation, and three newly defined species, *S. cariocanus, S. mikatae* and *S. kudriavzevii* (Naumov *et al.,* 2000). As well as the well-known prevalence in winemaking of *S. cerevisiae, S. bayanus* plays a special role in fermentations at low temperatures (Tamai *et al.,* 1998). In an indigenous population in Croatian vineyards, *S. paradoxus* with interesting oenological characteristics have been found in much higher numbers than *S. cerevisiae* (Redzepovic *et al,.* 2002).

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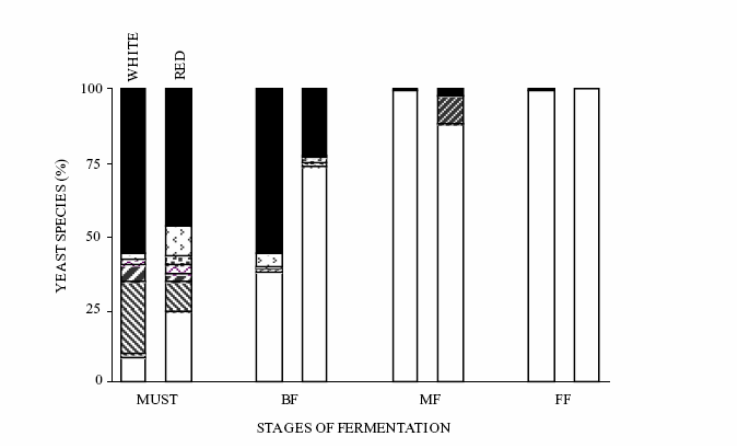

Fig.1. Biodiversity of yeast species during alcoholic fermentation analysed as a function of the type of vinification. BF: Beginning of fermentation, MF: Mid fermentation, FF: End of fermentation. (Torija *et al.,* 2001)

□ *Saccharomyces,* ■ *Candida stellata,* ▨ *Hanseniaspora uvarum,* ⁘ *Candida colliculosa,* ◪ *Metschnikowia pulcherrima,* ▩ *Schizosaccharomyces* spp., ⁙ *Zygosaccharomyces bailii,* ⊠ *Kluyveromyces thermotolerans,* ? non identified).

The initial population of yeast in freshly extracted grape juice is 10^3-10^6 cfu/ml. *Hanseniaspora (Kloeckera)* spp. are often the predominant species on the surface of grape berries, accounting for 50-75% of the total yeast population. Less prevalent are the species of *Candida, Pichia, Cryptococcus, Bettanomyces, Rhodotorula, Metschnikowia, Kluyveromyces* and *Hansenula* (Romano *et al.,* 2006). Non-*Saccharomyces* species are more prevalent at the beginning of white fermentations than red ones (Fig.1). However, in mid fermentation they have only been detected in red fermentations (Torija *et al.,* 2001). The predominant species and the diversity of species changed with the degree of ripeness. The ripeness of the grape increased the predominance of *Candida stellata* in the must. Other apiculate yeast such as *H. osmophila* overcame *H. uvarum* and *C. stellata* in some fermentation conditions. There was a direct relationship between high sugar content in the must (higher ripeness) and the presence of *H. osmophila* (Hierro *et al.,* 2006). The most interesting oenological characteristic of *C. stellata* is that they are highly fructophilic (Mills *et al.,* 2002). Ciani and Ferraro (1998) demonstrated that mixed fermentations containing *C. stellata* and *S. cerevisiae* utilized sugars more completely and they postulated that the cause was the preferential utilization of fructose by *C. stellata*. However, it has been seen to increase volatile acidity when it is present throughout alcoholic fermentation (Llauradó *et al.,* 2002).

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Although it occurs little in natural habitats such as vineyards, *S. cerevisiae* is abundant in the grape juice and must-coated surfaces of winery equipment. Those strains which are the natural microbiota in a winery are called "residential" or "winery" flora (Fleet and Heard, 1993). But the yeast population also changes and the appearance of new strains from year to year suggests that there is a high diversity of resident microbiota (Torija *et al.,* 2001). An ecological 6-year follow-up study (Beltran *et al.,* 2002), carried out in a completely new experimental winery, shows the rapid imposition of *Saccaromyces* over non-*Saccharomyces* species in spontaneous fermentations over the years. In the first 3-4 vintages, non-*Saccharomyces* species lasted as long as 2 to 6 days in competition with *Saccaromyces* species. However after four vintages, when the cellar environment had already been "contaminated" with commercial *Saccharomyces cerervisiae* yeast strains, *Saccharomyces* dominated from the very early stages of fermentation. Variability is a good parameter for evaluating the number of *S. cerevisiae* strains actively involved in fermentation. Variability changes enormously in each ecological study. Values ranging from 8.6%, (Querol *et al.,* 1994) to 20.7% (Nadal *et al.,* 1996) and 22% (Torija *et al.,* 2001) have been reported. This diversity strongly depends on the killer factor: yeasts with killer or neutral phenotypes mean that variability is lower. Variability was highest in those studies in which no yeasts had the killer phenotype and only a few neutral strains were present. As a result, the fermentation was not carried out by a predominant strain but by a large number of strains fermenting sequencially (Torija *et al.,* 2001).

The use of active dry wine yeasts (ADWY) is an effective way of controlling the yeast population in the winemaking process. Inoculation of selected ADWY reduces the number of indigenous *Saccharomyces* strains in favour of the starter. This reduction in diversity of natural strains is observed even when spontaneous fermentations take place in cellars which have previously been inoculated (Constantí *et al.,* 1998). This inoculation strategy using starters decreases the lag phase, minimizes the influence of wild yeast by imposition of the inoculated yeast strain, ensures rapid and complete grape must fermentation and, as a result, improves the reproducibility of the wine production (Bauer and Pretorius, 2000; Fleet and Heard, 1993).

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1.2. Requirements for Active Dry Wine Yeast (ADWY)

Traditionally, indigenous yeast populations have been used in winemaking. However, in the last 20 years, the use of active dry wine yeast (ADWY) in winemaking has increased considerably. The kinetics of spontaneous wine fermentation is fairly haphazard. Fermentation speed and degree of completion depends on the indigenous strain present. To avoid any possible risk, grape juice should be inoculated with ADWY at a concentration of 15-20 g/Hl or 10^6 cells/ml of juice, immediately after clarification (Ribéreau-Gayon *et al.,* 2004).

Empirical criteria have been used to select ADWY strains for their oenological aptitudes in different winemaking regions of the world. The natural yeast strains of a particular region are completely adapted to the prevailing climatic conditions (Martini, 1993). Wine yeast strains were first selected by isolating strains with successful spontaneous fermentations. Among the criteria proposed for selecting the strains are several aspects related to stress resistance (Degré 1993). Degré (1993) proposed that a good wine yeast strain should conduct a vigorous fermentation with a short latent phase and ensure must dryness (low to no residual fermentable sugar left). It should also have reproducible fermentation characteristics; behave predictably; be tolerant to ethanol, high pressure, SO_2 and temperature; and produce glycerol and β-glycosidase but no off-flavours. A simply method of selection based on the measurements of viability under oxidative and ethanol stress has been proposed (Zuzuarregui and del Olmo, 2004).

Additionally, nutrient deficiencies in the natural fermentation media, inhibitory substances or certain technological practices (i.e. fermentation at low temperatures) may lead to stuck or sluggish fermentations (Alexandre *et al.,* 1998, Bisson 1999). The manufacturing process of yeast itself requires some specific adaptations, in particular the ability to efficiently produce biomass in aerobic conditions and to survive long periods of storage, either dried or frozen (Bauer and Pretorius 2000). Thus, efficient industrial yeast must be able to adapt to all the conditions listed in table 1.

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Wine fermentation	Industrial production
Chemical parameters	
High sugar (>200 g/l in batch)	Low sugar (<1 g/l in fed-batch)
Limitation of essential nutrients	Supplementation of limiting nutrients
Low oxygen concentration	High oxygen rate
Presence of SO_2	No SO_2
High ethanol	No or very low ethanol
High levels of CO_2	Very low CO_2
Potential presence of toxic metals (copper)	Absence of toxic metals
Highly variable supply of nitrogen sources	Constant supply
Biological parameters	
Presence of competing microorganisms	Sterile environment
Potential presence of toxins	Absence of toxins
Xenobiotics	Absence of xenobiotics
Physical parameters	
High density (hyperosmotic pressure)	Low density environment
Temperature changing, mostly below optimal	Temperature adjusted for efficient growth
Low pH (3-3,4)	Optimal pH (around 5)
	Dessication, freezing-thawing
Growth conditions permanently changing with Avancing fermentation (nutrients, ethanol, CO_2)	Growth conditions maintained constant
Yeast metabolic activity	
Efficient fermentative metabolism	Efficient respiratory metabolism
Efficient growth in suboptimal conditions	Efficient growth in optimal conditions
Wanted end products	
High ethanol	No ethanol
Low biomass	High biomass

Table 1. Comparison of growth conditions during wine fermentation and industrial production process (Bauer and Pretotius, 2000)

Table 1 clearly shows that the conditions encountered during the two processes differ fundamentally: low sugar and high oxygen during yeast production *vs* high sugar and low oxygen during wine fermentation. However, there is a common denominator that can be used to describe most of the requirements that the yeast must fulfil in both conditions: stress resistance.

1.3. Industrial production of ADWY

Once a yeast strain has been selected from its natural habitat, the process of ADWY production starts by verifying the absence of contamination and the positive identification of the strain (karyotyping). Next, the cells are inoculated into a small volume of pre-culture, typically 250 ml, from which they are re-inoculated after 24-48 hours into a large batch fermentation tank. The production process itself finally takes place in large fermentation tanks using fed-batch conditions. These conditions are optimised for the production of biomass. The medium contains abundant nutrients but a low concentration of glucose, which is added continuously to the growth media, usually

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in the form of diluted molasses and key nutrients (nitrogen and phosphate sources, and some minerals and vitamins).

At the same time, oxygen concentrations are kept high, which results in a respiratory metabolism that produces a considerable amount of biomass and little or no ethanol (Bauer and Pretorius, 2000, Attfield 1997). High oxygen concentrations also induce a higher production of unsaturated fatty acids by yeast. The fatty acid composition of ADWY shows high levels of unsaturation (between 70% and 80%), while medium-chain fatty acids are not detected. In fact, only four fatty acids of this profile have been found: two saturated fatty acids, palmitic (C16:0) and stearic (C18:0), and two unsaturated fatty acids, palmitoleic (C16:1) and oleic (C18:1). Moreover, oleic acid was practically 50% of the total cell fatty acids and the unsaturated/saturated fatty acid ratio was higher than 2 (Torija *et al.,* 2003). Since wine yeasts are grown under fed-batch conditions in the presence of air, they will contain a reservoir of unsaturated fatty acids and sterols needed for limited number of generations that occur during the fermentation grape must (Degre 1993). Thus, the high content of unsaturated fatty acids in dry yeasts may be related to good fermentation (Rozès *et al.,* 1988).

When biomass has reached the desired level, the yeast is briefly deprived of oxygen and carbon, which induces some stress response mechanisms. This mainly increases the intracellular trehalose content and the accumulation of stress protection proteins. Manufacturers of ADWY cultivate their yeast in such a way that the maximum amount of storage carbohydrates (trehalose and glycogen) is accumulated in the yeast cells. For commercial baker's yeasts, the levels of trehalose are 15–20% of the dry weight and, for wine yeast, levels range from 10-20% of the dry weight for trehalose and 4-15% dry weight for glycogen (Pretorius 2000, Roustan and Sablayrolles, 2002). During the production run, the yeast is concentrated through centrifugation, then washed and dried. Both washing and drying create intense stresses, which require the protective presence of trehalose, stress-related proteins and specific membrane constituents (Van Dijck *et al.,* 1995, Sales *et al.,* 2000). After desiccation, the yeast is vacuum packed under nitrogen and sold as ADWY to winemakers. In this dried state, yeast cells will remain viable for considerable periods with no metabolic activity.

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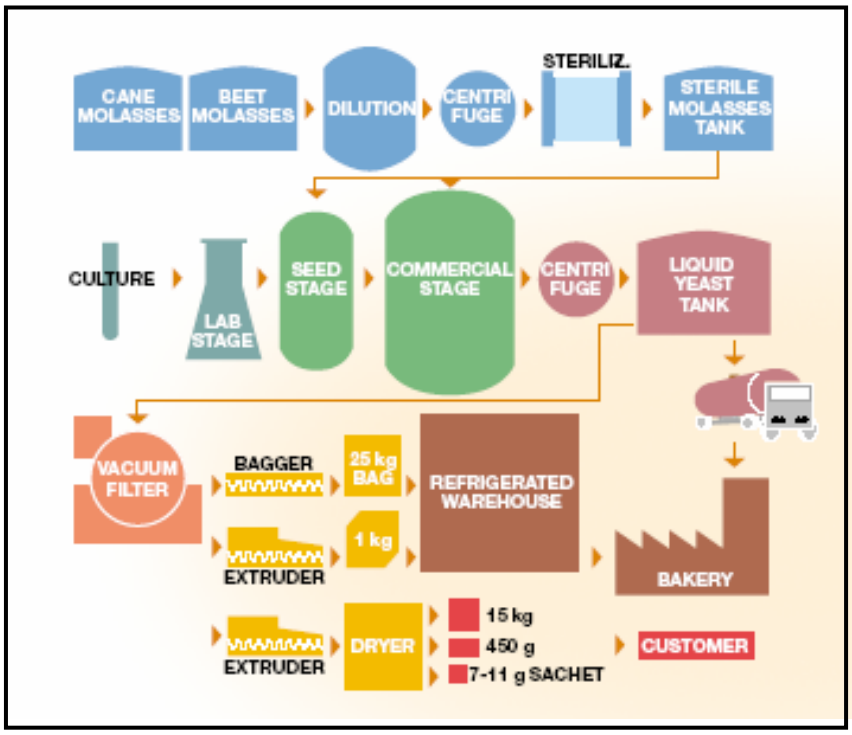

Fig. 2. Schematic flowchart of a commercial yeast plant (from Lallemand)

Pure strains are kept in the laboratory and then transferred to the plant process, where they are grown in a nitrogen-enriched molasses medium fortified with vitamins and minerals for optimum cell activity. After the appropriate number of generations, the mature cell mass is separated from the spent liquid and made into liquid cream, filtered and sold as compressed block yeast, or filtered and dried into instant dry yeast.

1.4. General Stress Response

1.4.1. Metabolic activation

Yeasts have evolved to become extremely responsive and adaptive to environmental changes. They need to be able to sense the availability of nutrients in their surroundings and to respond rapidly to changes in the nutritional status. This adaptation of cells is associated with rapid post-translationally induced changes in enzyme activity or gene expression (Thevelein and de Winde, 1999). For this purpose, many different mechanisms are used in *S. cerevisiae,* particularly to sense the availability of carbon and nitrogen (Gagiano *et al.,* 2002). These mechanisms are mitogen-activated protein kinase (MAPK) cascade, the nutrient-response signalling

cascades RAS/cyclic AMP (cAMP) pathway, the rapamycin-sensitive Tor pathway and the Fermentable-growth-medium-induced (FGMI) pathway.

Mitogen-activated protein kinase MAPK cascades are some the most intensively studied signal transduction pathways. *S. cerevisiae* has five MAPK pathways that mediate responses to hyperosmotic stress, heat, and hypotonic stress, α factor stimulation and nutrient starvation (Proft and Struhl, 2004). On being subject to stress, various kinases are sequentially activated by phosphorylation, culminating in the generation of an enzymatically active MAP kinase. Activated MAP kinases directly phosphorylate DNA binding transcriptional activators and repressors, which alters gene expression at the level of transcriptional initiation. MAP kinases also phosphorylate a wide variety of proteins that are not transcriptional regulators (Chen *et al.,* 2001).

The RAS/cyclic AMP (cAMP) pathway plays a major role in controlling growth and metabolism response to nutrients. The core of the pathway consists of the guanine nucleotide exchange factor *CDC25*, which responds to nutrients by activating small GTPases Ras1 and Ras2. Ras, in turn, activates adenylate cyclase *CDC35*, which produces cAMP and activates protein kinase A (PKA) (Crespo and Hall, 2002). This leads to transcription and posttranslational events that support growth, but also to the repression of stress-induced genes. The activation of PKA causes transient changes in several systems including trehalose and the glycogen metabolism, glycolysis and gluconeogenesis (Thevelein and de Winde, 1999). Examples of fast transient responses are trehalose degradation, which is the result of posttranslational activation of trehalase in response to nutrient up-shift (François and Parrou, 2001), and the activation of glycogen synthase (Gsy2p) by dephospholylation, under nutrient limitation and mediated by Snf1 kinase (Sanz 2003).

The rapamycin-sensitive target of rapamycin (TOR) kinase is a key component in a conserved signalling network that links cell growth to nutrient availability. It is especially important for nitrogen availability but it also seems to play a role in carbon metabolism (Schemlzle and Hall, 2000). In favourable growth conditions TOR is active, and results in initiation and progression from G_1. The TOR pathway can be deactivated by nutrient limitation, which results in several physiological changes characteristic of starved cells: inhibition of translation initiation, inhibition of ribosome biogenesis,

specific changes in transcription, sorting and turnover of nutrient permeases, accumulation of storage carbohydrates and induction of autophagy (Crespo and Hall, 2002). Both TOR and PKA kinases interact functionally in a number of different ways and have positive and negative effects on gene expression including ribosomal proteins, carbon metabolism and genes required for entry into the stationary phase (Chen and Powers, 2006).

Adding a nitrogen source to yeast cells starved of nitrogen on a glucose-containing medium triggers the activation of PKA targets through a pathway that, for activation to be sustained, requires both a fermentable carbon source and a complete growth medium (fermentable-growth-medium-induced or FGM pathway) (Thevelein and Hohmann, 1995). Trehalase is activated; trehalose content, glycogen content and heat resistance drop rapidly; STRE-controlled genes are repressed and ribosomal protein genes are induced. The rapid effect of amino acids on these targets specifically requires the general amino acid permease Gap1p (Donato *et al.,* 2003).

1.4.2. Transcriptional response

Common protection and repair mechanisms can lead to tolerance of a broad range of environmental stresses which define the *general stress response* (Ruis and Schüller, 1995). This system regulates the coordinated induction of many stress genes through a common *cis* element in their promoter (known as the *st*ress *r*esponse *e*lement, STRE whose consensus sequence is 5'-CCCCT-3'), which is induced by various environmental or metabolic stresses (Estruch 2000). Genome-wide transcriptional profiling has shown that ~10% of the genome is induced or repressed in this response. These genes are defined as the environmental stress response, ESR (Gasch *et al.,* 2000) or common environmental response, CER (Causton *et al.,* 2001), and the two groups overlap considerably. Induced ESR/CER genes are involved in such cellular functions as transport and carbohydrate metabolism, cellular redox reactions, DNA damage repair and autophagy. Among the induced ESR/CER genes we found the classical heat shock genes which encode molecular chaperones to facilitate protein folding, genes related to protein degradation and genes involved in detoxification of reactive oxygen species. Repressed genes generally function in cell growth-related processes, including RNA

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metabolism, nucleotide biosynthesis, secretion and ribosomal performance (Gasch *et al.,* 2000, Causton *et al.,* 2001).

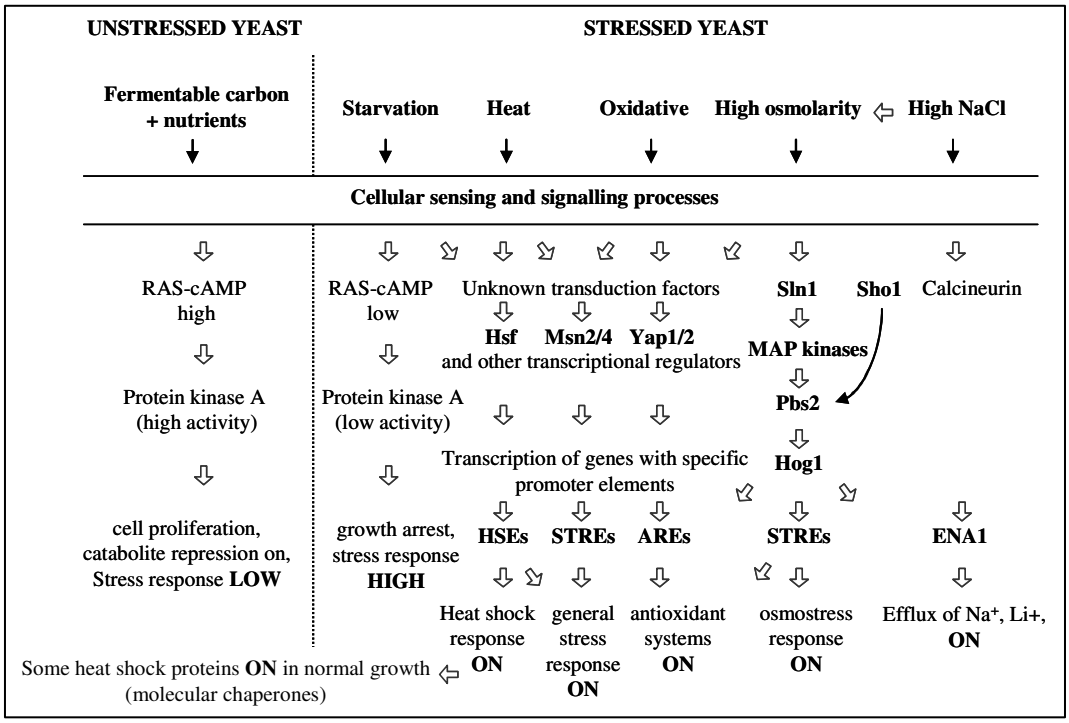

Fig. 3. A simplified summary of stress response processes in *Saccharomyces cerevisiae* (adapted from Attfield 1997)

When cells are growing exponentially by fermentation under optimum conditions the Ras-adenylate cyclase pathway is fully operative, which leads to cAMP-dependent activation of protein kinase A (PKA). The components of the cAMP-PKA pathway (Fig.4) have been studied extensively. Adenylate cyclase can be activated by Ras1p and Ras2p or by the G protein-coupled receptor system Gpr1p-Gpa2p. Activation of the Gpr1/Gpa2 pathway requires both extracellular glucose detection by the Gpr1-Gpa2 G-protein-coupled receptor system and intracellular glucose phosphorylation by one of the sugar kinases Hxk1p, Hxk2p or Glk1p (Rolland *et al.,* 2002). The cAMP produced activates the cAMP-dependent PKA by binding to the regulatory subunit (encoded by *BCY1*) and dissociating the catalytic subunits (encoded by *TPK1, TPK2* and *TPK3*) (Thevelein and Winde, 1999). Additionally, the function of the Ras proteins might be related to the sensing and transmission of stress signals through direct interaction with cytosolic Hsp70p. The accumulation of denatured proteins in stress

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conditions would reduce the activity of the cAMP-PKA pathway by recruiting Hsps proteins and reducing their interaction (Estruch 2000).

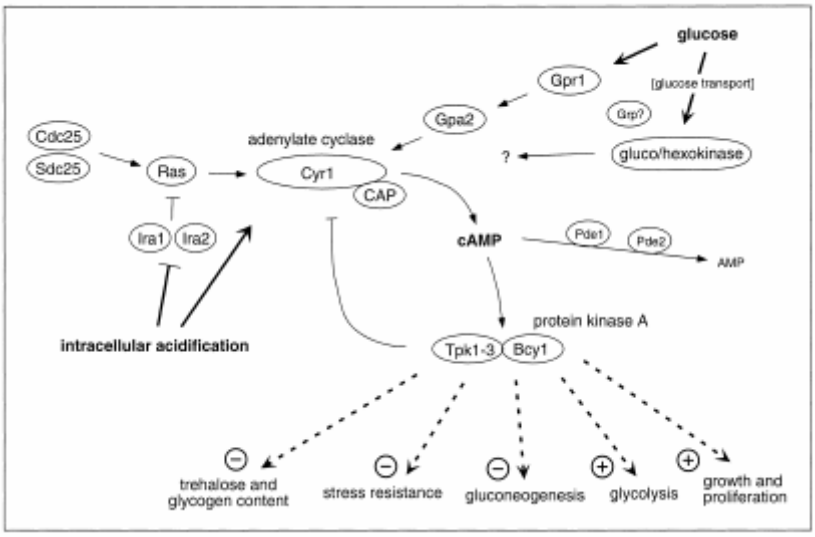

Fig. 4. Model that proposes the mechanisms by which glucose and intracellular acidification control adenylate cyclase activity. Glucose probably binds to the Gpr1p receptor, which activates cAMP synthesis through Gpa2p, while intracellular acidification acts through the Ras proteins. The presence of an active glucose kinase is essential, but the reaction product, glucose-6-phosphate, as well as further downstream metabolites do not seem to be involved as activators. The transient cAMP accumulation triggered by glucose activates PKA, which causes transient changes in several systems containing components controlled by PKA-mediated phosphorylation (Thevelein and Winde, 1999)

When yeast enters suboptimal growth conditions, the responses may be complex. In *S. cerevisiae* there are at least three positive transcriptional elements activated by stresses. These are heat-shock elements (HSEs), stress-response elements (STREs) and AP-1 responsive elements (AREs). There are specific factors involved in the activation/expression of HSE-, STRE- or ARE-controlled genes. These include heat-shock transcription factor (HSF), STRE-transcription factors (designated Msn2/4) and transcriptional activators (Yap1/2) that bind to AREs (Attfield 1997)**.**

Although well-known HSF target genes encode heat shock proteins (HSPs), which function as molecular chaperones in protein folding, stabilization, activation, trafficking and degradation (Parsell and Lindquist, 1993; Pirkkala *et al.,* 2001), newly identified HSF targets suggest that HSF has a broad, central role in orchestrating the multitude of cellular reprogramming events that occur in response to stress and normal cell growth and differentiation. Approximately 165 direct HSF target genes have been identified

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(Fig. 5) (Hahn *et al.,* 2004). It has been demonstrated that HSF, both directly and indirectly through Pdr3p, activates *RPN4* gene expression, and encodes a direct transcriptional activator of the genes that encode subunits of the yeast 26S proteasome (Hahn *et al.,* 2006). This type of regulation involves a transcription factor that regulates a second transcription factor which binds to a common target promoter with the first regulator and has been defined as a feed-forward loop (Lee *et al.,* 2002, Shen-Orr *et al.,* 2002).

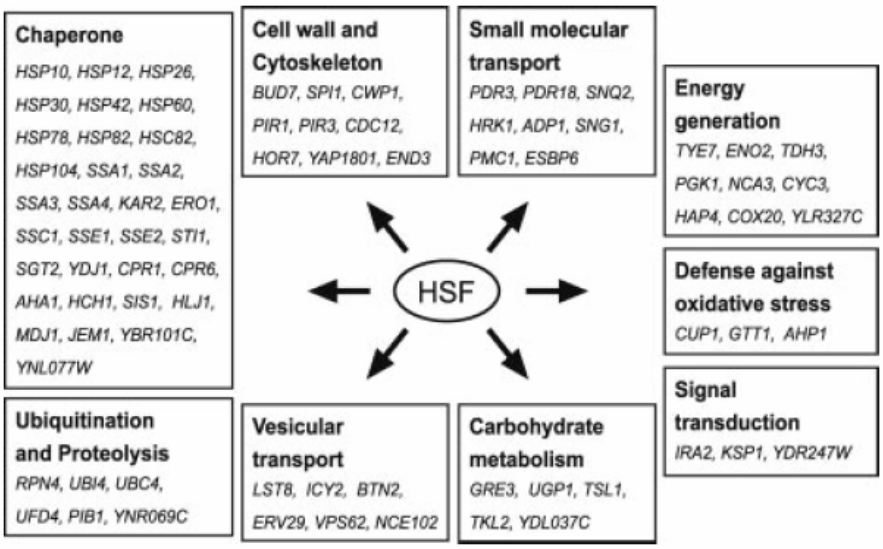

Fig. 5. HSF target genes in the yeast genome. Representative HSF targets are categorized according to their known or hypothetical functions based upon sequence homology (Hahn *et al.,* 2004)

At least two signalling pathways converge on the STRE: the HOG1 pathway, the activation of which positively regulates STRE-dependent gene expression, and the Ras-cAMP pathway, the activation of which negatively regulates STRE-dependent gene expression. Msn2p and Msn4p bind the STRE element to induce the transient expression of genes that make it possible to adapt to less favourable growth conditions. These genes are involved in carbohydrate metabolism, molecular chaperones, antioxidant proteins and protein degradation (Gasch *et al*. 2000, Causton *et al.,* 2001, Hasan *et al.,* 2002). Under normal growth conditions, Msn2p and Msn4p are localised in the cytoplasm. Upon stress they migrate to the nucleus. However, subcellular localization is not the only level of control in the response of Msn2p and Msn4p to stress (Estruch 2000). Several aspects have been studied; for example, the role of Msn2p and Msn4p hyperphosphorylation in controlling their stability (Bose *et al.,* 2005) or the recent localization of an activating domain in the N-terminal half of Msn2p (Boy-Marcotte *et al.,* 2006).

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Finally, Yap1p is a transcriptional factor that regulates many key antioxidant genes. Exposure of cells to H_2O_2 leads to the accumulation of Yap1p in the nucleus and altered transcription of ~70 genes, including *TTR1, CTT1, TRX2, SOD1, CCP1* and those involved in glutathione metabolism (Gasch 2000). However, oxidative stress is more a set of related stresses that require several constitutive cellular processes if they are to be resisted. In some cases, the promoters of the relevant genes in oxidative response have been found to respond to various combinations of transcription factors including Yap1p, Msn2p/Msn4p, Skn7p and factors which regulate mainly respiratory functions (Hap1p, Hap2p, Hap3p, Hap4 and Hap5p) (Temple *et al.,* 2005).

1.4.3. Yeast stress responses during winemaking

Stress response is a key issue in winemaking not only during alcoholic fermentation but also during ADWY production. During the yeast production stage, yeasts are exposed to various stressful situations (see table 2) that induce multiple intracellular changes. Osmotic and oxidative stresses are two of the main adverse conditions that *S. cerevisiae* senses during the process of biomass propagation to produce ADWY. Increased expression of the *GPD1* gene (specific response to osmotic stress) and *TRX2* gene (marker of specific response to oxidative stress) has been observed. Additionally, induction of *HSP12* throughout the different stages of yeast production means that yeast senses stress continuously (Pérez-Torrado *et al.,* 2005). Inoculation into molasses generates osmotic shock, shown by *GPD1* induction. Interestingly, the *TRX2* gene is induced during the first hours of batch stage meaning that an oxidative stress response is required when growing in high aeration conditions. These dynamic environmental injuries affect the biomass yield, the fermentative capacity, the vitality and the viability of the cells (Attfield 1997).

When ADWY are inoculated into the must they are subjected to considerable hyperosmotic stress because of the high sugar content of the must (~ 200 g/l of an equimolar mixture of glucose and fructose) and as fermentation proceeds they have to cope with progressive nutrient depletion and ethanol production (Pretorius 2000). Whereas initial osmotic stress is temporal and transient (Pérez-Torrado *et al.,* 2002), in the stationary phase the stress response is general and contains numerous stress signals (ethanol, osmotic, acid, nutrient depletion) (Rossignol *et al.,* 2003). To adapt to these

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changing conditions, yeast sequentially activates and represses large numbers of genes involved in critical metabolic pathways. Glycolysis, ergosterol uptake and thiamine biosynthesis are strongly expressed throughout the fermentation process (Rossignol *et al.,* 2003, Beltran *et al.,* 2006). In wine fermentations, yeast growth is never restricted by carbon limitation, but always by another nutrient, frequently nitrogen. The principal mechanisms involved in sluggish fermentations have been fully elucidated: nitrogen deficiency, thiamine depletion of the must, lack of oxygen, excessive clarification of juice, and inhibition of yeast cells by fermentation by-products (especially octanoic and decanoic acids) killer toxins and pesticides (Sablayrolles *et al.,* 1996). Depending on the winemaking process, other stress factors can be found: temperature increase, cold stress and high levels of CO_2 (particularly in sparkling wine fermentations) (Bauer and Pretorius, 2000).

The paradigm for a stress response involves: (1) a cellular sensor that recognizes the specific stress, (2) a signal transduction pathway that often involves one or more distinct enzymatic activities, (3) modification of specific transcriptional activator and repressor proteins by the end product of the signal transduction pathway and (4) direct alteration of gene expression, thereby resulting in the production of proteins that directly relieve the stress (Proft and Struhl, 2004).

1.4.3.1. Early adaptation: the lag phase

Inoculation of yeast cells into a complete fermentable growth medium delays growth: this known as the ***lag phase***. In this phase, the yeast must rapidly change its metabolism so as to take maximum advantage of the new enriched environment (Brejning and Jespersen, 2002). Cells grow more and increase their capacity for protein synthesis at a higher rate until they can begin a new cell cycle (Baroni *et al.,* 1994). The changes occurring during the lag phase are characterised by an overall change in protein synthesis and reflect the physiological condition of the yeast, which affects its fermentative capacity and fermentation performance (Quain 1988, Blomberg 1997).

The rate of protein synthesis increases considerably immediately after transfer to a new glucose-enriched minimal medium. However, only a reduced number of proteins are induced (*ADO1, ALD6, ASC1, GPP1, SAM1, SSB1* and *YKL056c*) (Brejning and

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Jespersen, 2002). At 1 and 3h after inoculation, the proteins encoded by *ADO1, ALD6, ASC1, CYS3, GDH1, LYS9, RPS0A, RPS0B, SAM1, SAM2, SSB1* and *YKL056c* have similar gene and protein induction factors. For the first 20 min after inoculation, however, strong gene inductions do not correlate with strong protein induction for *ADO1, ASC1, LYS9, RPS0B, SAM2* and *YKL056c* (Brejning *et al.,* 2003). This poor correlation between changes in gene and protein expression indicates that regulatory mechanisms are operative at the post-transcriptional level when yeasts are exposed to environmental changes (Brejning *et al.,* 2005). Much of the transcriptional response observed is due to the presence of glucose in the new medium. Genes involved in protein synthesis machinery, including RNA synthesis and processing, are highly induced during the lag phase, with expression being highest 20 min. after inoculation. Amino acid induction, nucleotide metabolism, and ammonia and inorganic phosphate uptake were also found (Brejning *et al.,* 2003).

Changes in gene expression and proteins after inoculation in a fermentable growth medium are independent of the exact conditions of growth and the yeast species studied. When yeast initiates or shifts its carbon metabolism towards glycolysis in the presence of oxygen, the synthesis of sterols and other membrane constituents is especially active (Higgins *et al.,* 2003; Brejning *et al.,* 2005). In winemaking, yeast must have certain features that adapt and complete alcoholic fermentations. Aspects related to carbohydrate and sulphur metabolism, nitrogen transport, stimulus response and sterol biosynthesis and transport may explain some of the differences in the fermentative behaviour of wine yeast (Zuzuaregui *et al.,* 2006).

1.4.3.2. Osmotic stress

Immediately after inoculation into grape must, yeast faces hyperosmotic conditions that trigger a fast and transient osmotic stress response, characterised by an increase in the mRNA levels of the *GPD1* gene involved in glycerol production (Pérez-Torrado *et al.,* 2002). Hypertonic conditions lead to an efflux of water cells, and lower turgor pressure and water availability. Yeast cells respond to such a hyperosmotic shock through a number of mechanisms, including the modification of the cell wall and the cytoskeleton (Slaninova *et al.,* 2000) and the accumulation inside the cell of a compatible compound, glycerol, to re-establish an osmotic equilibrium (Hohmann

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2002). The amount of glycerol usually formed by *Saccharomyces cerevisiae* in wine varies between 2 and 11 g/l although normal concentrations are in the range 4-9 g/l (Michnick *et al.,* 1997, Remize *et al.,* 2000).

In *Saccharomyces cerevisiae*, any increase in the environmental osmolarity is perceived by one of two osmosensors (see Fig. 3), Sln1p (the only known yeast histidine kinase sensor) or Sho1p (a transmembrane protein) (Hohmann 2002). Recently, a third osmosensor Msb2p, which is partially redundant with Sho1p, has also been characterized (O'Rourke *et al.,* 2002). Once the osmosensors are activated, they transmit the signal through different elements. This signal is integrated at the level of the MAPKK Pbs2p. Then, Pbs2p phosphorylates the Hog1p MAPK, which gives its name to the osmoregulatory MAPK cascade: the HOG (high osmolarity glycerol) pathway. This pathway is rapidly stimulated in response to changes in osmolarity, and leads to the phosphorylation of the MAP kinase Hog1p and its subsequent translocation to the nucleus. Once in the nucleus, Hog1p regulates the expression of numerous genes (~ 600 genes) by controlling the activity of such transcriptional activators and repressors as Sko1p, Hot1p, Msn2p and Msn4p (Posas *et al.,* 2000, Rep *et al.,* 2000, O'Rourke *et al.,* 2002).

Although the HOG pathway has classically been considered as specific to osmotic stress, recent studies have revealed that it has new functions, supporting the idea that the HOG pathway plays a more general role in stress response in yeast. The HOG pathway is essential for regulating the adaptation to citric acid stress (Lawrence *et al.,* 2004), it is also involved in methylglyoxal resistance (Aguilera *et al.,* 2005), in the distribution of proteins within the Golgi and in cell-wall maintenance (Reynolds *et al.,* 1998). In addition, HOG pathway mediates the transmission of the cold signal, regulates the expression of a sub-set of cold-induced genes and determines viability on freezing (Panadero *et al.,* 2006). An entirely different effect caused by osmotic stress is that it inhibits transport processes. It strongly diminishes the uptake of several amino acids, and expression analysis of cells fully adapted to and actively growing under osmotic/salt stress might reveal an amino acid starvation response (Norbeck and Blomberg, 1998, Pascual-Ahuir *et al.,* 2001).

2. TREHALOSE METABOLISM IN YEAST

Budding yeast accumulates two types of glucose stores: glycogen and trehalose. Glycogen is a high molecular mass branched polysaccharide of linear α(1,4)-glucosyl chains with α(1,6)-linkages. Trehalose, α-D-glucopyranosyl(1-1)-α-D-glucopyranoside is a non-reducing disaccharide composed of two glucose moieties with an α(1,1) linkage. Both, glycogen and trehalose are typical hall-marks of yeast cells rapidly adapting to changing environmental conditions. Since the major function of glycogen is to provide carbon and energy to maintain cellular activities when nutrients are scarce, trehalose has an interesting dual role as a storage carbohydrate and as a stress protector (Thevelein 1996, François and Parrou, 2001).

Trehalose was first discovered incidentally in 1832 by Wiggers from ergot, a fungal crop blight. A quarter of a century later, Berthelot found the same sugar in cocoons of the beetle *Larinus* that were used for medicinal purposes in what was then Persia and the Ottoman Empire. These cocoons were called "Trehala", inspiring Berthelot to name the sugar Trehalose. Trehalose has since been found in a great variety of species, most notably the anhydrobionts. These unusual organisms, which include "resurrection plants", certain brain shrimp, nematodes and *Saccharomyces cerevisiae* yeast, are able to withstand almost complete desiccation. They endure long periods in seemingly suspended animation, returning from dormancy to full activity once water again becomes available (Singer and Lindquist, 1998).

The success of trehalose in nature compared to other sugars can be explained by its peculiar structure. In addition to being non-reducing, it possesses several unique physical properties, which include high hydrophilicity and chemical stability, non-hygroscopic glass formation and the absence of internal hydrogen bond formation. These features account for the principal role of trehalose as a stress metabolite (Argüelles 2000). Two specific lines of research have received considerable attention. The first is the control of trehalose mobilization during the initiation of growth in resting cells and the secondly is the possible role of trehalose as a stress protector. Trehalose mobilization appears to be a part of the developmental program initiated in the presence of nutrients to induce growth (Thevelein 1996). In stress resistance, although such other players as molecular chaperones participate in synergy with

trehalose, it has two unique properties that make it a stress protector. The first is its capacity to protect membranes from dessication and the second is its ability to exclude water from the protein surface and hence to protect proteins from denaturation in hydrated cells (François and Parrou, 2001)

2.1 Trehalose physiology in yeast

Classical studies in yeast suggested that trehalose was a major reserve compound. However, this is a point of controversy and several reports have been published both supporting and refuting this role. Thevelein (1996) postulated that trehalose had a dual function as a storage carbohydrate and as a stress protection metabolite. If trehalose is a reserve carbohydrate, why do cells mobilise their large trehalose reserves when they are inoculated in a media full of glucose? It has been argued that a trehalose reserve is essential for providing energy and carbon to the preceding developmental stages in situations in which external nutrients are restricted (Thevelein 1996). However, the amount of glucose released from intracellular trehalose mobilization accounts only for a minimal percentage of the actual energy, compared to that supported by high concentrations of the external carbon source (Wiemken 1990). It has also been argued that the persistence of high levels of trehalose interferes with the reactivation of denatured substrates, explaining the need to degrade trehalose during recovery (Singer and Lindquist, 1998, Kandror *et al.,* 2004). Trehalose is predominantly synthesized at the onset of reduced growth periods and not when there is an excess of exogenous energy source, provided that availability of other essential nutrients is not a limiting factor (Argüelles 2000).

In *S. cerevisiae*, the trehalose content and the general level of stress resistance of the cells vary as a function of changes in the environmental conditions and undergo drastic changes during the life cycle. The level of trehalose is very low in yeast cells growing exponentially and it increases when cells enter the stationary phase or undergo stressing conditions (Thevelein 1984). The accumulation of trehalose appears to be associated with periods of reduced growth, such as when cells are starved of nitrogen, phosphate or sulphur, as well as during the stationary phase of growth on glucose (Lillie and Pringle, 1980). Trehalose accumulation is directly related to the duration of the G_1 phase and is induced at a G_1 phase of 5 h or more. Thus, in response to non-optimal

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environmental conditions in batch cultures, the growth rate is reduced and trehalose begins to accumulate (Paalman *et al.,* 2003).

Trehalose contributes to survival during environmental stress conditions, dehydration, and heat stress. In these conditions, dramatic changes in cell viability are accompanied by the aggregation of macromolecules and the denaturation of proteins. It seems likely that trehalose reduces protein aggregation and maintains polypeptide chains in a partially folded state, thus increasing tolerance to thermal and desiccation stresses (Thevelein 1984, Wiemken 1990, Singer and Lindquist, 1998). Yeast was found to be protected against severe heat shock and glucose starvation when the intracellular trehalose was as low as 1.5% (wt/wt) of the cell dry mass (Plourde-Owobi *et al.,* 2000).

Under adaptive treatments, trehalose is synthesized inside the cell and is transported through the plasma membrane, which protects both sides and stabilizes intracellular components (Eleutherio *et al.,* 1993, Herdeiro *et al.,* 2006).

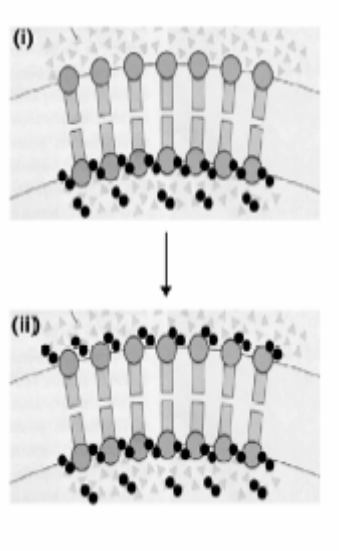

Fig. 6. Proposed model of the mechanism of trehalose protection during the adaptive treatments to mild heat: (i) during 40 °C adaptation, trehalose is synthesized and intracellular accumulation takes place, as well as transport (ii), protecting the outer side of the membrane during stress conditions (Herdeiro *et al,*. 2006)

Accumulation of trehalose increases the stress resistance in yeast growing on non-fermentable carbon sources (Van Dijck *et al.,* 1995), resistance to freezing (Soto *et al.,* 1999) and resistance of endocytosis to ethanol (Lucero *et al.,* 2000). It also protects yeast cells and cellular proteins from damage caused by H_2O_2 (Benaroudj *et al.,* 2001), reduces intracellular ROS concentration and decreases in vivo lipid oxidation during exposure to menadione (Herdeiro *et al.,* 2006). Trehalose is involved in the survival of yeast exposed to severe osmotic stress conditions in the absence of growth (Hounsa *et*

al., 1998). In near-freezing response, multiple genes for trehalose synthesis (*TPS1, TPS2*, *TPS3* and *TSL1* genes) are induced up to 7-fold after 24 h at 0ºC. (Kandror *et al.,* 2004). During yeast storage (35 days at 5ºC) the amount of trehalose is economised and consumed slowly. This is in sharp contrast to glycogen, which is almost depleted after the storage period (Jorgensen *et al.,* 2002).

Few studies have been made of trehalose accumulation in a winemaking context, in spite of the importance of this industrial process and the wide variety of stresses that characterise it. In microvinification, trehalose accumulation begins when glucose concentration is still very high. At the end of fermentation, after the glucose has been exhausted, trehalose content drops and it is undetectable after five days of glucose deprivation. This mobilization of trehalose is simultaneous to a loss of cell viability (Gimeno-Alcañiz *et al.,* 1999).

Of all the nutrients required by yeast during the fermentation of grape must, carbon sources (glucose and fructose) and nitrogen are quantitatively the most important. Trehalose metabolism in *S. cerevisiae* is differentially controlled by nitrogen and glucose limitations. Complete exhaustion of the nitrogen source under nitrogen limitation appeared to be the primary condition to trigger the accumulation of trehalose. Similar results could be expected when exponentially growing cells on glucose are starved of nitrogen. When glucose was limited, trehalose began to accumulate only at the onset of the diauxic shift, after the glucose had been depleted. In this condition, trehalose accumulates and glycogen content starts to decrease. Thus, cells use the glycogen pool while they are starved of carbon to terminate proliferation and accumulate trehalose (Parrou *et al.,* 1999, Jorgensen *et al.,* 2002).

Growth of *S. cerevisiae* on trehalose is possible and it is preceded by a long lag phase due to the "induction" of trehalose uptake (Basu *et al.,* 2006). The ability of yeast to take up exogenous trehalose is associated with the maltose fermenting trait of the cells (Plourde-Owobi *et al.,* 2000).There are at least two trehalose transporters in *S. cerevisiae*: the high-affinity H^+-trehalose symporter Agt1p (K_m = 2-4 mM) and a low-affinity transporter system (K_m > 50 mM) whose corresponding gene remains to be characterized. The high affinity transporter is repressed in glucose and highly expressed

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in maltose-containing media, whereas the low affinity transporter is insensitive to the carbon source (Stambuk *et al.,* 1996, Stambuk *et al.,* 1998).

Trehalose can be assimilated by two distinct pathways. The most important one is associated with acid trehalase, and two alternatives have been proposed. Nwaka *et al.* (1996) hyphothesized that trehalose reaches the vacuole by an endocytotic process in which it is degraded by the vacuolar acid trehalase. Jules *et al.* (2004), however, proposed a simpler explanation based on the finding that more than 90% of total acid trehalase in *S. cerevisiae* is extracellular and cleaves extracellular trehalose into glucose in the periplasmic space. A second pathway for trehalose assimilation couples trehalose transport by Agt1p with trehalose hydrolysis by Nth1p (Jiang *et al.,* 2000; Jules *et al.,* 2004; Parrou *et al.,* 2005).

2.2. Trehalose metabolism

2.2.1. Trehalose biosynthesis

In yeasts, trehalose is synthesized through a pathway that was elucidated by Cabib and Leloir 50 years ago. The biosynthesis of trehalose is catalysed by a two-step process involving trehalose-6-phosphate (Tre6P) synthase and Tre6P phosphatase on a multimeric protein complex (see fig. 7). The Tre6P synthase activity of the complex displays affinities for Glucose-6P (K_m ~ 5-20 mM) and UDP-Glc (K_m ~ 1.5 mM) which are 3-10 times higher than the bulk concentration in the cells. This enzyme is also strongly non-competitively inhibited by Pi (K_i = 2 mM) while fructose-6P acts as a potent activator. At 50ºC these effects are much reduced but the enzyme is more active. In contrast to other enzymatic systems involved in reserve carbohydrate metabolism, the trehalose synthase complex is not subject to reversible phosphorylation (review in François and Parrou, 2001). This complex consists of four different subunits encoded by *TPS1, TPS2*, which catalyze the reactions of trehalose biosynthesis, and *TSL1* and *TPS3*, which have no catalytic activity but which stabilize the trehalose synthase complex (Cabib and Leloir, 1958, Vuorio *et al.,* 1993, Bell *et al.,* 1998). Thus, the complete enzyme will consist of Tps1p, Tps2p, Tps3p and Tsl1p which will act as both synthase and phosphatase activities.

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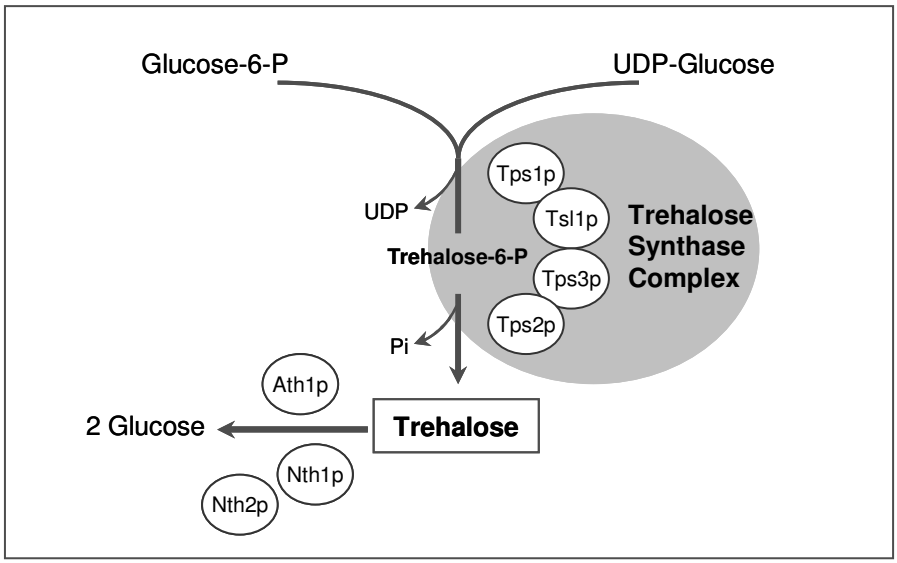

Fig. 7. Trehalose metabolic pathway in the yeast *Saccharomyces cerevisiae*

Trehalose biosynthesis is catalysed by the trehalose synthase complex consisting of four subunits. The trehalose-6-phosphate synthase subunit (Tps1p) produces trehalose-6-P from UDP-glucose and glucose-6-P, which is dephosphorylated in trehalose by the trehalose-6-P phosphatase subunit (Tps2p). Tps3p and Tsl1p are two regulatory subunits that stabilize the complex. Trehalose is degraded by the neutral (Nth1p) or the acid (Ath1p) trehalases. The role of Nth2p in this degradation process has not yet been clarified. (Adapted from François and Parrou, 2001).

Deleting the *TPS1*, the gene that encodes trehalose-6-phosphate synthase, means that cells cannot synthesize trehalose or grow on glucose but Δ*tps1* mutants can grow on galactose. Since the metabolic steps of catabolism are identical for both sugars after glucose-6-P, the cause of the lack of growth on glucose is likely to be either at the transport level, the hexokinase step or in some system involved in glucose sensing. So far, the inhibition of the major hexokinase PII isoenzyme activity by sub-millimolar concentrations of Tre6P is the only connection that has been found between trehalose biosynthesis and glycolysis (François and Parrou, 2001, Gancedo and Flores, 2004). A part of the Tps1p is also present in the cells as a free monomeric protein or, at least, it is not bound to the trehalose synthase complex. The dual location of Tps1p might reflect a dual function. The Tps1p in the trehalose synthase complex may be involved only in trehalose synthesis, whereas the free Tps1p might have a function in controlling the influx of glucose into glycolysis (e.g. by interacting with hexokinase and/or sugar carriers). All genes encoding subunits of the trehalose synthase complex display

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significant sequence homology to the *TPS1* gene. However, none of the other subunits can take over the function of Tps1p in synthesizing Tre6P or in controlling glucose influx into glycolysis (Bell *et al.,* 1998).

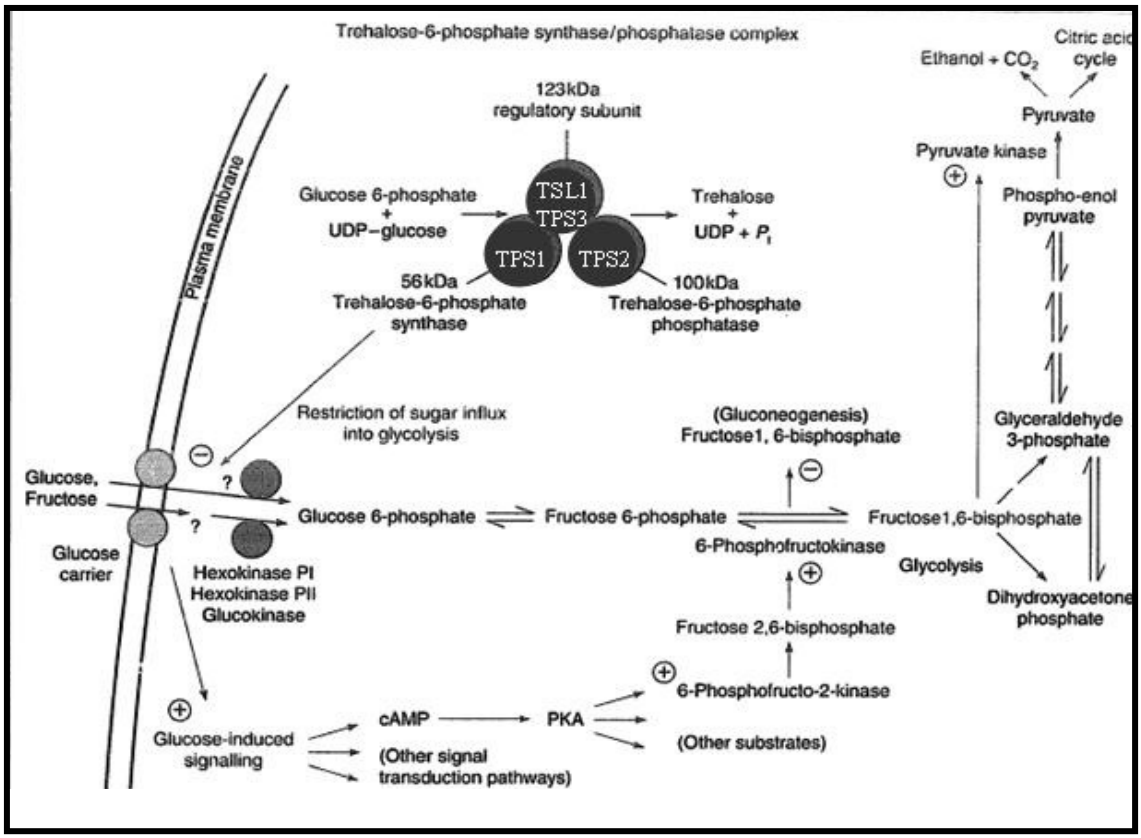

Fig. 8. The trehalose-6-phosphate synthase/phosphatase complex in *Saccharomyces cerevisiae* and its connection with glycolysis and glucose-induced signalling

Only deleting *TPS1* results in the inability to grow on glucose, because of the uncontrolled influx of glucose into glycolysis. Glucose activates several signalling pathways of which the RAS-cyclicAMP pathway appears to be important for the control of the switch from gluconeogenesis to glycolysis. This pathway stimulates the accumulation of fructose-2,6-bisphosphate, an allosteric activator of 6-phosphofructokinase and inhibitor of fructose-1,6-bisphosphatase. Fructose-1,6-bisphosphate in turn is an allosteric activator of pyruvate kinase. This control is strengthened by a variety of other mechanisms that act at the post-translational or at the transcriptional level. PKA, cAMP-dependent protein kinases (adapted from Thevelein and Hohmann, 1995).

A disruption of the *TPS2*, the gene that encodes a 102-kDa polypeptide, eliminates the trehalose-6-phosphatase activity, and results in a temperature sensitive growth phenotype accompanied by a hyperaccumulation of Tre6P. The phenotype produced by mutations in *TPS2* is conspicuous and probably indicates unknown regulatory phenomena (Gancedo and Flores, 2004). Elliot *et al.* (1996) suggest that the

accumulation of Tre6P is the main cause of thermosensitivity in Δ*tps2* mutants. They found that the heat shock resistance of a Δ*tps1* single mutant and a Δ*tps1tps2* double mutant was similar to that of the wild-type. However, a Δ*tps2* single mutant was extremely sensitive to the treatment and accumulated a considerable amount of Tre6P. Moreover, a suppressor that did not restore trehalose synthesis but decreased the accumulation of Tre6P suppressed the thermosensitive phenotype.

Individual deletion of the *TSL1* and *TPS3* genes does not markedly affect the trehalose content of the yeast but a double deletion of Δ*tsl1tps3* decreased it by more than 50%. Deletion of these subunits also changed the effect of phosphate on Tre6P synthase activity: it was activated by phosphate not inhibited by it (Bell *et al.,* 1998).

2.2.2. Trehalose degradation

The most widely distributed catabolic system in fungi is the irreversible hydrolysis of trehalose into glucose by trehalases. Yeasts have two types of trehalose hydrolases, known as *acid* and *neutral* trehalases because of their optimal pH for activity (4.5-5.0 and 6.8-7.0 respectively), or *extracellular* or *cytosolic* trehalases because of their localization in the cell.

S. cerevisiae have two genes, *NTH1* and *NTH2*, that encode the *neutral* (or *cytosolic*) trehalases, two protein isoforms with 77% identity. The neutral trehalase Nth1p is responsible for the intracellular mobilization and/or recycling of trehalose. There is a paradoxical situation with respect to Nth1p, since its expression is low while its activity is high in glucose-growing cells whereas the opposite is observed in the post-diauxic phase (review in François and Parrou 2001). On entry into diauxic shift, transcription of *NTH1* is maximal and the activity of neutral trehalase drops, suggesting a strong inactivation of this enzyme (François *et al.,* 1987; San Miguel and Argüelles, 1994). High levels of "inactive" trehalase during and after the diauxic growth shift is may allow yeast cells to rapidly resume growth under favourable conditions by mobilizing their endogenous stores (Parrou *et al.,* 1999). Neutral trehalase rapidly hydrolyses endogenous trehalose (K_m ~ 5-35 mM) in response to developmental programs (during spore germination, for example) when nutrients are added to starved cells or after a down-shift in temperature. Neutral trehalase exists in two

interconvertible forms by reversible phosphorylation. PKA is the only protein kinase known to directly phosphorylate and activate Nth1p. However, nothing is known about the protein phosphatase that desphosphorylates the phosphorylated form of Nth1p (review in François and Parrou, 2001).

It has been reported that deletion of the gene *NTH1* in yeast is associated with a lack of measurable "in vitro" trehalase activity (Nwaka and Holzer, 1998). In contrast, no change in trehalose and trehalase activity was observed in the Δ*nth2* mutant. The only phenotypic feature found for the Δ*nth2* mutant is its lower thermotolerance, which was also found for the Δ*nth1* mutant. *NTH2* is expressed at low levels in exponentially growing cells on glucose and at high levels in the stationary phase after glucose exhaustion (Nwaka *et al.,* 1995).

Acid (or *extracellular*) trehalase, encoded by *ATH1*, is active as a monomer in yeast. It is highly specific for trehalose (K_m ~ 0.8-5 mM), and has a high temperature optimum and high thermostability. Moreover, it does not appear to be regulated by a post-translational mechanism. Firstly, it was thought that Ath1p is only localised in the vacuole but the protein could be also found in the cell surface, in the periplasmic space. However, the mechanism by which Ath1p is exported to the cell surface and the way in which it is retained still have to be identified (Parrou *et al.,* 2005). The extracellular localization of acid trehalase seems to preclude its activity on endogenous trehalose. Deletion of *ATH1* in the Δ*nth1tnh2* mutant results in the accumulation of extracellular trehalose. Degradation of endogenous trehalose by Ath1p may involve the transport of trehalose out of the cells and its hydrolysis by external trehalose (Parrou *et al.,* 2005). It has been shown that this enzyme is required for growth on trehalose in all fungi including yeast S. *cerevisiae*. Nwaka *et al.* (1996) reported that the *ath1* mutant cannot grow on trehalose as a sole carbon source.

2.3. Regulation of trehalose metabolism

In general, a close correlation is observed between the trehalose content and the stress resistance of the cells (Wiemken 1990). Genes related to trehalose metabolism have at least one stress responsive element (STRE) in their promoter, which is under the

positive control of the transactivator Msn2p/Msn4p (Estruch, 2000). However, the mere presence of one or several STREs in the promoter does not explain the increase in trehalose level, and other factors must be considered. In fact, increases in the amount of enzymes, changes in enzymatic activities caused by allosteric effectors or covalent modification, and increases in the levels of substrates must be taken into account to explain the accumulation of trehalose in a specific stress condition (François and Parrou, 2001). In vivo changes in trehalase activity appear to be mediated by phosphorylation/dephosphorylation (Thevelein, 1996).

Intracellular levels of trehalose are the result of a well-controlled balance between enzymatic synthesis and degradation. Because the synthesis of 1 mol of trehalose from glucose requires 3 mol of ATP, but no ATP is produced when the disaccharide is hydrolysed by trehalase, a futile cycle will probably arise during simultaneous synthesis and degradation of trehalose (François *et al.,* 1991). As pointed out by Holzer (1976), the rapid inactivation of enzymes should be an excellent way of saving cells and circumventing this problem.

In the yeast *S. cerevisiae*, the trehalose content varies as a function of nutrient availability, stress and the activity of the Ras-cAMP pathway. The addition of glucose triggers a cAMP-dependent protein phosphorylation cascade which activates neutral trehalase and mobilizes trehalose. This supports the idea that glucose does not specifically trigger the depletion of trehalose, but that trehalose is just one part of a developmental program initiated in the presence of glucose (Thevelein 1996). Addition of glucose alone causes a transient activation of trehalase as well as a partial trehalose mobilization followed by a re-synthesis of the disaccharide. Under this condition, the activities of Tre6P synthase and Tre6P phosphatase were not significantly modified. However, glucose combined with yeast extract or with bacto-peptone caused a complete degradation of trehalose which was not followed by a resynthesis of the disaccharide. Tre6P synthase and Tre6P phosphatase activities decreased progressively to reach only 15% of their initial value after 2h of incubation (François *et al.,* 1991). Glucose-induced activation of Tre6P synthase is probably triggered by the same fermentable-growth-medium-induced pathway which appears to activate the catalytic subunits of cAMP-dependent protein kinase in a cAMP-independent way (Thevelein 1996).

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2.4. Role of trehalose in anhydrobiosis

Many anhydrobiotic organisms (a living state which results in dry but viable organisms), including *Saccharomyces cerevisiae*, accumulate large quantities of trehalose (as much as 20% of the dry weight) in the absence of water. The analogue of trehalose in higher plants appears to be sucrose, which may accumulate as much as 50% of the dry weight (Crowe *et al.,* 1992). About 2-3% of intracellular trehalose is indispensable if compressed yeast is to survive to any significant extent. Only those yeast cells in which trehalose has fully replaced bound water can survive in the lyophilized state (Sano *et al.,* 1999). It seems that a basic level of trehalose is necessary to protect yeast cells and that this level is the amount required to replace bound water and to fully cover the yeast cell. However, trehalose may be produced during desiccation only when yeast is exposed to ample glucose supplies. Under glucose-limiting conditions trehalose is not available in sufficient amounts to provide significant biophysical protection of the membranes, which suggests that the response may be related to the amount of carbohydrate available to the organism (Singh *et al.,* 2005). Singh *et al.* (2005) also proposed that a peptide or a small protein such as the hydrophilin Sip18p (overexpressed during their desiccation study) could also function in a similar role.

Trehalose alters the physical properties of membrane phospholipids in the dry state in ways that may explain the remarkable stability of membranes in anhydrobiotic organisms. Two different explanations of the action of trehalose in dry yeast have been put forward: the ***vitrification hypothesis*** and the ***water replacement hypothesis***. These hypotheses do not describe mutually exclusive mechanisms; indeed, it is believed that they are both required to preserve the labile components of the cells of anhydrobiotes in the dry state (Crowe *et al.,* 1992, Koster *et al.,* 1994, Crowe *et al.,* 1998).

2.4.1. Water replacement hypothesis

By careful drying, the water content of yeast cells can decrease from about 70% to about 7%. This dried yeast retains its enzymatic activity and has much better storage

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properties than fresh yeast (Van Steveninck and Ledeboer, 1974). Two primary stresses destabilize bilayers during drying: fusion and lipid phase transitions. If the liposomes become mobile, they may come into contact with each other and undergo fusion. Fusion is damaging because it not only increases the size of the vesicles, it also often results in leakage during the fusion event (Crowe *et al.,* 1998). Trehalose and other sugars will inhibit fusion between the vesicles during drying, but this alone is not sufficient to preserve the dry vesicles. Only a small amount of trehalose is sufficient to inhibit fusion completely, but much more is required to prevent the water-soluble marker from being lost (Crowe *et al.,* 1992).

Polar head groups of phospholipids are normally hydrated to some extent, and the head groups are separated from each other by these water molecules. Thus, when phospholipids are dehydrated, the packing density of the head groups increases, thereby increasing opportunities for van der Waals interactions among the hydrocarbon chains. As a result, the phase transition temperature (T_m, the temperature at which the hydrocarbon chains undergo a change form gel to liquid crystalline state) increases enormously: in other words, dehydrated lipids enter the gel phase at temperatures at which hydrated lipids are in the liquid crystalline phase (Crowe *et al.,* 1984). In the case of the lipids used in the liposome dehydration experiments, T_m increases from about -10 to about 60ºC (Crowe *et al.,* 1992). Fully hydrated egg phosphatidylcholine (PC) has a transition temperature of about -7ºC. When this phospholipid is fully dehydrated, T_m increases to about 70ºC. As a result, when the dry lipids are placed in water they would be expected to undergo a phase transition. The significance of this phase transition during rehydratation is that when phospholipids pass through such transitions, the bilayer becomes transiently leaky. Thus the leakage that normally accompanies this transition must be avoided if the contents of membrane vesicles and whole cells are to be retained. During drying this is not a problem because T_m is not affected until all the bulk water has been removed. However, during rehydration it is a serious problem; the membranes are placed in water and undergo the phase transition in the presence of excess bulk water. For instance, dry cells are known to require rehydration at elevated temperatures, about 40ºC. Membrane lipids in the dry yeast cells have a phase transition between 30 and 38ºC. If the cells are rehydrated at lower temperatures, they leak their contents and are killed during the rehydration. But if they are hydrated at 40ºC or higher, they do not leak (Leslie *et al*. 1994, Leslie *et al.,* 1995, Crowe *et al.,* 1998).

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Thus, when a membrane is dehydrated at a temperature at which it would normally be in a liquid crystalline state, PC would be expected to enter a gel phase as dehydration progresses. When PC enters a gel phase because of reduced temperature, the result is lateral phase separation of the PC, other lipid classes, and intra-membrane proteins. It follows that damage to the membrane might be alleviated if the initial event in this sequence, transition of PC to its gel phase, is prevented. The most likely mechanism for inhibiting the phase transition by trehalose is interaction between trehalose and phosphate head groups, which increases the spacing between head groups. This is the "***water replacement***" hypothesis (Crowe and Clegg, 1973). One of the predictions of this hypothesis is that in the presence of trehalose dry lipids have physical properties similar to those of dehydrated lipids. They measured T_m for mixtures of dipalmitoyl phosphatidylcholine (DPPC) and trehalose. The dry and hydrated DPPC show T_m's of 68ºC and 41ºC, respectively. When even small amounts of trehalose were added to the DPPC before it was dried, the endotherm broadened and displaced to a lower temperature. With increasing concentrations of trehalose, T_m decreased steadily until, at the highest concentration, it was actually below that of fully hydrated DPP. In other words, addition of trehalose to dry phospholipids in some respects mimics the addition of water.

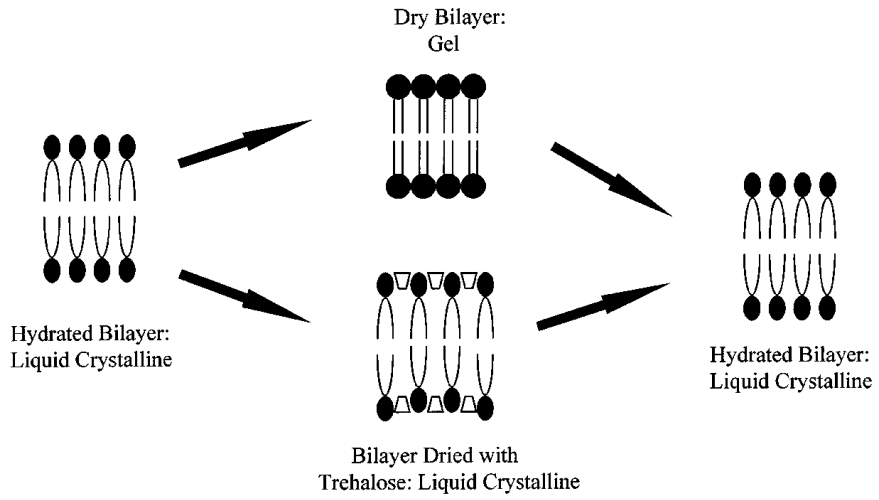

Fig. 9. Diagram illustrating the mechanism for stabilizing liposomes in the dry state by sugars. Similar results have been obtained for intact cells (Crowe *et al,* 1998).

Similar studies have been made on several other carbohydrates and show that the ability of these carbohydrates to preserve membrane structure in the dry state is

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correlated with their relative efficiencies at decreasing T_m and interacting with the phosphate head group. Glycerol depresses T_m although not to the same extent as trehalose; 5.2 moles of glycerol per mole of DPPC were required to reduce T_m to that of hydrated DPPC, but with trehalose only 2.0 moles per mole of DPPC were required to achieve the same m_c. In contrast to the results with trehalose, T_m in the presence of glycerol was not depressed below the T_m for fully hydrated DPPC. However, while the ability of a molecule to depress the melting temperature of dry phospholipid is probably important in preserving the biomembrane structure in the dry state, it is not sufficient in itself. Glycerol depresses T_m, but its mode of interaction with the phospholipids (probably intercalation between the head groups) involves deleterious side effects such as fusion. As a result, glycerol does not preserve dry membranes. These side effects are not found with trehalose, probably because its interactions with phospholipids involve hydrogen bonding between OH groups in the trehalose and the phosphate head group. These hydrogen bonds may replace the same or similar hydrogen bonds between the lipid and water that occur in bulk water. The relative specificity of the bonding between trehalose and phosphate head groups may therefore be an important factor in the ability of this molecule to replace water around the head group of a phospholipid and thereby to stabilize dry membranes (Crowe *et al.,* 1984).

Trehalose also preserves labile proteins during drying (Carpenter and Crowe, 1989). For example, phosphofructokinase (PFK) is a tetramer that irreversibly dissociates into inactive dimers during drying. Many molecules that stabilize proteins in solution will prevent this dissociation when excess water is present, but when the water that makes up the hydration shell of the protein is removed, the specificity for the stabilization is very high. Only disaccharides are effective at stabilizing the protein during extreme dehydration. For instance, when PFK is partially dried, it is inactivated. If proline, which stabilizes PFK in solution, is added, the enzyme is stabilized during this partial dehydration, but as the dehydration proceeds this stabilization is lost. In contrast, if trehalose is added, the enzyme is completely stabilized even after extreme dehydration. Evidence has been provided (Carpenter and Crowe, 1989) to show that, as with membranes, trehalose interacts directly with the dry protein, probably by hydrogen bonding of –OH groups to polar residues in the protein.

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However, it is not clear yet how this interaction leads to stabilization. The fact that trehalose is a non-reducing sugar is important, but cannot in itself affect stabilization. Reducing sugars show a browning reaction with dry proteins, which leads to denaturation. Since trehalose is non-reducing, this reaction does not occur with dry proteins, and browning of the protein by glucose can even be inhibited (Crowe *et al.,* 1990). Since other non-reducing compounds (like glycerol) are not effective at stabilizing dry proteins, it follows that the fact that the sugar is non-reducing is not in itself sufficient to affect stabilization (Crowe *et al.,* 1992).

2.4.2. Vitrification hypothesis

An alternative viewpoint is based on the fact that both sucrose and trehalose form glass in the dry state. It has been suggested that glass formation (vitrification) is often required to stabilize dry biomolecules, but is in itself insufficient to accomplish the stabilization. A glass is a liquid of such high viscosity that it is capable of slowing chemical reactions or even stopping them altogether. In so doing, it has been suggested by Bruni and Leopold (1991) that the glassy state may assure quiescence and stability in a living system for lengthy periods. Viscous glass can, nevertheless, be readily melted by adding water, thus restoring conditions that allow normal metabolism. This latter point is particularly important when considering the stability of cells in the dry state. Commercial active dry yeast contains glass (of which the main component is probably trehalose), which proves the existence of a vitreous state (cytoplasmic vitrification) in ADWY (Schebor *et al.,* 2000).

Koster *et al.* (1994) proposed that vitrification of the sugar inhibits the increase in T_m during dehydration, perhaps by limiting lateral stresses in bilayers during the process. One of their key findings is that T_g (the glass-transition temperature for the dry sugar) must exceed T_m in order to depress T_m in the dry lipid. T_g for trehalose (115ºC) is much higher than for sucrose (65ºC). As a result, the addition of small amounts of water to sucrose by absorption in moist air decreases T_g to below the storage temperature, whereas at the same water content, T_g for trehalose is above the storage temperature. Under these conditions, one would expect the sample dried with sucrose to be degraded more rapidly. Aldous *et al.* (1995) have suggested an additional interesting property of trehalose. Because the crystalline structure of trehalose is a dihydrate, some of the sugar

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might, during adsorption of water vapour, be converted into the crystalline dihydrate, thus sparing the remaining trehalose from contact with the water. This suggestion turned out to be right; after adding small amounts of water, the crystalline dehydrate immediately appeared, and the T_g for the remaining glassy sugar remained unexpectedly high.

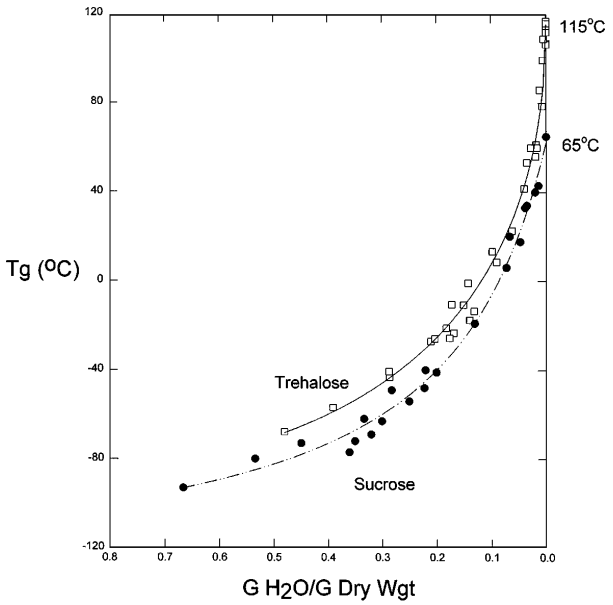

Fig. 10. State diagrams for sucrose and trehalose. Note that T_g for dry trehalose is approximately 50ºC higher than for sucrose (data from Crowe *et al.,* 1996)

3. YEAST REHYDRATION

Life takes place only in the presence of water. The water content of a fully rehydrated living system is 4-5 g H_2O/g dry weight (Crowe *et al.,* 1990). Yeasts need high concentrations of water for growth and enzymatic activity. The amount of bound water is hardly affected by the total water content but, therefore, it drastically decreases when the trehalose content is greater than 2-3%, suggesting that most of the intracellular bound water has been replaced by trehalose (Sano *et al.,* 1999). In contrast, numerous organisms can survive more or less complete dehydration, a condition known as ***anhydrobiosis*****.** They commonly survive even when >99% of their body water is removed. They remain in this living state until water again becomes available, when they rapidly swell and resume active life (Crowe *et al.,* 1992).

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The water permeability (*Lp*) of the membrane cell is considered to be an important parameter which is specific to the cell species and growth conditions. The volume of cells subjected to osmotic shift is first modified through the passive mechanisms of water or solute transfer and then the cell recovers its initial volume through active mechanisms. The characteristic inner sizes of yeast cells depend on growth time and are 2.3, 3.0 and 2.7 μm for yeast incubated for 9 h (exponential growth phase), 24 h (end of exponential growth phase) and 48 h (stationary growth phase), respectively. Considering the outer sizes obtained by electron microscope data, the yeast wall thickness is estimated as approximately 0.5 μm (Suh *et al.,* 2003).

In the bibliography, there are considerable discrepancies about the cell volume time response and the water permeability measurement. For *Saccharomyces cerevisiae*, time responses (for 50% of the cell volume variation) have been found to vary between 3.7 seconds and 15 minutes, determined with different devices and different temperatures (Niedermeyer *et al.,* 1977, Morris *et al.,* 1986, Gélinas *et al.,* 1991). The water membrane permeability for *S. cerevisiae* is very high. *Lp* values depend on the mixing chamber used and on the conditions of the assay and they are calculated from irreversible thermodynamic theory (Kedem and Katchalsky, 1958: K-K model), using the equation below:

$$J_v = -\ 1/A(dV/dt) = Lp\ \sigma\ \Delta\Pi$$

Where:
dV/dt is the rate of volume change ($m^3 \cdot s^{-1}$)
A is the cell area (m^2), considered constant during the shift
ΔΠ is the osmotic pressure between the extra and intracellular medium (Pa)
$\mathbf{J_v}$ is the volume flux ($m \cdot s^{-1}$)
σ is the reflection coefficient for a solute (σ=1, for a semi-permeable membrane)
Lp is the hydraulic or water membrane permeability ($m \cdot s^{-1} \cdot Pa^{-1}$)

Lp values of $6x10^{-15}$ $m \cdot s^{-1} \cdot Pa^{-1}$ and $1.4x10^{-15}$ $m \cdot s^{-1} \cdot Pa^{-1}$ were calculated for two different chamber and mixing conditions (Martínez de Marañón *et al.,* 1997).

S. cerevisiae has two genes that encode aquaporins (water channels) *AQY1* and *AQY2*. However, water permeability can also be regulated through membrane channels

or carriers that transport water molecules in addition to their main substrate (Zeuthen and MacAulay, 2002). The physiological role of aquaporins has yet to be elucidated. Over-expression of aquaporins protects cells under conditions of rapid freezing (Tanghe *et al.,* 2004), they facilitate most of the water influx at low temperatures (below 10ºC) (Tanghe *et al.,* 2006), Aqy1p might be involved in spore maturation by enabling water outflow (Sidoux-Walter *et al.,* 2004) and they have also been proposed as sensors of osmotic or turgor pressure (Hill *et al.,* 2004).

Resistance to drying is a specific characteristic of microorganisms and depends on taxonomical and physiological properties (Becker and Rapoport, 1987). Cells tolerate desiccation because they can protect vital components of their cellular machinery from damage and/or repair them quickly upon rehydration (Potts 2001). Desiccation-tolerant cells implement structural, physiological and molecular mechanisms to survive severe water deficit. These mechanisms are poorly understood. Dehydration causes a rapid efflux of water through the cell membrane, resulting in the collapse of the cytoskeleton. This anhydrobiosis may deleteriously affect yeast cell physiology by altering the structure and function of vacuolar, nuclear and cell membranes (Walter and van Dijck, 2006). The ability of microbial cells to survive dehydration and be reactivated at higher water activities in their environment is of fundamental importance to food technology and biotechnology. In particular, yeast viability after rehydration is a main concern in oenological industry for both yeast producers and winemakers.

3.1. Damages produced by the drying/rehydration process

The processes of drying and rehydrating essentially affect the structural organization and metabolism of microbial cells. Dessication induces such changes in the cellular environment as the reduced hydration of macromolecules and the consequent conformational changes, reduced cytoplasmic and intracellular transport, shifts in cytoplasmic pH and ion concentrations, and accumulation of organic and inorganic ions (Senaratna and Mc Kersie, 1986). Dehydration causes an intensive folding of the cytoplasmic membrane, and damage to the nuclear membrane rigidity, which is manifested by a considerable widening of nuclear pores when the dehydrated yeast is rehydrated (Becker *et al.,* 1984). One of the molecular mechanisms that can lead to

death in desiccation-sensitive cells when they are dried is free radical attack on phospholipids, which leads to lipid peroxidation (Leprice *et al.,* 1994). Dehydration causes oxidative damage and mechanisms that scavenge these free radicals are important components of the desiccation tolerance responses. In this respect, the two isoforms of the catalase (*CTA1* and *CTT1*) are required to protect cells against dehydration (França *et al.,* 2005).

On a molecular scale, the physical principles underlying the mechanism of damage from extreme dehydration are the same, regardless of whether organisms are an animal, microbe or plant. They accumulate large amounts of disaccharides which stabilize membranes and macromolecules upon dessication. Many anhydrobiotic organisms, including *Saccharomyces cerevisiae*, accumulate large quantities of trehalose in the absence of water (see chapter 2). Trehalose alters the physical properties of membrane phospholipids in the dry state in ways that may explain the remarkable stability of membranes in anhydrobiotic organisms (Crowe *et al.,* 1992). Yeast anhydrobiosis investigations mainly aim to detect the different reconstructions of intracellular structures and to identify protective reactions that maintain organism viability (Rapoport *et al.,* 1997).

Hyperosmotic shock results in a loss of turgor pressure and a rapid decrease in cytoplasmic water content and cell volume (i.e. cells shrink). Conversely, hypoosmotic shocked cells increase in volume because of the high water permeability of the plasma membrane and because they also lower the intracellular levels of potassium and glycerol (Walter and van Dijck, 2006). When yeast cells change from a state of vital activity to anhydrobiosis there are a number of structural and functional rearrangements in the cells. The resulting changes may be classified into damage of macromolecules, structures, organelles and defensive intracellular reactions. Of special interest are the membrane changes. Intracellular membranes in viable cells are repaired during their reactivation from anhydrobiosis. During rehydration, this membrane damage leads to an increase in cellular membrane permeability (Beker and Rapoport, 1987).

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3.2. Membrane permeability during rehydration

The organization of membrane lipids is dramatically altered as a result of thermotropic and dehydration-induced phase transitions. Among the alterations are the phase separation of membrane constituents (leading to such phenomena as the aggregation of non-bilayer phases), fusion between adjacent bilayers and transient permeability changes (resulting in leakage of the cellular content) (Review in Crowe *et al.,* 1989). The site of imbibitional damage is the plasma membrane and the damage can be explained by the phase behaviour of phospholipids. In particular, the formation of non-bilayer phases (and specifically the hexagonal phase II (H_{II}), although it forms only in extremely restricted areas of the membrane) in phospholipids during dehydration can result in leakage (review in Crowe *et al.,* 1992, Rapoport *et al.,* 1995).

Hydration-dependent phase diagrams have been prepared for only a few phospholipids, so it is not possible to generalize about the magnitude of the increase in the phase transition temperature (T_m) as a result of dehydration. In the case of DPPC, T_m increases from 41ºC to 120ºC, while in the case of POPC, T_m increases from -3ºC to 57ºC (Crowe *et al.,* 1986). Consequently, phosphatidylcholines like POPC that are in the liquid-crystalline phase at room temperature (T_m= -3ºC) are in the gel phase when dry (T_m= 57ºC), and return to the liquid-crystalline phase during rehydration. POPC vesicles are not expected to lose their contents during dehydration since the liquid-crystalline to gel transition occurs after bulk water was removed. However, during rehydration, when the vesicles undergo the gel to liquid-crystalline transition, they would be expected to leak into the surrounding medium. It follows that the gel to liquid-crystalline phase transition is likely to be a primary vector for leakage across dry bilayers as they are rehydrated (Crowe *et al.,* 1989).

Trehalose prevents this leakage by depressing the phase transition temperature of the dry lipids (see chapter 2.4.1) (Crowe and Clegg, 1973). At a_w=0.117 (dry state), the temperature of the dry gel to liquid crystal phase transition in yeast is around 60ºC. Nevertheless, adding trehalose before drying lowers the transition temperature from around 60 to around 40ºC. This allows rehydration to a physiologically tolerable temperature (Leslie *et al.,* 1994). As a result, dry lipids do not undergo a transition from gel to liquid crystalline during rehydration and they do not leak (Fig. 11).

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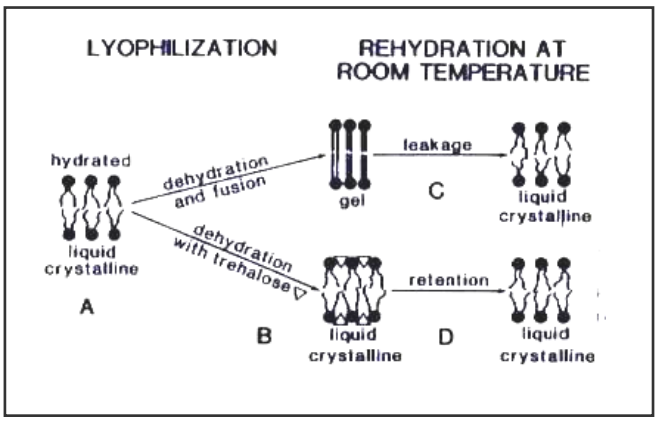

Fig. 11. Diagram illustrating the mechanism by which trehalose is thought to stabilize dry phospholipid bilayers.

It inhibits fusion during dehydration, but also reduces the gel to the liquid crystalline transition temperature in dry phospholipids. As a result of the latter effect, passage through the phase transition during dehydration can be avoided (Crowe *et al.*, 1992).

A corollary to the mechanism proposed is that if saturated lipids are used to prepare the liposomes, the mechanisms of preservation are quite different. For instance, the T_m of dipalmitoylphosphatidylcholine is 41ºC when it is fully hydrated. When it is dried without trehalose, T_m> 110ºC, and with trehalose it rises to about 50ºC. In this case, the lipid would be in gel phase at all times during dehydration-rehydration at room temperature. Thus the hydration-dependent phase transition is not expected to be a problem, and it can be preserved only by inhibiting fusion (Crowe *et al.,* 1989*b*). It seems probable that the high ergosterol concentration in yeast cell membranes has a similar effect to cholesterol, keeping the hydrocarbon chains of the phospholipids in an intermediate fluid condition, and preventing phase transitions (Van Stevenick and Ledeboer, 1974).

3.3. Leakage of intracellular components

When anhydrobiotic organisms are first placed in water they imbibe rapidly, and then there is a slower phase of imbibition until they become fully hydrated. During the early stages of imbibition, these organisms leak solutes into the surrounding medium. This leakage, which has been unambiguously ascribed to the loss of intracellular constituents, often results in extensive damage to the organism, and even to death (Crowe *et al*. 1992, 1988). It has been established that the permeability of dried yeasts increases sharply during rehydratation. The total losses of cell components can amount to 20-30% of the dry weight (Beker *et al.,* 1981). This increase in cytoplasmic membrane permeability and a substantial leakage of intracellular substances explain the formation of bubbles, drops and clusters of various forms and sizes on the surface of

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rehydrated cells. Among the substances that leak from the cell there are amino acids, vitamins, nucleotides, inorganic compounds, etc (Becker and Rapoport, 1987).

Minimum amounts of dry matter, nucleotides and potassium ions are lost during the rehydration of dry yeast cells at 35-45ºC. The release of potassium and magnesium ions from dried cells also notably increases. The amount of these ions excreted from the cells during rehydratation does not correlate with the viability. It has been found that, at the optimal rehydration temperature of 40ºC, the K^+ loss was of 25%. K^+ loss is maximum after about 5 min., coinciding with the moment that the yeast is fully suspended. This time can be decreased by more vigorous shaking. Viable cells uptake K^+ from the medium because of the start of metabolic activity (Van Steveninck and Ledeboer, 1974). A certain amount of trehalose leaks into the medium during rehydration. Towards the middle of the reactivation process, up to 60% of the leaked trehalose is observed to be reabsorbed by the cells again (review Beker and Rapoport, 1987). It has been proposed that the rate of extracellular trehalose consumption during rehydration is more important than the concentration of this compound in AWDY for a rapid start to alcoholic fermentation (Roustan and Sablayrolles, 2002). A considerable increase in the free amino acid pool in cells in a complex medium during reactivation indicates that the change in permeability also accounts for reverse processes, the entering of substances into the cell. Dried yeasts lose about 50% of nicotinic acid and small amounts of thiamine and riboflavin after dehydration. A considerable increase in dried cell permeability has been found for compounds with peptide bonds with a typical absorbance at 200 nm and for nucleotides absorbing at 260 nm (Beker and Rapoport, 1987).

The amount of leakage of cell components during rehydratation depends on the composition of the medium. In dried yeasts that are to be rehydrated, the maximum permeability of the cytoplasmic membrane for nucleotides was observed for a 3-5% concentration of NaCl or KCl in the medium. Since monomolar solutions of NaCl are known to re-orient phospholipid polar head groups, it is assumed that in the case of solutions with similar salt concentrations the disorientation of lipid components in biomembranes increases because of the removal of some bound water at the dehydration stage. When the rehydration medium is saturated with salt, there is a further decrease in

cytoplasmic membrane permeability that can most possibly be explained by the increasing osmotic pressure of the medium (Becker *et al.,* 1984).

Calcium ions and glucose also have a stabilizing effect on membranes during rehydration. Binding to the polar head groups of two neighbouring phospholipids, calcium ions form salt bridges, and increase the rigidity of the membrane structure. This is also required for to repair damaged cytoplasmic membranes. Glucose may permeate into the cell and stimulate the formation of protein gels that prevents diffusion rate of intracellular substances. On the other hand, at higher temperatures glucose may accelerate sugar-amine reactions and thus increase cell permeability. Calcium ions may diminish this negative effect of glucose. Becker et *al.* (1984) showed that 3 mM calcium decreases the nucleotide losses by 8%. Glucose is equally effective at a concentration of 30 mM. The most effective decrease in cell permeability was obtained when both compounds were applied simultaneoulsy (3mM Calcium and 150 mM glucose) (Beker and Rapoport, 1987).

3.4. Recovering damage of the dried cells: viability and vitality

The situation of yeast during rehydration is difficult to analyse, since the cells emerge from a metabolically inactive state and may not be able to properly perceive and respond to stress conditions (Bauer and Pretorius, 2000). It has been shown that survival depends on the physiological condition of the cells before the beginning of the drying process (Eleuterio *et al.,* 1995) and on the temperature and the kinetics of rehydration (Poirier *et al.,* 1999). The storage conditions of the dried cells are also very important in the preservation of cell viability. Conservation is improved at low temperatures and under vacuum or nitrogen atmosphere (Becker and Rapoport, 1987).

The viability of cells after rehydration depends on the composition, osmotic pressure and temperature of the rehydration medium. Viability increased significantly when cells were rehydrated with a protectant solution instead of water. Survival was 60 and 85% with the best combinations of treatments. For *Candida sake*, a mix of 10% lactose + 10% skimmed non-fat milk as protectant during freeze-drying and 10% of skimmed non-fat milk as rehydration media increased viability to 85% after the freeze-drying process (Abadias *et al.,* 2001). Maréchal and Gervais (1994) have shown the

importance of dehydration kinetics on the viability of *Saccharomyces cerevisiae*. Slow dehydration kinetics increased the viability observed during rapid dehydration, probably by allowing the accumulation of trehalose (Leslie *et al.,* 1994). Cells rehydrated (reaching a water activity of a_w= 0.990) immediately after dehydration (a_w= 0.117) and their viability was 28%. However, samples slowly rehydrated to a_w=0.455 for 14 days, and viability after total rehydration was 91% (Poirier *et al.,* 1999).

The viability of *S. cerevisiae* subjected to hyperosmotic shock is strongly dependent not only on shock intensity, but also on the temperature at which it takes place. A relationship has been found between mortality following rapid dehydration or rehydration, and water flow through an unstable membrane in phase transition. For low osmotic pressure shifts, the water flow resulting from the osmotic shock does not induce extensive damage to the membrane because of this low intensity, and the cell can survive such a stress (Laroche and Gervais, 2003).

As far as the importance of rehydration temperature on cell viability is concerned, it has been found that survival rate increases with rehydration temperatures between 9 and 50ºC. Poirier *et al.* (1999) found that if rehydration is carried out at 9ºC, 2% of the cells are preserved whereas rehydration at 50ºC preserved 65%. For *S. cerevisiae*, 43ºC was found to be the optimal rehydration temperature (Becker and Rapoport, 1987). Van Stevenick and Ledeboer (1974) showed that *S. cerevisiae* rehydrated at temperatures below 38-40ºC suffer imbibitional damage and leak cytoplasmic contents into the rehydratation medium and, consequently, suffer high mortality. These results led Van Stevenick and Ledeboer to propose that a gel to liquid crystal phase transition occurs when *S. cerevisiae* cells are rehydrated at low temperatures. Therefore, the positive effect of increasing the rehydration temperature is that it avoids the phase transition. Heating dry cells above the transition temperature before they are rehydratated obviates the hydration-dependent phase transition and they do not leak. Indeed, at T_m (temperature of the main membrane phase transition), a portion of the phospholipid species is in a gel state, and the rest is in a liquid-crystalline state (due to the heterogeneous nature of the lipids in the membrane). These domains can cause packing defects at the boundaries between the liquid-crystalline and gel phases (Cameron and Dluhy, 1987), so the membrane may not provide adequate barrier properties. Modifying the osmotic pressure creates a water flow. If a simultaneous

conformational change in phospholipids results in a low barrier property, this water flow could lead to membrane injury and leakage of the cytoplasm constituents into the surrounding medium, and, ultimately, cell death. Hence, mortality should only occur if there are conformational changes to the phospholipids during dehydration and, for treatment temperatures that are outside the phase transition zone, cell viability should be preserved (Laroche and Gervais, 2003).

Cellular membranes rapidly repaired themselves during rehydration, and the lipid storage within the cells mobilized quickly (Becker and Rapoport, 1987). 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 (Benchekroun and Bonaly, 1992; Rosi and Bertuccioli, 1992). During rehydration, yeast cells can incorporate a limited amount of lipophilic probes or solubilized sterols in their cellular membranes. Rehydration of ADWY in the presence of a high concentration (about 150 g/l (dw)) of inactive dry yeast increases the fermentation rate and thus diminishes the duration of fermentation, suggesting a transfer of sterols between inactive and active dry yeasts (Trioli 1996, Dulau *et al.,* 2002, Soubeyrand *et al.,* 2005). Used at high concentrations, inactive yeasts release fragments that can form micelle-like particles. These can act as bioemulsifiers by decreasing the total surface tension of water and by solubilizing a specific fraction of inactive yeast sterols in the rehydration medium (Soubeyrand *et al.,* 2005). During wine fermentation, yeast incorporates grape phytosterols which promote normal yeast growth. However, they perturb the yeast membrane properties. Supplementing the culture medium with small amounts of solubilized ergosterol compensates for the detrimental effects of phytosterols (Luparia *et al.,* 2004).

3.5. Transcriptional responses during water deficit and rehydration

Complete genome data bases are enabling novel ways of determining if different organisms have evolved similar biochemical solutions to environmentally stressful conditions. Resurrection plants, seeds and desiccated vegetative tissues accumulate *L*ate *E*mbryogenesis *A*bundant (LEA) proteins that may play a role in protecting cytoplasmic structures during dehydration. LEA proteins are usually rich in hydrophilic amino acids and high in glycine content. They are water soluble and can be divided into six different

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groups based on sequence similarities and properties (Bernacchia and Furini, 2004). LEA-like proteins (hydrophilins) are also found in fungi and bacteria, and are usually <0.2% of the total proteins. They seem to be involved in pathways that respond to water deficit. In *Saccharomyces cerevisiae*, 12 hydrophilins have been identified. Three of them have been reported to be induced by osmotic stress (*HSP12, SIP18* and *GRE1*) and, of the other nine genes, functional characterizations have been reported only for *STF2, IF1A* and *RL44* (Garay-Arroyo *et al.,* 2000).

Singh *et al*. (2005) suggest that desiccation and rehydration processes are similar to the stationary-phase growth. Cells are in a "holding pattern" (i.e., cells are lost in the S phase and concomitantly increase in equiescence (G_0/G_1). Entry into the quiescent state can be also induced by caloric restriction or by reaching the stationary phase of growth (Gray *et al.,* 2004, Martinez *et al.,* 2004). A transcriptional analysis of the stationary phase of growth identified 127 genes that are essential for survival at 37ºC. Many of these genes are involved in the mitochondrial function, posttranslational protein modification and resistance to oxidative stress (Martinez *et al.,* 2004). More than half of these genes have been found to be up-regulated during desiccation and rehydration. These genes included *CIT3, CRC1, CTA1, FDH1, GRE1, MLS1* and *SIP18* (Singh *et al.,* 2005).

The *Saccharomyces* Genome Database (http://www.yeastgenome.org) currently lists 10 genes under the category "response to desiccation" and only 3 (*GRE1, SIP18* and *YJL144w*) of them are marked enhanced during desiccation and rehydration. Genes related to fatty acid catabolism, gluconeogenesis and the glyoxylate and methylcitrate cycles are activated during dessication and subsequent rehydration. Elevated expression levels of *TUB3* and *SRO9* suggest that changes in the cytoskeleton may occur in the rehydration phase (Singh *et al.* 2005). They also suggest that cell wall organization and composition are critical for desiccation tolerance. A reduction in cell wall rigidity may allow the cell to distribute stabilizing factors more effectively.

The transcriptional response to yeast rehydration is likely to differ significantly depending on the exact rehydration conditions. Rossignol *et al.* (2006) observed a rapid induction of ribosomal protein genes and a decrease in the stress genes, which is consistent with an activation of protein kinase A and related to the presence of glucose in the rehydration medium. In fact, rehydration conditions were carried out in a 50 g/l

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glucose solution. When yeast is rehydrated in plain water, this kind of response is not expected.

3.6. Yeast rehydration in an oenological context

In the last decades, spontaneous grape must fermentations, traditionally used in winemaking, have been replaced by the inoculation of commercial ADWY. Before selected ADWY are added to musts in the fermentation tanks they must be rehydrated. The rehydration process is required to recover the metabolic activity and membrane functionality, since the water activity of the dried yeast is too low to maintain any of these processes (Bauer and Pretorius, 2000). Both the rehydration phase and the subsequent inoculation process are two traumatic steps for yeast, mainly because of the osmotic and thermal shocks that are produced. The activity of the yeast is rapidly recovered during this short rehydration period and the large population size and the cell activity shortens the lag phase and controls the fermentation kinetics and performance better. Thus, yeast rehydration is a key step if winemakers are to avoid stuck and sluggish fermentations. In spite of the importance of this rehydration step in winemaking, the oenological community has a standard protocol and this is normally carried out by following traditional practises which have never been questioned. In fact, there is a complete lack of knowledge about what this process represents in the whole winemaking context and what kind of practises could be developed to improve it.

The cells are activated beforehand for 30 min. in water or in a must/water (1:1) mixture at 37-43ºC (Ribéreau-Gayon *et al.,* 2004). Some protocols use both plain and sugar-enriched water (5 g/l or 50 g/l), but the rehydration must never take more than 30 minutes. Yeasts are also rehydrated directly in the grape must but, in general, the quantity of yeast needed is diluted in 10X of warm water. The solution should be stirred lightly every 15 min to allow the yeast to suspend. Vigorous mixing leads to higher mortality. However, the negative effect of vigorous mixing was reduced by allowing cells to pre-soak before mixing (Attfield *et al.,* 2000). Different “optimised” protocols for yeast rehydration are provided by the AWDY producers. The use of high cell concentrations during rehydration appears to have multiple effects on the leaching of cellular components by damaged cells. The release of solutes can have opposite effects; some tend to protect the cells (amino acids, nucleotides), while others can trigger

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specific stress responses (organic acids) (Rossignol *et al.,* 2006). Several kinds of nutrients used in this starting period are also available on the market. They consist mainly of mixtures of nitrogen sources (basically ammonium salts and thiamine), and inactive dry yeast and yeast extract both of which are interesting because they supply yeast sterols. Other advice given by ADWY producers is that rehydration in distilled or deionized water is lethal to the yeast. The cell walls require the presence of some minerals (sodium, calcium, magnesium and/or potassium) during rehydration. Tap water at a hardness of 250 ppm is optimum. The presence of 1/2% yeast extract, yeast hulls, autolyzed yeast or peptone in the rehydration water will give the yeast an added boost that will get it through its lag phase quicker. A solution with 5% of sugar helps to avoid osmotic shock.

The only parameter that winemakers always respect is the concentration of yeast cells inoculated in the must. Must juice is inoculated with active dry yeast at a concentration of 10-15 g/Hl or 10^6 cells/ml of juice, immediately after clarification (Ribéreau-Gayon *et al.,* 2004). Such a huge inoculum leads to a clear and expected imposition of the chosen yeast strain, which is of great interest of both winemakers and ADWY producers. However, nothing has been done to optimise the quantity of the inoculated cells and/or to improve the metabolic activity of yeast at the moment of inoculation. Better knowledge of the micronutrient composition of the rehydration medium and its evolution during rehydration would be helpful to improve our understanding of yeast behaviour.

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CHAPTER I

Changes in wine yeast storage carbohydrate levels during preadaptation, rehydration and low temperature fermentations

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ABSTRACT

The metabolism of glycogen and trehalose was analysed in a wine yeast strain fermenting at 25ºC and 13ºC. Trehalose and glycogen degradation was completed during the lag phase of fermentation. Ammonia was taken up rapidly and once it had been reduced to negligible amounts, the synthesis of trehalose started. Glycogen followed a similar pattern. If trehalose synthesis was taken as a stress indicator, the fermentation at 13°C could not be considered stressful, because the maximum concentrations are similar at both temperatures. In industrial fermentations, and after a preadaptation in grape must for several hours at 18°C, the lag phase was reduced significantly, and this may be why trehalose and glycogen were completely depleted at the beginning of the low temperature fermentation. Various preadaptation conditions were tested so that their influence on trehalose and glycogen degradation could be determined. The presence of fermentable carbon sources such as glucose or fructose triggered the mobilisation and use of trehalose. However, just increasing the osmotic pressure did not reduce the trehalose content. No such differences were observed in glycogen metabolism.

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INTRODUCTION

The wine industry has managed to control fermentation temperature effectively. Wines produced at low temperatures (10 to 15ºC) are known to develop certain characteristics of taste and aroma (Feuillat *et al.,* 1997) and the improved quality of these wines is probably because there are fewer higher alcohols and a greater proportion of acetate and ethyl esters among the total volatile compounds (Argiriou *et al.,* 1996). The volatile profile of wines is dominated by those components that are formed and retained most during fermentation, since these compounds are present in the highest concentrations (Pretorius, 2000). Another interesting aspect is that low temperatures notably reduce the growth of acetic and lactic acid bacteria and they can make it easier to control alcoholic fermentation. However, the optimal growth temperature for *Saccharomyces cerevisiae* is 25ºC, while 13ºC is restrictive and increases the risks of stuck or sluggish fermentations (Meurgues, 1996). Low temperatures increase the duration of alcoholic fermentation, decrease the rate of yeast growth and modify the ecology of wine fermentation. Indigenous non-*Saccharomyces* species are more likely to make a stronger contribution to fermentations conducted below 20ºC. Such ecological influences should be reflected in the chemical composition and sensory properties of the wine (Fleet and Heard, 1993). Thus, although low temperature fermentation has interesting applications in the enological industry, it also has an adverse effect on cell growth, increasing the yeast stress during wine production.

Wine technology also makes it possible to inoculate a selected yeast to control the alcoholic fermentation better. This selected yeast comes in the form of dry yeast that needs to be rehydrated before being added to musts in the fermentation tanks. Rehydration of the yeast is the first step to ensuring healthy cells and a good fermentation. Several scientific and technical reports have analysed the techniques involved in this step (Meledina *et al.,* 1978; Kraus *et al.,* 1983; Poirier *et al.,* 1999; Bauer and Pretorius, 2000). Rehydration in water at temperatures between 35 and 40 ºC is probably the most frequently used protocol for winemaking. However, various rehydration media have been tested (Degre, 1993) and given different results for yeast activity. Within the last 20 years, active dry wine yeast has been used more and more and has replaced the traditional practice of spontaneous fermentations in many wineries.

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During vinification, yeast cells are subjected to several environmental factors which have an adverse effect on growth; *i.e* the cells grow under different stress conditions. Main conditions that affect the yeast during wine production are high sugar content and, therefore, high ethanol concentration, nitrogen starvation, oxygen limitation, pH, temperature, SO_2 treatments, etc. (Ivorra *et al.,* 1999). Clearly, the survival and environmental adaptation (and, indeed, industrial performance) of yeast depends on the severity of stresses, the physiological condition of yeasts at the time of the stress (*i.e*. its degree of intrinsic robustness), genetic potential (strain-dependent), and prevailing conditions after cells have adjusted physiologically. It is clear that stress response is essential for survival and growth in natural environments and industrial applications (Attfield, 1997).

Glycogen and trehalose are the main storage carbohydrates in yeast cells (Panek, 1991) and recent extensive work is proving how important these carbohydrates are for the viability, vitality and physiological activity of yeasts (O'Connor-Cox *et al.,* 1996; Pretorius, 2000). Trehalose has been associated to (i) nutrient-induced control of cell cycle progression, (ii) control of glucose sensing, transport and initial stages of glucose metabolism, (iii) protection against dehydration, freezing, heating and osmotic stress, and (iv) protection against toxic chemicals, such as ethanol, oxygen radicals and heavy metals (Thevelein, 1996). Trehalose protects cells by preserving the integrity of biological membranes and stabilising proteins in their native state (Lucero *et al.,* 2000). Glycogen provides a readily mobilizable carbon and energy source while the yeasts adapt to a new growth medium (Pretorius, 2000). Besides, glycogen breakdown is accompanied by sterol formation, which is essential for yeast vitality and successful fermentation (François *et al.,* 1997).

However, trehalose and glycogen metabolism have mainly been studied in laboratory strains and/or synthetic fermentation media, with few exceptions (O'Connor-Cox *et al.,* 1996; Gimeno-Alcañiz *et al.,* 1999). Although this has intrinsic fundamental value, more work needs to be done on this issue. If the impact of these carbohydrates on industrial yeast applications is to be understood, industrial conditions should be reproduced. For this reason, the aim of the present work has been to study trehalose and glycogen metabolism in wine yeasts during vinification at low (13°C) and optimal (25°C) temperatures. The performance of dry yeasts in low temperature fermentations

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may be affected by rehydration so, we have also analysed how different rehydration conditions affect glycogen and trehalose metabolism as an indicator of early metabolic activation.

MATERIALS AND METHODS

Yeast strain and fermentation conditions

Laboratory and industrial fermentations were performed by inoculating 2 10^6 cells ml^{-1} with the commercial wine *Saccharomyces cerevisiae* strain QA23 (Lallemand Inc, Montreal, Canada). Active dry yeasts were rehydrated in warm water (37ºC) for 30 minutes, according to manufacturer especifications.

In laboratory conditions, concentrated must was diluted to 230 g sugar l^{-1} with distilled water and supplemented with 0.1 g l^{-1} of *Vitiamine* (thiamine and ammonium phosphate, Martin Vialatte, France), which yielded a total ammonia concentration of 94 mg l^{-1} and available nitrogen of 150 mg l^{-1}. Musts were fermented in flasks capped with cotton with a working volume of 2,000 ml. Fermentations proceeded at control temperature (25ºC) and at low temperature (13ºC) after the yeast had been inoculated. *S. cerevisiae* cells were harvested after the flask had been shaken.

Industrial fermentations (Caves Gramona, Sant Sadurni de Noia, Spain) were conducted at low temperature (13ºC) in a closed 15,000-litre tank filled with Muscat grape must, which had a sugar concentration of 180 g l^{-1}, a pH of 3.12 and 40 mg SO_2 l^{-1} was added. In the winery, after initial rehydration, yeasts were cultured in grape must at 18ºC for several hours and seed into the newly filled tanks to reach a population of 2 10^6 cells ml^{-1}.

Laboratory test for rehydration conditions

After 30 minutes of rehydration in warm water (37ºC) cells were transferred to different preadaptation media. In one set of experiments the preadaptation medium was a synthetic must medium (Riou *et al.,* 1997) containing as a carbon source either 10% (w/v) glucose plus 10% fructose (A), 10% glucose plus 10% sorbitol (B), 10% fructose plus 10% sorbitol (C) or 20% sorbitol (D). Sorbitol, a non assimilable carbon source for

yeast, was added to have osmotic pressure similar to that found in the medium with 10% glucose plus 10% fructose. The other set of experiments was done in distilled water with single carbon sources, *i.e.* only glucose (E), only fructose (F) or only sorbitol (G) in the preadaptation media at 20% in all the cases. Both sets of experiments were performed at room temperature and, distilled water was used as a control (W). The pH of the synthetic medium was 3.3.

Analytical methods

Fermentations were monitored by measuring the density and reducing sugar content by an enzymatic method (Boehringer Mannheim kit, Mannheim, Germany). Cell viability was estimated after plating on YEPD, and used to determine the growth phase. Trehalose and glycogen were determined by the method described by Parrou and François (1997). Assimilable nitrogen was determined by the Dukes and Butzke (1998) method and ammonia content was determined by the enzymatic method using the Boehringer Mannheim kit (Mannheim, Germany).

Statistical analysis

The results were statistically analysed by one-way ANOVA and the Duncan test when appropriate from the statistical software package SPSS 10.0. The statistical degree of significance was set at $P<0.05$.

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RESULTS

Trehalose and glycogen patterns during alcoholic fermentation in laboratory conditions

Most commercial wine strains are sold as active dried yeasts (< 5% water) and before the inoculation they are rehydrated. The trehalose and glycogen values at day 0 (that is to say, their concentration in dry yeasts before inoculation) were 91.6 µg trehalose 10^{-7} viable cells and 72 µg glycogen 10^{-7} viable cells. Because cellular death occurs throughout fermentation, dry cell weight is not related to a viable population and, thus, our data is expressed as µg carbohydrate 10^{-7} viable cells.

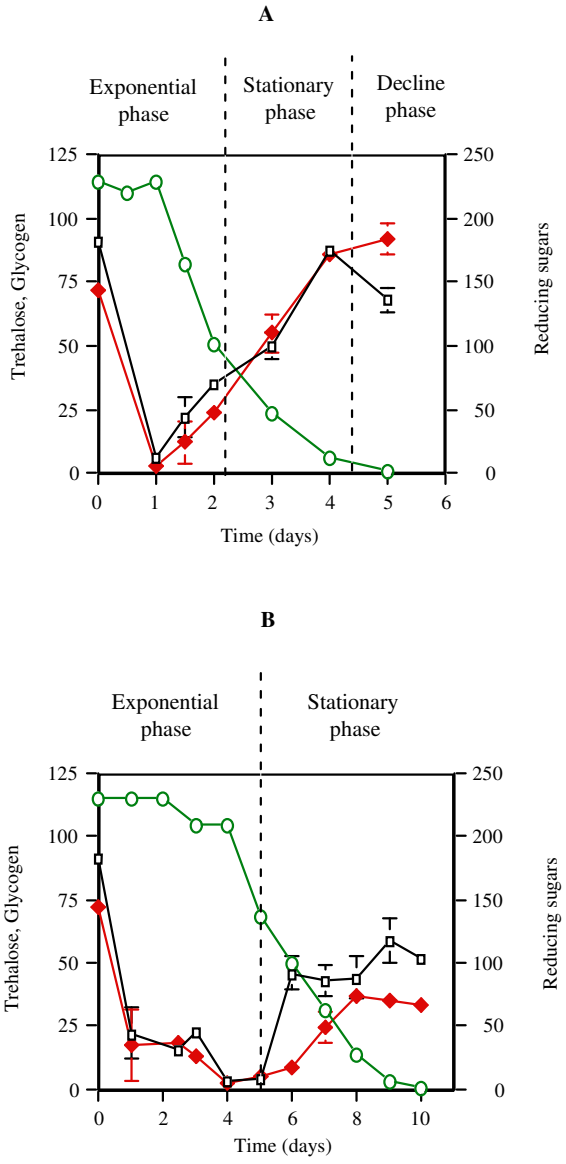

Figure 1. Effect of fermentation temperature on the metabolism of trehalose and glycogen in QA23 yeast strain. The results are the average ± s.d. of two separate experiments. Trehalose (□) and glycogen (◆) are expressed in µgx10^{-7} cells; reducing sugars (○) in g l^{-1}. A: 25ºC, B: 13ºC

Regarding the fermentation at 25ºC, most of the initial trehalose and glycogen were degraded during the first day of fermentation (Fig. 1). As for the fermentation at 13ºC, both carbohydrates took longer to disappear and the minimal concentration was delayed until the fourth day of fermentation. After these lowest concentrations, trehalose and glycogen increased throughout the process (with some exceptions in the final stages). Ammonia concentration in the must was 94 mg l^{-1} and accounted for more than 60% of the total nitrogen content. On the day after the inoculation at 25ºC, the yeast population had increased 10-fold up to 2-5 10^7 viable cells ml^{-1} and ammonia was undetectable while sugar concentration remained practically identical (Fig. 1A). The trehalose content was seen

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to decrease on the last day of fermentation at 25ºC, when the glucose had been almost completely consumed. Instead, at 13ºC no decrease in trehalose or cell viability was observed at the end of fermentation.

Trehalose and glycogen degradation patterns under industrial conditions at 13ºC

In industrial conditions, yeasts were previously preadapted in grape-must at 18ºC. When yeasts were inoculated after preadaptation, the intracellular trehalose and glycogen were already depleted (Fig. 2). However, after rehydration, the levels in the dry yeast were as high as usual (cells were from the same batch as before).

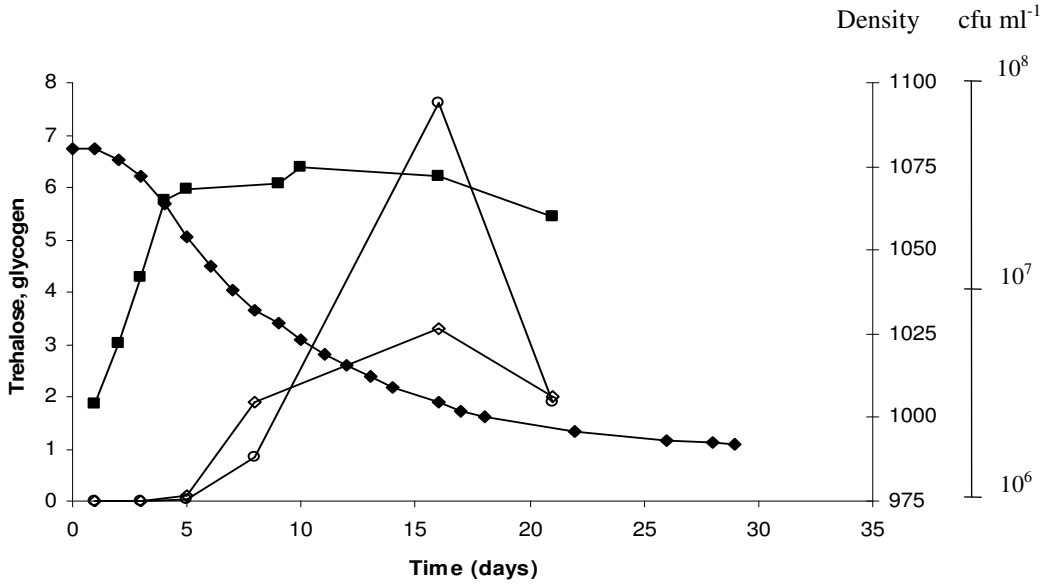

Figure2. Industrial fermentation. The results show must density (g l^{-1}) (♦), yeast population (cfu ml^{-1}) (■), trehalose (○) and glycogen (◊) concentrations (µg × 10^{-7} cells).

Thus, during the few hours of the preadaptative period, both carbohydrates were completely consumed by the yeasts. Once the yeasts had been inoculated in the new must, sugar consumption was not delayed as it was in laboratory conditions at 13ºC. Furthermore, the levels of glycogen and trehalose recovered only slightly during fermentation.

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Trehalose and glycogen content related to dry yeast hydration and preadaptation

The preadaptation period was analysed in the laboratory by measuring the levels of trehalose and glycogen in cells transferred to several synthetic media after rehydration. During the rehydration stage no significant differences were observed in the levels of trehalose and glycogen (Fig 3).

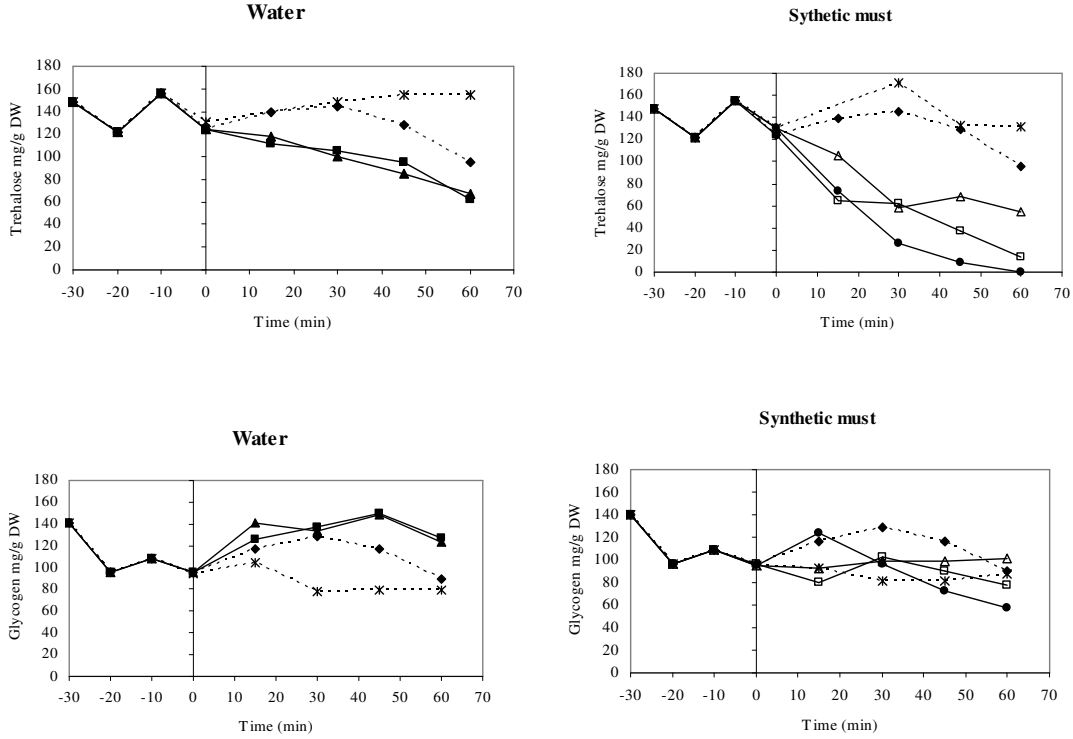

Figure 3. Effect of rehydratation and preadaptation media on the changes of trehalose and glycogen intracellular levels. The different conditions analysed in synthetic must were as follows: Glucose +Fructose (A, ●) Sorbitol + Glucose (B, □), Sorbitol + Fructose (C, Δ), Sorbitol (D,*). In water the conditions were as follows: Glucose (E, ■), Fructose (F,▲), Sorbitol (G, *) Distilled water (W,♦) was used as control in both condition.

The presence of glucose (E) or fructose (F) in water yielded a reduction in the trehalose levels. However, this reduction was higher when these sugars were in synthetic must (A, B, C). The concentration of these sugars in the medium was also important because the trehalose consumption was faster in the synthetic medium with glucose plus fructose (A) than the medium with glucose or fructose plus sorbitol (B, C). Thus, and additive effect of sugar concentration and the presence of nutrients was observed and was sustained by the statistical analysis. In fact, after the statistical treatment, three clusters were constituted: (i) the synthetic must with glucose plus fructose (A); (ii) the synthetic must medium with glucose or fructose plus sorbitol (B, C) along with those with

fermentable source in water (E, F); and (iii) presence of sorbitol (D, G) or only water (W). No such differences were seen in glycogen levels, which remained stable throughout the period analysed.

DISCUSSION

In dry yeasts, the accumulated amount of trehalose and glycogen depends on many variables during industrial production (O'Connor-Cox *et al.* 1996). Glycogen was the major storage polysaccharide in brewing yeasts, accounting for up to 30% of the dry weight of cells while the levels of trehalose did not exceed 6% (O'Connor-Cox *et al.* 1996). However, trehalose levels of 15 to 20% of the dry weight were also reported for commercial baker's yeasts (Van Dijck *et al.,* 1995), in which the stored trehalose was higher than glycogen.

Thevelein (1996) postulated that trehalose had a dual function, acting both as a storage carbohydrate and as a stress protection metabolite. However, if trehalose is a reserve carbohydrate, why do cells mobilise their large trehalose reserves when they are inoculated in a media full of glucose? Thevelein (1996) argued that a trehalose reserve was essential for providing energy and carbon to the preceding developmental stages. This suggests that trehalose can provide carbon and energy so that cells can adapt quickly to a new growth media. In wine fermentations, rehydrated yeasts are inoculated in a media with more than 200 g l^{-1} of reducing sugars, a pH between 3 and 3.5 and a low content of nitrogen compounds. This is obviously not an ideal medium for yeast growth, and it causes a delay in the start of fermentation between 1 and 3 days. During this adaptation or lag phase, sugars are hardly consumed but nitrogen compounds, mainly ammonia, are depleted and the cell population size also increases. In our hands, the patterns of degradation and accumulation of trehalose and glycogen during must fermentation in laboratory conditions agreed with those previously reported (Attfield, 1997; Gimeno-Alcañiz *et al.,* 1999). The inoculation of yeast cells in a fermenting sugar medium triggered rapid mobilisation of glycogen and trehalose (Thevelein, 1984; Van Dijck *et al.,* 1995).

Trehalose degradation probably enabled cells to adapt the metabolic machinery to the new medium. High trehalose contents in active dry yeasts mean that cells would adapt

better to the grape musts. At low temperatures, alcoholic fermentations had a longer lag phase, which corresponded with a prolonged decrease in trehalose concentration (Figure 1B). It also took longer for the ammonia consumption and the population to increase than in the fermentation at 25ºC. Therefore, there may be a correlation between the length of the lag phase and the consumption of trehalose. In addition, glycogen degradation was practically identical to that of trehalose and it may have a similar role. At an early stage in fermentation, the catabolism of glycogen was considered to be the major endogenous source of energy for sterol and unsaturated fatty acid biosynthesis (O'Connor-Cox. *et al.,* 1996).

The highest growth rate correlated with the lowest trehalose concentrations while the highest concentrations were found in the stationary phase. The same was observed for glycogen. Accumulation of trehalose and glycogen started almost simultaneously with the end of the exponential phase, which suggested a link between the inhibition of growth and the triggering accumulation (Parrou *et al.,* 1999). The triggering of this synthesis happened simultaneously with total ammonia consumption. Parrou et al. (1999) have already stated that complete exhaustion of the nitrogen source appears to be the primary condition for triggering the activation of both the glycogen and trehalose anabolism, when nitrogen is the limiting nutrient. Winemaking fermentations are considered to be limited by nitrogen, with ammonia as the primarily nitrogen source and easily consumed by yeasts. Therefore, ammonia concentration would be a key metabolite for triggering trehalose and glycogen synthesis in *S. cerevisiae* in wine fermentations, although other sources of nitrogen may still be available. Further research is needed to establish the link between nitrogen or ammonia limitation and storage carbohydrate synthesis. Better understanding of this link would probably lead to better fermentation performance and control. On the other hand, sugar concentration was not involved in the activation of trehalose and glycogen production in our conditions, which were characterised by an excess of sugar. Trehalose and glycogen synthesis started in the presence of a considerable amount of sugar in the medium.

Wiemkem (1990) argued against the idea that trehalose primarily functions as a reserve carbohydrate, because it is synthesised under conditions of starvation, even for glucose. Instead, glycogen is indeed produced when excess glucose is available. Our results showed a similar trend for both compounds, except in the last stage of the fermentation

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at 25ºC. Trehalose content decreased on the last day of fermentation when glucose had almost been completely consumed while glycogen concentration increased. It has been reported that cells use glycogen during prolonged incubations in the stationary phase (Singer and Lindquist, 1998) while trehalose is degraded long after glycogen stores have been depleted and started with the onset of cell death (Lillie and Pringle, 1980; Wiemken, 1990). In our hands, trehalose concentration decreased at the end of fermentation and thus trehalose was primarily metabolised in wine fermentation conditions, as similar microvinification experiments have also reported (Gimeno-Alcañiz *et al.,* 1990).

Yeast cells grown at temperatures higher than 30°C are known to contain greater trehalose concentrations (Grba *et al.,* 1979). We assumed that yeast cells growing at suboptimal temperatures, in this case at 13°C, would also be stressed and would produce more trehalose. However, the concentration of trehalose was highest at 25ºC. Glycogen levels were also highest after the fermentation at 25ºC. The effect of low temperature was to increase the length of the fermentation, which was twice as long as at 25°C. Surprisingly, viable cells remained at maximum levels, without any indication that they were entering a decline phase. This, along with the low concentrations of trehalose, might indicate that this 'low' temperature was not enough to trigger a stress response.

However, in industrial fermentations, trehalose synthesis took even longer to start and was not quantitatively as important as in the laboratory experiments at 13°C. In these conditions of fermentation, this increase also occured when the cells enter the stationary phase (days 7-8 of fermentation). Most importantly, industrial fermentations showed that preadaptation provided a shorter lag phase and better performance at low temperatures (Fig 2). It is noteworthy that although the initial levels of both carbohydrates were high in the dry yeast, the levels after the preadaptation were negligible. This supports the opinion that trehalose and glycogen provide initial energy for yeast metabolism, rather than exogenous sugars, and are therefore important for yeast activation after drying (Van Dijck *et al.,* 1995; O'Connor-Cox *et al.,* 1996; Thevelein, 1996; Attfield, 1997).

As already mentioned, the most usual protocol in the wine industry is to rehydrate the active dry yeast in warm water and to inoculate the grape must after 30 minutes.

Therefore, yeast rehydrated in a hypoosmotic medium has to cope with a new medium with a high sugar concentration and, obviously, high osmolarity. This shock reduces cell viability and fermentation performance (Poirier *et al.,* 1999). One way of improving the rehydration process is to increase the activation of the yeast metabolism before inoculation. In this study, the preadaptation of yeast in grape must between rehydration and inoculation significantly reduced the lag phase and improved wine fermentation. This was probably due to more active cells at the time of inoculation. Bearing in mind that trehalose and glycogen mobilisation are indicators of yeast metabolism activation, we tested various preadaptative conditions to see how they influenced the patterns of these storage carbohydrates.

By no means are all the media equally appropriate for succesful rehydration, at least as far as the fast recovery of yeast metabolic activity is concerned. This recovery also depends on there being suitable substrates in the media to make activation worthwhile. The presence of assimilable substrates and/or stress conditions could easily be signals to activate the mechanisms for metabolic activity. It is known that success in yeast rehydration is related to the temperature of water to prevent cell leakage (van Steveninck and Ledeboer, 1974), probably related to membrane lipid phase transitions (Crowe *et al.,* 1992). In fact, this leakage has also been observed to affect distribution between the cells and the medium of intracellular trehalose. Increased trehalose leakage also decreases cell viability (Zikmanis *et al.,* 1988). Furthermore, after rehydration, yeast cells face such new challenges as a high density and high osmolarity medium. These conditions are probably related to leakage and, thus, to water efflux from the cell (Poirier *et al.,* 1999), which would reduce water availability (Bauer and Pretorius, 2000). Our observed small, though non-significant, reduction of glycogen and trehalose immediately after rehydration could also be related to these rehydration effects. Likewise, the similar levels of both carbohydrates in the presence of water or sorbitol suggest that cells did not undergo further leakage after initial rehydration.

However, the presence of a fermentable carbon source in the preadaptative medium (A, B, C, E, F) quickly reduces trehalose levels, as was observed during the fermentation. It is noteworthy that glycogen levels are unchanged in these conditions, which only last for the first hour after rehydration. Trehalose seems to be the most easily usable reserve and the initial use of trehalose may indicate the start of metabolic activation. The

presence of other nutrients as well as fructose and glucose (A) was the most effective signal to trigger the use of trehalose as a source of carbon and energy, as previously reported (Thevelein, 1984; Van Dijck *et al.,* 1995). Moreover, the concentration of the fermentable carbon source seems to be important in the velocity of this degradation. From our results, it is clear that this signal is not related with osmotic pressure because the presence of sorbitol did not trigger the trehalose mobilisation. The intracellular contents in the dry yeast of these reserve carbohydrates are probably high enough to sustain a limited growth, provided the yeasts are in the appropriate medium. Mobilisation of these carbohydrates would allow the cells to divide and use the nutrients available in the medium. These observations, then, agree with the proposed role of trehalose as a primary provision of energy and carbon in the developing yeast (Thevelein, 1996).

This preliminary work used an industrial strain and a grape must to study the trehalose and glycogen metabolism in *S. cerevisiae* in winemaking conditions. Although some studies about this subject have been made in recent years, the regulation of reserve carbohydrate metabolism under these conditions still needs to be understood much better. This understanding could improve the yeast performance in industrial processes. Although rehydration in plain water at warm temperatures (35-40 °C) is the most recommended protocol for winemaking, full cell metabolic activity may depend on the presence of other substrates. Further work on rehydration protocols and how they affect trehalose and glycogen metabolism are currently under way. It is clear from our results that quick mobilisation of reserve carbohydrates can result in a shorter lag phase, which is highly desirable in problematic wine-making conditions. Changes in trehalose and/or glycogen concentrations may be an early indication of cell metabolic activity. Thus, further knowledge of rehydration protocol could lead to greater inoculation efficiency in industrial winemaking.

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CHAPTER II

Effect of nitrogen limitation and surplus upon trehalose metabolism in wine yeast

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ABSTRACT

Trehalose metabolism in yeast has been related to stress and could be used as an indicator of it. Winemaking conditions are stressful for yeast and understanding trehalose metabolism under these conditions could be useful for controlling alcoholic fermentation. In this study we analysed trehalose metabolism of a commercial wine yeast strain during alcoholic fermentation by varying the nitrogen levels from low (below adequate) to high (excess). We determined trehalose, nitrogen, sugar consumption and expression of *NTH1* and *NTH2*, and *TPS1*. Our results show that trehalose metabolism is slightly affected by nitrogen availability and that the main consumption of nitrogen occurs in the first 24 hours. After this period, nitrogen is hardly taken up by the yeast cells. Although nitrogen and sugar are still available, no further growth is observed in high concentrations of nitrogen. Increase of gene expression involved in trehalose metabolism occurs mainly at the end of the growth period. This could be related to an adaptive mechanism for fine tuning of glycolysis during alcoholic tumultuous fermentation, as both anabolic and catabolic pathways are affected in this expression.

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INTRODUCTION

Alcoholic fermentation is the principal transformation during winemaking and is conducted by yeast of the *Saccharomyces cerevisiae* species. During vinification yeast cells are subjected to several stress conditions, and their survival depends on their ability to adapt quickly to the changing environment. The main change that yeasts may face is a progressive decrease in the essential nutrient concentrations. This leads to a permanent adjustment of the genetic and metabolic machinery of the cell. However, vinification includes other stress conditions such as low pH, osmotic stress due to the high content of sugars in the must, nitrogen limitation, increasing ethanol concentration and carbon starvation at the end of the process (Ivorra *et al*., 1999).

The cellular machinery for controlling stress conditions involves the rapid synthesis of protective molecules and the activation of signal transduction systems, which induce the activation of enzyme activities and the transcription of genes encoding factors with protective functions. Of these, Trehalose has remarkable stress protection properties and may determine the survival response of yeasts under extreme environmental conditions (Singer and Lindquist 1998). Trehalose is present in the yeast cell as a storage carbohydrate and as a stress protectant. One of the main functions of trehalose is to protect cells against the denaturation and aggregation of proteins during periods of stress. Concomitant with this function, yeast cells accumulate trehalose when exposed to adverse conditions, such as nutrient depletion or heat stress (Van Vaeck 2001).

The biosynthesis of trehalose is catalysed by a two-step process involving trehalose-6-phosphate (Tre6P) synthase and Tre6P phosphatase on a multimeric protein complex encoded by four genes (*TPS1, TPS2, TSL1* and *TPS3*). The Tps1p subunit catalyses the formation of Tre6P from UDP-glucose and glucose-6-phospate (Glu6P), which is then dephosphorylated to trehalose by the Tps2p subunit. The Tsl1p subunit and its homologue (Tps3p) have a regulatory function. As well as its role in trehalose synthesis, Tps1 has an additional function in the regulation of sugar influx into glycolisis (Van Vaeck 2001). For the degradation, three trehalase genes have been described in *Saccharomyces cerevisiae*. These are *NTH1, NTH2* and *ATH1*. Degradation of trehalose is mediated by neutral trehalase encoded by the *NTH1* gene. The function and expression of the *NTH2* gene, which is highly homologous to the *NTH1* gene, is still not

fully understood. Neither delection nor overexpression of *NTH2* influences trehalose levels or trehalase activity. *S. cerevisiae* also contains an acid trehalase encoded by the *ATH1* gene, which displays no sequence homology to neutral trehalase. Ath1p is located in the vacuole and appears to be important for growth on media containing trehalose as a carbon source. (Wera *et al*., 1999).

Because of the potential applications in industry, most attention has focused on the biochemical and genetic control of trehalose metabolism in yeast, but using laboratory models and well-controlled systems. However, winemaking conditions are different in several ways. The first difference is the yeast, which is normally diploid or aneuploid. The second difference is that the culture media is not what may be considered optimal. We determined trehalose metabolism and its gene control of a commercial wine yeast strain during alcoholic fermentation. Also, due to the nitrogen limitation in the must and winemaking conditions, we used three very different nitrogen levels to determine the importance of this nutrient for regulating the trehalose metabolism.

MATERIALS AND METHODS

Yeast strain, fermentation conditions and sampling

Laboratory fermentations were performed by inoculating 2 10^7 cells ml^{-1} with the commercial wine *Saccharomyces cerevisiae* strain QA23 (Lallemand Inc, Montreal, Canada). Before inoculation, active dry yeast was rehydrated in warm water (37ºC) for 30 minutes. Fermentations were carried out at 25ºC. Ten-ml samples were taken by homogenizing the sample after gentle shaking at 1, 3, 6, 8, 11, 17 and 24 hours, then everyday until the end of fermentation.

The medium used was a synthetic must (Riou *et al.,* 1997) but without anaerobic factors and with 200 g l^{-1} of reducing sugars (glucose and fructose in a 50:50 ratio) at pH 3,3. As fermenters we used 1,800 ml-volume flasks capped with cotton, which produces semianaerobiosis during fermentations, thus simulating winemaking conditions.

We tested three conditions with different yeast assimilable nitrogen (YAN) contents. The control medium contained 300 mg l^{-1} YAN (120 mg YAN l^{-1} derived from ammoniacal nitrogen and 180 mg YAN l^{-1} from amino acids), which corresponds to the

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nitrogen content of a nitrogen-plentiful grape must. The high-nitrogen medium (HN) contained 1200 mg YAN l^{-1}. The proportions of the different amino acids and ammonium were maintained in this medium. The low-nitrogen medium (LN) contained 60 mg YAN l^{-1} derived solely from amino acids and did not contain ammoniacal nitrogen.

Analytical methods

We monitored fermentations by measuring the density and, in the latter stages, the reducing sugar content by an enzymatic method (Boehringer Mannheim kit, Germany). The maximum fermentation rate was the maximum slope from the representation of sugar consumption towards fermentation time and was expressed as concentration of consumed sugars (g l^{-1}) per day. Cell viability was estimated after plating on YEPD and used to determine the growth phase. Assimilable nitrogen was determined by the Aerny (1996) method and ammonia content was determined by the enzymatic method using the Boehringer Mannheim kit (Germany). The quantitative determination of trehalose was analysed by the method described by Parrou and François (1997).

RNA extraction, purification and cDNA synthesis

RNA was isolated from yeast samples as previously described by Siekstra *et al.* (1992) and resuspended in 50 µl of DEPC-treated water. Contaminating genomic DNA was removed in order to purify total RNA suspensions. To achieve it, we used the High Pure Isolation kit (Roche) in accordance with the manufacturer's protocol. Purified RNA concentrations were determined using a GenQuant spectrophotometer (Pharmacia) and verified electrophoretically on 0.8% agarose gels.

cDNA synthesis was done using the reserve transcriptase Superscript™ II (Invitrogen) in a GenAmp PCR System 2700 (Applied Biosystems). 0,5 µl of Oligo$(dT)_{12\text{-}18}$ Primer (Invitrogen) was used with 0,8 µg of total RNA as template in a reaction volume of 20 µl. Following the manufacturer's protocol, after denaturation at 70 ºC for 10 minutes, cDNA was synthesized at 42ºC for 50 minutes. Finally, the reaction was inactivated at 70ºC for 15 minutes.

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Gene expression analysis

The gene expressions of *TPS1, NTH1* and *NTH2* were determined by using the real-time quantitative PCR technique. Actin (*ACT*) was used as a housekeeping reference gene. The PCR primers (Table 1) were all designed with the available GenBank sequence data and the Primer Express software (PE Applied Biosystems, Cheshire, UK) according to the PE Applied Biosystems guidelines.

Table 1. Primers used in this study

Primer	Nucleotide sequence (5' to 3')----------
ACT-F	TGGATTCCGGTGATGGTGTT
ACT-R	CGGCCAAATCGATTCTCAA
TPS1-F	TCCATTACCATCCTGGTGAGAT
TPS1-R	TTGGTGAACGTCTGGTTTGC
NTH1-F	GTTTGGACCGCATCTCCAA
NTH1-R	AATACCGAGACCGTTAGGATGGT
NTH2-F	CGATCAGGCAATGGGATTA
NTH2-R	GCAGATAAACCTTTCCAGGCTAATA

For each gene, a standard curve was determined with yeast genomic DNA. DNA extraction was performed as described by Querol *et al.* (1992), digested by RNase and isolated by two-fold phenol-cloroform extractions and ethanol precipitation. DNA concentration was determined using a GenQuant spectrophotometer (Pharmacia). We prepared a series of 10-fold dilutions of DNA, which produced a ranking of concentrations from 4 to $4 \cdot 10^{-4}$ ng/µl. These dilution series were amplified (in duplicate) by SYBR PCR for each gene. 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 gene (Actin).

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Real-Time Quantitative PCR reaction was performed using SYBR® Green I PCR (PE Applied Biosystems). In SYBR PCR, amplification is monitored by the fluorescence gain of the double-strand specific DNA-binding dye SYBR green. The 25µl SYBR PCR reaction contained 300 nM of each PCR primer (Table 1), together with 1 µl cDNA (or 5 µl of each DNA serial dilution for standard tubes) and one time SYBR master mix (PE Applied Biosystems, UK).

Possible mistakes by DNA contamination during PCR were rejected by analysing No Amplification Controls (NAC) for each sample. In the NAC tube, no reverse transcriptase was added, thus no cDNA synthesis occurs. Threshold cycles for NAC did not differ from the No Template Control (NTC) ones, which confirmed the absence of genomic DNA contamination. Alternatively, the absence of nonspecific amplication was confirmed by analysing the generated dissociation curve on the GeneAmp 5700 Sequence Detector and by checking the PCR product with agarose gel.

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 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. The coefficient of variation in all analysed samples was less than 10%.

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RESULTS

Fermentation kinetics at different available nitrogen levels

The fermentation kinetics are shown in Figure 1. It can be seen that the population size reached very similar levels in the three fermentations, although in the fermentation with higher nitrogen availability (HN) this maximum population was reached within few hours, whereas in both control (C) and low nitrogen (LN) fermentations this maximum was achieved after one day. In order to collect enough cells for the analysis in the first hours of the study, we decided to inoculate a yeast population size 10-fold greater than the usual one used in wineries, to compensate for the drained biomass.

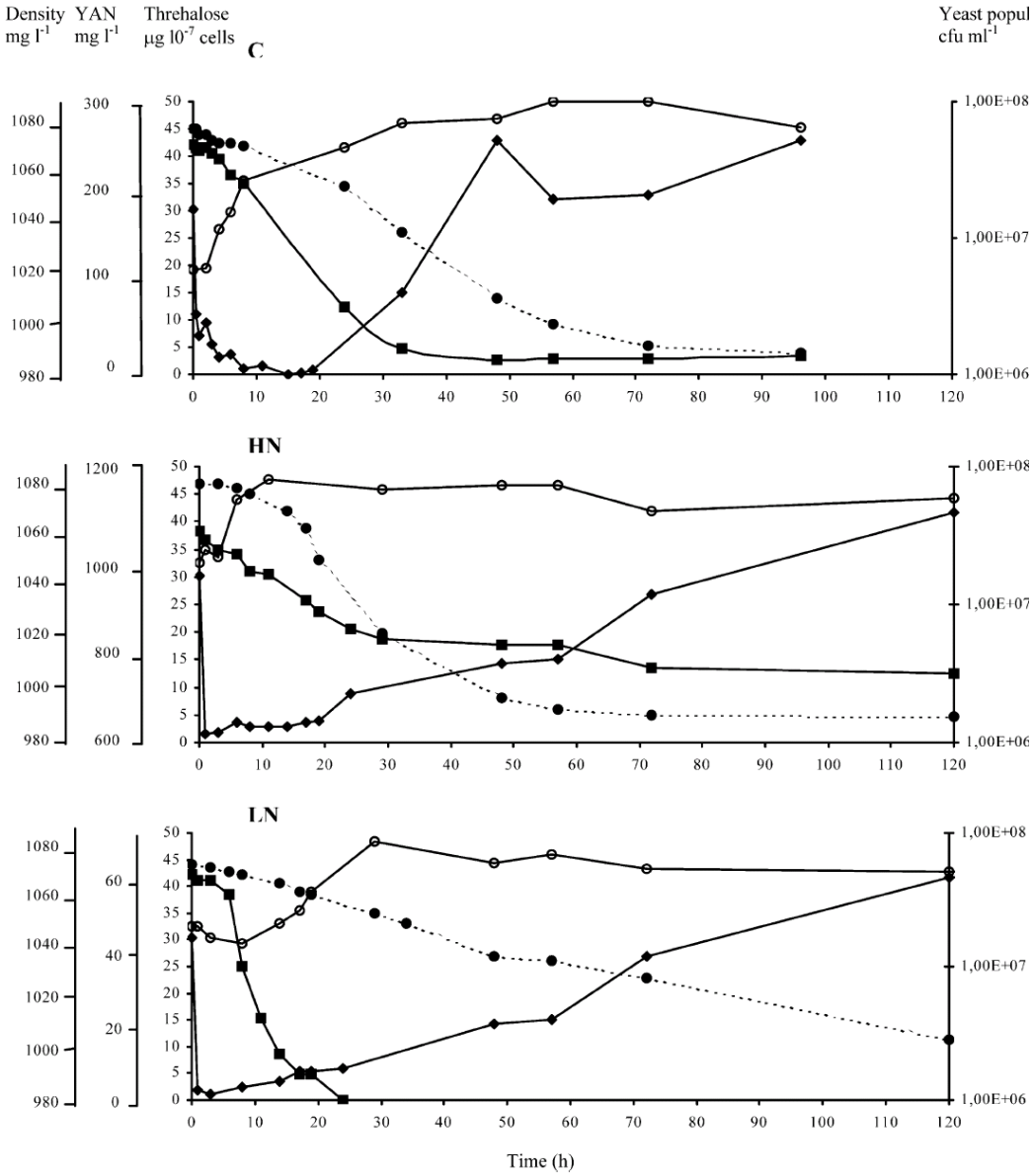

Fig.1 Changes in trehalose levels (µg/10^7 cells, *filled diamonds*) and yeast assimilable nitrogen (YAN) (mg l^{-1}, *filled squares*) and cell population (cfu ml^{-1}, *open circles*) and density (mg l^{-1}, *filled circles*) during control (*C*), high nitrogen (*HN*) and low nitrogen (*LN*) fermentations.

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The maximum fermentation rate was dependent on the amount available nitrogen in the media, yet it did not maintain a direct relation. In fact, the fermentation rate was very similar between HN and C fermentations, yet at LN the fermentation was slower. This was also reflected in the ending of fermentation that was similar for both HN and C fermentations, yet delayed in LN. Oddly enough, the rate of nitrogen consumption was about the same in the three conditions, although the differences in total N content were dramatic. Furthermore, in the LN fermentation no nitrogen was left after few hours, whereas in C fermentation some nitrogen was left in the medium. In the HN fermentation only 1/3 of the total nitrogen was consumed, mostly in the form of ammonia (results not shown).

Trehalose metabolism

To properly analyse the metabolism of trehalose, we used a controlled medium that may be considered grapemust-like (in terms of pH, the level of reduced sugars and acidity, etc). In these conditions, we determined the intracellular concentration of trehalose and the expression of the genes involved in trehalose metabolism during the alcoholic fermentation.

In the control fermentation, most of the intracellular trehalose was degraded within the first hour of fermentation and was undetectable thereafter (Figure 1). During this first hour no sugar consumption from the medium was detected and the yeast population did not increase. One day after the must inoculation, trehalose levels started to increase. The triggering of this synthesis was simultaneous with the consumption of most of the nitrogen, whereas sugar concentration was only slightly reduced. Most of the yeast grew during this period but did not yet reach the stationary phase. At the end of both the exponential phase and the total uptake of nitrogen, the intracellular levels of trehalose reached the maximum, which was slightly above the levels of the initial yeast. This general pattern was very similar in the three fermentations, although the amount of nitrogen, and the time for nitrogen exhaustion was very different. Thus, in all the fermentations the total consumption of initial trehalose occurred before the uptake of external sugar, although trehalose synthesis was not related with nitrogen exhaustion (which did not occur in the HN fermentation). The amount of trehalose at the end of the fermentations was similar in all conditions ($\approx$ 45 $\mu g/10^7$ cells). Only slight differences were found in the synthesis rates.

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Gene expression was referred to the expression at time 0 (expression value = 100), i.e. the expression in the yeast cell at the end of the rehydration period, just before inoculation in the must. Initially, all the genes had a very similar expression level, which was about one third of that of the reference gene, actine (data not shown). All the genes showed a reduction of the expression after inoculation. In control fermentation, *TPS1* gene was strongly induced between 8 and 17 hours of fermentation (Fig 2C). The *TPS1* induction was not accompanied by an immediate increase in trehalose. A delay was observed between the genetic induction and the accumulation of trehalose. *TPS1* showed the highest change in its expression during the period studied, while both neutral trehalase genes, especially *NTH2*, showed smoother changes. *NTH2* had the lowest basal expression and did not change during fermentation. The expression of *NTH1* increased simultaneously with the expression of *TPS1*, recovering the initial expression when the cells reached the stationary phase.

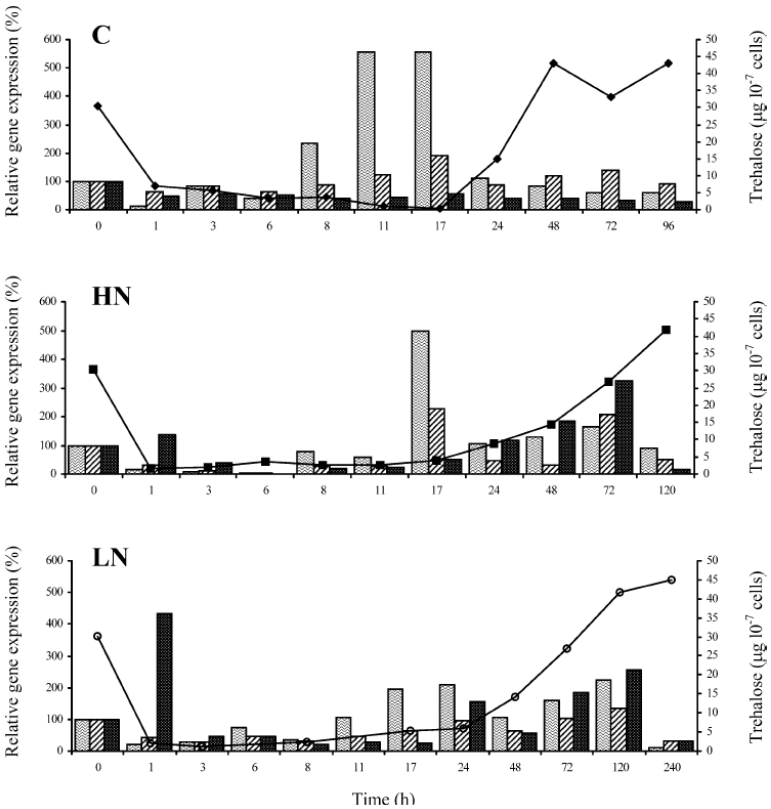

Fig.2. Changes in gene expression during *C, HN* and *LN* fermentations.

***TPS1* (*shaded bars*), *NTH1* (*cross-hatched bars*) and *NTH2* (*black bars*) are expressed relative to their expression at time 0 (prior to must inoculation). Trehalose values at the sampled timepoints are indicated.**

This gradual increase in trehalose was not simultaneous with the expression of *TPS1* gene in any fermentation. As in the control, in both nitrogen-restricted and nitrogen-excess conditions, the increase in *TPS1* expression appeared much earlier than the increase in trehalose. Generally, when trehalose started to accumulate the *TPS1* expression was decreased, except in LN when the expression was maintained. During

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this period of trehalose synthesis in HN and LN fermentations the expression of both trehalases were also increased, in a very different pattern than that of C fermentation. In the three situations, the expression of the studied genes was very low at the end of the fermentation.

DISCUSSION

Under winemaking conditions, yeasts must survive a changing environment, which is mainly characterised by a high sugar (200 g l^{-1}) content and a low nitrogen (<300 mg l^{-1}) content. Yeast growth represents a progressive depletion of essential nutrients, leading to a number of physiological changes that result in the change to a stationary phase (Pretorius 2000). In our previous study, glucose did not show any relation to trehalose accumulation (Novo *et al*., 2003), while nitrogen was exhausted when trehalose synthesis was triggered. Parrou *et al.* (1999) have already stated that the complete exhaustion of the nitrogen source appears to be the primary condition for triggering the activation of trehalose anabolism when nitrogen is the limiting nutrient. We also confirmed these results, but the possible link between nitrogen level and trehalose metabolism had to be further analysed (Novo *et al.,* 2003). We therefore studied the trehalose metabolism in the presence of low nitrogen (LN, 5-fold less YAN than the control media) and high nitrogen (HN, 4-fold YAN than control).

As expected, the three fermentations proceed similarly, with a slower fermentation when nitrogen was very limiting. Control fermentation could not be considered as nitrogen-limited because biomass and fermentation rate was very similar to the HN fermentation. Instead, LN fermentations were clearly nitrogen-limited, as for the slow rate and the longest time of total fermentation. However, in all the fermentations the nitrogen uptake rate was the same and occurred at the beginning of the fermentation, which could be understood that in the three fermentations nitrogen uptake proceeds at the maximum possible rate.

We expected that in the low N fermentations, as nitrogen starvation occurs earlier, trehalose accumulation would proceed faster, while in the high N fermentations there would be no trehalose accumulation, or at least that it would be delayed. Surprisingly, our results showed that the nitrogen content in the media did not affect trehalose

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metabolism. All the conditions had the same trehalose pattern even if nitrogen was in large excess or completely consumed. However, there was a common feature: trehalose accumulation started when cells entered the stationary phase. We can therefore conclude that the slow down in growth was the key point in trehalose metabolism during alcoholic fermentation. Recent studies in fed-batch cultures showed that cells growing at low carbon fluxes accumulated trehalose and glycogen concomitant with an increase in the duration of the G_1 phase (Paalman *et al.,* 2003).

The net accumulation of trehalose implies several mechanisms, such as (i) moderate induction of Tps1p activity, (ii) inactivation of neutral trehalase and (iii) increased availability of glucose-6P, the substrate of Tre6P synthase (François *et al*., 1991; Huang *et al.,* 1997). The expression of most of the genes encoding enzymes of trehalose metabolism is co-regulated. At the transcriptional level, *STRE* elements have been identified at their promoters—three for *NTH1* and six for *TPS1* (Estruch 2000; Parrou *et al.,* 1997; Zähringer *et al.,* 2000). Our data show a clear induction of *TPS1* just before trehalose synthesis in all conditions. Coregulation between *TPS1* and *NTH1* is not so clear, however it could be observed in some given points (17 hours in Control and HN fermentations). On the other hand, both HN and LN conditions share a common trend at the end of fermentation which is an induction in the expression of all three genes.

Our results showed expression of these genes in a media with plenty of sugar. This induction argues against the claim that these genes are under 'catabolic repression'. A similar conclusion was previously reported by Parrou *et al.* (1999). Thevelein and Hohmann (1995) developed a concept which they called the 'fermentable growth medium induced pathway' (FGMIP). This is defined as one or more signal transduction pathways, which are activated by a specific combination of nutrients, readily fermentable sugars and all the other nutrients required for growth. The authors considered that this was different from the general glucose repression and RAS-adenylate cyclase pathways. In these conditions a fine regulation of glycolisis is probably needed to adjust the rate to the changing conditions of nutrient availability. Trehalose metabolism, and especially trehalose synthase and its product Trehalose 6 Phosphate, have been related to the fine tuning of glycolysis (Blazquez *et al*., 1993). In this situation, increased levels of both enzymes would most likely be required, so the induction of gene expression should be expected. As the alcoholic fermentation

proceeded after 24 hours of inoculation (fast uptake of glucose and ethanol production), increased expression of the trehalose genes was observed between 14 and 19 hours. This can be an enzyme accumulation system to allow fine-regulation afterwards by the most efficient mechanism of activation/inactivation, which would tune the glycolysis pathway during the fermentation.

Clearly, however, in the winemaking conditions, nitrogen deprivation is not the only limiting factor. Other nutrients or growth conditions are needed to understand the changes in trehalose metabolism and, therefore, the changes from the initial growth phase to the stationary phase that involves the massive glucose uptake and its metabolisation characteristic of alcoholic fermentation. However, from our results it is also clear that changes in trehalose metabolism are strongly related to the metabolic activity of the yeast cells and the shift from the exponential phase to the stationary phase during fermentation. Therefore, if we could better understand the trehalose metabolism we could better analyse the possible performance and characteristics of an alcoholic fermentation. Further studies are under way in our laboratory to determine how a better understanding of the trehalose metabolism can improve yeast performance during alcoholic fermentation.

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CHAPTER III

Early transcriptional response of wine yeast after rehydration: osmotic shock and metabolic activation

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ABSTRACT

The inoculation of Active Dry Wine Yeast (ADWY) is one of the most common practices in winemaking. The molecular adaptation that yeasts undergo from the ADWY to the fermenting state has not been investigated. We have therefore used DNA microarrays technology to examine the genetic expression patterns of a commercial ADWY strain after rehydration in water and exposure to different media. After ADWY rehydrated for 30 minutes, a further hour in water after rehydration did not lead to any relevant changes in the global gene expression. Expression changes in rehydrated cells upon incubation for one hour in a sorbitol solution at the same osmotic pressure as in complete must were rather limited, while the presence of fermentable carbon sources or the complete medium (synthetic must) produced very similar transcriptional responses. The two main responses were the activation of some genes of the fermentation pathway and of the non-oxidative branch of the pentose pathway, and the induction of a huge cluster of genes related to ribosomal biogenesis and protein synthesis. The presence of cycloheximide in fermentable medium produced a similar but stronger transcriptional response. These major modifications were probably the result of the sugar-induced activation of both the Ras-cAMP and the Tor1/2 -dependent signalling pathway. While the viability of rehydrated cells incubated for one hour in these different media was similar, yeast vitality, which expresses the fermentative capacity of the yeast, showed a positive correlation with the availability of a fermentable carbon source.

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INTRODUCTION

Using Active Dry Wine Yeast (ADWY) is a widespread practice in wine technology. It replaces spontaneous fermentations in order to obtain more reproducible wines by better control of alcoholic fermentation [1]. ADWY is obtained from selected natural wine yeast that is propagated in molasses and subjected to a dessication process. The molecular response of yeast to this propagation and desiccation processes is being studied [2,3], but it is well established that this desiccation process causes a water deficit that leads to the arrest of cellular functions. A rehydration period is therefore required for the resumption of these cellular functions and for the full recovery of membrane functionality [4]. One of the main characteristics for the optimal performance of wine fermentation is a set of physiological and metabolic changes occurring immediately upon the inoculation of yeast cells in the musts [5]. Using ADWY, the current process is to rehydrate the dried yeast by incubation in water at 37°C for a short period of time, followed by seeding the rehydrated cells into large fermentation tanks. Due to the high sugar concentration in the musts, *ca*. 200 g l^{-1} of an equimolar mixture of glucose and fructose, these rehydrated yeast are subjected to very high osmotic pressure, as documented by a rapid induction of *GPD1* [6,7]. However, adaptation to osmotic stress is probably not the only mechanism elicited in rehydrated yeast since glucose and fructose themselves are also strong elicitors for the induction of other signalling systems. Among these signalling systems, there are the Ras-cAMP pathway [8], characterised by the rapid mobilisation of trehalose [9], the Snf1-dependent glucose repression pathways [10] and the so-called "Fermentable-growth-medium induced pathway" [11] which likely corresponds to the Tor1/Tor2 mediated signalling pathway [12]. How these different signalling pathways behave upon rehydration of ADWY and subsequent inoculation of rehydrated cells into wine medium is a challenging question.

Given the economic importance of reproducible end products in winemaking, there is enormous interest in achieving the fast metabolic recovery of the "active" wine dry yeast, which ensures a rapid imposition of the inoculated strains. This requires a thorough molecular and biochemical analysis of the rehydration process. When ADWY are inoculated into the must, they are subjected to low pH and high hyperosmotic stress due to the high sugar content. As the fermentation proceeds, they also have to endure progressive nutrient depletion (oxygen, nitrogen, carbon) and ethanol production [5].

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Transfer to a new glucose-enriched medium leads to an increase in the transcription of genes related to protein synthesis. However, only a small number of proteins are effectively synthesised [13]. Much of the transcriptional response observed during the lag phase is due to the presence of glucose in the new medium [14]. Initial stresses do not lead to a permanent stress response, since the response to osmotic stress after inoculation is rapid and transient [6]. Only from the stationary phase, when yeast integrates numerous stress signals (ethanol, osmotic, acid, nutrient depletion), is a general stress response established [15-17].

Successful ADWY adaptation implies a metabolic reorganization in order to maintain cell activity (vitality). Most aspects of this molecular response to rehydration and early adaptation to musts remain unknown. The only two published transcriptional studies on yeast rehydration have been conducted in the presence of glucose, so their transcriptional responses are mainly related to this factor [3,18]. However, the transcriptional response strictly due to the rehydration process, which is using plain water, has not been studied yet.

DNA microarray is a powerful tool to deal with this problem at genome scale. By DNA microarray, we are able to examine the transcriptional changes that take place during the rehydration of dried yeast in water and subsequent molecular events in the early stage after the inoculation of rehydrated cells in the must medium. Because of the possible superposition of molecular signals (i.e. osmotic pressure, sugars, nitrogen sources), we considered it useful to examine these molecular events taking place in this process in selective media, beginning with the simplest (water) to the most complex synthetic must medium. Finally, cycloheximide (CYH), which acts as a protein synthesis inhibitor by translation blocking at 80S peptidyl transferase [19], was also used in our DNA array experiments in order to study the transcriptional effect of limiting the protein synthesis. In addition, we also related the metabolic changes to the translational events, which were analysed by yeast vitality.

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MATERIALS AND METHODS

Strain and rehydration media

The *Saccharomyces cerevisiae* strain QA23 (Lallemand S.A., Canada) supplied as ADWY was rehydrated in water at 37ºC for 30 minutes in accordance with the manufacturer's instructions. The rehydrated yeast was then inoculated at 10^8 cells ml^{-1} in 250 ml of different sterilized media contained in a 500ml-Erlenmeyer flask plugged with cotton. Incubation at 20ºC lasted for 1 hour without shacking. The following media were used: (i) water, (ii) water containing 200 g l^{-1} of sorbitol, (iii) water containing 100 g l^{-1} of glucose and 100 g l^{-1} of fructose, and (iv) a synthetic must (SM) prepared according to Belly *et al*. (1990) [20] containing 200 g l^{-1} of reduced sugars (glucose and fructose at 50%) without anaerobic factors and buffered at pH 3.3 with NaOH. In addition, cycloheximide (CYH) was added to (v) water and (vi) synthetic media (SM + CYH) at a final concentration 100 µg ml^{-1}.

Yeast Sampling, RNA extraction and cDNA synthesis

Yeast samples (50 ml) were harvested by centrifugation 3000 x g for 3 min at 4°C. The cell pellet was sucked into the remaining few µl of medium liquid by a Pasteur pipette, thrown as individual drops directly into liquid nitrogen and stored at –80 ºC until use. For RNA extraction, three frozen droplets were disrupted in microDismembrator (Braun, Melsungen) via mechanical disruption. A commercial kit (RNAeasy mini kit, Qiagen) was used to extract the RNA from the pulverized cells in accordance with the manufacturer's instructions. The quantity and the quality-control of the extracted RNA were checked by micro capillary electrophoresis using a Bioanalyzer 2100 (Agilent Technologies, Wilmington, DE, USA). Fluorophore-labelled cDNA probes were prepared from total RNA (20 µg per reaction) using the CyScribe First-Strand cDNA Labelling (Amersham Biosciences) in the presence of either Cy3-dUTP or Cy5-dUTP. Non-reacting mRNA was degraded by RNAse and non-incorporated nucleotides were removed using a CyScribe GFX Purification Kit (Amersham Biosciences).

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Microarray experiments

The DNA chips were manufactured at the Biochip platform of Toulouse-Genopole on dendrislides using 70-mer oligonucleotides representing ~ 99 % of the yeast genome purchased from Operon Inc (the list of corresponding genes is accessible at http://biopuce.insa-toulouse.fr/oligosets/). Hybridization was carried out in an automatic hybridization chamber (Discovery from Ventana Medical System, Inc) for 10h at 42°C. After hybridization, slides were washed in 2 x SSC/0.2% (v/v) SDS, immersed briefly in isopropanol and dried under a stream of air. To reduce systematic and biological variability, each experiment was done twice from independent ADWY, which was rehydrated in water for 30 min. Total RNA was extracted for these two similar conditions but from two independent cultures. One of these was reverse transcribed and labelled with dCTP-CY3 and the other was labelled with dCTP-CY5 (the so-called dye-switch method). Every gene was thus represented by 4 raw data from two independent DNA arrays. For all slides, yeast cells that had remained for one hour in water after rehydration were taken as a reference. After hybridization, DNA arrays were sequentially washed at room temperature in 2X SSC, 1X SSC and isopropanol, and then dried using compressed air. The hybridization signal was detected by scanning using a GenePix 4000B laser Scanner (Axon Instruments), and the signal quantification was converted into numerical values using the integrated GenePix software version 3.01.

Microarray data analysis

The raw data are presented at http://biopuce.insa-toulouse.fr/supdata/yeastrehydration/, which provides full details of normalisation and statistical regimes using our home-made Bioplot software. This software is an online web service available to all users of the Biochips platform. 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 the 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. Common changes in gene expression were analysed by a Venn diagram using Bioplot software. Genespring version 4.2 software (Silicon genetics Inc, USA) was used for visualization and hierarchical clustering.

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Viability and vitality assay

Cell viability was determined after plating on YPD agar using an automatic spiral plater (AES Laboratoire, Combourg, France). Colony formation units (CFU) were counted using the ProtoCOL SR/HR counting system software version 1.27, supplied by Synbiosis (Cambridge, UK). To measure cell vitality during incubation in the growth media, the *Bac*-Trac® 4300 microbiological analyzer (SY-LAB Instruments, Austria) was used. This device is based on the electric impedance measurement and detects the CO_2 produced by an indirect technique [21]. To this end, yeast cells were inoculated in a vial containing 5ml of YPD (20 g l^{-1} glucose, 20 g l^{-1} peptone and 10 g l^{-1} yeast extract) at a level of $2x10^7$ cells ml^{-1}. The CO_2 produced reacts with a solution of KOH 0.02% (w/v), which leads to measurable changes in the electric conductivity of this solution. The impedance level was monitored every 10 minutes by *Bac*-trac and a curve expressing the percentage decrease in impedance over time was drawn. As an arbitrary parameter we chose the time needed to reach a 50% decrease in total impedance at 13ºC. We worked at this low temperature because the metabolism is slower and differences in vitality are clearly shown. Viability and vitality assays were carried out in triplicate. The results were statistically analysed by one-way ANOVA and the Scheffé test from the statistical software package SPSS 13.0. Statistical significance was set at $P < 0.05$.

Gene expression analysis by real-time RT-PCRq

The gene expression of *GON7, RPS22A, GCY1, PYK1* and *HSP12* were determined using the real-time quantitative PCR technique. Actin (*ACT1*) was used as the reference gene, since the expression of this gene was found to be relatively unchanged. The PCR primers (Table 1) were all designed with the available GenBank sequence data and the Primer Express software (PE Applied Biosystems, Cheshire, UK) according to the PE Applied Biosystems guidelines. RNA was extracted from 10^8 yeast cells as previously described by Siekstra *et al.* (1992) [22] and resuspended in 50 µl diethylpyrocarbonate (DEPC)-treated water. To purify total RNA, genomic DNA was removed using a High Pure Isolation kit (Roche, Mannheim, Germany) according to the manufacturer's protocol. Purified RNA concentrations were determined measuring at 260 nm using a GenQuant spectrophotometer (Pharmacia, Uppsala, Sweden) and verified electrophoretically on 0.8% agarose gels. cDNA synthesis was carried

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out using reverse transcriptase Superscript II (Invitrogen, Carlsbad, Calif.) in a GenAmp PCR System 2700 (Applied Biosystems, Foster City, Calif.), following the protocol previously described by Novo *et al.* (2005) [23]. All samples were analysed in triplicate and the coefficient of variation in all analysed samples was less than 10%.

Primers	Nucleotide sequence (5' to 3')
ACT1-F	TGGATTCCGGTGATGGTGTT
ACT1-R	CGGCCAAATCGATTCTCAA
GON7-F	GCCCAGTGACCATGTGCTAA
GON7-R	CGTGCGTTCCGGTTCTG
RPS22A-F	AGACAATTCGGTTACGTCATCTTG
RPS22A-R	AACGTGCTTTCTTCTGGCTTCT
GCY1-F	TTGAAAGATGGCTACCGACACA
GCY1-R	TTGATGGCTTGACCGACTTG
PYK1-F	ATGTCGATTTGCCAGCTTTGT
PYK1-R	GACCATGTGGACACCGTTCTT
HSP12-F	GGCAGACCAAGCTAGAGATTACATG
HSP12-R	AACATATTCGACGGCATCGTT

Table 1. Primers used in this study

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RESULTS AND DISCUSSION

Experimental design and global overview

The usual winemaking practice of seeding grape musts in fermentation tanks with exogenous yeast is to rehydrate ADWY for 30 min in water at 35-40ºC and then transfer these rehydrated cells to the must. Our aim was to examine, under several physiological conditions (see materials and methods), the transcriptomic modifications that take place one hour after the rehydration. Thus, we have carried out a microarray to compare rehydrated yeast (30 min. at 35-40ºC) with the permanence of yeast an extra hour in water. This microarray revealed minor transcriptional effects since the expression of only 9 genes was modified. This condition was therefore taken as a control (see http://biopuce.insa-toulouse.fr/supdata/yeastrehydration/ for further details). This indicates that 30 minutes of rehydration were enough to rebuild the 'water-deficit' of dried yeast. The global changes in gene expression in the three other conditions (i.e. transfer to sorbitol, transfer to a concentration of 200 g l^{-1} obtained with equimolar amounts of glucose and fructose, and transfer to a complete synthetic must) showed that the presence of fermentable sugars alone induced larger changes in gene expression than the presence of synthetic must (121 genes compared to 71), while transfer to sorbitol produced a weaker gene change in expression (47 genes). With regard to functional classification according to MIPS [24], the differentially expressed genes were mainly divided into three categories: protein synthesis, metabolism and unclassified proteins (Table 2). We can see that the vast majority of genes involved in protein synthesis were overexpressed, which is consistent with the fact that the presence of sugar promotes growth [25].

	SM		Gluc/fruct		Sorbitol		SM + CYH	
Functional categories	**Over**	**Under**	**Over**	**Under**	**Over**	**Under**	**Over**	**Under**
Unclassified proteins	4	10	15	17	12	2	63	16
Cellular transport	4	8	5	7	9	2	19	2
Metabolism	9	6	11	21	3	2	23	9
Transcription	7	1	10	6	2	4	20	5
Cell cycle and DNA processing	4	0	5	10	3	1	12	8
Cell type differentiation	1	3	3	2	2	1	11	4
Protein synthesis	22	0	23	4	2	1	47	7
Protein fate	11	7	6	10	2	3	12	6
Ionic homeostasis	0	0	0	2	3	0	4	4
Cell rescue, defense and virulence	2	4	2	12	4	0	2	6
Energy	3	4	3	8	2	1	2	8
Cellular communication	0	0	0	0	0	0	6	3

Table 2. Distribution of the number of modified genes according to the functional categories of MIPS.

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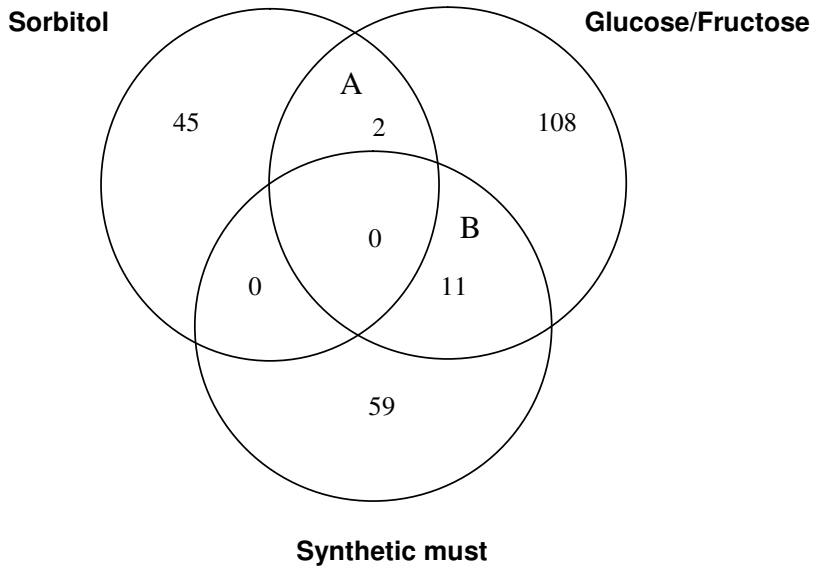
As we can see in the Venn diagram in Figure 1, surprisingly no gene was found in all three conditions. Only two genes *CDC55* and *YLR057w* were commonly up-regulated in response to sorbitol and to reducing sugars, and none was commonly altered by both sorbitol and synthetic must. *CDC55* encodes for a regulatory subunit of protein phosphatase 2A, which is indispensable for spindle checkpoint activity [26] and is involved in the initial steps of glucose induction [27]. The phosphatase activity of the phosphatase 2A complex is regulated by the TOR-kinase pathway. Activation of the Tor kinases leads to the inhibition of the corresponding phosphatases [28]. This phosphatase activity would prevent some of the most important events of glucose induction, such as *HXT1* activation [27]. Therefore, the induction of the *CDC55* in response to sorbitol and to reducing sugars (without the presence of nitrogen) can be attributed to inactivation of the TOR-kinase pathway.



A

ORF	Gene	Description	Sorbit.	G/F	SM
YLR057W		Hypothetical ORF. Mutants present intermediate defect while growing on minimal media	2.32	2.32	2.13
YGL190C	**CDC55**	Protein phosphatase 2A regulatory subunit B. Involved in cellular morphogenesis. Cell Division Cycle	2.29	2.4	1.83

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B

ORF	Gene	Description	Sorbit.	G/F	SM
YJL190C	**RPS22A**	Ribosomal Protein of the Small subunit	-1.12	10.78	11.16
YLR249W	**YEF3**	Translation elongation factor 3. Contains twoABC cassettes, and binds and hydrolyses ATP	-1.30	4.14	4.09
YAL038W	**PYK1**	Pyruvate kinase, functions as a homotetramer in glycolysis to convert phosphoenolpyruvate to pyruvate, the input for aerobic (TCA cycle) or anaerobic (glucose fermentation) respiration. Cell Division Cycle	-1.11	3.43	3.83
YPL220W	**RPL1A**	N-terminally acetylated protein component of the large (60S) ribosomal subunit, nearly identical to Rpl1Bp and has similarity to E. coli L1 and rat L10a ribosomal proteins; rpl1a rpl1b double null mutation is lethal	1.07	3.36	3.31
YBR181C	**RPS6B**	Protein component of the small (40S) ribosomal subunit; identical to Rps6Ap and has similarity to rat S6 ribosomal protein	1.20	3.22	2.85
YHR162W		Hypothetical ORF. Mitochondrion	1.35	2.69	2.38
YOR133W	**EFT1**	translation elongation factor 2 (EF-2). Ribosome	-1.06	2.08	2.27
YGL080W	**FMP37**	Found in Mitochondrial Proteome	-1.06	-2.22	-2.04
YKR018C		Hypothetical ORF.	-1.45	-2.44	-2.56
YOR008WB		Not found	1.71	-2.94	-2.56
YNL194C		Hypothetical ORF.	-1.33	-3.45	-3.23

Figure 1. Venn diagram and list of the genes involved in the common responses to osmotic shock and the availability of different carbon sources with *p*-value< 0.05.

On the other hand, the number of differentially expressed genes shared between glucose/fructose medium and the synthetic must was 11, 7 of which were overexpressed and 4 were underexpressed. Five of the overexpressed genes corresponded to up-regulation of the protein synthesis machinery and the other two genes were implicated in glucose metabolism. The down-regulated genes encoded proteins of still unknown functions.

Analysis of the concordance between the three conditions that include fermentable sugars (synthetic must, SM+CYH and reducing sugars; see Figure 2) shows a significant overlap in the expression of 30 genes. A third of these were coding for proteins of unknown function and another third (including the two common genes for the three conditions) were ribosomal proteins (8) or elongation factors (2). All these common genes involved in protein synthesis were over-expressed. Three genes were related to ubiquitin or ubiquitin-like processes (*HBT1, UBC8, UBP13*) and two genes

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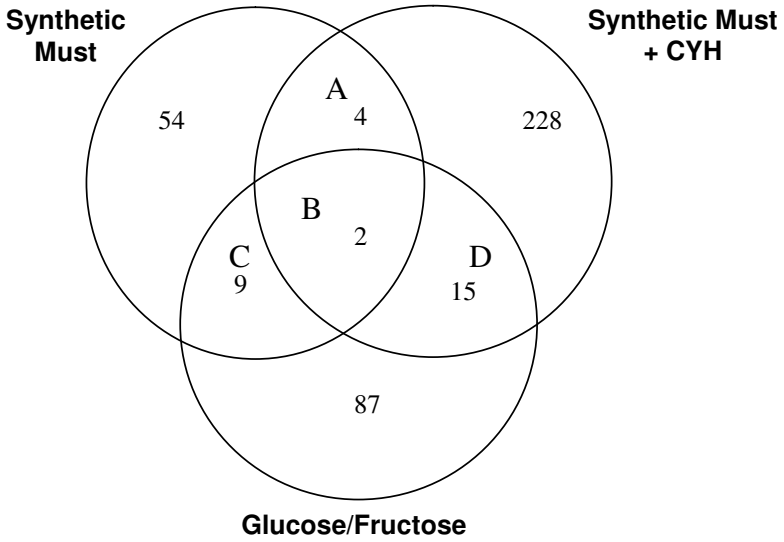
were involved in glucose metabolism (*HXT5* and *PYK1*). Finally, *ARO10*, which is the first specific step of the Ehrlich Pathway, was under-expressed.



A

ORF	Gene	Description	G/F	SM	SM+CYH
YDL223C	**HBT1**	Substrate of the Hub1p ubiquitin-like protein that localizes to the shmoo tip (mating projection)	-3.33	-3.13	-2.56
YEL012W	**UBC8**	Ubiquitin-conjugating enzyme that negatively regulates gluconeogenesis	-2.63	-2.27	-2.33
YDL083C	**RPS16B**	Protein component of the small (40S) ribosomal subunit	4.08	3.36	4.36
YLR167W	**RPS31**	Fusion protein that is cleaved to yield a ribosomal protein of the small (40S) subunit and ubiquitin.	2.92	4.44	3.54

B

ORF	Gene	Description	G/F	SM	SM+CYH
YBR181C	**RPS6B**	Protein component of the small (40S) ribosomal subunit	3.22	2.85	2.9
YJL190C	**RPS22A**	Protein component of the small (40S) ribosomal subunit	10.78	11.16	10.89

C

ORF	Gene	Description	G/F	SM	SM+CYH
YNL194C		Hypothetical protein	-3.45	-3.23	-3.33
YOR008WB		Hypothetical protein	-2.94	-2.56	-3.03
YKR018C		Hypothetical protein	-2.44	-2.56	-1.32
YGL080W	**FMP37**	The non-tagged protein was localized to the mitochondria	-2.22	-2.04	1.00
YOR133W	**EFT1**	Elongation factor 2 (EF-2), catalyzes ribosomal translocation during protein synthesis	2.08	2.27	2.8
YHR162W		Hypothetical protein	2.69	2.38	2.18
YPL220W	**RPL1A**	Component of the large (60S) ribosomal subunit	3.36	3.31	2.48
YAL038W	**PYK1**	Pyruvate kinase	3.43	3.83	2.37
YLR249W	**YEF3**	Translational elongation factor, stimulates binding of aminoacyl-tRNA to ribosomes by releasing EF1from the ribosomal complex	4.14	4.09	4.64

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D

ORF	Gene	Description	G/F	SM	SM+CYH
YGR085C	**RPL11B**	Protein component of the large (60S) ribosomal subunit	-2.63	-1.18	-5.26
YPL017C		Putative S-adenosylmethionine-dependent methyltransferase of the seven beta-strand family	-2.13	-2.44	-3.13
YBR147W		Putative protein of unknown function; YBR147W is not an essential gene; resistant to fluconazole	-6.67	-4.55	-3.03
YHR096C	**HXT5**	Hexose transporter with moderate affinity for glucose, expression induced by a decrease in growth rate.	-5.88	-4.76	-2.86
YGR043C		Putative protein of unknown function	-7.69	-4.35	-2.70
YBL067C	**UBP13**	Putative ubiquitin-specific protease	-2.94	-1.56	-2.63
YDR380W	**ARO10**	Phenylpyruvate decarboxylase, catalyzes decarboxylation of phenylpyruvate to phenylacetaldehyde.	-5.00	-3.45	-2.50
YOL052CA	**DDR2**	Multistress response protein, expression is activated by a variety of xenobiotic agents or physiological stresses	-3.33	-4.35	-2.44
YML128C	**MSC1**	Protein of unknown function	-4.17	-3.23	-2.17
YBL092W	**RPL32**	Protein component of the large (60S) ribosomal subunit	2.04	1.7	3.21
YCR043C		Hypothetical protein	3.39	2.24	3.36
YFL034CA	**RPL22B**	Protein component of the large (60S) ribosomal subunit	2.76	2.77	3.6
YLR042C		Hypothetical protein	4.81	3.06	4.02
YHR139CA	**SPS100**	Protein required for spore wall maturation; expressed during sporulation; may be a component of the spore wall	2.69	1.56	5.02
YHR126C		Hypothetical protein	9.02	5.25	5.77

Figure 2. Venn diagram of the responses related to the presence of cycloheximide, comparing Synthetic must, Synthetic must with Cycloheximide and glucose/fructose conditions.

Transcriptional changes due to rehydration of dried yeast: extended period in water

Surprisingly, after the initial rehydration and for a further 60 minutes in water, the expression profile hardly changed. The under-expressed genes presented about -2.5 expression ratios with miscellaneous functions such as UDP-glucose epimerase (*GAL10*), protein binding for cytoskeleton stability (*SAC2*) or mRNA binding (*SRO9*) and splicing (*PRP8*). Rehydration protocols of 30 minutes in warm (35-40ºC) water are generally recommended by the ADWY suppliers and this is evidently a sufficient period of time. However, the near absence of changes in the transcriptional profiles clearly indicates that the cell is fully recovered from the dessication-rehydration process. In

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addition, the relative short lives of most of the yeast mRNA [29] could lead us to conclude that is no further degradation of mRNA after ADWY rehydration.

Transcriptional changes as caused by osmotic pressure to rehydrated yeast.

As a specific response to sorbitol, it is relevant to notice that the transcriptional response was relatively mild and it implies an expression change of genes belonging to various categories (metabolism, transport, transcription, protein synthesis, etc). The functional category with the highest number of affected genes (13, all of which were up-regulated), however, was the transport facilitation and transport route. Particularly relevant in this list of genes were *MEP3*, which encodes a low affinity ammonium permease, and *AGP3*, which encodes a protein of the amino-acid permease family, plus several genes encoding cation transporters (*CTR2* for copper, *CCH1* for calcium and *SMF3* for vacuolar iron) and drug exporters such as *YOR378w*, which encodes a protein with a strong similarity to the aminotriazole resistance protein, and *NFT1*, which encodes an ABC transporter. Altogether, this suggests that the control of ion homeostasis is critical for osmoadaptation. We also found genes related to vesicular trafficking, such as *ARL3* (a GTP-binding protein/GTPase), *VTH1* (a potential membrane glycoprotein), and *ERV25* (a component of the COPII-coated vesicles). In this sorbitol cluster, we identified one gene, *GON7*, which encodes a protein related to the broader class of proteins referred to as hydrophilins and which also includes *SIP18* and *GRE1*. While the expressions of *SIP18* and *GRE1* were not significantly modified, *GON7* exhibited a specific induction of 2.95 after 1 hour's incubation in sorbitol medium. Gon7p is a protein of unknown function that is reported to be involved in the transfer of mannosylphosphate groups onto N-linked oligosaccharides and is also reported to be involved in responding to osmotic stress [30].

Difference in the adaptation of rehydrated cells to the presence of fermentable carbon sources and to a complete growth medium

In yeast, glucose and related rapidly fermentable sugars trigger several signal transduction pathways that ultimately result in the activation of growth [31]. An essential signalling mechanism is the Ras-cAMP pathway, which exerts a positive

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control on the ribosomal protein synthesis and carbon metabolism while negatively affecting the expression of genes implicated in stress [25]. Accordingly, the first noticeable change after exposure of rehydrated cells to glucose/fructose and SM media was a pronounced activation of the protein synthesis machinery, as witnessed by the up-regulation of 109 genes encoding ribosomal proteins and elongation factors in either of the two conditions. These results were consistent with previous microarray data [18, 31, 32] that showed that the induction of ribosomal genesis is one of the early events triggered by glucose added to non-growing cells. The TOR-pathway controls the expression of ribosomal protein genes. Under favourable growth conditions, the TOR-pathway signals via PKA to inhibit the kinase *YAK1*, and this effect leads to the inactivation of several repressors of ribosomal protein genes, thus resulting in an activation of its transcription [33]. However, the fact that only 5 genes that belong to this family were found in both conditions indicated that the presence of other nutritional elements in the SM medium may have some impact on these transcriptional modifications. In accordance with this assumption, it is known that the activation of genes encoding ribosomal proteins upon glucose up-shift comprises two phases: an initial and transient response independent of the actual growth potential, and a sustained response that is dependent on growth and requires both glucose and an adequate nitrogen source [34]. Under favourable growth conditions, the TOR-pathway is active, resulting in initiation and progression from G_1. Inactivation of the TOR-pathway occurs because of nutrient limitation and results in several physiological changes that are characteristic of starved cells, including the inhibition of translation initiation, the inhibition of ribosome biogenesis, specific changes in transcription, sorting and turnover of nutrient permeases, the accumulation of storage carbohydrates and the induction of autophagy [35]. The TOR-pathway is therefore considered as a central nutrient sensing that is especially important with respect to nitrogen availability but that also seems to play a role in carbon metabolism [12].

With regard to carbon metabolism (Figure 3), we observed a potent activation (albeit without statistical significance, with *p*-values between 0.06 and 0.28) of the two isoforms of glycerol 3-phosphatase (*GPP1* and *GPP2*) after incubation in glucose/fructose (ratios 6.99 and 8.00 for *GPP1* and *GPP2*, respectively), in SM (ratios of 7.9 and 10.36, respectively) and in SM with CYH (ratios of 9.45 and 9.16, respectively) media, but not upon incubation in sorbitol medium. If we take into account

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that *GPP2* can be regulated both by PKA and by osmotic stress, these results indicate that the up-regulation of *GPP1* and *GPP2* was due to activation of the PKA pathway. Though these results suggest a possible flux-up of the glycerol pathway in response to a fermentable carbon source, it is odd that the transcriptional modification was on *GPP1* and *GPP2* and not in the increase of *GPD1* and *GPD2*, which encode the rate-limiting enzyme in glycerol metabolism [36].

The activation of glycolysis was suggested by the up-regulation of *HXT1*, which encodes low affinity glucose, and a set of glycolytic genes, including *PFK2* and *PYK1*. In accordance with this activation, and expected as a response to assimilable sugars, we observed a common and important induction of the pyruvate kinase gene *PYK1*, also named *CDC19*, which is a key enzyme in glycolysis and is necessary for cell cycle progression. In the glycolytic pathway, *PYK1* catalyses the irreversible reaction which converts phosphoenolpyruvate to pyruvate. The other key reaction in the control of glycolytic flux is catalysed by phosphofructokinase, which is encoded by *PFK1* and *PFK2* (belonging to the regularory and the catalytic subunits, respectively), catalyses for the first irreversible step and is specific for glycolysis [37]. Our results showed that *PFK1* was induced in conditions in which sugar was present and was not modified in the presence of sorbitol. *PFK2* was nearly repressed in the presence of sorbitol and a tendency to induction was found in the presence of carbon sources. At the end of the glycolysis, pyruvate can be further degraded via pyruvate dehydrogenase or pyruvate decarboxylase. The pyruvate dehydrogenase complex, encoded by *PDA1*, was found to be downregulated upon incubation in sugar medium, while the gene encoding the main pyruvate decarboxylase (*PDC1)* was upregulated, which is consistent with a stimulation of fermentation under these conditions. Interestingly, we found that only in the presence of glucose/fructose was the synthesis of acetyl-CoA synthesis probably reduced. This is indicated by the repression of *PDA1* and *PDA2*, which encode pyruvate dehydrogenase (mitochondrial production of acetyl-CoA), *ALD3* and *ACS1*, which encode aldehyde dehydrogenase and acetyl-coA synthetase, respectively (cytosolic production of acetyl-CoA) (Figure 3). According to carbon catabolite repression, the specific gluconeogenic gene, *PCK1*, presented a clear tendency to repression in all conditions in which sugar was present, except in the medium with CYH.

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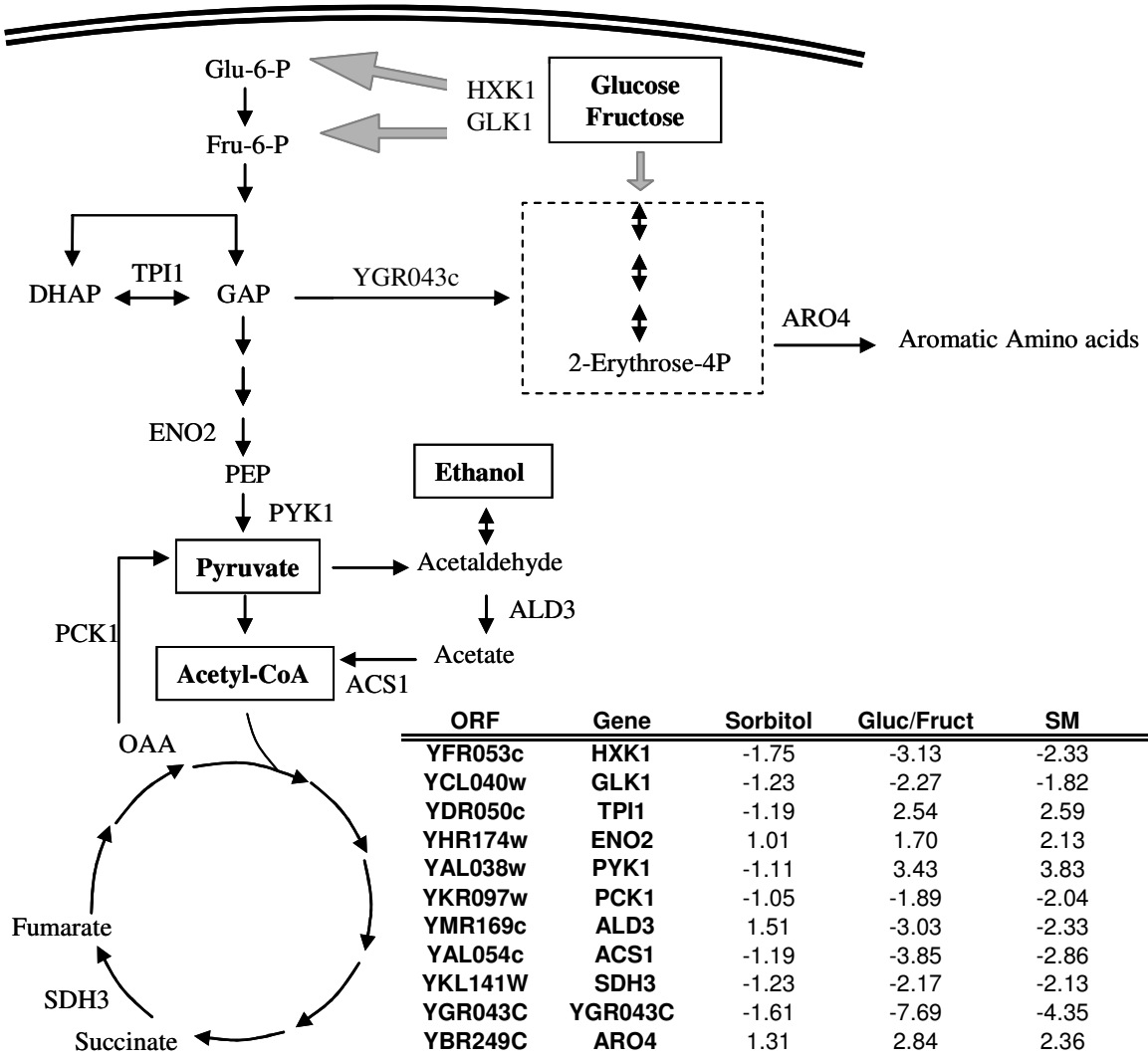
Taking into account the collateral pathways directly related to the pentose phosphate pathway, we also found a common behaviour of gene expression in the conditions in which sugar was present. This is the induction of *ARO4*, which catalyzes the first step in aromatic amino acid biosynthesis. Also, though not induced in a significant way, we found a coordinated induction of *PRS1, 4* and *5*, which are 5-phospho-ribosyl-1(alpha)-pyrophosphate synthetases involved in the super-pathway of histidine, purine and pyrimidine biosynthesis.



ORF	Gene	Sorbitol	Gluc/Fruct	SM	SM + CYH
YFR053c	HXK1	-1.75	-3.13	-2.33	-1.89
YCL040w	GLK1	-1.23	-2.27	-1.82	-1.82
YDR050c	TPI1	-1.19	2.54	2.59	2.38
YHR174w	ENO2	1.01	1.70	2.13	1.40
YAL038w	PYK1	-1.11	3.43	3.83	2.38
YKR097w	PCK1	-1.05	-1.89	-2.04	1.92
YMR169c	ALD3	1.51	-3.03	-2.33	-2.56
YAL054c	ACS1	-1.19	-3.85	-2.86	-1.69
YKL141W	SDH3	-1.23	-2.17	-2.13	-1.45
YGR043C	YGR043C	-1.61	-7.69	-4.35	-2.70
YBR249C	ARO4	1.31	2.84	2.36	2.17

Figure 3. Scheme of carbon metabolism of *S. cerevisiae*, showing the gene expression levels of those genes significantly modified in this study. There are represented the conditions containing a carbon source, this is Sorbitol, Glucose/Fructose, Synthetic must and Synthetic must with cycloheximide. Abbreviations: DHAP, dihydroxyacetone phosphate; GAP, glyceraldehyde-3-phosphate; PEP, phosphoenolpyruvate, OAA, oxaloacetate.

Trehalose mobilisation is part of the developmental program initiated by the cell in the presence of nutrients in the culture medium [38]. The metabolism of trehalose is tightly controlled by trehalases and trehalose synthase through activation by phosphorylation mediated by the stimulation of cAMP-dependent PKA activity, which is a typical response to nutrient availability [39]. However, it seems that protein activation mediated by phosphorylation is the main mechanism of trehalose regulation since no changes in the gene expression of any of the trehalose-regulating enzymes were observed either in this or in our previous study [23].

Importance of genome-wide expression change in the resumption of yeast vitality after rehydration

As our results clearly indicate an activation of the translation machinery upon incubation of rehydrated cells into a sugar medium, we evaluated the true transcriptional effect of cycloheximide. Cycloheximide is widely used as an inhibitor of eukaryotic protein synthesis *in vivo* and *in vitro*, though it is known that cycloheximide interferes with other cellular functions including respiration, ion absorption, amino acid uptake and the interconversion of pyrimidine nucleotides. This effect is often believed to be easily reversible, yet some studies have led to the opposite conclusion and, in fact, CYH kills yeast at a rate which is proportional to their metabolic activity [40]. In this sense, our results seem to confirm that there are no differences at transcriptional level when CYH is present in water. Instead, a considerable effect is seen when present in synthetic medium (SM + CYH). The main category of classified proteins is protein synthesis, most of which are clearly overexpressed genes and related to ribosome biogenesis some with very high ratios (the maximal ratio is 13.9 for *RPS1A*). The presence of cycloheximide in the synthetic must condition did not lead to different behaviour in ribosomal protein gene expression.

The viability and vitality of the cells after the treatments were analysed by plate culturing (viability) and by monitoring impedance changes (vitality). As we started with a pure culture of yeasts that grew well in solid media, plate counting was equivalent to viability (counts under the microscope were also done and the results were similar). There were no differences in viability as a response to permanence in the different

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condition media, since in all cases yeast populations were in the order of $1x10^8$ cfu ml^{-1} (results not shown). In fact, the time tested is too short to produce cell division. Moreover, none of the conditions produced a significant decrease in viable cells.

As for vitality, we considered the capacity of the cells to enter to a full metabolic activity at 13ºC and we found significant differences among the tested media (Figure 4). The condition that significantly reduced the activation time was the inoculation in the synthetic must. This reduction was consistent when tested against any other condition. Also, the presence of reducing sugars increased the vitality of the cells, as is seen by the significant differences referred to both control condition and synthetic must. The extended permanence of the yeast cells in water significantly reduced the vitality in comparison with rehydrated cells, which is the usual practice in cellars. This again indicates the suitability of using this time period. On the other hand, the presence of cycloheximide was the most limiting condition, especially when it was added to water, though vitality recovered faster when it was added to the synthetic must. Interestingly, the lack of a transcriptional response in water with CYH led to a slower vitality, whereas the higher transcriptional response in synthetic must was better for fermentation.

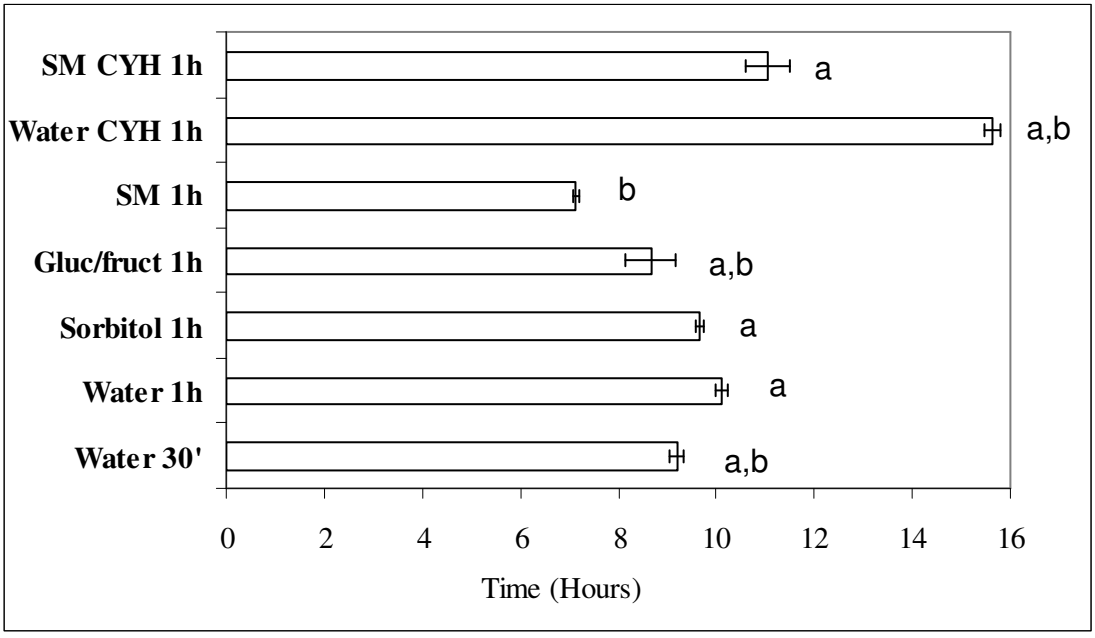

Figure 4. Vitality of yeast after permanence of one hour in the various conditions of study. Statistical analysis: *a* significantly different from the SM condition and *b* significantly different from 1h in water.

Confirmation of the gene transcriptional results by real time RT-PCRq

To validate our results, we compared the expression levels of selected genes obtained from the microarray technique to those determined by RT-PCRq (see Table 3). Genes were selected because of their significant changes in the various conditions and for their relevance in the conditions tested. Both *PYK1* and *RPS22A* were affected when reducing sugars were present in the media. *GON7* is reported to be involved in responding to osmotic stress [30], whereas *GCY1* is an indicator of genotoxicity [41] and *HSP12* is a marker of general stress.

ORF	Gene	Technique	Sorbitol	Gluc/fruct	SM	SM +CYH	Water+CYH
YAL038w	**PYK1**	Array	-1.11	3.43*	3.83*	2.37	-1.27
		PCRq	1.67	4.42*	5.11*	3.28*	1.11
YJL190c	**PRS22A**	Array	-1.12	10.78*	11.16*	10.89*	-1.43
		PCRq	2.19	79.38*	129.67*	486.03*	1.47
YJL184w	**GON7**	Array	2.97*	2.25	1.75	2.07	1.24
		PCRq	-1.16	1.26	1.21	2.97*	1.45
YOR120w	**GCY1**	Array	1.00	-1.11	1.13	2.74*	1.56
		PCRq	1.32	-2.87*	-2.26*	-1.44	1.9
YFL014W	**HSP12**	Array	-1.89	-5.88*	-4.76	-3.33	1.77
		PCRq	1.48	-2.15*	-1.55	1.19	-1.05
YFL039c	**ACT1**	Array	1.05	1.1	1.34	1.35	0.96

* Results with statistically significant differences (p-value < 0.05).

Table 3. Real-time RT-PCRq and microarray analysis of selected genes.

Microarrays and RT-PCR use different standardisation protocols, so it is understandable that the actual numbers could be different, although the trends should be similar. The standardisation of RT-PCR was the expression of actine gene (*ACT1*), which is normally considered to be a housekeeping gene. Instead of the expression of actine gene, the standardisation of microarrays is done by the general expression levels of the whole genome, which is why the value of the *ACT1* gene is included in table 3. In fact, we can see that the levels of *ACT1* hardly change in any of the conditions tested. However, some values deserve further comment because this general reason is not consistent.

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The values of expression changes in *PRS22A* are considerably higher in RT-PCR than in microarrays but even in microarrays the values are among the highest values found. Because of the normalisation used in microarrays, the changes are likely to be "buffered" when these values are so high. In a recent study with large differences between test and reference conditions, normalization with *ACT1* made possible to detect an increase in the overall rate of transcription compared to normalization to all ORFs [14].

The similarity in tendencies and expression levels is clearly observed in most cases. When there are significant differences in one of the techniques, there are also significant or similar (though not significant) changes in the other technique. Only in two conditions (*GON7* in sorbitol and *GCY1* in must with cycloheximide) were significant differences by microarray not confirmed by RT-PCR.

CONCLUSIONS

The rehydration of ADWY is a straightforward mechanism for the yeast to recover its functionality. However, the reaction of the yeast depends on the new medium it encounters after rehydration. The first aspect we have addressed in this study is yeast permanence in water for an extended period. It is obvious that 30 minutes of rehydration is enough for the yeast to fully recover and that longer times in this medium are detrimental. With regard to the transcriptional response, after the first 30 minutes in water the changes are more related to metabolic adaptation and activity than to mRNA degradation.

The yeast transcriptional switch is the presence of fermentable sugars, and this is mostly related to ribosome and protein synthesis. These changes are more outstanding than those already observed and can be considered as a metabolic adaptation. Changes such as those on trehalose metabolism, which are known to develop after glucose sensing, are more related to pre-existing enzymes than to transcriptional changes. Limited, though relevant, changes in glucose metabolism are observed after one hour in glucose medium.

At a transcriptional level, the presence of cycloheximide is especially noticeable when a fermentable carbon source is present. However, the cells can recover easily after treatment with cycloheximide (indicated by yeast vitality) when this is simultaneous with exposure to the fermentable carbon source.

We should point out that initial exposure to full fermentable medium (SM) does not produce too many transcriptional changes, but the response in vitality is faster. Thus, yeast gives an optimal response in terms of economy of transcription related to metabolic activation. Adverse conditions, such as the presence of cycloheximide or only carbon sources, produce a wider transcriptional response.

In terms of recovery of full metabolic activity, the presence of the various media produces notable changes. The estimation of yeast vitality by means of CO_2 production can be useful for testing rehydration conditions that can improve yeast performance, especially when the yeast face adverse developing conditions. This may be the case in winemaking when the must has limited or uneven nutrient concentration, high osmotic pressure, or technological conditions such as low temperatures or high atmospheric pressure.

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GENERAL CONCLUSIONS

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General conclusions:

- Fermentation at 13ºC slows down fermentation kinetics as expected, but does not trigger a stress response by the yeast. In fact, viability is maintained at maximum levels until the end of fermentation and the concentration of trehalose is lower than fermentation control at 25ºC, the optimal temperature.

- The levels of carbohydrates after preadaptation in a complete medium are negligible and produce a shorter lag phase. Thus, intracellular trehalose and glycogen provide initial energy for yeast metabolism, which is important for yeast activation after drying.

- Trehalose is the most easily usable reserve carbohydrate, since glycogen levels are unchanged during the first hour after rehydration. Thus, this initial use of trehalose may indicate the start of metabolic activation.

- Although rehydration in plain water at warm temperatures (35-40ºC) is the most recommended protocol for winemaking, full cell metabolic activity may depend on the presence of other substrates.

- During winemaking, as in other previously studied growth conditions, the highest growth rate correlated with the lowest concentrations of trehalose and glycogen while the highest concentrations were found in the stationary phase.

- Slow-down in growth is a key point in trehalose metabolism during alcoholic fermentation.

- Accumulation of trehalose and glycogen start to accumulte almost simultaneously with the end of the exponential phase, which suggests a link between the inhibition of growth and the triggering of accumulation.

- Neither glucose nor nitrogen exhaustion are involved in the activation of trehalose and glycogen production in winemaking conditions.

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- Fermentation under nitrogen limitation leads to a slow fermentation rate and the total fermentation takes longer. However, the nitrogen uptake rate is not affected by the concentration of nitrogen available in the medium.

- *TPS1*, the gene encoding trehalose-6-phosphate synthase is induced just prior to trehalose synthesis, independently of the concentration of nitrogen found in the medium.

- Under winemaking conditions, co-regulation of *TPS1* and *NTH1*, encoding neutral trehalase, is not clear, and is only observed at certain points.

- Both, nitrogen limitation and nitrogen excess share a common induction pattern for all the genes related to trehalose metabolism (*TPS1, NTH1* and *NTH2*) at the end of fermentation. This suggests that excess nitrogen could also be detrimental to fermentation activity.

- During the early phases of adaptation to the must, the metabolism of trehalose is not regulated at the transcriptional level. Post-translational mechanisms of phosphorylation are responsible for the tightly control of trehalose metabolism.

- ADWY rehydration for 30 minutes is long enough to ensure full yeast recovery. However, an extended period in water is detrimental to the yeast physiology since vitality is significantly reduced.

- Yeast gives and optimised response in terms of economy of transcription related to metabolic activation. Adverse conditions, such as presence of cycloheximide or only carbon sources produce a wider transcriptional response than in synthetic must.

- The transcriptional response of yeast to the presence of fermentable sugars is to switch on the protein synthesis machinery by inducing genes related to ribosomal proteins and elongation factors.

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➢ The major transcriptional modifications are likely the results of the sugar-induced of both the RAS-cAMP and the Tor1/2-dependent signalling pathway.

➢ When glucose is present, the two isoforms of glycerol-3-phosphate (*GPP1* and *GPP2*) are induced. However, in sorbitol this induction does not take place. Thus, we conclude that *GPP1* and *GPP2* genes are regulated by the activation of the PKA pathway as a response to glucose, independently of osmotic stress.

➢ Common responses to glucose/fructose and sorbitol media are related to the inactivation of the TOR-pathway (induction of *CDC55)*, which is only activated in the presence of both carbon and nitrogen sources.

➢ Specific response to sorbitol is limited, because cell transport is the functional category with the highest number of affected genes. Additionally, sorbitol induces the expression of *GON7*, a gene involved in osmotic stress.

➢ Key glycolytic genes (*PYK1, PFK1* and *PFK2*) are induced when fermentable carbon sources are present in the medium. Additionally, ethanol synthesis is stimulated by repressing pyruvate dehydrogenase (*PDA1*) and by inducing pyruvate decarboxylase (*PDC1*).

➢ The specific gluconeogenic gene *PCK1* tends to repress when fermentable sugars are present. However when cycloheximide (CYH) is added to the medium, its transcriptional behaviour is reversed, which results in gene overexpression.

➢ The collateral pathway to glycolysis, the pentose phosphate pathway, is also induced in presence of fermentable sugars. This induction involved the biosynthesis of amino acids, histidine, purine and pyrimidine.

➢ Cycloheximide induces a transcriptional response in a complete medium, capable of ensuring cell growth. The lack of transcriptional response in water with CYH leads to slower vitality whereas the higher transcriptional response in synthetic must with CYH is better for fermentation.

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DISCUSSION & PERSPECTIVES

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Discussion and perspectives:

Wine industry is placed in a very dynamic and competitive global market. During the last century, fundamental innovations in winemaking practises revolutionised oenology and today the forces of *market-pull* and *technology-push* continue to challenge the tension between tradition and innovation (Pretorius, 2000). Among these innovations, the use of selected Active Dry Wine Yeast (ADWY) and the control of fermentation temperatures are important factors to improve quality and reproducibility of wine, especially in white and *rosé* vinifications. Alcoholic fermentations at low temperatures are used to enhance production and retention of flavour volatiles. However, low temperatures are restrictive to yeast growth and increases the risk of stuck and sluggish fermentations. Especially affected are the initial stages of fermentation, *lag* phase, which takes longer at low temperatures. This phase implies adaptation of yeast to the new media and start of metabolic activity, clearly delayed at low temperature. In addition, this long *lag*-phase leads to a higher contribution of non-*Saccharomyces* species in the composition and sensory properties of wine. Thus, a critical point in order to improve winemaking kinetics at low temperatures is the reduction of *lag*-phase.

This reduction in *lag* could be reflected by enhance in the yeast vitality during the first stages of fermentation, i.e. after inoculation into must. In this thesis, we have used the impedance technique to determine the robustness of yeast after rehydration and permanence in different media. Usually, winemakers do not test the physiological status of their yeasts prior inoculation. Impedance techniques devices such as *Bac*-trac® are becoming available in wineries and used to quantify viable cells. These methods could be used to check yeast robustness and to predict early metabolic activity. On the other hand, we have also studied transcriptional responses as an approach to know the early adaptation of wine yeast to the must. From an oenological point of view, this technique does not supply yet specific information to conclude if yeasts are physiologically active or not. However, we have found several categories of genes that could be considered as markers to predict yeast adaptation to must. Among these genes, we found glycolytic genes (*PYK1, TPI1*), genes related to protein synthesis (*PRS22A, RPS6B*) and elongation factors (*YEF3, EFT1*). The early over-expression of these genes after

inoculation into a fermentable medium could be used as markers in the selection of yeast strains for winemaking.

During winemaking, yeast cells are subjected to several stress conditions and their survival depends on their ability to adapt quickly to the changing environment. The use of selected ADWY involves the rehydration before addition to musts in the fermentation tanks. Both rehydration and the subsequent inoculation are two traumatic steps for yeast, mainly due to the osmotic and thermal shocks that they undergo. Winemaking is, in addition, a process where a wide diversity of stresses takes place as low pH, nitrogen limitation, increasing ethanol content, carbon starvation and technological practises (as fermentation at low temperatures) (Bauer and Pretorius, 2000). Therefore, stress response is essential for yeast survival and growth during winemaking. In yeast, glycogen and trehalose levels are metabolic hallmarks in response to changes in growth conditions and to various environmental stresses. Thus, the knowledge of trehalose and glycogen metabolisms as stress markers is of great interest to determine the critical points in the whole winemaking process, from yeast production until the end of alcoholic fermentation. So, one of the main objectives was to characterise the metabolism of the reserve carbohydrates (trehalose and glycogen) during winemaking, comprising the rehydration of ADWY and the whole alcoholic fermentation.

We have carried out all the studies with a commercial wine yeast strain and we have studied both, industrial and laboratory conditions. We were mainly interested in mimicking as much as possible the real conditions of winemaking. In this context, we have also studied the impact that low temperatures (13ºC) induce to the reserve carbohydrates metabolism (trehalose and glycogen). As expected, low temperature affects the kinetics of fermentation (at 13ºC fermentation was 2-fold longer than at 25ºC). Keeping in mind that the oenological practise to ferment at low temperatures could be a source for additional stress in the whole process when compared to optimal conditions (25ºC), higher level of reserve carbohydrates at 13ºC were expected. However, the concentration of trehalose and glycogen were highest in fermentation at 25ºC. In addition, viable cells remained at maximum level for longer period of time at 13 ºC. These features led us to conclude that temperature of 13ºC is not a stressful

condition for wine yeast. In fact, 13ºC leads to a slow metabolic rate but a higher viability of yeast cells throughout the fermentation.

Trehalose mobilization is a part of the developmental program initiated by the cell in the presence of nutrients in the culture medium. Only when yeasts are inoculated in a good growth media, the intracellular trehalose is degraded, indicating the start of the yeast metabolic activity. Poor media without fermentable carbon sources (permanence in water and sorbitol) results in a conservation of the intracellular levels of trehalose, indicating that yeast cell remains in a non-proliferating status. Osmotic stress itself does not induce a mobilisation of trehalose, as shown by the lack of changes in trehalose content in presence of only sorbitol in the medium. Taking into account our transcriptional data (obtained both by microarray technique and by real time quantitative RT-PCR), no changes were found in the expression of genes related to trehalose metabolism during the earlier responses of yeast after inoculation. It points out that the main mechanisms of trehalose regulation are post-translational, through activation of trehalases by phosphorylation mediated by stimulation of PKA activity, as typical response to nutrient availability. However, several signalling pathways should be involved in this tightly metabolic control, since the speed of trehalose degradation depends on the availability of both nutrients and carbon sources. Glucose is the preferred carbon source to induce trehalose breakdown and it results more efficient in presence of essential nutrients (nitrogen, vitamins, amino acids, etc). Trehalase activity plays a role in removing trehalose and bringing the cell back to homeostasis once the stress is over. Certainly, trehalases deserve more investigation to understand its implication in energy, carbon metabolism and in better understanding the roles of trehalose in yeasts.

We have checked that an old oenological practice, such as yeast proliferation in must prior inoculation into fresh musts, is not a stressful condition for yeast. In fact, trehalose is completely depleted and yeasts are already fully active once they are inoculated into the must. Industrial fermentations share the same pattern of trehalose and glycogen accumulation, however their final concentration in storages carbohydrates is lower. This could be probably due to the scale factor between laboratory and industrial conditions. In addition, a higher tank volume could imply some features that could decrease the stressful conditions for yeast during winemaking (*i.e.*, oxygen availability,

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homogeneous yeast population by heat convection produced during fermentation, etc) comparing to our laboratory conditions.

Yeast growth represents a progressive depletion of essential nutrients, leading to physiological adaptations that produce the entering into the stationary phase. Related to trehalose metabolism, at transcriptional level, we have observed a more important induction of trehalose-6-phosphate synthase gene (*TPS1*) than trehalases (*NTH1* and *NTH2*) before the beginning of trehalose synthesis. Once trehalose synthesis begun, transcript levels of *TPS1* decreased. In addition, neutral trehalase *NTH1* showed co-induction with *TPS1*, although it reached slower levels of induction. We have found that neither glucose nor nitrogen exhaustion are responsible for the beginning of trehalose biosynthesis during winemaking. In fact, this process is completely related to growth arrest. This growth arrest may be due to the exhaustion of some nutrients or to the presence of growth inhibitor as ethanol. Therefore, to deeply understand the changes in trehalose and glycogen metabolism and their relationship to cell growth, other studies focused on the change from *lag* to stationary phases are needed.

At the later stages of fermentation at 25ºC there is a beginning of trehalose mobilization, coinciding with both glucose exhaustion and decline phase of growth. An interesting aspect is that fermentation at 13ºC shows higher viability which lasts until the end of fermentation and, even under glucose exhaustion, neither decline phase nor later trehalose degradation are detected. It is possible that this later trehalose mobilization at 25ºC is an attempt to continue growth when viability decreases. Again, a link between cell growth and trehalose metabolism is established.

The cellular machinery to control stress conditions involves rapid synthesis of protective molecules and the activation of signal transduction systems that induce the activation of enzyme activities and the transcription of selected genes. Much has been attributed to the protective effects of trehalose. However, recent studies start to reconsider the degree of importance that trehalose could play in stress response. For instance, it has been found that correlation between the degree of dessication tolerance and trehalose level is poor, and, although high intracellular trehalose concentration can quantitatively improve tolerance, trehalose itself is neither necessary nor sufficient for survival (Ratnakumar and Tunnacliffe, 2006). Thus, continued focus on this

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disaccharide detracts from otherwise important and alternative mechanisms that contribute to the tolerance of desiccation and other stresses, such the *l*ate *e*mbryogenesis *a*bundant (LEA) proteins or a widespread group of proteins: the *hydrophilins.* The *S. cerevisiae* genes *HSP12*, *SIP18* and *GRE1* belong to this late group (Singh *et al.,* 2005). Thus, the study of these alternative mechanisms of protection under winemaking conditions (especially during rehydration and *lag* phase) could allow us to better understand the performance and characteristics of wine yeast metabolism.

Yeast rehydration could be a key step for winemakers to avoid stuck and sluggish fermentations. However, in spite of its importance, there is a complete lack of knowledge about what this process may represent in the whole winemaking context and what kind of practises could be developed to improve it. We have characterised a little bit this process but much more efforts are needed to better understand it. Yeast rehydration results in a stressful process which mainly compromise yeast viability and vitality. Also, it is a quick process involving morphological restoring that leads to an important loss of intracellular contents. This leakage goes in detriment of cell viability and vitality. One important aspect that should be considered concerns the timing and kinetics of rehydration. The widespread protocol used in winemaking is rehydration in plain water at 37ºC during 30 minutes. However, alternative rehydration media could be tested in an attempt to improve yeast physiology prior inoculation into the must.

We have verified by transcriptional approach that remaining yeast more time in water does not lead to transcriptional changes, suggesting that 30 minutes is enough to induce all the required arrangements. In addition, longer permanence in water is detrimental to vitality, suggesting that yeast contact in plain water is a negative situation that must be limited. So, one interesting aspect would be to determine the minimal time needed to reach the total transcriptional modifications, as a way to control the changes that take place during rehydration. Functional genomic approaches, such as microarray profiling, are powerful tools for the analysis of gene expression at a whole genome scale, providing a comprehensive view of yeast physiology. Several studies have analysed yeast gene expression profile during the rehydration (Singh *et al.,* 2005; Rossignol *et al*. 2006) but none of them focussed on the changes during rehydration in plain water, the widespread protocol used in winemaking. Therefore, the knowledge of transcriptional

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changes during rehydration could give us interesting clues in order to decide for better strategies to improve this process.

Further knowledge of rehydration protocol could lead to greater inoculation efficiency in industrial winemaking. Not only in laboratory but in real industrial conditions must be tested. In order to improve rehydration and winemaking efficiency, several questions have to be assessed. One question to further study is the way to reduce effectively the period of *lag*-phase, especially in fermentations at low temperatures. It could be interesting to define if it is more efficient to induce budding during rehydration in order to reach the maximal yeast population earlier in the *lag* or if it is better to induce greater cell vitality which could lead to a faster adaptation to the must. Another question is related to thickness of cell envelope. Large-sized (mother) cells have a more resistant cell envelope than small-sized (daughter) cells in order to maintain the same pressure gradient across the cell envelope (Martinez de marañón *et al.,* 1996). Thus, it could be interesting to control and minimize the number of budding cells at the final of yeast production process.

It is interesting to study the events that take place during industrial production of ADWY and during rehydration in order to draw a landscape about the response mechanisms of yeasts. Meanwhile yeast production is characterised by low sugar content, aerobic metabolism which leads to oxidative stress, nutrient limitation and a mix of different stresses (thermic, osmotic, etc) when drying. Rehydration and inoculation into the must is characterised by high sugar, osmotic shock and fermentative metabolism. Almost opposite events are taken place through these two processes.
Rehydration could not be treated as an isolated process itself, but previous steps in yeast production have to be also considered. From a biothecnological point of view, strategies linking both processes must be studied and protocols of production and rehydration/inoculation have to be designed considering the two processes as a whole. The suggested hypothesis is that if both yeast production and rehydration/inoculation process share the same physiological yeasts responses, the viability and vitality of yeasts when inoculating into the must could be improved.

We have characterised the earlier transcriptional responses of yeast after inoculation into the must. In addition, the main factors that yeast found after inoculation (osmotic

shock and presence of carbon sources) were examined in an independent set of experiments, studying global gene expression changes due to presence of sorbitol and glucose/fructose, respectively.

The simple presence of fermentable carbon sources in the medium, irrespective to the presence of nitrogen, leads to an induction of genes related to protein synthesis, ribosomal genesis, glycolytic genes (*PYK1, TPI1*, more detail in Annex I), repression of glyconeogenic genes (*PCK1*) and the previously mentioned trehalose breakdown. Some of these events are controlled by the signalling pathway, the Ras-cAMP pathway, which controls the activation of protein kinase A (PKA) that regulates those transcriptional and post-translational events and it result in the activation of growth. We have additionally found a common induction of the two isoforms of the glycerol-3-phosphatase (*GPP1, GPP2*) in the media where glucose was present. This finding is interesting because indicates that osmotic stress does not induce the *GPP1, GPP2* expression, and, in fact, it is the PKA pathway the responsible for its transcriptional control. On the other hand, we have been able to detect the specific regulation induced by the different selective media (sorbitol and glucose/fructose). An interesting aspect is the common response found in these media (induction of *CDC55*) which points out the inactivation of TOR-kinase pathway. This finding is consistent with the fact that TOR-kinase signalling pathway is only induced when both, carbon and nitrogen sources are present in the medium. Thus, these results indicate that the major transcriptional changes of yeast after inoculation are the result of the complete media-induced activation of both the Ras-cAMP and the TOR-dependent signalling pathway.

One of the main responses to inoculation in must was the induction of protein synthesis. We have studied the transcriptional and physiological effects produced by the addition of a protein synthesis inhibitor, the drug cycloheximide. Cycloheximide dramatically affects yeast vitality in plain water coincident with a total lack of transcriptional response. Only in synthetic must, a rich medium where yeast growth is promoted, cycloheximide induces considerable transcriptional changes and vitality is recovered. In fact, it was found important induction of genes related to ribosomal subunits and protein synthesis, the metabolic target of cycloheximide. Thus, it seems that in response to cycloheximide, yeast over-produces those elements that are attacked by the drug (*i.e.* machinery of protein synthesis). Cells try to counteract the negative effects of

cycloheximide by possibly reaching a threshold level of those proteins involved in the blocking effect of cycloheximide. As a result, cycloheximide effect is diminished and yeast vitality could be recovered.

To sum up, this work has focused on the stress metabolic responses that wine yeast strain develops during winemaking process. We have taken into account rehydration, metabolic adaptation just after inoculation and the whole fermentation. We have also studied the widespread practise in white and rosé vinifications that is fermentation at low temperatures (13ºC). Breakdown of trehalose could be considered as a marker of metabolic activation of yeast and it is related to the presence of growth factors in the medium. The beginning of its synthesis is related to the arrest of growth, irrespective of the amount of carbon and nitrogen content present in the must at this moment. In addition, the landscape of the initial transcriptional responses of the yeast after inoculation has been defined. Moreover, these responses were characterised according to selective media beginning from the simplest (water) to the most complex (synthetic must). The next step should be to study further the yeast physiology of active dry wine yeast after rehydration in an attempt design alternative rehydration protocols to improve the initial stages of fermentation.

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ANNEXE I

Additional data of the transcriptional study about wine yeast response after rehydration

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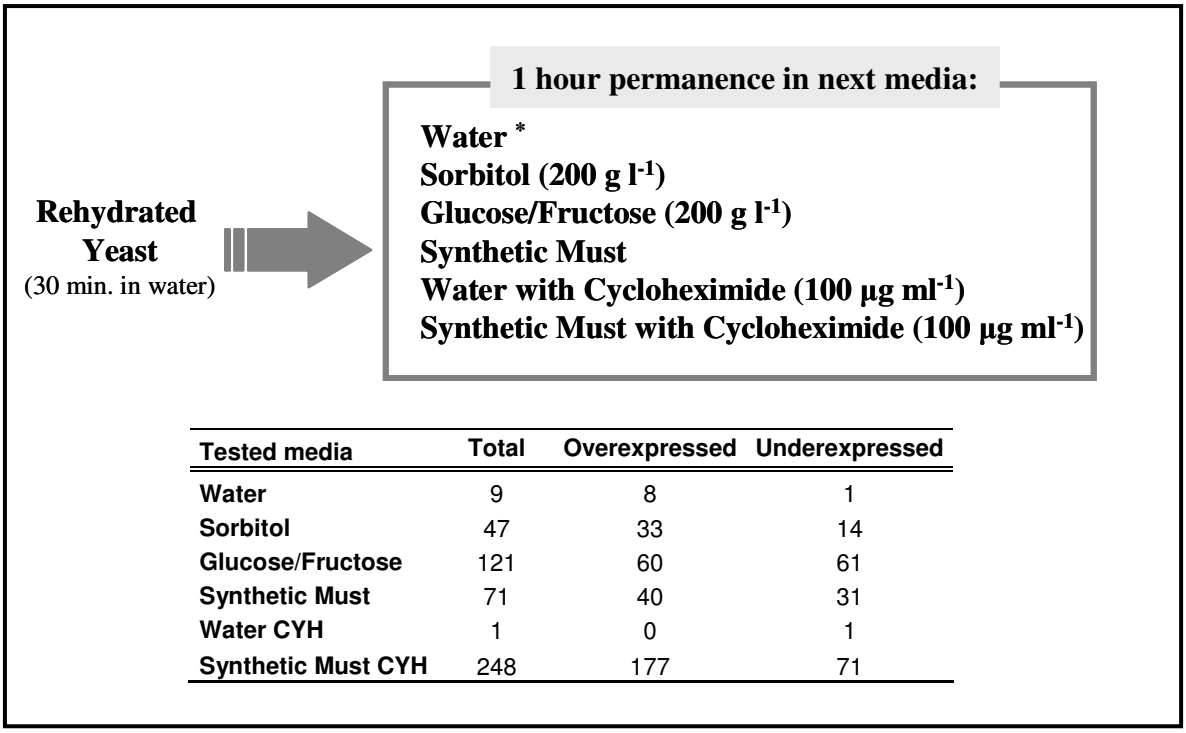
Diagram of the experimental design used in the transcriptional study and number of genes with modified expression in the different tested media.



Tested media	Total	Overexpressed	Underexpressed
Water	9	8	1
Sorbitol	47	33	14
Glucose/Fructose	121	60	61
Synthetic Must	71	40	31
Water CYH	1	0	1
Synthetic Must CYH	248	177	71

* Permanence in water was considered the control condition. In addition, an array comparing transcriptional response between rehydrated yeast and permanence in water was also performed (results showed below).

List of the 9 modified genes (>2-fold variation) after the permanence of yeast in plain water for 1 hour after rehydration. Information about the gene description was taken from *Saccharomyces* Genome Database (www. yeastgenome.org).

	Gene id	Ratio	Gene description
YJR075w	**HOC1**	**-2.79**	Mannosyltransferase complex, Golgi-localized. Type II integral membrane protein
YBR008c	**FLR1**	**-2.74**	Fluconazole resistance1. Membrane-associated transporter acting as multidrug efflux pump
YCL037c	**SRO9**	**-2.34**	RNA-binding protein, Cytoplasmic. Roles: translating ribosomes, organizing actin filaments
YBR019c	**GAL10**	**-2.34**	UDP-Glucose-4-epimerase. UDP-Glucose to UDP-Galactose (vicev)
YDR484w	**SAC2**	**-2.21**	Component of the GARP (Golgi-associated retrograde protein) complex which is required for recycling of proteins form endosomes to the late Golgi. Involved in localization of actin and chitin.
YJR053w	**BFA1**	**-2.17**	GTPase activator. Mitotic spindle checkpoint that control exit from mitosis
YHR165c	**PRP8**	**-2.15**	mRNA splicing. Component of the U4/U6-U5 snRNP complex, involved in the second catalytic step of splicing
	YMR075C-A	**-2.09**	Hypothetical protein
	YKR073C	**2.04**	Delections: sensitivity growing in 1M NaCl and in medium of pH8

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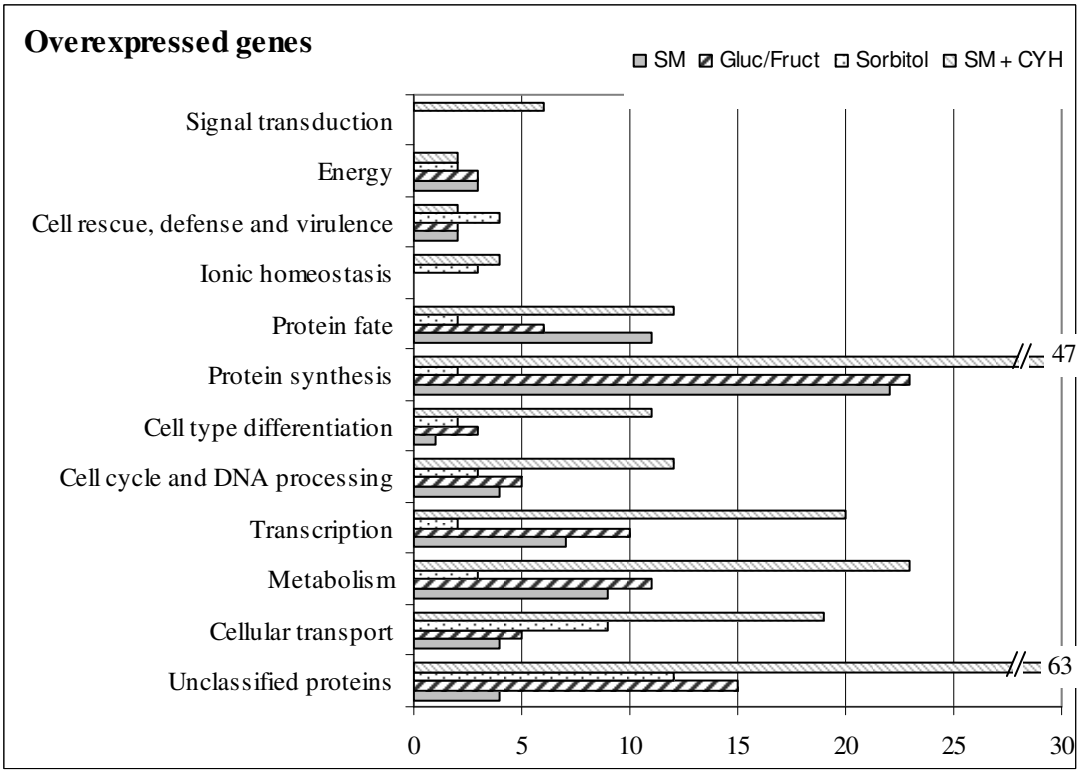
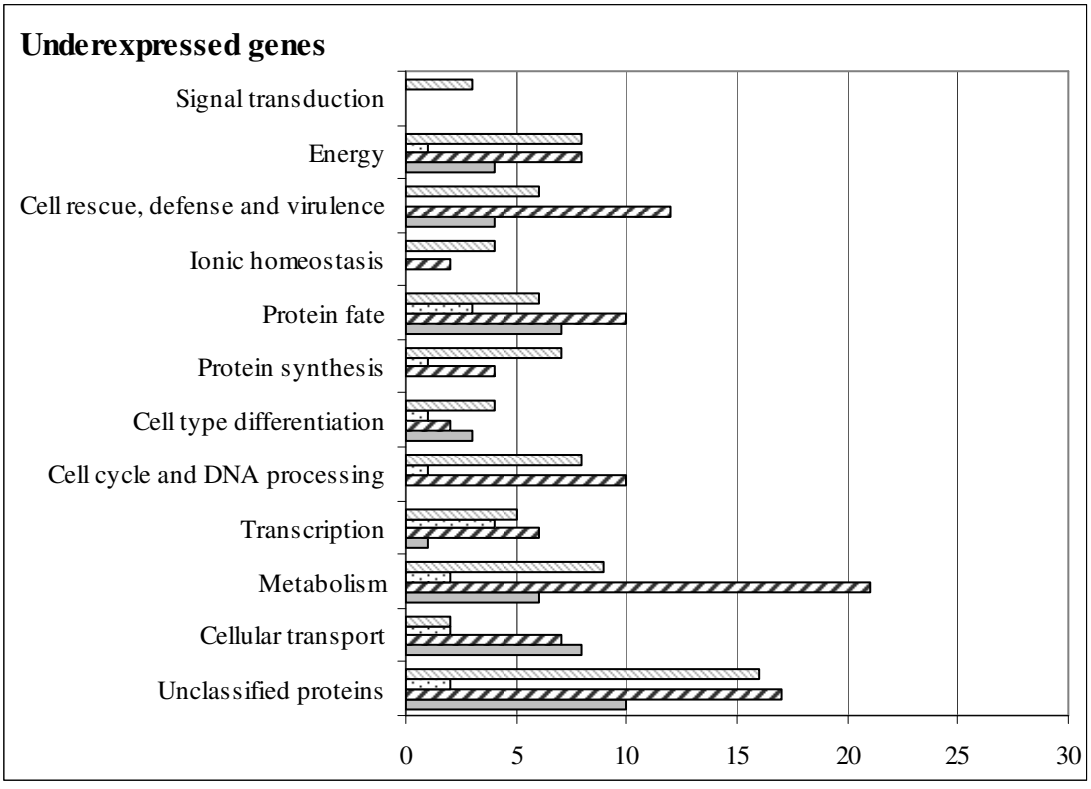
Number of genes significantly over- and underexpressed (*i.e.* >2-fold variation and Student's *t*-test with *p*-value<0.05) in each functional category (MIPS). The results correspond to the following conditions: Synthetic must (SM), Glucose and Fructose (Gluc/Fruct), Sorbitol and Synthetic must with Cycloheximide (SM + CYH).





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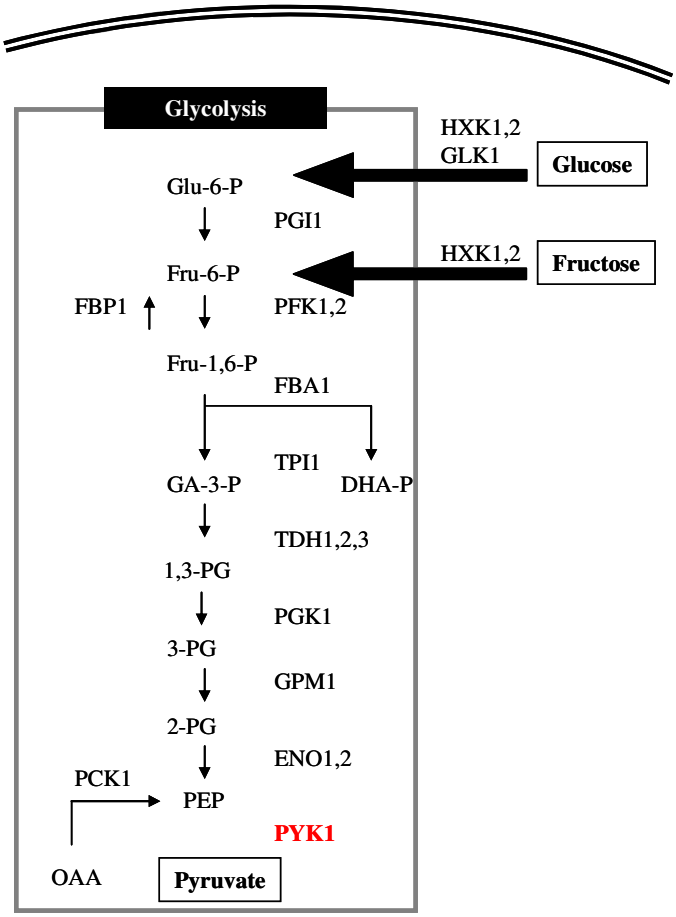
Expression levels of the glycolytic and glyconeogenic genes in response to both carbon sources media (Sorbitol, Glucose/Fructose and Synthetic Must, SM) and presence of cycloheximide. In bold are those ratios with good *p*-value (≤ 0.05)



ORF	Gene	Sorbitol	Gluc/Fruct	SM	SM + CYH	Water + CYH
	Glycolytic genes					
YFR053c	**HXK1**	-1.75	**-3.13**	**-2.33**	-1.85	1.36
YGL253w	**HXK2**	1.02	1.15	1.25	1.88	-1.03
YCL040w	**GLK1**	-1.23	**-2.27**	-1.82	-1.82	-1.45
YBR196c	**PGI1**	1.02	2.79	2.41	2.50	1.25
YGR240c	**PFK1**	1.06	2.72	2.41	**-2.56**	1.21
YMR205c	**PFK2**	-1.85	1.51	1.67	2.47	1.40
YKL060C	**FBA1**	-1.54	1.45	2.07	1.57	-1.03
YDR050c	**TPI1**	-1.19	**2.54**	**2.59**	2.38	-1.03
YJL052W	**TDH1**	1.00	1.89	2.36	1.33	1.10
YJR009C	**TDH2**	-1.43	1.40	1.95	1.93	1.28
YGR192C	**TDH3**	1.07	1.39	1.54	-1.82	-1.59
YCR012w	**PGK1**	-2.04	-1.02	1.28	1.99	2.16
YKL152c	**GPM1**	1.04	3.54	4.20	2.89	-1.19
YGR254w	**ENO1**	1.31	-1.20	1.08	-2.86	-1.18
YHR174w	**ENO2**	1.01	1.70	**2.13**	1.40	1.19
YAL038w	**PYK1**	-1.11	**3.43**	**3.83**	2.37	-1.27
	Glyconeogenic genes					
YKR097w	**PCK1**	-1.05	-1.89	**-2.04**	-1.92	-1.33
YLR377c	**FBP1**	-1.15	-1.52	-1.43	-1.30	-1.16

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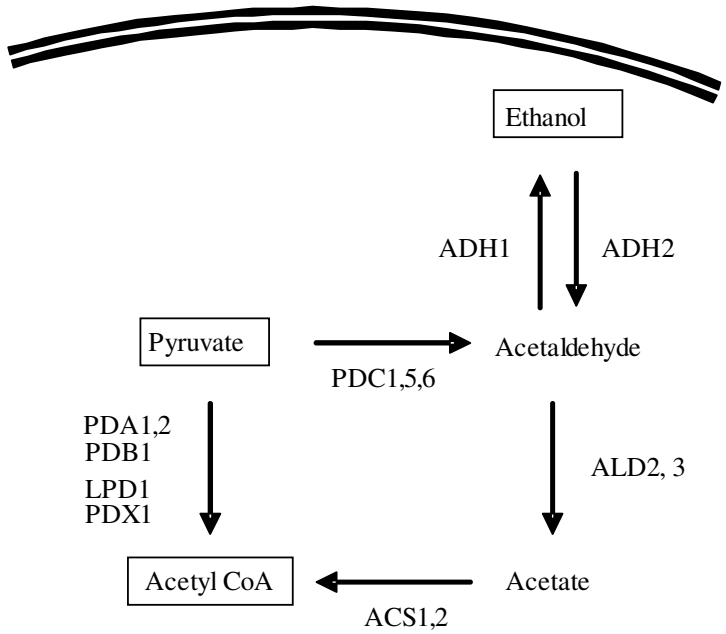
Expression levels of the genes involved in pyruvate degradation in response to carbon sources media (Sorbitol, Glucose/Fructose and SM) and in response to presence of cycloheximide. In bold are those ratios with good *p*-value (≤ 0.05).



ORF	Gene	Sorbitol	Gluc/Fruct	SM	SM + CYH	Water + CYH
	Ethanol production genes					
YLR044c	**PDC1**	-1.75	1.60	2.14	1.78	1.09
YLR134w	**PDC5**	-1.25	1.37	1.05	2.05	1.47
YGR087c	**PDC6**	-1.37	1.36	1.44	-1.75	-1.37
YOL086c	**ADH1**	-1.89	1.32	1.24	2.19	1.29
YMR303c	**ADH2**	-1.10	1.90	2.48	1.63	-1.15
	Acetyl-CoA production genes					
YER178w	**PDA1**	-1.15	-1.85	-1.33	**-1.79**	-1.25
YNL071w	**PDA2**	-1.28	-2.08	-2.33	-1.25	-1.16
YBR221c	**PDB1**	-1.11	-1.54	-1.61	-1.19	-1.09
YFL018c	**LPD1**	-1.30	-1.67	**-1.54**	-1.54	-1.33
YGR193c	**PDX1**	3.21	1.53	1.06	-2.44	1.07
YMR170c	**ALD2**	1.48	-1.09	-1.39	-1.43	1.06
YMR169c	**ALD3**	1.51	-3.03	**-2.33**	-2.56	-1.06
YAL054c	**ACS1**	-1.19	-3.85	**-2.86**	-1.69	-1.12
YLR153c	**ACS2**	1.90	1.32	1.23	1.92	1.10

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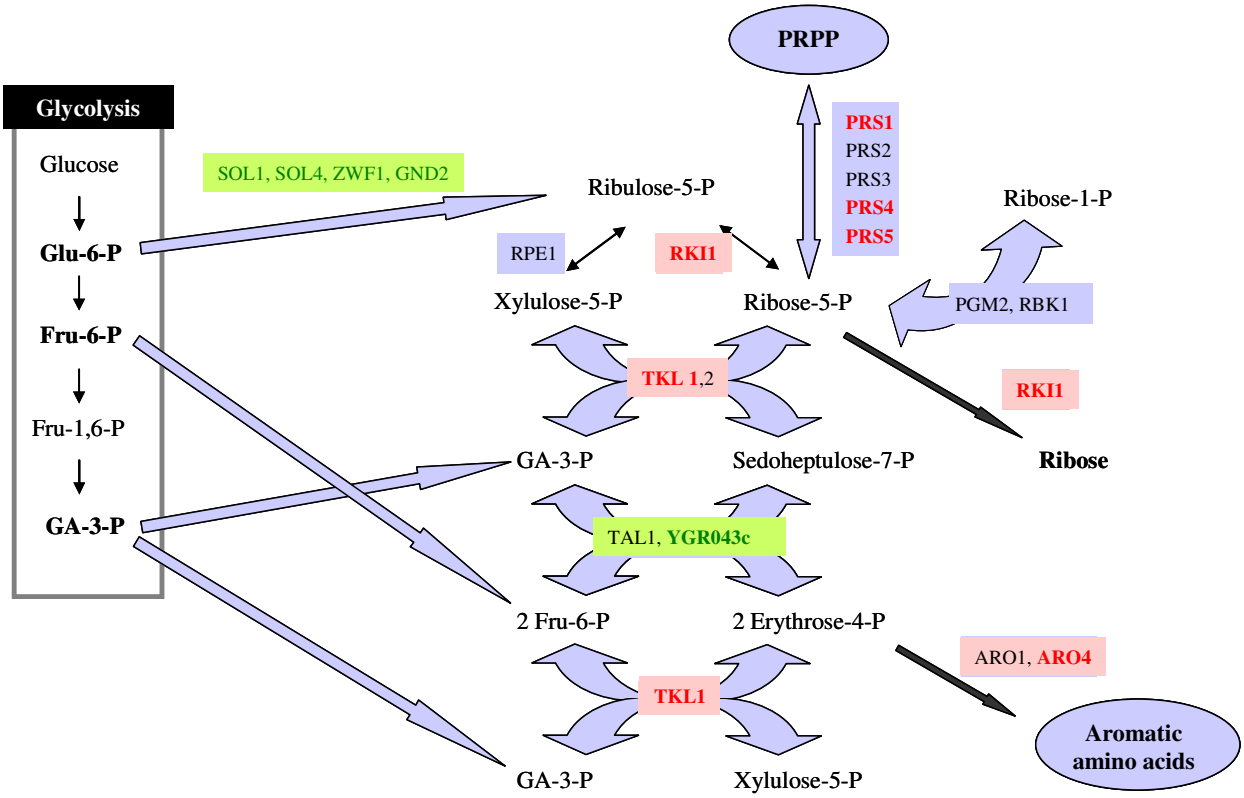
Gene expression changes of the genes involved in the pentose phosphate pathway in *S. cerevisiae*, showing the connexions with glycolysis. In bold are those ratios with good *p*-value (≤ 0.05).



ORF	Gene	Sorbitol	Gluc/Fruct	SM	SM + CYH	Water + CYH
Oxidative branche of Pentose phosphate pathway						
YNR034w	**SOL1**	1.23	1.4	1.21	1.86	1.03
YGR248w	**SOL4**	-1.09	-1.03	1.34	-1.56	-1.49
YNL241c	**ZWF1**	-1.82	-4	-4.55	-2.13	-1.18
YGR256w	**GND2**	-1.15	-1.10	1.27	-2.22	-1.08
Non-oxidative branche of Pentose phosphate pathway						
YJL121c	**RPE1**	-1.09	1.13	-1.12	2.04	1.09
YOR095c	**RKI1**	-1.35	2.02	2.23	2.76	1.15
YPR074c	**TKL1**	-1.16	3.86	3.37	2.45	-1.64
YBR117c	**TKL2**	-1.04	-1.39	-1.47	-1.32	-1.22
YLR354c	**TAL1**	-1.03	1.42	-1.28	-1.35	-1.41
YGR043c	**YGR043C**	-1.61	**-7.69**	-4.35	**-2.70**	1.03
YMR105c	**PGM2**	-1.12	1.18	-1.25	1.58	1.25
YCR036w	**RBK1**	1.14	-1.18	-1.06	-1.06	-1.16
YDR127w	**ARO1**	-1.01	1.04	1.14	1.55	1.04
YBR249c	**ARO4**	1.31	**2.84**	2.36	2.17	-1.15
YKL181w	**PRS1**	-1.06	1.88	2.03	2.25	-1.02
YER099c	**PRS2**	1.11	1.27	1.14	1.91	1.16
YHL011c	**PRS3**	-1.79	-1.52	-1.54	-3.23	-1.49
YBL068w	**PRS4**	1.4	2.58	2.7	2.49	-1.25
YOL061w	**PRS5**	1.92	3.49	3.37	2.83	-1.03

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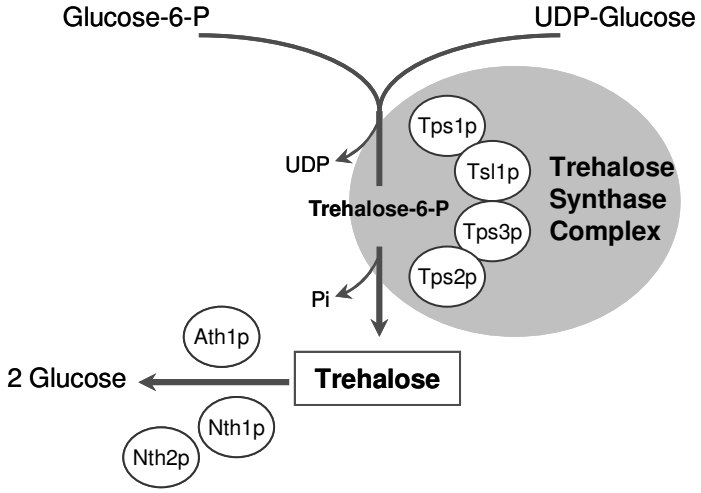
Expression levels of the genes involved in synthesis and degradation of trehalose for all the studied conditions. The ratio values are not good *p*-value and in only two cases ratio were >2-fold variation.



ORF	Gene	Water 1h	Sorbitol	Gluc/Fruct	SM	SM + CYH	Water + CYH
	Trehalose synthase complex						
YBR126c	**TPS1**	-1.12	1.12	1.05	-1.09	1.55	1.24
YDR074w	**TPS2**	1.01	1.31	1.04	-1.32	-1.12	-1.04
YMR261c	**TPS3**	1.13	1.32	-1.03	-1.01	1.31	-1.18
YML100w	**TSL1**	-1.09	-1.30	-2.00	-2.08	-1.41	-1.41
	Trehalases						
YDR001c	**NTH1**	-1.02	-1.06	-1.25	-1.18	-1.39	-1.01
YBR001c	**NTH2**	1.05	-1.05	-1.14	-1.22	-1.28	-1.18
YPR026w	**ATH1**	-1.15	-1.20	-1.41	-1.27	1.40	1.93

Expression levels of the genes involved in glycerol shyntesis. Glycerol is produced in two steps from the glycolytic intermediate dihydroxyacetone phosphate. In the first step, dihydroxyacetone phosphate is reduced by the NAD-dependent glycerol 3-phosphate dehydrogenase (Gpd) to glycerol 3-phosphate, which is immediately dephosphorylated to glycerol by glycerol 3-phosphatase (Gpp). In *S. cerevisiae*, both glycerol 3-phosphate dehydrogenase and glycerol 3-phosphatase are encoded by two differentially expressed isogenes, *GPD1*/*GPD2* and *GPP1*/*GPP2*, respectively.

ORF	Gene	Sorbitol	Gluc/Fruct	SM	SM + CYH	Water + CYH
YDL022w	**GPD1**	1.08	1.05	1.22	1.3	1.04
YOL059w	**GPD2**	-1.04	-1.04	1.05	1.3	1.07
YIL053w	**GPP1**	1.23	6.99	7.9	9.45	-1.15
YER062c	**GPP2**	-1.03	8	10.36	9.16	-1.04

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Expression levels of the glucose carriers in *S. cerevisiae* in the conditions of the transcriptional study. In bold are the ratio values with a good *p*-value (< 0.05).

Gluc. affinity	ORF	Gene	Sorbitol	Gluc/fruct	SM	SM + CYH	Water + CYH
Low	**YHR094C**	**HXT1**	-1.28	1.9	2.3	2.62	1.51
	YDR345C	**HXT3**	-1.67	-1.35	-1.23	1.92	3.02
Moderate	**YMR011W**	**HXT2**	-1.25	1.6	1.91	**3.51**	1.45
	YHR092C	**HXT4**	-1.67	-1.12	-1.22	**2.18**	2.85
	YHR096C	**HXT5**	-1.23	**-5.88**	-4.76	**-2.86**	-1.08
High	**YDR343C**	**HXT6**	-1.16	-1.14	-1.39	1.73	2.43
	YDR342C	**HXT7**	-1.72	-1.37	-1.25	1.66	2.44
	YLR081W	**GAL2**	-1.56	-1.69	-1.79	1.39	1.92
	YFL011W	**HXT10**	1.03	-1.14	-1.03	1.61	1.15
	YJL214W	**HXT8**	-1.10	-1.41	-1.54	1.55	1.00
	YJL219W	**HXT9**	-1.92	1.11	1.44	**2**	1.69
Drug pumps	**YOL156W**	**HXT11**	-1.89	1.06	1.38	2	1.63
	YIL170W	**HXT12**	-1.72	-1.03	1.39	1.47	1.22
	YEL069C	**HXT13**	-1.43	-2.80	-1.52	1.67	1.59
Unknown	**YNL318C**	**HXT14**	2.66	1.2	1.02	-3.03	1.05
	YDL245C	**HXT15**	-1.06	2.39	1.45	**2.5**	2.19
	YJR158W	**HXT16**	1.88	**4.5**	2.33	3.6	1.76
	YNR072W	**HXT17**	-1.27	-1.03	1.22	2.18	1.17

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ANNEXE II

Materials and methods

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1. Medios y cepa utilizados

1.1. Mosto sintético (Bely *et al.,* 1990)

El medio elegido en las fermentaciones de laboratorio sirve para modelizar las condiciones enológicas ya que imita la composición de un mosto de uva. Se caracteriza por una elevada concentración de azúcares (200 g/l), un contenido relativament bajo de nitrógeno (300 g/l) y un pH ácido (3,3). A diferencia con el medio de referencia (Bely *et al.,* 1990), en nuestro caso no añadimos los factores de anaerobiosis**.**

A) Composición de un litro de MS:

Glucosa	100 g
Fructosa	100 g
Ácido cítrico	5 g
Ácido málico	0.5 g
Ácido tartárico	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

B) Añadir después de autoclavar (20 min. a 120ºC):

NH_4Cl	0.460 g
Solución madre aminoàcidos (1)	13.09 ml
Solución madre oligoelementos (2)	1 ml
Solución madre vitaminas (3)	10 ml
H_2O destilada	Qsp 1 L

(1) Aminoácidos: Preparar la solución en un tampón de Na_2CO_3 2%. Es necesario calentar la solución a 70ºC para disolver Tirosina, Triptófano e Isoleucina. 3,4 g/l Asp; 9,2 g/l Glu; 6 g/l Ser; 38,4 g/l Gln; 2,6 g/l His; 1,4 g/l Gly; 5,8 g/l Thr; 28,3 g/l Arg; 11,2 g/l Ala; 1,5 g/l Tyr; 1,6 g/l Cys; 3,4 g/l Val; 2,4 g/l Met; 13,4 g/l Trp; 2,9 g/l Phe; 2,5 g/l Ile; 3,7 g/l Leu; 1,3 g/l Lys; 46,1 g/l Pro.

(2) Oligoelementos: 4 g/l $MnSO_4 \cdot H_2O$, 4 g/l $ZnSO_4 \cdot 7H_2O$, 1 g/l $CuSO_4 \cdot 5H_2O$, 1 mg/l KI, 0,4 g/l $CoCl_2 \cdot 6H_2O$, 1 g/H_3BO_3, 1 g/l $(NH_4)_6Mo_7O_{24}$.

(3) Vitaminas: 2 g/l Myo-Inositol, 150 mg/l Calcium pantothenate, 25 mg/l Thiamin, 200 mg/l Nicotinic acid, 25 mg/l Pyridoxine, 3 ml Biotin (stock solution: 100 mg/l).

C) Finalmente, ajustar el pH a 3,3 con NaOH

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1.2. Mostos naturales y mosto concentrado

En algunas fermentaciones a escala de laboratorio, se utilizó mosto concentrado de uva blanca (Concentrados Pallejà, Riudoms, Tarragona) diluído 1:4 con agua destilada, hasta una concentración de 230 g/l de azúcares y suplementado con 0,1 g/l de *Vitiamine* (Tiamina y fosfato amónico, Martin Vialatte, Francia) con lo cual se alcanzan 94 mg/l de amonio y 150 mg/l de nitrógeno asimilable por las levaduras.

Para la realización de estudios a escala industrial utilizamos dos bodegas diferentes, la bodega de Caves Gramona (Sant Sadurní d'Anoia, Barcelona) y la de la Finca experimental Mas dels Frares, propiedad de la Universitat Rovira i Virgili (Constantí, Tarragona). Utilizamos mostos naturales provinentes del prensado y desfangado posterior de la variedad de uva blanca Moscatel de Alejandría.

1.3. Condiciones de rehidratación

Como protocolo básico de rehidratación de la levadura seca activa (LSA), se siguieron las especificaciones recomendadas por el fabricante, recogidas en la ficha técnica de la cepa Enoferm QA23:

- Rehidratar la levadura en 10 veces su peso en agua (destilada) a 37ºC.
- Espolvorear, al cabo de 20 minutos agitar e inocular el medio cuando la levadura lleva 30 minutos en contacto con el agua.
- En ningún caso dejar las levaduras rehidratándose más de 30 minutos en ausencia de azúcares.

1.4. Condiciones ensayadas en el estudio transcriptómico

Para el análisis transcriptómico, primero se rehidrataron 2,5 g de LSA (cepa QA23) en tubos falcon de 25 ml de agua destilada durante 30 min., en un baño a 37ºC. Después de la rehidratación, las células se centrifugaron y se transfirieron a las diferentes condiciones de estudio, siguiendo la metodología siguiente.

250 ml de medio (volumen final) en erlenmeyers de 500 ml, cubiertos con un tapón de algodón
Los medios se esterilizaron mediante autoclave.
Nivel de inoculación: **10^8 células/ml** (los 25 ml del tubo falcon)
Incubación durante **1 hora** sin agitación a 20ºC (Novotron HT, Bottminger, Switzerland)

Medios utilizados:

- **Medio agua:** 250 ml de agua destilada
- **Medio Sorbitol:** 250 ml de agua destilada con 200 g/l de Sorbitol
- **Medio Glucosa/Fructosa:** 250 ml de agua destilada con 100 g/l de glucosa y 100 g/l de fructosa
- **Mosto sintético:** 250 ml de mosto sintético (composición apartado 1.1)
- **Mosto sintético con Cycloheximida:** 250 ml de mosto sintético con 100 µg/ml de cycloheximida

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- **Medio agua con Cycloheximida:** 250 ml de agua destilada con 100 µg/ml de cycloheximida

1.5. YEPD (Yeast Extract Peptone Agar) agar

Composición del medio YEPD agar:

- 20 g/l Glucosa, 20 g/l Peptona, 10 g/l Extracto de levadura, 15 g/l Agar (Tipo E, Cultimed), disuelto en agua destilada, autoclavado y repartido en placas Petri.

1.6. Características técnicas de la cepa vínica utilizada: Enoferm QA23

Para estudiar las fermentaciones alcohólicas a baja temperatura, en nuestro laboratorio se escogió como levadura de referencia la cepa vínica comercial **Enoferm QA23** elaborada por la empresa Lallemand S.A. Es una cepa perteneciente a la especie *Saccharomyces cerevisiae*, seleccionada por la Universidad de Tras os Montes e Alto Douro (UTAD), Portugal.

A) Caraterísticas fermentativas:

- ✓ Cinética fermentativa calmada y regular. Cepa muy segura al final de la fermentación, incluso en condiciones difíciles.
- ✓ Fermentaciones en amplia gama de temperaturas, desde 12 a 35ºC.
- ✓ Resistencia a bajas temperaturas, acortándose su fase de latencia en estas condiciones.
- ✓ Rendimiento alcohólico muy elevado, próximo al rendimiento teórico, incluso a baja temperatura.
- ✓ Poco exigente en nutrientes. Muestra siempre una actividad muy intensa en mostos que sufren fuertes tratamientos prefermentativos, como centrifugación, flotación o filtración al vacío.
- ✓ Producción muy reducida de acidez volátil, aun en condiciones particulares nunca supera 0,30 g/l expresado en ácido acético.

B) Efecto sobre la composición del vino

- ✓ Alta producción de glicerina.
- ✓ Sensación de redondez y volumen en boca.
- ✓ Incremento de la fracción aromática.

C) Efectos organolépticos

- ✓ Vinos blancos afrutados, dotados de notable frescor, con perfumes suaves y francos partiendo de variedades neutras.
- ✓ En variedades aromáticas, el amplio espectro de actividades enzimáticas permite realzar los caracteres varietales de la uva. En Chardonnay, aporta notas de fruta exótica, piña madura y tonos cítricos.
- ✓ Aromas muy estables en el tiempo, permaneciendo en el vino hasta su comercialización.

D) Campo de aplicación

- ✓ Elaboración de vinos blancos jóvenes en general, tanto de uvas neutras como aromáticas.
- ✓ Fermentaciones de vinos blancos varietales en barricas, (crianza sobre lías).
- ✓ Vinos rosados con volumen en boca.

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E) Afinidad varietal

- ✓ Variedades neutras: Verdejo, Airén, Pedro Ximenez
- ✓ Variedades aromáticas: Albariño, Malvasía, Chardonnay, Sauvignon blanc.
- ✓ Variedades tintas: Garnacha, Monastrell.

2. Seguimiento de la cinética fermentativa

Se determinaron las cinéticas de las fermentaciones mediante control diario de la pérdida de densidad del mosto, debida a la formación de CO_2 producto de la fermentación de los azúcares. Asimismo, se monitorizaron el consumo del nitrógeno asimilable por las levaduras y el amonio

2.1. Determinación de la densidad del mosto.

En fermentaciones industriales, el seguimiento de la densidad se efectuó mediante el método habitual en bodega, es decir, uso de un densímetro de entre 980-1120 g/l. En fermentaciones de laboratorio, se realizó mediante pesada, por triplicado, de un volumen de 5 ml de muestra, utilizando pipetas aforadas.

2.2. Determinación de los azúcares reductores: Método GAB

Los azúcares mayoritarios en el mosto son la glucosa y la fructosa, no obstante, también aparecen otros azúcares no fermentables, básicamente pentosas, que permanecen incluso al haberse consumido completamente los azúcares fermentables. En el contexto enológico, se considera que la fermentación está finalizada cuando la concentración de azúcares reductores en inferior a 2 g/l.

El método GAB (García-Barceló 1990) es una modificación del método Rebelein (1973), el cual se fundamenta en la reducción de un reactivo cúprico-alcalino. Se hace reaccionar la muestra sobre una cantidad determinada de dicho reactivo y se valoran por iodometría (con tiosulfato) los iones de cobre restantes. En las bodegas se suele utilizar un kit suministrado por GAB Sistemática Analítica, S.L. (Moja-Olèrdola, Barcelona)
Para verificar el final de las fermentaciones, se utilizaron dos determinaciones diferentes.

Protocolo:

- En un erlenmeyer de 250 ml, se vierten 10 ml de la solución cúprica (medidos exactamente con la pipeta de doble enrase), 5 ml de solución alcalina y 2 ml de la muestra a analizar (no debe contener más de 28 g/l de azúcares). Añadimos piedra pómez.

- Se tapa el erlenmeyer con un vidrio de reloj y se lleva la muestra a ebullición en un calefactor eléctrico previamente calentado. Se hace hervir durante un minuto y medio exactamente, enfriando rápidamente al chorro de agua fría.

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- Se añaden al erlenmeyer: 10 ml de ioduro, 10 ml de almidón, 10 ml de ácido sulfúrico 16% (la muestra queda negra).

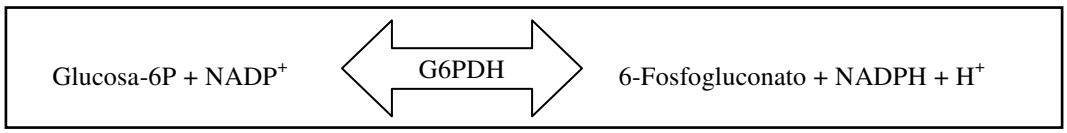
- Se valora con solución de tiosulfato sódico, hasta un color crema claro, utilizando una bureta GAB invertida, diseñada especialmente para el análisis o con una bureta de 30 ml. La concentración en azúcar se lee directamente (en g/l) de la bureta de valoración, considerando únicamente el factor de dilución.

2.3. Determinación de glucosa y fructosa

El contenido en glucosa y fructosa se determinó mediante kit enzimático (Boehringer Mannheim), siguiendo el protocolo del fabricante. El principio del método se basa en las siguientes reacciones:

1.- La D-glucosa y la D-fructosa son fosforiladas por el enzima hexoquinasa y ATP.

2.- En presencia del enzima Glucosa-6-P-deshidrogenasa (G6PDH), la glucosa-6-P es oxidada a 6-Fosfogluconato con la reducción de $NADP^+$ a NADPH. La cantidad de NADPH es estequiométrica a la cantidad de glucosa y su aumento se mido por absorbancia a las longitudes de onda 334, 340 ó 365 nm.



3.- Por otra parte, la Fructosa-6-P es convertida a Glucosa-6-P por la Fosfoglucosa isomerasa. La Glucosa-6-P se transformará en 6-Fosfogluconato como en la reacción anterior.

2.4. Determinación del nitrógeno asimilable por las levaduras (YAN). Determinación del índice de formol. (*Aerny, J.*, 1996)

La adición de formaldehído libera un ion H^+ por molécula de aminoácido. La valoración se efectúa potenciométricamente mediante una solución de hidróxido sódico. El grupo amino secundario de la histidina no reacciona y los de la prolina y la hidroxiprolina lo hacen parcialmente. El nitrógeno terciario y los grupos guanidina tampoco reaccionan.

A) Materiales, reactivos y equipos.

- pHmetro y buretas
- Solución de NaOH 0,25M (para 500 ml, pesar 5g) y solución de NaOH 0,025M
- Solución de formaldehído pura (min. 35%) con pH ajustado a 8,1 inmediatamente antes de su utilización.
- Agua oxigenada al 30% (si la muestra está sulfitada)

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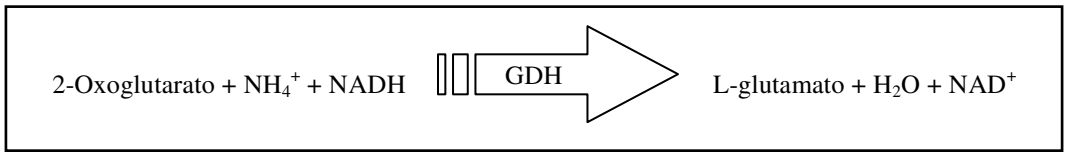
B) Protocolo:

- Introducir 25 ml de la muestra en un vaso de precipitados pequeño (si se dispone de suficiente muestra, se pueden utilizar 50 ml modificando los cálculos posteriores, multiplicando por 5 en lugar de por 10)
- Si la muestra contiene sulfuroso, añadir unas gotas del agua oxigenada para eliminarlo.
- Neutralizar la muestra hasta pH 8,1 con la solución de NaOH 0,25M (o bien con NaOH concentrada). Añadir a continuación 10 ml de formaldehído a pH 8,1 y homogeneizar.
- Dejar reaccionar durante un minuto, valorar la muestra hasta pH 8,1 con la solución de NaOH 0,25M. Si la valoración requiere más de 20 ml de NaOH, repetir la dosificación añadiendo 15 ml de solución de formaldehído.
- **CÁLCULOS:** Índice de formol = 10 (ó 5) x ml de NaOH 0,25M
  N total asimilable (mg N/l) = 14 x índice de formol

Si a priori se sospecha que la muestra contiene menos de 200 mg/l de N total (como es el caso de nuestros mostos), es más preciso valorar con NaOH 0,025M, se gastará 10 veces más cantidad y se disminuirá el error. No obstante, en este caso, el índice de formol corresponde directamente con el valor del N total asimilable.

2.5. Determinación del contenido en Amonio.

El contenido en amonio se determinó mediante kit enzimático (Boehringer Mannheim), siguiendo el protocolo del fabricante. El principio del método se basa en la reacción siguiente: en presencia del enzima glutamato deshidrogenasa (GDH) y de NADH, el amonio reacciona con el 2-oxoglutarato formando L-glutamato y agua, mientras que el NADH es oxidado a NAD^+. La cantidad de NADH oxidada en la reacción es estequiométrica con la cantidad de amonio. La disminución de NADH se determina mediante la medida de absorbancia a 365 nm.



3. Control poblacional a lo largo de la fermentación.

Para determinar la fase de crecimiento de las levaduras a lo largo de las fermentaciones estudiadas, se realizaron los controles, tanto de la población total como de la población viable. Asimismo, se determinó la imposición de la levadura inoculada, identificando y tipificando las levaduras a nivel de especie y de cepa.

3.1. Recuento de la población total

El recuento de la población total de levaduras en todos los experimentos, se realizó mediante el uso de la Cámara de Neubauer (Brand), en un microscopio visible con aumento 40X.

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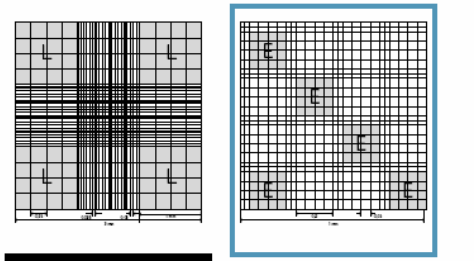
La profundidad de la cámara es de **0,1 mm**.. Los 4 cuadrados grandes de las esquinas señalados con una "L" están divididos en 16 cuadrados con aristas de 0,25 mm. El cuadrado grande central está dividido en 16 cuadrados medianos, estando cada cuadrado mediano subdividido en 16 cuadrados pequeños con aristas de 0,05 mm y una superficie de 0,0025 mm^2, y una profundidad de 0,1 mm. La superficie de recuento de los cuadrados medianos está delimitada por la línea exterior de las tres líneas.

Para el recuento de las levaduras, se contaba la diagonal señalada con una "E" dentro del cuadro central, contando un total de 64 cuadrados pequeños. El cálculo utilizado para determinar las células/ml fue:

$$\text{Nº células/ml} = \text{Células contadas} \times 4 \cdot 10^6 \times \text{Dilución} / \text{Cuadros contados}$$



3.2. Recuento de la población viable.

El seguimiento de la viabilidad celular, se realizó mediante siembra en placas petri en medio YEPD-agar (previo banco de diluciones, consiguiendo sembrar en placa entre 20-200 ufc) y posterior recuento de las unidades formadoras de colonias (UFC), tras 48 h de incubación en estufa a 28ºC.

Este control de la viabilidad se realizó la mayor parte del tiempo de desarrollo de la tesis a mano (utilizando bolas de vidrio y contaje manual), pero el laboratorio adquirió unos aparatos para automatizar y simplificar el proceso. Para la siembra se utilizó el sembrador espiral semiautomático AES laboratoire, Combourg (France). Para el recuento, se utilizó el software ProtoCOL SR/HR counting system software version 1.27, suministrado por Synbiosis (Cambrige, UK).

3.3. Determinación de la viabilidad mediante microscopía de fluorescencia

En los estudios de rehidratación, la viabilidad también de determinó mediante microscopía de fluorescencia, utilizando el kit commercial LIVE/DEAD *Bac*Light (Molecular Probes, USA). Si bien este método fue concebido en su origen para la detección de la viabilidad en bacterias, recientemente se ha comprobado su validez en el caso de las levaduras (Zhang and Fang, 2004). El kit comprende dos fluorocromos que tiñen ácidos nucleicos: SYTO®9, que emite fluorescencia en verde, y Propidium Iodide (PI), emisión en rojo. Estos fluorocromos difieren tanto en sus características espectrales (excitación/emisión máximas son: 480/500 nm para SYTO9 y 490/635 nm para PI) como en su capacidad en penetrar membranas celulares dañadas. SYTO9 marca todas las células, independientemente de su estructura de membrana, y PI penetra únicamente en las células con membrana dañada, produciendo la reducción de SYTO9.

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Así, células con membranas intactas emitirán en verde y células dañadas emitirán rojo/anaranjado.

Preparación de la muestra:

- Partimos de una población de $1 \cdot 10^7$ cel./ml.
- Mezclamos en un eppendorf: 0.5 ml de células + 1.5 µl SYTO 9 + 0.7 µl PI
- Homogeneizamos y contamos en Cámara de Neubauer tanto la población total (contaje en campo claro) como las células teñidas (contaje bajo fluorescencia).

3.4. Identificación de las levaduras a nivel de cepa: Análisis del perfil de restricción (RFLPs) del DNA mitocondrial (Querol *et al.,* 1992)

El DNA mitocondrial (mtDNA) de *S. cerevisiae* es una molécula pequeña de entre 65 y 80 kb, cuyo grado de variabilidad puede ser mostrado mediante su restricción. La técnica utilizada está basada en el hecho de que el mtDNA contiene un 75% de A y T, aunque se han encontrado unas 200 regiones ricas en GC. Por tanto, digestiones del DNA total con enzimas cuya diana es GCAT no reconocen las secuencias ricas ni en GC ni en AT. Dado el pequeño número de puntos de corte en el mtDNA y el gran número de puntos de corte en el DNA nuclear, este último es cortado en pequeños fragmentos, lo que permite la visualización de las bandas correspondientes al mtDNA. Los enzimas que permiten la identificación a nivel de cepa en *S. cerevisiae* son *HinfI* y *HaeII* (Fernández-Espinar *et al.,* 2006).

A.- Extracción del DNA:

- Hacer crecer las levaduras durante una noche en 1,5 ml de **YEPD** a 25ºC, para obtener cultivo fresco.
- Centrifugar 2 min.a 10.000 rpm. Eliminar el sobrenadante.
- Limpiar el *pellet* con 1 ml de agua destilada estéril.
- Centrifugar 2 min.a 10.000 rpm. Eliminar el sobrenadante.
- Resuspender el *pellet* en 500 µl de **Tampón 1** (Sorbitol 0,9M, EDTA 0,1M pH 7.5)
- Añadir 30 µl de solución **Zymoliasa 60.000** (1.5 mg zymoliasa en 1300 µl Tampón 1). Incubar durante 20 minutos, en un baño a 37ºC.
- Centrifugar 2 min.a 10.000 rpm. Eliminar el sobrenadante.
- Resuspender las células en 500 µl de **Tampón 2(**Tris 50mM pH 7.4, EDTA 20 mM)
- Añadir 13 µl de **SDS 10%,** agitar e incubar en un baño a 65ºC durante 5 min.
- Añadir 200 µl de **Acetato potásico 5M**, homogeneizar. Incubar en hielo 5 min.
- Centrifugar en frío (4ºC), 10 min a 12.000 rpm.
- Transferir el sobrenadante a un nuevo eppendorf, añadir 700 µl de **Isopropanol**, incubar a temperatura ambiente 5 min. (en este paso, podemos guardar a -20ºC)
- Centrifugar 10 min.a 12.000 rpm. Eliminar el sobrenadante (pipeteando).
- Añadir 500 µl de **etanol 70%** (v/v), centrifugar 5 min.a 12.000 rpm. Eliminar el sobrenadante.
- Secar el precipitado mediante SpeedBac, al vacío. Resuspender el DNA en 15 µl de **TE** (Tris 10mM pH 7.4, EDTA 1mM, pH 8.0)

B.- Digestión del DNA total:

- Preparación del mix de digestión en un volumen final de 25 µl:

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10-12 µl de DNA
2.5 µl Tampón H (Boehringer Mannheim)
1 µl enzima de restricción *Hinf*I (Boehringer Mannheim)
Agua mili-Q hasta el volumen de 25 µl

- Incubar a 37ºC durante toda la noche.

C-. Electroforesis

- Preparar un gel de agarosa al **0.8%** (p/v) en tampón TBE (Tris base 89 mM, Ácido bórico 89 mM, EDTA 2 mM, pH 8). Añadir Bromuro de Etidio, una concentración final de 0.4 µg/ml (1µl por cada 25 ml de TBE).
- Preparación de la muestra para "pinchar" el gel: a 25 µl de muestra se le añaden 4 µl de tampón de carga (solución 10X: 0.25% (p/v) azul de bromofenol, 1% (p/v) SDS, 20% (v/v) glicerol, 0.1 M Na_2EDTA, pH 8). Como marcador del peso molecular, utilizamos una mezcla al 50% (v/v) de los marcadores: DNA Molecular Weight Marker II y DNA Molecular Weight Marker III (0.12-21.1 kpb, ADN del fago λ digerido con EcoRI i Hind III) (Boehringer Mannheim).
- Aplicar un voltaje entre 25-30 V en TBE 1X durante 8 h.
- Visualización de las bandas mediante transiluminador de luz UV. Fotografía mediante cámara Polaroid.

3.5. Identificación de las levaduras a nivel de especie. Análisis del perfil de restricción (RFLPs) del DNA ribosomal (Guillamón *et al.,* 1998)

Los genes ribosomales (5.8S, 18S y 26S) están agrupados en tándem formando unidades de transcripción que se repiten en el genoma entre 100 y 200 veces. En cada unidad de transcripción existen otras dos regiones, ITS (Internal Transcribed Spacers) y ETS (External Transcribed Spacers), que se transcriben pero no se procesan. Asimismo, la unidades codificantes están separadas por regiones intergénicas, las NTS. Tanto los genes ribosomales como los ITS y NTS son muy buenas herramientas para establecer relaciones filogenéticas e identificar especies (Fernández-Espinar *et al.,* 2006). La técnica utilizada está basada en amplificación por PCR de un fragmento del DNA ribosomal que posteriormente es digerido por diversas enzimas de restricción, originando perfiles distintos para cada especie de levadura. De esta manera, podemos distinguir las cepas *Saccharomyces* de las no-*Saccharomyces*.

A.- Reacción en cadena de la Polimerasa (PCR)

- Mix de la reacción de PCR (V_{final}= 50 µl):
  1 µl de los primers (cada uno 10 pM):
    ITS1 (5' TCCGTACGTGAACCTGCGG 3')
    ITS4 (5' TCCTCCGCTTATTGATATGC 3')
  4 µl dNTPs, de un mix que contiene 1 µl de cada uno (10 mM)
  3 µl $MgCl_2$ (solución 100 mM, Ecotaq)
  5 µl Tampón Taq 10X, sin Mg (Ecotaq)
  33 µl Agua mili-Q estéril
  3 µl DNA extraído
  0.5 µl Taq-polimerasa (Ecotaq)

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- Condiciones del programa de PCR (termociclador)

  5 min 95ºC (Desnaturalización)

  30 seg. 95ºC (Desnaturalización)
  1 min. 52 ºC (Hibridación)
  1 min. 72 ºC (Extensión)
  } **35 ciclos**

  7 min. 72 ºC (Extension final)

B.- Gel de agarosa para visualizar el amplificado

- Preparar un gel de agarosa multipurpose al **1.2%** (p/v) en tampón TBE (Tris base 89 mM, Ácido bórico 89 mM, EDTA 2 mM, pH 8). Añadir Bromuro de Etidio, una concentración final de 0.4 µg/ml (1µl por cada 25 ml de TBE).
- Preparación de la muestra para "pinchar" el gel: a 5 µl del DNA amplificado se le añaden 4 µl de tampón de carga (solución 10X: 0.25% (p/v) azul de bromofenol, 1% (p/v) SDS, 20% (v/v) glicerol, 0.1 M Na_2EDTA, pH 8). Como marcador del peso molecular, utilizamos el marcador 100 pb DNA Ladder (Gibco BRL).
- Aplicar un voltaje entre 50-100 V en TBE 1X durante 2 h.
- Visualización de la banda mediante transiluminador de luz UV. Fotografía mediante cámara Polaroid.

C.- Digestión del amplificado mediante CfoI, HaeIII y HinfI

- Preparar el mix de digestión (uno por enzima de restricción utilizado; V_{final}= 25 µl)

  8 µl del DNA amplificado (0.5-1 µg DNA)
  2 µl de tampón, uno por cada enzima
  5 unitats de enzima: CfoI, HaeIII y HinfI (Boehringer Mannheim)
  Agua mili-Q hasta 25 µl.
- Incubar a 37ºC un mínimo de 3 h (normalmente, toda la noche)

B.- Gel de agarosa para visualizar la digestión del amplificado

- Preparar un gel de agarosa al **2%** (p/v) en TBE 1X y 0.4 µg/ml de bromuro de etidio
- A 25 µl de muestra de la digestión, añadir 4 µl de tampón de carga. Como marcador del peso molecular, utilizamos el marcador 100 pb DNA Ladder (Gibco BRL).
- Aplicar un voltaje entre 25-30 V en TBE 1X durante 8 h.

4. Técnicas analíticas para vinos

4.1. Determinación de la Acidez total (Ough and Amerine, 1988)

La O.I.V. (Oficina Internacional de la Viña y el Vino), define la acidez total como la suma de los ácidos valorables, hasta pH 7.0, por adición de solución de hidróxido sódico. Esta valoración incluye a los ácidos procedentes del mosto (tartárico, málico, etc) y los formados durante la fermentación (succínico, láctico, acético, etc). El ácido carbónico y el dióxido de azufre no se deben incluir en la expresión de la acidez total.

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- Eliminar el gas carbónico mediante agitación y haciendo el vacío con una trompa de agua.
- Colocar **10 ml** de muestra en un erlenmeyer, añadir 20 ml de agua destilada.
- Valorar con **NaOH 0,1N**, utilizando **azul de bromotimol** (0,4%, en etanol al 20%) como indicador (viraje a verde/azulado).
- Cálculo: $N_{muestra}$ = (ml NaOH x 0,1 N)/ 10 ml muestra
  $N_{muestra}$ (eq/l) x 1 eq. Tartárico/2 eq. NaOH x 150 g Tart./1 eq. Tart

Acidez total (g/l Ac. Tartárico) = ml NaOH x 0.75

4.2. Determinación del pH

El conocimiento del pH del vino tiene gran interés, ya que es muy importante sobre el crecimiento microbiano, color, sabor, potencial red-ox, quiebras férricas, etc. El método utilizado en la medida potenciométrica mediante el pHmetro.

4.3. Determinación de la Acidez volátil (método García-Tena)

En los vinos, se define como acidez volàtil al contenido de ácidos grasos como acético, fórmico, propiónico, butírico, etc. El constituyente principal de la acidez volátil es el ácido acético. Cantidades normales de 0.3-0.8 g/l pueden considerarse normales. La legislación actual no permite valores superiores a 1 g/l (García-Barceló, 1990).

El método García-Tena, se basa en una destilación fraccionada del vino (probetas de 5,1 ml y 3,2 ml), utilizando un placa calefactora eléctrica, y valoración de la segunda fracción recogida.

- Encender la placa calefactora unos 15 minutos previos al anàlisis. Desgasificar la muestra.
- Colocar 10 ml de la muestra en el matraz de destilación, añadiendo piedra pómez.
- Se recupera la primera fracción de 5,1 ml (que se deshecha) y se recoge luego la segunda fracción de 3,2 ml.
- El líquido de esta segunda fracción, se valora con NaOH 0,0204 M utilizando fenolftaleína (1% en etanol) como indicador.
- Cálculo:
  g/l Ác. Acético= N x 0,0204 x 60 x 100/ V x 33,3 V= Volumen de muestra (10 ml)
  N = ml consumidos de NaOH

Acidez volàtil (g/l Ac. Acético) = ml NaOH x 0.366

4.4. Determinación de Dióxido de azufre (SO_2). (método Ripper simplificado)

Desde muy antiguo, se emplea el dióxido de azufre como antiséptico y antioxidante en los vinos. Normalmente, se distingue entre sulfuroso libre y sulfuroso total. El sulfuroso libre es el que presenta una actividad más significativa. El dióxido de azufre tiende a combinarse con el etanol (formando bisulfito), con la función aldehídica de los azúcares y con compuestos fenólicos. A la suma de sulfuroso libre y sulfuroso combinado se denomina sulfuroso total.

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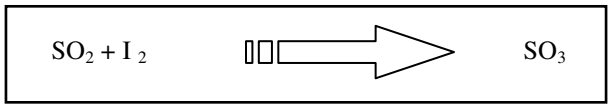
Utilizamos un método comercializado por la empresa GAB Sistemàtica Analítica, S.L. (Barcelona), basado en el método Ripper. El SO_2 libre se mide mediante una valoración iodométrica directa. El SO_2 combinado, mediante una valoración iodométrica después de hidrólisis alcalina sobre el vino.



Sulfuroso libre:

- En un erlenmeyer, se vierten 25 ml de muestra, 2-3 ml de indicador (almidón 2%) y 5 ml de ácido (H_2SO_4 33% (p/v))
- Se valora con el líquido de valoración (Yoduro potásico, como fuente de yodo), hasta viraje color azul.
- Cálculos: SO_2 libre (mg/l) = V (ml líquido valoración) x 10

Sulfuroso total:

- En un erlenmeyer, verter 25 ml de muestra y 10 ml de solución alcalina (NaOH 1M)
- Para evitar la pérdida de SO_2 gas, se tapa, agita y deja en reposo 10-15 min.
- Se añaden 2-3 ml de indicador (almidón 2%) y 5 ml de ácido (H_2SO_4 33% (p/v))
- Se valora como en el caso anterior.
- Cálculos: SO_2 total (mg/l) = V' (ml líquido valoración) x 10

4.5. Determinación del grado alcohólico por ebullometría (Método GAB)

Se basa en la disminución del punto de ebullición que experimentan las soluciones hidralcohólicas a medida que augmenta el porcentaje de alcohol (ley de Raoult). Se utiliza el ebullímetro de la firma GAB (Moja-Olèrdola, Barcelona).

- Conectar el sistema de refrigeración del aparato (muy importante para conservar la resistencia que calienta la muestra!!)
- Rellenar con 50 ml (hasta la línia de enrase) de agua destilada el recipiente del aparato y conectar el calefactor.
- Tomar nota de la temperatura de ebullición del agua.
- Rellenar ahora el recipiente con la muestra de vino y proceder como con el agua.
- Ajustar la regleta con la temperatura de ebullición del agua e interpolar el grado alcohólico mediante la temperatura de ebullición del vino.

5. Determinación del contenido en Trehalosa y Glucógeno intracelular en levaduras. (Parrou J.L. and François J., 1997)

A) Materiales, reactivos y equipos.

- Baño a 95ºC, baño a 37ºC, horno de hibridación con sus cilindros para introducir las muestras. Espectofotómetro de visible. Tubos Eppendorf con rosca.
- Na_2CO_3 0,25M, Ácido acético 1M: para 100 ml, 5,9 ml de ácido acético 17M, Acetato sódico 0,2M a pH=5,2

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- Trehalasa 0,64 U/ml: para 500 µl, añadir 100 µl de 3,22 U/ml (Sigma Cat.No. T-8778, 3,22 U/ml)
- Amyloglucosidasa 31,2 U/ml: para 1 ml, pesar 3,6 mg de Amyloglucosidasa 500U (Roche 1 202 332)
- Kit enzimático para la determinación de glucosa (Boehringer Mannheim, Ref. 0139106)

B) Protocolo:

- Partimos de *pellet* (2-5·10^8 células) congelado en nitrógeno líquido.
- Resuspender las células en 250 µl de Na_2CO_3 0,25M
- Incubar durante **4 hores a 95 ºC** (así se permeabilizan las células para la entrada de los enzimas y la salida de la glucosa)
- Añadir 150 µl de Ácido acético 1M y 600 µl de Acetato sódico 0,2 M
- Incubar la mitad de la suspensión para la determinación de Trehalosa con Trehalasa 0,05 U/ml **durante una noche a 37ºC. Añadir 40 µl de la solución de trehalasa 0,64 U/ml.**
- Incubar la otra mitad para la determinación de Glucógeno con *A. niger* Amyloglucosidasa 1,2 U/ml **durante una noche a 57ºC con agitación constante** (utilizar el horno de hibridación). **Añadir 20 µl de la solución de amyloglucosidasa 31,2 U/ml.**
- Centrifugar 3 min. a 5000 g.
- Determinación de la glucosa formada mediante el kit enzimático de glucosa de Boehringer Mannheim.
- Para expresar el contenido de Trehalosa y Glucógeno en mg (carbohydrato)/célula, determinar la población viable (ufc/ml) en el momento del muestreo.

6. Extracción de los metabolitos intracelulares (González *et al.,* 1997)

- Centrifugar las células (aprox. 10^8 cel./ml)
- Añadir rápidamente al *pellet* de 3 a 5 ml de etanol puro diluído al 75% con Hepes (70 mM, pH 7.5) hirviendo
- Incubar durante 3 minutos a 80ºC
- Concentrar hasta la sequedad y resuspender el precipitado en 1 ml de agua millipore
- Centrifugar a 5000 rpm durante 10 minutos a 4ºC
- Recuperar el sobrenadante, donde están disueltos los metabolitos intracelulares. En el *pellet* se retienen las paredes y membranas celulares.

7. Determinación de la actividad metabólica de las levaduras: *Bac*-trac® 4300

Para determinar la vitalidad de las levaduras después de su permanencia en los diferentes medios ensayados, utilizamos el *Bac*-trac 4300 microbiological analyzer (SY-LAB Instruments, Austria). Esta técnica está basada en la medida de la impedancia eléctrica, utilizada para la detección indirecta del CO_2 producido (Ribeiro *et al.,* 2003).

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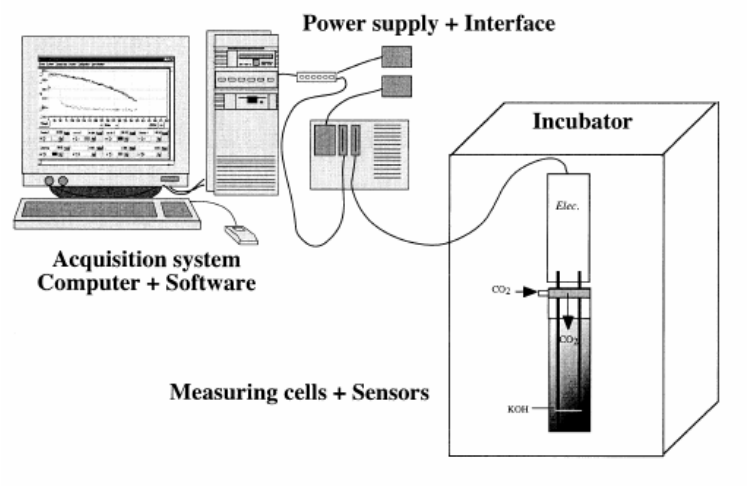

Las levaduras se inocularon en 5 ml de YEPD líquido (20 g/l glucosa, 20 g/l peptona, 10 g/l extracto de levadura) a un nivel de $2 \cdot 10^7$ cél./ml. El CO_2 producto de la metabolización de la glucosa reacciona con una solución de KOH 0,02%, originando cambios en la conductividad eléctrica de esta solución. Estos cambios se traducen en una pérdida de impedancia que es medida cada 10 minutos y memorizada por el aparato. La variación de la impedancia está relacionada cuantitativa y cualitativamente con la producción de CO_2, según la reacción:

$$2\ KOH + 2\ CO_2 \longrightarrow 2\ KHCO_3$$

La conductancia molar de la reacción ($\Delta\Lambda_0$), por mol de CO_2 se calcula mediante:

$$\mathbf{\Delta\Lambda_0 = \Lambda_0(HCO_3^-) - \Lambda_0(OH^-) = 44{,}5 \cdot 10^{-4} - 198{,}3 \cdot 10^{-4} = -153{,}8 \cdot 10^{-4}\ S\ m^2/mol}$$

Como resultado, se dibuja una curva que relaciona el % de impedancia disminuída en función del tiempo. Una mayor vitalidad, implica una formación más precoz de CO_2 que conlleva una variación de impedancia más acelerada, llegando antes a la saturación de la solución de KOH. Como parámetro para comparar la vitalidad, escogimos las horas requeridas para llegar a un umbral de variación de impedancia del -50.

8. Determinación de la expresión génica mediante RT-PCR cuantitativa a tiempo real.

El análisis del mRNA se ha visto ampliamente extendido a partir del surgimiento de la metodología de reacción en cadena de la polimerasa (PCR) en la década de los 80. Se realiza mediante la metodología conocida como RT-PCR, la cual conjuga la técnica de PCR y la reacción catalizada por la enzima transcriptasa inversa (RT), una polimerasa capaz de sintetizar una cadena de DNA complementario (cDNA) a partir de una molécula de RNA molde (transcripción inversa). Posteriormente se amplifica mediante PCR este cDNA sintetizado por la RT, así la cantidad del producto amplificado obtenido es directamente proporcional a la cantidad de moléculas del mRNA presente inicialmente en el material de partida.

La PCR cuantitativa a tiempo real está basada en la monitorización de la PCR usando técnicas de fluorescencia. Utilizamos como fluoróforo el SYBR-Green I (Applied Biosystems) que se une con gran afinidad al surco menor del DNA bicatenario. La señal

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fluorescente es proporcional a la cantidad de producto de PCR y permite la visualización en directo del perfil completo de amplificación de la diana. El número de copias de la diana al inicio de la reacción se puede cuantificar a través del ciclo umbral (Ct). El Ct es el número de ciclos necesarios para que se produzca un aumento de fluorescencia significativo con respecto a la señal de base, y es inversamente proporcional a la cantidad inicial de moléculas molde. De esta manera, utilizando muestras patrones que contienen diluciones seriadas de concentraciones conocidas del gen diana, se construye una recta de calibración que relaciona los Ct con el logaritmo de la cantidad inicial de molde (N_0). Extrapolando en esta recta los Ct obtenidos para las muestras problema, se puede estimar el valor de sus N_0.

A) **Extracción del RNA** (Sierkstra *et al.,* 1992)

Partimos de unos *pellets* congelados procedentes de diferentes puntos de fermentación que contienen entre 10^7-10^8 cél./ml. Es muy importante tener en cuenta que desde la extracción del RNA hasta la obtención del cDNA ha de trabajarse en condiciones libres de RNAsas.

- Limpiar el *pellet* con 1 ml de **Extraction Buffer** (100 mM Tris-HCl pH 7,4; 100mM LiCl; 0,1 mM EDTA).
- Centrifugar 5 min. a 10000 rpm a 4ºC y eliminar el sobrenadante.
- Resuspender el pellet en 0,5 ml de **Vortex Buffer** (100 mM LiCl, 10 mM EDTA, 0,5% LitiumDodecylSulfat, pH 7,4).
- Romper las células. Para ello, se han utilizado dos metodologías con buenos resultados:

  - **Perlas de vidrio:** Transferir la suspensión en Eppendorfs de rosca de 2 ml en donde hemos introducido previamente 1g de perlas de vidrio. Vortexar en intervalos de tiempo de 30 segundos vórtex/30 segundos en hielo. Realizar de 8 a 10 repeticiones.
  - **Minibeat beater:** Exactamente el mismo procedimiento que con la rotura en vórtex, pero solamente 5 repeticiones.

- Centrifugar 10 min. a 10000 rpm 4ºC.
- Transferir la fase acuosa (la superior) a otro eppendorf y añadir (v/v) **fenol/cloroformo/alcohol isoamílico** (25:24:1). Este paso ha de repetirse varias veces para asegurar la limpieza de la muestra, repetir hasta que no aparezca interfase.
- Centrifugar 5 min. a 10000 rpm a 4ºC, transferir la fase acuosa (superior) y añadir **cloroformo** (v/v).
- Centrifugar y añadir a la fase acuosa (superior): 1/10 del volumen de muestra de **NaAc 3M** (pH 5,6) y 2,5 del volumen de **Etanol Absoluto.**
- Precipitar 15 min. a -80ºC.
- Centrifugar 30 min a 4ºC y limpiar el *pellet* con 500 µl de **Etanol 70%** (para diluir el etanol utilizar **agua DEPC**!!). Centrifugar 5 min. a 10000 rpm a 4ºC.
- Secar el *pellet* al aire. Resuspender con 50 µl de **agua DEPC** durante la noche a 4ºC.

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Preparación de los Buffer:

Extraction Buffer: (250 ml):	25 ml Tris-HCl 1M
	1,06g LiCl
	50 µl EDTA 0,5 M
Vortex Buffer:(100 ml):	0,42 g LiCl
	0,5 g LDS
	2 ml EDTA 0,5M
	Ajustar a pH 7,4 con Li OH
Agua DEPC 0,1%:	50 ml agua miliQ
	50 µl Diethylpyrocarbonate (DEPC)

- Homogeneizar muy bien mediante fuerte agitación. Pero mucho cuidado, hasta que no se autoclave, es **altamente tóxico**. Dejar actuar durante una noche dentro de una campana y autoclavar.

B) Purificación del RNA

Es importante purificar el RNA de la muestra, eliminando los posibles restos de DNA que puedan quedar y que interferirían en los resultados del análisis. Realizamos la digestión del DNA existente en la muestra mediante el kit comercial High Pure Isolation kit (Roche Applied Science, Germany), siguiendo es protocolo suministrado en el kit.

C) Cuantificación del RNA y verificación de su pureza

- **Cálculo de la concentración del RNA obtenido:** Se realiza obtiene el barrido del espectro comprendido entre 200 y 300 nm (230, 260, 280 nm) Y se calcula:
  RNA (µg/ml)= ABS 260 x 2 (Cubeta 0,5mm) x 40 µg/ml/1 U x Dilución 1:50
  $Abs260/Abs230 \geq Abs\ 260/Abs280 \geq 1,8$

Para cuantificar DNA:
DNA (µg/ml)= ABS 260 x 2 (Cubeta 0,5mm) x 50 µg/ml/1 U x Dilución 1:50

- **Comprobación de la digestión del DNA:** Comprobaremos si han quedado restos de DNA mediante un gel de agarosa al 0,8% (0,4 g Agarosa, 5 ml TBE10X, 45 ml agua dest. y 2 µl Bromuro de etidio): 1 µl de muestra en 4 µl de agua-DEPC y 1 µl de colorante. Como marcadores del peso molecular, utilizamos una mezcla al 50% de DNA Molecular Weight Marker II y Weight Marker III (0,12-21,1 kpb, ADN del fago λ digerido con EcoRI y HindIII), de Boehringer Mannheim. En la parte inferior aparecen 2 bandas (el RNA), si apareciese banda en la parte superior sería DNA y tendría que repetirse la digestión.

  También se ha utilizado para este propósito de verificación de la pureza del RNA el bioanalizador RNA 6000 Nano Assay (Bioanalyzer 2100, Agilent Technologies).

D) Realización de la RT ⇒ Obtención del cDNA

Realizamos la reacción en el termociclador Perkin Elmer GeneAmp PCR System 2400

- Diluímos las muestras para obtener una concentración de 320 ng/µl*
- Repartimos la dilución en dos eppendorf **A** y **B** (2,5 µl en cada uno).

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- Añadiremos en cada eppendorf: (V_{final}= 20µl con 40 ng/µl de RNA)

A (RT)	**B (no RT)**
2,5 µl muestra	2,5 µl muestra
1 µl Oligo-dT12-18 Primer (Invitrogen)	1 µl agua-DEPC
8,5 µl agua-DEPC	8,5 µl agua-DEPC

- Dejamos las muestras durante 10 min. a 70ºC y después 1 min. en hielo
- En ambos eppendorfs añadimos:
  4 µl de 5xPCR buffer (Invitrogen), 1 µl de dNTPs y 2 µl de DTT.
- Incubamos 5 min. a 42ºC.
- En el eppendorf **A** añadimos 1 µl de Superscript (la transcriptasa inversa) (Invitrogen) y en **B** 1 µl de agua-DEPC.
- Ponemos los dos eppendorfs a 42ºC durante 50 min. Y para parar la reacción se dejan durante 15 min. a 72ºC.
- Añadimos 80 µl de agua miliQ al cDNA. Así diluímos 1:5 y tenemos la muestra lista para realizar la PCR.

* Si queremos partir de otra concentración de RNA muestra y sabiendo que para la RT necesitamos 800 ng de RNA (2,5µl x 320ng/µl), recalculamos el volumen de muestra a añadir y el agua restante.

E) Realización de la PCR cuantitativa a tiempo real

Realizamos la reacción en el termociclador Applied Biosystems GeneAmp 5700 Sequence Detection System.

- El volumen final en el pocillo de la placa será de **25 µl.**
- Reactivos a añadir (MIX):
  - ✈ 12,5 µl SYBR Green Mix (2x)
  - ✈ PRIMERS: Añadir una concentración de 300 nM → 1,5 µl primers 5 µM
  - ✈ Agua hasta volumen de 20 µl
  - ✈ 5 µl de la muestra cDNA (diluída 1:5 después de la RT)

- Muestras control en la PCR cuantitativa:

NAC (No Amplified Control): ***No RT***
UNKN (Unknown): Muestra RT
NTC (No Template Control): Agua

Lista de primers utilizados:

El diseño de los primers se realizó a partir de las secuencias de los genes obtenidas en *Saccharomyces* Genome Database (http://www.yeastgenome.org) y mediante el software Primer Express (Applied Biosystems). Este programa tiene definidos unos parámetros por defecto que dan lugar a combinaciones de primers que entran en el protocolo estándar de la reacción de PCR: 40 ciclos de 15 seg. a 95 ºC y 1 min. a 60ºC

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Secuencia de nucleótidos de los primers (5′–3′)

ACT1-F	TGGATTCCGGTGATGGTGTT
ACT1-R	CGGCCAAATCGATTCTCAA
TPS1-F	TCCATTACCATCCTGGTGAGAT
TPS1-R	TTGGTGAACGTCTGGTTTGC
NTH1-F	GTTTGGACCGCATCTCCAA
NTH1-R	AATACCGAGACCGTTAGGATGGT
NTH2-F	CGATCAGGCAATGGGATTA
NTH2-R	GCAGATAAACCTTTCCAGGCTAATA
GON7-F	GCCCAGTGACCATGTGCTAA
GON7-R	CGTGCGTTCCGGTTCTG
RPS22A-F	AGACAATTCGGTTACGTCATCTTG
RPS22A-R	AACGTGCTTTCTTCTGGCTTCT
GCY1-F	TTGAAAGATGGCTACCGACACA
GCY1-R	TTGATGGCTTGACCGACTTG
PYK1-F	ATGTCGATTTGCCAGCTTTGT
PYK1-R	GACCATGTGGACACCGTTCTT
HSP12-F	GGCAGACCAAGCTAGAGATTACATG
HSP12-R	AACATATTCGACGGCATCGTT

F) Cálculo de la expresión génica a partir de las rectas patrón

Primero tenemos que determinar la recta patrón para cada uno de nuestros genes de estudio. Para ello, partimos de DNA genómico de la misma cepa QA23 y del cual hacemos diluciones (1/10) seriadas desde 400 a $4 \cdot 10^{-3}$ ng/µl. Para cada concentración de DNA se obtiene una Ct determinada, a partir de las cuales obtenemos las rectas patrón para cada gen estudio. Las rectas patrón son válidas para cada prueba, se deben repetir en cada experimento. Para saber si la eficiencia de la PCRq ha sido la correcta, tenemos la referencia de la pendiente. Eficiencias del 100% dan un valor de pendiente de -3,2, de manera que nuestras rectas tienen que tener pendientes próximas a este valor.

Rectas patrones obtenidas en los estudios:

ACT1: Ct = -3,0644 log(x) + 26,3022

TPS1: Ct =-3,0789 log(x) + 26,8080

NTH1: Ct =-2,8528 log(x) + 23,8282

NTH2: Ct =-3,0114 log(x) + 28,0621

GON7: Ct =-3,0668 log(x) + 27,5350

RPS22A: Ct =-3,2197 log(x) + 28,6090

GCY1: Ct =-3,0025 log(x) + 28,0745

PYK1: Ct =-3,1340 log(x) + 27,4530

HSP12: Ct = -3,0802 log(x) + 27,6484

Para analizar cambios relativos de la expresión génica de los genes de estudio, se eligió como referencia (*housekeeping*) el gen ***ACT1*** (Actina) cuya expresión no varía en los

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tratamientos analizados. Comparando la concentración del gen de referencia con el gen problema, determinamos cambios relativos en la expresión del gen problema.

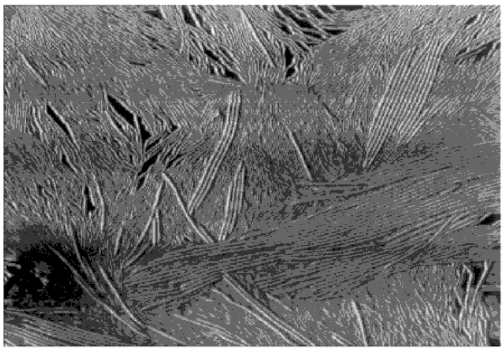
Expresión génica relativa= [gen estudio]/ [gen referencia]

9.- Determinación de la expresión génica global mediante Microarrays

Esta determinación se realizó en su totalidad en la Plataforma de Biochips perteneciente al Genopole Midi-Pyrénées de Toulouse (Francia), localizada en el INSA Toulouse y dirigida por el profesor Jean-Marie François (http://biopuce.insa-toulouse.fr)

Fundamento del método de microarrays

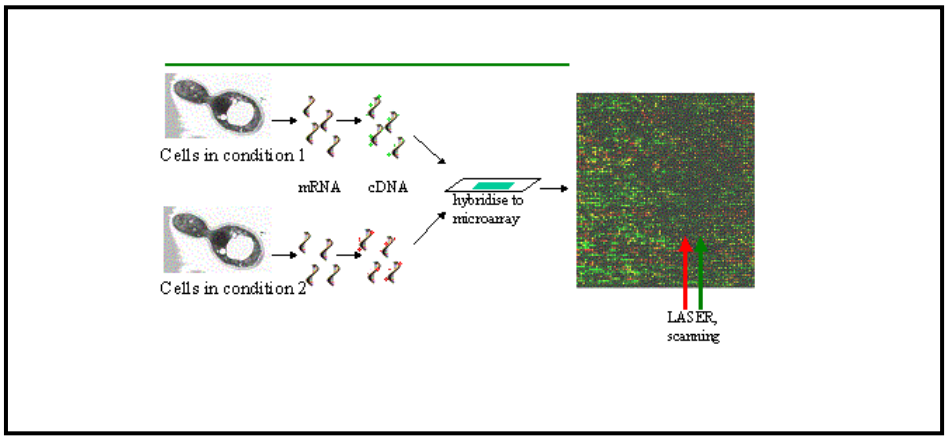
Un microarray es una lámina de vidrio (u otro material) sobre la cual se colocan moléculas de DNA en posiciones prefijadas (*spots*). Puede haber cientos de miles de spots en un array, conteniendo gran número de moléculas idénticas de DNA, de longitudes que oscilan de los veinte a cientos de nucleótidos.



Microscopía electrónica de un spot microarray: las hebras son moléculas de DNA depositadas. Foto de Duggan *et al., Nature Genetics* 21: 10-14, 1999.

Para estudios de expresión génica, cada una de estas moléculas idénticas identifica un gen o un exón del genoma.
Desde 1997, se dispone de microarrays que contienen la totalidad de los aproximadamente 6000 genes del genoma de la levadura.

Una de las aplicaciones de los microarrays es la comparación de los niveles de expresión génica entre dos diferentes muestras o condiciones (ver figura).



Esquema del uso de la tecnología de los microarrays para la comparación de la expresión génica entre dos condiciones de estudio

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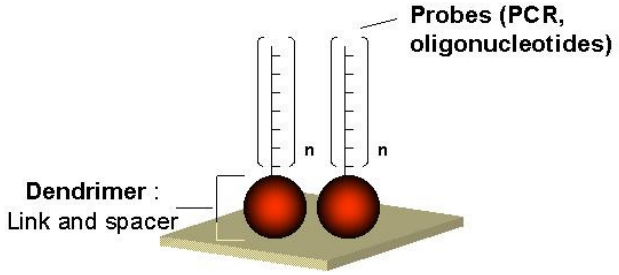
El mRNA total de las células en dos condiciones diferentes es extraído, transformado a cDNA y marcado con dos marcadores fluorescentes diferentes, uno verde (Cy3) y el otro rojo (Cy5). Ambos extractos se colocan sobre el microarray, donde los cDNA marcados hybridan con las secuencias complementarias sobre los spots correspondientes. La cantidad de muestra hibridada en cada condición se mide mediante la intensidad de fluorescencia emitida por el fluorocromo cuando éste es excitado mediante un láser. Si hay más cantidad de RNA de la condición 1, el spot se verá verde, y si es hay más cantidad de RNA de condición 2, entonces se verá rojo. Si ambos niveles de RNA son iguales, el spot aparecerá amarillo. Por tanto, a partir de las intensidades de las fluorescencias y de los colores de cada spot, se estima la expresión génica relativa en ambas muestras.

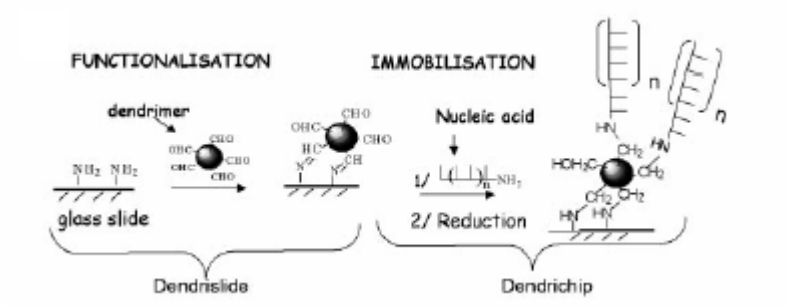
Tecnología utilizada

Las láminas utilizadas (*dendriláminas*) han sido diseñadas (patente Internacional WO 03/091304 A2, 2003) y producidas por el Laboratoire de Chimie de Coordination de Toulouse (LCC-CNRS). Se trata de un soporte para la detección de las hibridaciones, basado en la formación de un lecho uniforme, unido sobre la lámina de vidrio, sobre el cual se fijan mediante enlace covalente las sondas de DNA.



Representación esquemática de la tecnología de las *Dendriláminas*

El lecho está compuesto por dendrímeros esféricos que contienen un gran número de funciones aldehído en periferia. El método de preparación de estas dendriláminas se realiza en diferentes etapas y da lugar a un soporte activo y estable ya que el dendrímero aldehído es estable (Leberre *et al.,* 2003). Las dendriláminas presentan una superficie homogénea con una fuerte capacidad de inmovilización para las sondas de DNA.



Descripción de la activación de la superficie para fijar los dendrímeros e inmovilización mediante unión covalente de los ácidos nucleicos (sondas DNA)

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Las sondas de DNA son oligonucleótidos de 70 mers, representando el 99% del genoma de *Saccharomyces cerevisiae*. Las sondas se inmovilizan mecánicamente sobre la superficie de la lámina mediante un robot automático (Eurogridder from Virtek/Bio-Rad). Cada spot contiene ~2 nl de DNA y un diámetro de ~80 µm.

A) Muestreo y extracción de RNA total

Se recogieron 50 ml de los distintos medios (apartado 1.4), en tubos Falcon.

- Se centrifugaron (3000xg, durante 3 min) y resuspendieron las células en el mismo medio en un volumen inferior a 5 ml.
- Se resuspenden con una pipeta Pasteur y se distribuyen en forma de gotas sobre nitrógeno líquido. Se conservan a -80ºC.

Para la extracción de RNA, se utilizaron tres de las gotas congeladas. En el caso de la levadura seca, se pesaron directamente del paquete 15 mg (~10^8 cells).

- Se rompieron mecánicamente a 2600 rpm durante 2 min. en el microDismembrator (Braun, Melsungen) con una bola de tungsteno de 7 mm de diámetro dentro de un dispositivo de teflón (previamente inmerso en nitrógeno líquido).
- La extracción del RNA se efectuó mediante un kit comercial (RNeasy Midi kit, Qiagen). Se transfieren las células fragmentadas a un tubo de 15 ml con 1,9 ml buffer RTL y 19 µl β-mercaptoetanol.
- Centrifugar 5 min. a 4000 rpm a temperatura ambiente
- Seguir el protocolo del kit a partir del paso 6.
- El paso final de elución, se realiza en un eppendorf RNAsa free con agua-DEPC. Tenemos un volumen final de 500 µl.

Concentración del RNA:

- Añadir 50 µl de Acetato de sodio (3M, pH 5,2) y 500 µl de isopropanol. Se deja precipitar durante 2 horas a -20ºC.
- Centrifugar durante 30 min. a 12.000 rpm, a 4ºC
- Limpiamos dos veces con etanol 70%, centrifugando 5 min. a 12.000 rpm
- Secamos con bomba de vacío.
- Disolver el *pellet* con 50 µl de agua-DEPC.

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B) Cuantificación y comprobación de la pureza del RNA

Se verificó la pureza y se determinó la concentración de RNA extraída mediante RNA 6000 Nano Assay (Bioanalyzer 2100, Agilent Technologies). Se sigue el protocolo suministrado por la casa comercial. El ratio 28S/18S ≥ 1.7

C) Obtención, marcaje y purificación del cDNA

Para preparar las muestras de cDNA marcadas, utilizamos CyScribe First-Strand cDNA Labelling (Amersham Biosciences), conteniendo Cy3-dUTP y Cy5-dUTP fluorescent labels, usando 20 µg de RNA total.

- Añadir en un eppendorf: X µl RNA (màx. 9 µl)
  1 µl random monamers
  1 µl oligo(dT)
  Agua hasta 11 µl volumen
- Homogeneizar e incubar a 70ºC, 5 min. Dejar luego 10 min. a temperatura ambiente.
- Centrifugar 30 seg.
- Poner el eppendorf en hielo y añadir los componentes del marcaje: V_{total} = 20 µl
  4 µl 5X CyScript buffer
  2 µl DTT 0.1M
  1 µl dCTP nucletide mix
  1 µl dCTP CyDye-labelled nucleotides (Cy3-dCTP o Cy5-dCTP)
  1 µl CyScript Reverse Transcriptase
- Vortexar la mezcla y hacer un pulse de centrífuga de 30 seg.
- Incubar la reacción a 42ºC durante 1.5 horas. Conservar el cDNA marcado hasta su purificación en hielo o a -20ºC, protegido de la luz.

Una vez se obtiene el cDNA, ha de purificarse. Para ello, primeramente degradamos los restos de mRNA en pequeños oligómeros mediante un tratamiento alcalino. Luego, estos oligómeros junto con los nucleótidos libres que no se han incorporado, se eliminan mediante columna, utilizando el kit CyScribe GFX Purification Kit (Amersham Biosciences).

<u>Degradación alcalina del mRNA:</u>

- Añadir 2 µl de NaOH 2.5M en cada uno de los tubos que contienen el cDNA marcado.
- Mezclar los tubos con vórtex y centrifugar 30 seg.
- Incubar las muestras a 37ºC durante 15 minutos
- Añadir 10 µl de HEPES free acid 2M en cada tubo de reacción.
- Mezclar los tubos con vórtex y centrifugar 30 seg.

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D) Hibridación en las *dendrilames*

- Las láminas (microarrays) se prehibridan con 3 ml de una solución de BSA 1%, SCC 2X, SDS 0.2%, durante 30 min at 42ºC. Se deja en la cámara de hibridación automática (Discovery™, Ventana).
- Se hibrida la muestra sobre la lámina:

En un eppendorf añadimos: 10 µl cDNA marcado Cy3
10 µl cDNA marcado Cy5
180 µl RiboHybe™ (Ventana)

- Recuperamos el volumen con un pulse de centrífuga, depositamos la mezcla sobre la lámina e hibridamos en la cámara de hibridación a 42ºC durante 10-14 horas.

- Después de la hibridación, las láminas se limpian secuencialmente a temperatura ambiente, sumergiendo la lámina en tubos falcon que contienen las siguientes soluciones: 2X SSC, 1X SSC e isopropanol. Luego se secan con aire comprimido.

E) Análisis de las imágenes y de los datos

Las láminas se escanearon mediante Genepix 4000 (Axon Instrument, CA.) y las señales se analizaron mediante el software Genepix 3.0. Los datos obtenidos a partir de Genepix, fueron normalizados y tratados estadísticamente mediante el software Bioplot, agrupados mediante la aplicación Diagrama de Venn, ambos desarrollados por el servicio informático de la Plateform Transcriptome-Biochips, y GeneSpring (Silicon Genetics).

En el análisis de la lámina, las intensidades se expresan en *log*10. Se corrige la intensidad media de cada *spot* respecto el ruido de fondo local alrededor del *spot* (local background substraction). Se normaliza la intensidad de cada *spot* respecto a la intensidad media global de toda la lámina (All spot's mean). Se calculan las medias aritméticas de los ratios entre las réplicas de los genes existentes en una lámina. Asimismo, se hacen las medias de cada gen entre las dos láminas de la misma prueba pero en las que se cambió el orden de los fluorocromos de marcaje. Para determinar los genes que, significativamente, presentaban diferente expresión se utilizó el Test de Student, considerando una p-value del 5%. Para ratios Cy3/Cy5 superiores a 2, se consideraron genes inducidos, y para ratios inferiors a 0,5, genes reprimidos.

10. Análisis realizados mediante Microscopía electrónica de barrido (SEM = Scanning Electron Microscopy)

El microscopio SEM es una potente herramienta que permite la observación y caracterización de materiales tanto orgánicos como inorgánicos a escala micrométrica. Es uno de los instrumentos más versátiles para el análisis de características microestructurales de muestras sólidas debido principalmente a su elevada resolución (el modelo de microscopio electrónico utilizado JEOL JSM-6400, tiene una resolución de 5 nm a 30 keV) y su gran capacidad de campo, que permite la obtención de imágenes tridimensionales.

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Hemos estudiado la morfología externa de la levadura seca activa durante el proceso de rehidratación mediante microscopia electrónica. De esta manera, determinamos las modificaciones estructurales de la levadura debidas al influjo del agua al interior de la célula. Las muestras se recogieron a los 30 segundos, 1, 3, 5, 10, 15, 20 y 30 minutos a lo largo de la rehidratación. Para cada tiempo de muestreo, se pesaron 0.01g de levadura seca activa y se rehidrataron en 0.1 ml de agua en un baño a 37ºC. Para la microscopía electrónica de barrido, las levaduras se fijaron durante 2 horas a 4ºC mediante una solución 6% de glutaraldehído y 0.1M de tampón fosfato, se centrifugaron las muestras 18'' a 3000 rpm y se limpiaron dos veces con el mismo tampón fosfato. Se resuspendieron en 1% tetróxido de osmio y deshidrataron mediante concentraciones crecientes de etanol. Después de varias limpiezas 100% etanol, las muestras se lavaron con concentraciones crecientes de amilacetato en una solución etanol:amilacetato, hasta alcanzar el 100% de amilacetato. Después, las células se secaron en un secador de punto crítico, se cubrieron con una fina capa de oro y se observaron en el microscopio electrónico de barrido (JSM-6400 Scanning microscope. JEOL, Japan). Se tomaron fotos de 50 levaduras porda muestra y se midieron mediante el software INCA 304 (Oxford Instruments Analytical).

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Funspec: A Web-Based Cluster Interpreter for Yeast
http://funspec.med.utoronto.ca/
FatiGO: data mining with Gene Oncology
http://fatigo.bioinfo.cnio.es/
KEGG Pathway Database
http://www.genome.jp/kegg/pathway.html

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ANNEXE III

Fermentaciones a bajas temperaturas: análisis químico y sensorial

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Fermentaciones a bajas temperaturas: análisis químico y sensorial

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Se postula que los vinos blancos o rosados de elevada expresión aromática fermentados a bajas temperaturas pueden aumentar de forma considerable su potencial aromático. No obstante, esta propuesta choca con dos aspectos fundamentales: en primer lugar la falta de pruebas concretas, tanto a nivel químico como sensorial de dichas diferencias como la dificultad fermentativa. En el presente trabajo se presentan resultados experimentales que avalan las diferencias entre fermentaciones a 13 y 18°C así como propuestas prácticas para la realización de fermentaciones a 13°C con los mínimos riesgos posibles.

1. Introducción

Actualmente el consumidor pide vinos blancos y rosados con marcada tipicidad varietal y dotados de una complejidad aromática y frescura que reflejen la concepción de las vinificaciones en blanco. Las posibilidades técnicas actuales en estas vinificaciones y la personalidad de cada productor introducen una enorme variabilidad en el esquema tradicional de esta vinificación. Desde hace poco más de 30 años que se han incorporado nuevos axiomas en este tipo de vinificación, como son la práctica del desfangado, las fermentaciones a temperatura controlada de 17-18°C y la inoculación de levaduras, por ejemplo, que se han ligado ya a la producción de vinos de calidad.

En la actualidad, hablar de bajas temperaturas se refieren a las que se realizan entre 10°C y 15°C a diferencia de las más habituales en vinificaciones en blanco y en rosado, a las que nos referimos en general como temperatura controlada, a saber, entre 17°C y 20°C. La tendencia es pues, a bajar la temperatura de fermentación a temperaturas similares a las que se utilizan para las segundas fermentaciones del cava o las de las cervezas tipo *lager*.

La elección que tiene el productor para fermentar los mostos blancos o rosados para elegir la temperatura convencional (17°C-20°C) en lugar de una temperatura más baja (10-15°C) depende de dos motivos: la primera, la ausencia de unos resultados claros en las investigaciones a bajas temperaturas, y, en segundo lugar, los esfuerzos y dificultades para su realización por ahora no compensan los resultados obtenidos. Por tanto, está por ver si los posibles resultados cualitativos podrán compensar los esfuerzos a realizar.

En general se ha considerado que cuando se realizan las fermentaciones a bajas temperaturas, las levaduras son proclives a aumentar las concentraciones de compuestos aromáticos. Adicionalmente, se debería producir una mayor retención de aquellos compuestos aromáticos primarios, es decir aquellos provenientes de la uva. No obstante, lo cierto es que a pesar de que algunas levaduras son capaces de sintetizar más compuestos aromáticos a bajas temperaturas, otros presentan concentraciones muy similares a diferentes temperaturas (Tablas I, II y III). Los compuestos de mayor interés desde el punto de vista organoléptico y de calidad global del vino producido podemos destacar los alcoholes superiores, especialmente el 2-feniletanol, y sus correspondientes acetatos, así como los ésteres de ácidos grasos, que son sintetizados a lo largo de la fermentación alcohólica y que dependen estrictamente de la cepa de levadura y las condiciones de vinificación (mosto, temperatura, desfangado, etc.).

La selección de la levadura a utilizar en las fermentaciones de

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Tabla I. Concentración de algunos compuestos volátiles (mg/l) de un vino de moscatel (autores) y de Macabeo [22]

Variedad y lugar de fermentación	Moscatel URV		Macabeo, Rioja	
Temperatura de fermentación	13°C	18°C	14°C	18°C
Esteres etílicos de ácidos grasos	2,94	2,64	2,15	2,25
Acetatos de alcoholes superiores	0,66	0,65	1,62	2,21
Alcoholes isoamílicos y feniletanol	621	69	146	177
Terpenos	1,88	2,04	nd	nd

Tabla II. Compuestos volátiles (mg/l) de un vino de Pinot noir fermentado a 10°C y 25°C, con dos levaduras diferentes [17]

Cepa de levadura	Cepa SY055		Fermol Bouquet	
Temperatura de fermentación	10°C	25°C	10°C	25°C
Esteres etílicos de ácidos grasos	5,7	5,0	4,7	5,5
Acidos grasos de cadena media	4,3	4,8	4,9	5,5
Acetatos de alcoholes superiores	5,0	4,5	3,9	4,5
Alcoholes isoamílicos + feniletanol	516	497	271	235

los denominados mostos limpios como son los blancos y rosados es crucial, ya que tiene una influencia directa en la cinética fermentativa y la formación de compuestos volátiles en los vinos. Actualmente se encuentra disponible en el mercado una amplia oferta de levaduras secas activas (L.S.A), algunas de ellas seleccionadas para producir vinos blancos a bajas temperaturas. Todas ellas pertenecen al género *Saccharomyces*. La importancia de esta selección ha conllevado la clasificación de un grupo de levaduras que se han denominado criotolerantes, capaces en principio de realizar la fermentación entre 6°C y 30°C con un óptimo < 30°C, dejando de lado los no-criotolerantes con temperaturas entre 12°C y 36°C [5]. Estos estudios se han realizado para aplicaciones en la producción de vinos blancos secos (Valle de del Loire Francia, Moldavia, Italia y España) como licorosos (Sauternes i Jurançon en Francia o Tokay en Hungría).

Las principales especies de levadura utilizadas y comercializadas son de las especies *S. cerevisiæ* y *bayanus*. Las levaduras criotolerantes se han designado como de raza fisiológica *S. uvarum* al utilizar la antigua nomenclatura, aunque en realidad corresponde a alguna de las especies de *S. cerevisiæ* o *S. bayanus*. Estas levaduras se considera que presentan algunas características específicas al fermentar a bajas temperaturas, especialmente dando vinos más ácidos, debido a que la síntesis de los ácidos málico y succínico es mayor que su degradación (Tabla III). A pesar de que el rendimiento de azúcares/alcohol es inferior a la de otras levaduras, se debe tener en cuenta que la producción de 2-feniletanol es más elevada.

Tabla III. Comparación de la producción de los principales productos de la fermentación alcohólica entre las levaduras criotolerantes y no-criotolerantes [5, 8 y 3]

Tipo de cepa de levadura	Criotolerante *S.cerevisiæ p.r. uvarum*	No-criotolerante *S. cerevisiæ*
Temperatura de fermentación	6 – 30°C	12 – 35°C
Tolerancia a etanol	< 14%	≥ 14%
Capacidad fermentativa	elevada	media
Acido málico	Síntesis de 0,5 – 1,5 g/l	Degradación de 10 – 30%
Acido acético	30 – 300 mg/l	100 – 800 mg/l
Acido succínico	1 – 2 g/l	0,5 – 1,5 g/l
Glicerol	4 – 8 g/l	3,5 – 6,5 g/l
2 fenil etanol	1.20-453 mg/l	47 mg/l

2. Análisis sensorial

No obstante, las diferencias en cuanto a la composición química adquieren su auténtica relevancia cuando se ven refrendadas a nivel sensorial. En nuestra bodega se han realizado fermentaciones a 13 y 18°C durante dos años consecutivos (campañas del 2001 y 2002) con la variedad moscatel, en las mismas condiciones (fermentadores semiindustriales de 100 litros, idéntica cepa de levadura, rehidratación y mismo mosto). Se han realizado para ambas campañas pruebas triangulares de identificación con catadores profesionales y no profesionales (Tabla IV), mostrando en todos los casos que se identificaron las diferencias unas preferencias claras por los vinos procedentes de las fermentaciones a bajas temperaturas. Adicionalmente, las diferencias se detectaron siempre por los catadores profesionales y significativamente por los no profesionales (probabilidad de error inferior al 5% en la campaña 2002).

3. Inconvenientes de las fermentaciones a bajas temperaturas

A pesar de las ventajas que se obtienen, las fermentaciones a bajas temperaturas son procesos muy conflictivos por varias razo-

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Tabla IV. Resultado de catas triangulares en la cata de vinos fermentados a 13 y 18°C

	Catadores profesionales		Catadores no profesionales	
Campaña	Número	Identificación	Número	Identificación
2001	4	4 (<1%)	8	5 (NS)
2002	5	5 (<1%)	10	7 (<5%)

En todos los casos se indicó la preferencia hacia los vinos fermentados a 13ºC. En la identificación se indica el grado de significatividad de la respuesta (probabilidad de error, en %, NS= no significativo).

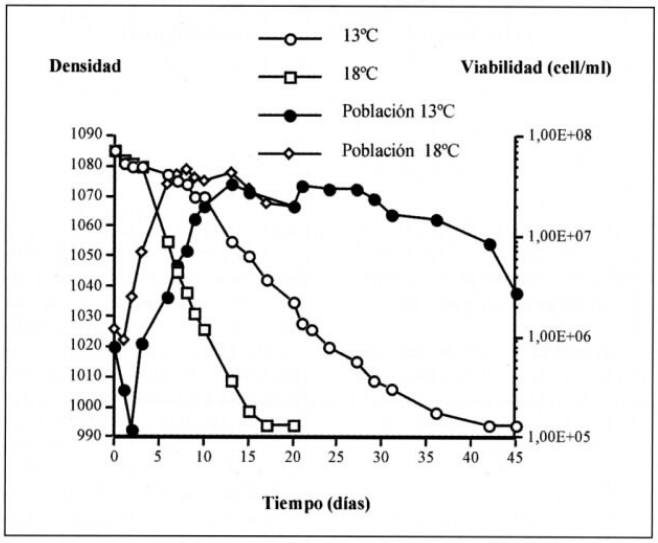

Figura 1. Fermentación de mosto Moscatel inoculado con QA23 (Lallemand) a 13°C y 18°C (Finca Mas dels Frares, Tarragona, 2000).

nes: el inicio de fermentación es más tardío y las fermentaciones son lentas (Fig. 1), llegando incluso a producir paradas de fermentación, lo que produce un alto grado de ocupación de los depósitos de fermentación. No obstante, un mejor conocimiento de las condiciones de fermentación puede hacer que las fermentaciones a bajas temperaturas sean más factibles. En el momento actual, se pueden mejorar de forma considerable mediante una acción coordinada que implique: compensación del nitrógeno fácilmente asimilable (NFA = Amonio y Aminoácidos excepto la prolina), nivel de oxígeno (aireación), y condiciones de rehidratación de las LSA previas a su utilización.

Uno de los factores relevantes y que puede condicionar sobre manera a las fermentaciones a bajas temperaturas es el contenido en NFA. Podemos encontrar diversas metodologías factibles para su uso en el ámbito de bodega para determinar el NFA [1 y 7] lo que nos permitiría hacer las adiciones pertinentes con amonio. Para valores de NFA < 150 mg N/l en mosto es preferible realizar adiciones de amonio,

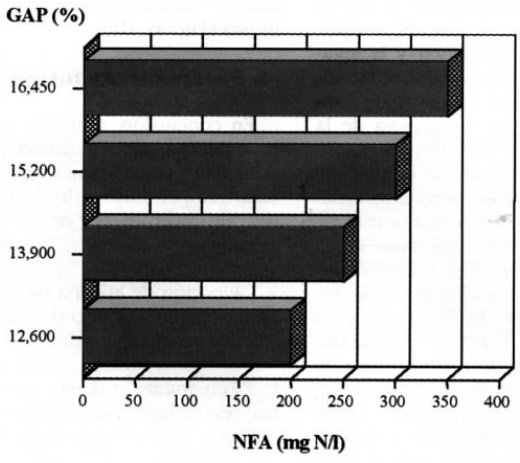

Figura 2 – Relación entre el Grado Alcohólico Probable (GAP) y las necesidades en Nitrógeno Fácilmente Asimilable (NFA) [4].

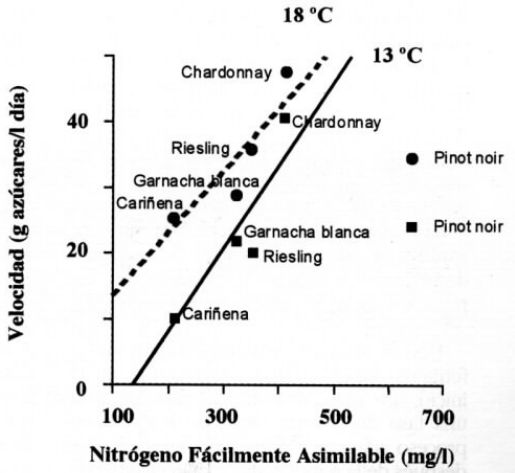

Figura 3 – Relación entre el contenido en NFA de diferentes mostos blancos y rosados, la temperatura de fermentación y la velocidad máxima de fermentación [16].

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aunque se ha de tener en cuenta que las concentraciones de azúcares condicionarán las necesidades de nitrógeno. Así cuanto mayores sean las concentraciones de azúcar en el mosto, mayores serán los requerimientos de nitrógeno para poder acabar la fermentación (Fig. 2). Tanto el NFA como la temperatura tienen influencia sobre la velocidad de fermentación (Fig. 3). A mayores concentraciones de NFA, mayor velocidad de fermentación [15 y 16]. Se puede observar, no obstante, alguna excepción, por ejemplo la observada con la variedad Pinot Noir. En esta variedad, la mayor parte del NFA está en forma de aminoácidos, a diferencia del caso más general, en que un 30% suele estar en forma de amonio. El aumento de velocidad de fermentación tiene un límite, superior al que se cita frecuentemente de 250 mg NFA/l, debido a la temperatura de fermentación (Fig. 3). Así, tanto a 13 como 18°C se puede observar como hay aumentos de velocidad de fermentación hasta 500 mg NFA/l, mientras que el límite de 250 se cita para fermentaciones a 25°C. No obstante, la concentración de NFA se debe referir a la concentración de azúcares en el medio, que es también un factor limitante.

El oxígeno también es un factor importante en vinificación y especialmente en el vino blanco. Las levaduras cuando crecen en anaerobiosis reducen su contenido en ácidos grasos insaturados y esteroles, que forman parte de la membrana plasmática [12 y 13]. La recuperación del óptimo funcional de las membranas (intercambios entre células y medio, multiplicación celular, reducción de la síntesis de ácidos de cadena mediana) requiere la nueva síntesis de ácidos grasos insaturados, para lo que es necesario la presencia de oxígeno [9]. Por eso se hace necesaria la aireación de los mostos para evitar paradas de fermentación. El mejor momento para la aireación (pequeño remontado) y la adición de nutrientes posiblemente sea la mitad de la fermentación, esto permitiría un consumo total de azúcares y la disminución de la duración total de la fermentación, especialmente para mostos conflictivos [21].

Uno de los problemas importantes en las fermentaciones a bajas temperaturas es el inicio, que generalmente es lento debido a una fase de latencia larga; en realidad un proceso adaptativo a las bajas temperaturas después de la rehidratación. La necesidad de este tiempo de adaptación es un ejemplo muy claro de las limitaciones de estas fermentaciones. Adicionalmente, otras levaduras *no-Saccharomyces* como *Kloeckera apiculata* y *Candida stellata* presentan buena viabilidad y podrían dificultar la imposición de *S. cerevisiæ* en estas condiciones [6].

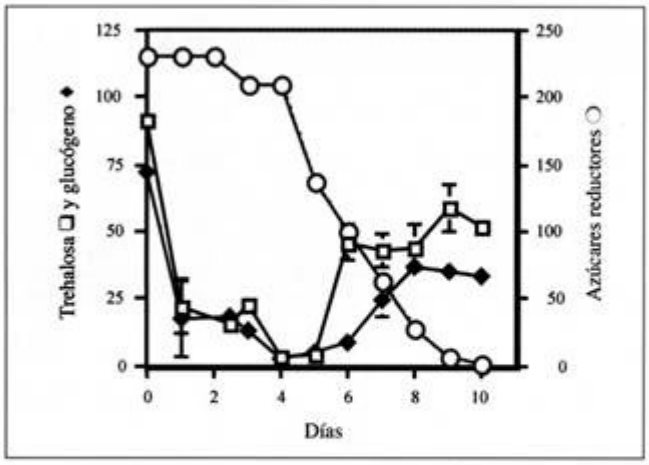

Figura 4. Concentraciones de trehalosa (❑) y glucógeno (◆) intracelulares (µg 10^7 células) durante la fermentación a 13ºC (azúcares reductores, mg/l, ○) en un medio sintético.

La rehidratación de las levaduras podría jugar un papel clave en la adaptación a bajas temperaturas. En general se recomienda utilizar agua tibia para la rehidratación (35°C – 40°C) durante un período de 30 minutos o en una mezcla de agua y mosto. Para evitar el choque térmico que podría conducir a la reducción de viabilidad de las levaduras se debería esperar a que la diferencia de temperatura entre el mosto a fermentar y la levadura rehidratada fuera la menor posible. Esto se consigue con una reducción progresiva de la temperatura de fermentación. No obstante, aún no hemos podido reducir de forma consistente la fase de latencia. Por esta razón nos hemos centrado en la investigación del metabolismo de sustancias glucídicas de reserva, como la trehalosa y el glucógeno. Especialmente la trehalosa está relacionada con la movilización de reservas intracelulares previa a la utilización de azúcares del medio por parte *S. cerevisiæ*, siendo crucial para su viabilidad, crecimiento y adaptación a condiciones de estrés, elementos imprescindibles para el desarrollo de la fermentación alcohólica. La trehalosa y el glucógeno son dos carbohidratos que sintetizan las levaduras y se acumulan en la célula para responder a diversas condiciones, tales como las altas temperaturas, choques osmóticos y presencia de etanol en el medio [14 y 2]. Las levaduras comerciales pueden contener en el momento de la rehidratación unas cantidades de estos carbohidratos que oscilan entre el 20 y 30% de su peso seco, que se consumen completamente en las primeras horas de desarrollo en un sustrato fermentable, como el mosto (Fig. 4), [18].

4. Perspectivas futuras

En conclusión, teniendo presente la investigación actual, para el éxito de fermentaciones a bajas temperaturas se deben tener en consideración los siguientes procesos:

- Elección de la cepa de levadura adecuada al tipo de mosto a fermentar.

- Rehidratación de las levaduras según las recomendaciones del proveedor previendo el choque térmico que se puede producir.

- Bajar lentamente la temperatura de fermentación.

- Controlar y rectificar la concentración en nitrógeno y practicar una aireación al final de la fase de crecimiento de las levaduras o a la mitad de la fermentación.

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ANNEXE IV

Revisión sobre las fermentaciones vínicas a baja temperatura. Perspectivas de futuro

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Revisión sobre las fermentaciones vínicas a baja temperatura. Perspectivas de futuro

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Nuestro grupo se ha dedicado al estudio de las levaduras vínicas tanto a nivel de identificación y seguimiento de fermentaciones industriales, como a nivel del metabolismo de* Saccharomyces cerevisiae *durante la fermentación alcohólica. A continuación, vamos a revisar algunos de los aspectos en los que se ha trabajado en relación a las fermentaciones vínicas a baja temperatura. Es decir, el efecto de la temperatura sobre la modificación de las poblaciones de microorganismos, su efecto sobre la cinética fermentativa y sobre el crecimiento y el metabolismo de* S. cerevisiae. *Al final, también apuntamos unas estrategias prácticas para optimizar el proceso desde un punto de vista enológico.

1. Introducción

El interés por la realización de la fermentación alcohólica a muy bajas temperaturas (10-15°C) surge una vez conseguido, por parte de la industria enológica, el control efectivo de la temperatura de fermentación. Es en los países denominados nuevos productores (Chile, Argentina, Sudáfrica, Canadá y California) donde primeramente se aplican estas temperaturas en la elaboración de vinos blancos y rosados, en busca de vinos con una mayor expresión de las variedades viníferas de gran potencial aromático.

Los beneficios que las bajas temperaturas aportan al vino se derivan principalmente de la mejora de su perfil aromático y sensorial, aunque hay pocos estudios al respecto con resultados claros y concluyentes. En general, se considera que las bajas temperaturas aumentan los aromas afrutados característicos de algunos ésteres (acetato de isoamilo) y disminuyen algunos compuestos que merman la calidad (ácido acético, acetaldehido y acetato de etilo) [9]. No obstante, las diferencias en cuanto a la composición química del vino adquieren su auténtica relevancia cuando se ven reflejadas a nivel sensorial. En nuestros estudios, hemos realizado pruebas triangulares de identificación con catadores profesionales y no profesionales, mostrando unas preferencias claras por los vinos procedentes de las fermentaciones a bajas temperaturas (13°C). Los vinos fermentados a baja temperatura fueron siempre identificados por los catadores profesionales y significativamente por los no profesionales [11].

Los compuestos aromáticos que están condicionados por la temperatura de fermentación son de dos tipos, los primarios, provenientes directamente de la uva, y los secundarios, productos del metabolismo de la levadura. Los aromas primarios se ven retenidos en mayor medida cuando bajan las temperaturas de fermentación. En el caso de los aromas secundarios, también llamados fermentativos, la temperatura es muy importante ya que condiciona totalmente el desarrollo de la levadura y, por tanto, la producción de unos compuestos u otros [7].

A pesar de todas estas ventajas cualitativas que aportan las bajas temperaturas de fermentación al producto elaborado, hay que tener presentes los inconvenientes que conllevan este tipo de fermentaciones. En realidad, todos los efectos negativos son el resultado de la dificultad con la que se encuentran las levaduras vínicas *Saccharomyces cerevisiae* para desarrollarse en unas condiciones adversas. En este tipo de fermentaciones, la capacidad de la levadura para crecer y desarrollar la fermentación alcohólica es clave.

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2. Efecto de la temperatura sobre la dinámica de las poblaciones de levaduras

El mosto de uva no es un substrato estéril y la fermentación alcohólica es un proceso bioquímico complejo en el que intervienen diferentes especies y cepas de levaduras. En nuestro grupo, hemos estudiado con diferentes técnicas moleculares la evolución de las diferentes poblaciones de levaduras durante la fermentación alcohólica durante varios años consecutivos y en diferentes regiones vitivinícolas [21 y 2]. En todos estos trabajos se pone de manifiesto un predominio de especies no-*Saccharomyces*, principalmente *Hanseniaspora uvarum* (o su fase imperfecta *Kloeckera apiculata*) y *Candida stellata*, en la uva, mosto y primeras fases de la fermentación alcohólica. Sin embargo, conforme avanza el proceso, la especies *S. cerevisiae* aumenta su proporción hasta ser claramente mayoritaria como consecuencia de una mayor capacidad fermentativa y mayor tolerancia a etanol y al SO_2. Durante la fermentación tumultuosa y hasta el final de la fermentación esta especie ya es claramente mayoritaria y la principal responsable de la transformación de los azúcares en etanol y CO_2. Sin embargo, otros grupos que han llevado a cabo estudios similares sobre las poblaciones de levaduras han comprobado que bajas temperaturas de fermentación aumentan la tolerancia de algunas especies no-*Saccharomyces* frente al etanol y por tanto, la permanencia de estas especies aumenta en estas fermentaciones y la imposición de *S. cerevisiae* no es tan rápida [6 y 3]. En estos estudios se comprobó que *Kl. apiculata* y *C. stellata* no morían y que incluso se mantenían en poblaciones viables muy elevadas (10^7-10^8 ufc/ml) a lo largo de toda la fermentación. Una mayor contribución de estas especies no-*Saccharomyces* durante el proceso tiene consecuencias en la composición química y sensorial del vino. Varios estudios han mostrado que las cepas de *K. apiculata* y *C. stellata* modifican el perfil aromático de los vinos producidos pero especialmente los niveles de ácido acético y de acetato de etilo (Tabla I).

3. Efecto sobre el crecimiento de las cepas de *Saccharomyces*

Al aumentar la temperatura, aumentan tanto el crecimiento de las levaduras como la cinética fermentativa. De esta manera, las fermentaciones conducidas a baja temperatura resultan más largas y lentas, aumentando los riesgos de paradas de fermentación. Además, una baja temperatura inicial puede limitar el crecimiento de las levaduras, teniendo una población insuficiente para una correcta fermentación [14]. *S. cerevisiae* no se caracterizan por su criotolerancia, especialmente si las comparamos con otras especies de levaduras. Sin embargo, no todas las cepas de *S. cerevisiae* presentan la misma capacidad fermentativa a diferentes temperaturas.

Torija y colaboradores [23] llevaron a cabo un estudio para conocer el efecto de la temperatura de fermentación sobre el crecimiento de diferentes cepas de *S. cerevisiae*. Para ello un total de 20 cepas eran inoculadas en el mismo mosto y en la misma proporción. Estos mostos se ponían a fermentar a temperaturas entre 15 a 35°C. Entre otros resultados, una de las conclusiones más importantes del trabajo fue que para cada temperatura de fermentación las cepas que aparecían al final del proceso eran diferentes o presentaban distinta proporción, es decir, que cada temperatura favorecía el desarrollo de un número de cepas distintas (Fig. 1). El análisis de los distintos vinos obtenidos también mostraban que la composición química de los mismos se modificaba en función de la temperatura de fermentación. La concentración de etanol era más alta en las fermentaciones a baja temperatura mientras que por el contrario la concentración de glicerol y acético aumentaban conforme aumentaba la temperatura (Tabla II).

4. Disponibilidad de levaduras criotolerantes

Como mostraba el anterior trabajo, ni todas las levaduras se comportan del mismo modo ni todas presentan las mismas características, es por esto que la selección de la levadura a utilizar es un punto crucial para el buen desarrollo de las fermentaciones a bajas temperaturas. Actualmente se encuentra disponible en el mercado una amplia oferta de levaduras secas activas (LSA), algunas de ellas seleccionadas para producir vinos blancos a bajas temperaturas. Estas levaduras se venden bajo la etiqueta de levaduras criotoleran-

Tabla I. Concentraciones de ácido acético, ésteres, acetaldehido, etanol y alcoholes superiores producidos por diferentes especies de levaduras vínicas [6]

Especies	Acido acético (g/l)	Acetato etilo (mg/l)	Acetato isoamilo (mg/l)	Acetal-dehído (mg/l)	Etanol %	Propanol (mg/l)	Isobutanol (mg/l)	Alcohol isoamílico (mg/l)
Saccharomyces Cerevisiae	0,3-0,8	10-100	0,1-16	50-120	6-23	9-170	5-78	17-330
Kloeckera apiculata	1-2,5	25-375	0,4-5	6-40	5-6,5	16-32	4-38	4-17
Candida krusei	1,0	220-730	<0,1	-	1-6,5	1-23	38-106	22-100
Candida stellata	1-1,3	7-25	0,1-0,4	-	4,5-6,5	4-8	13-21	-
Hansenula anomala	1-2	137-2.150	1-11	-	0,2-4,5	3-15	18-29	11-25
Metschnikowia	0,1-0-15	150-382	0,1-0,8	23-40	2	<1-43	37-123	21-243

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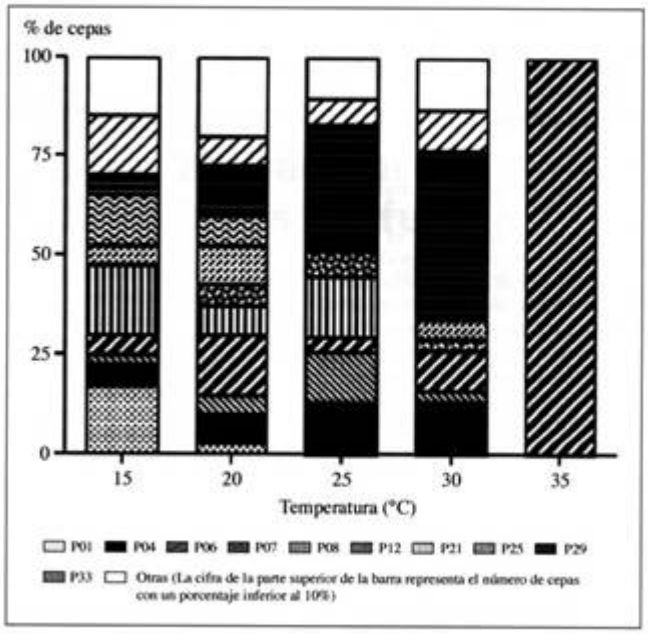
tes y designado tradicionalmente como de raza fisiológica *S. uvarum*, aunque en realidad corresponden a alguna de las especies *S. cerevisiae* o *S. bayanus*. En nuestro grupo hemos probado algún cultivo seleccionado como criotolerante y no siempre presentaban una buena capacidad fermentativa y sobre todo mostraban una baja tolerancia al etanol [22]. Por tanto, las compañías dedicadas a la comercialización de levaduras de uso enológico deberían aumentar la selección de levaduras con buena capacidad fermentativa a baja temperatura y prever específicamente los niveles de azúcares o etanol resultantes para minimizar riesgos.

5. Efecto de la temperatura sobre el metabolismo de la levadura

Las bajas temperaturas tienen su principal efecto en la membrana plasmática de las levaduras, reduciendo su fluidez y dificultando el transporte de sustancias. Hay que pensar que la membrana plasmática es el primer elemento de contacto entre la célula y su entorno. Por ello, se considera que juega un papel esencial en la respuesta adaptativa de la levadura [18]. En relación con esta respuesta adaptativa, realizamos un estudio sobre el efecto de la temperatura de fermentación sobre la composición de los ácidos grasos de membrana en dos cepas de *S. cerevisiae* y una cepa de *S. bayanus* [22]. La principal estrategia para mantener una fluidez de membrana adecuada fue aumentar el grado de insaturación de los ácidos grasos en las cepas de *S. cerevisiae*. Sin embargo, la cepa de *S. bayanus* adoptó una estrategia diferente que consistía en aumentar la concentración de ácidos grasos de cadena media (Tabla III). En este estudio también se llevo a cabo un análisis de los compuestos volátiles, probándose de nuevo una mayor concentración de estos compuestos a bajas temperaturas (Tabla IV). Aunque esta concentración

Figura 1. Porcentaje de cepas de Saccharomyces cerevisiae presentes al final de la fermentación [23].



también depende de la cepa o especie utilizada. Así, la cepa de *S. bayanus* se caracteriza por una elevada concentración de 2-fenil etanol en los vinos finales.

También relacionado con la composición de la membrana plasmática, Rozès y colaboradores [15] habían demostrado una disminución de la síntesis de esteroles. Otros problemas metabólicos puestos de manifiesto por otros investigadores es una disminución de la síntesis proteica de la célula como consecuencia de una menor velocidad catalítica de los enzimas [17]. En este caso la respuesta adaptativa de la célula consiste en aumentar el número de ribosomas celulares para compensar una menor velocidad de síntesis con un aumento de los orgánulos necesarios para esta síntesis proteica. Otros trabajos se han centrado en la identificación de genes de *S. cerevisiae* que aumentan la expresión en condiciones de baja temperatura. Entre ellos en-

Tabla II. Análisis químicos de los vinos finales fermentados a diferentes temperaturas [23]

Concentración (g/l)	15°C	20°C	25°C	30°C	35°C
Etanol	93,6	93,04	90,00	89,60	79,52
Glicerol	6,05	6,59	6,91	7,18	7,38
Acetaldehído	0,05	0,09	0,04	0,04	0,02
Ácido succínico	0,74	0,89	0,77	0,92	0,70
Ácido acético	0,08	0,13	0,14	0,13	0,22
Suma de productos	**100,52**	**100,74**	**97,86**	**97,87**	**87,84**
CO_2	89,53	88,99	86,08	85,70	76,06
Productos + CO_2	**190,05**	**189,73**	**183,94**	**183,57**	**163,90**

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Tabla III. Cambio de los perfiles de los ácidos grasos de membrana durante la fermentación. Los valores están expresados como porcentaje del total de ácidos grasos [22]

		Ácidos grasos	LSA	Mosta a ρ = 1.050	Mosta a ρ =1.000	Vino
S. cerevisiae	25°C	Cadena Media	0	13,65	17,29	23,18
		Saturados	20,48	60,42	64,95	61,73
		Insaturados	79,52	25,93	17,76	15,09
		Longitud media	17,05	15,76	15,64	15,23
		Insat./Satur.	3,88	0,43	0,27	0,24
	13°C	Cadena Media	0	25,18	26,04	21,99
		Saturados	20,48	50,73	50,98	50,25
		Insaturados	79,52	24,09	22,98	27,76
		Longitud media	17,05	14,97	14,96	15,12
		Insat./Satur.	3,88	0,47	0,45	0,55
S. bayanus	25°C	Cadena Media	0	24,30	21,61	22,13
		Saturados	26,16	52,93	63,25	59,78
		Insaturados	73,85	22,78	15,14	18,09
		Longitud media	17,00	15,05	15,23	15,29
		Insat./Satur.	2,82	0,43	0,24	0,30
	13°C	Cadena Media	0	34,45	33,20	36,09
		Saturados	26,16	43,62	45,36	44,34
		Insaturados	73,85	21,93	21,44	19,57
		Longitud media	17,00	14,20	14,39	14,30
		Insat./Satur.	2,82	0,50	0,47	0,44

Tabla IV. Contenido de los compuestos volátiles en los vinos (mg/l). El símbolo (-) son no detectados [22]

	S. cerevisae		*S. bayanus*	
	25°C	13°C	25°C	13°C
2-fenil etanol	**13,91**	**15,48**	**49,22**	**61,55**
Acetato de isoamilo	-	3,14	-	-
Acetato de hexilo	-	-	-	0,03
Acetato de 2-feniletil	0,23	0,29	0,51	1,12
∑ Acetatos	**0,23**	**3,43**	**0,51**	**1,15**
Butirato de etilo	-	0,51	-	-
Caproato de etilo	-	0,40	0,06	-
Caprilato de etilo	0,17	0,20	0,04	0,23
Caprato de etilo	0,04	0,07	0,04	0,09
∑ Etilos	**0,21**	**1,17**	**0,14**	**0,32**
Acido butírico	0,74	3,02	0,62	4,21
Acido hexanoico	3,37	5,50	2,03	4,36
Acido octanoico	2,63	6,01	2,59	3,31
Acido decanoico	0,03	0,36	0,32	0,16
Acido dodecanoico	-	0,04	-	-
∑ ácidos grasos de cadena media	**6,77**	**14,92**	**5,57**	**12,04**

contramos genes relacionados con el procesamiento del ARNr y la biosíntesis ribosomal, un grupo de genes inducibles por la temperatura (TIP, TIR) de funciones aún por determinar, genes relacionados con la captación de nutrientes y otro gen relacionado con la saturación de los ácidos grasos [17 y 20].

La información que se dispone sobre el metabolismo de la levadura durante la fermentación alcohólica está creciendo exponencialmente en los últimos años. Este aumento de la información se debe fundamentalmente a la disponibilidad desde el año 1996 de toda la secuencia del genoma de *S. cerevisiae*. La secuencia nucleotídica de todos los genes de la levadura ha permitido llevar a cabo análisis sobre la importancia de cada uno de estos genes en los procesos industriales conducidos por este organismo. Entre estas técnicas ha tenido gran importancia lo que se conoce como la tecnología de los *chips* de DNA o *microarray* [13]. Esta técnica se ha utilizado para comparar los patrones de expresión del genoma de la levadura en diferentes condiciones durante la fermentación alcohólica [4, 5 y 19]. Nuestro grupo ha llevado a cabo un análisis de la expresión global de una levadura vínica durante fermentaciones alcohólicas industriales a 13°C y 25°C. Con los datos que estamos obteniendo de este análisis nos permitirá conocer mucho mejor las principales dificultades metabólicas de la levadura cuando crece a temperaturas bajas. Un mayor conocimiento también nos permitirá en el futuro diseñar estrategias para adaptar o mejorar la capacidad fermentativa de las levaduras en fermentaciones en frío.

6. Herramientas para mejorar las fermentaciones a baja temperatura

En el momento actual, y hasta que no se tenga un mayor cono-

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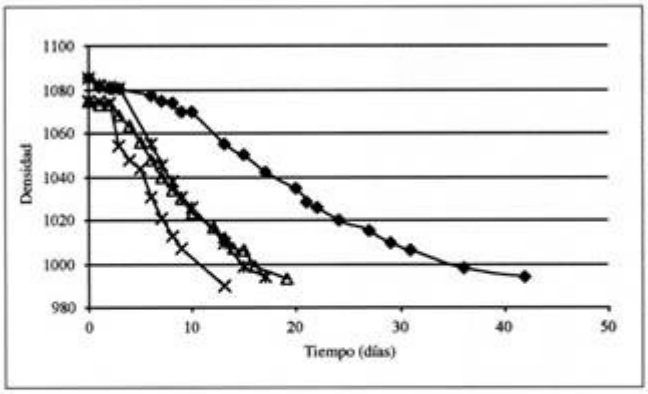

Figura 2. Cinéticas de las fermentaciones 2001 vs. 2002, a dos temperaturas. En el segundo año, realizamos las acciones que se indican y logramos reducir significativamente la duración de la fermentación a 13ºC 13ºC 2001, X, 18ºC 2001, ▲ 13ºC 2002, ◆18ºC 2002.

cimiento del proceso adaptativo de la levadura, las fermentaciones a baja temperatura se pueden mejorar de forma considerable controlando ciertos parámetros (Fig. 2). En realidad se trata de una acción coordinada que implica varios aspectos: (i) compensación del nitrógeno fácilmente asimilable (NFA= Amonio y Aminoácidos excepto la prolina), (ii) nivel de oxígeno (aireación), (iii) condiciones de rehidratación de las LSA previas a su utilización y (iv) modificación gradual de la temperatura [11].

Tanto el NFA como la temperatura tienen influencia sobre la velocidad de fermentación. A mayores concentraciones de NFA, mayor velocidad de fermentación (9 y 10). Para valores de NFA<150 mgN/l en mosto es preferible realizar adiciones de amonio. Pero el aumento de velocidad de fermentación tiene un límite, superior al que se cita frecuentemente de 250 mg N/l debido a la temperatura de (Fig. 3). Así, tanto a 13°C como a 18°C se puede observar como hay aumentos de velocidad de fermentación hasta 500 mg N/l, mientras que el límite de 250 se cita para fermentaciones a 25°C.

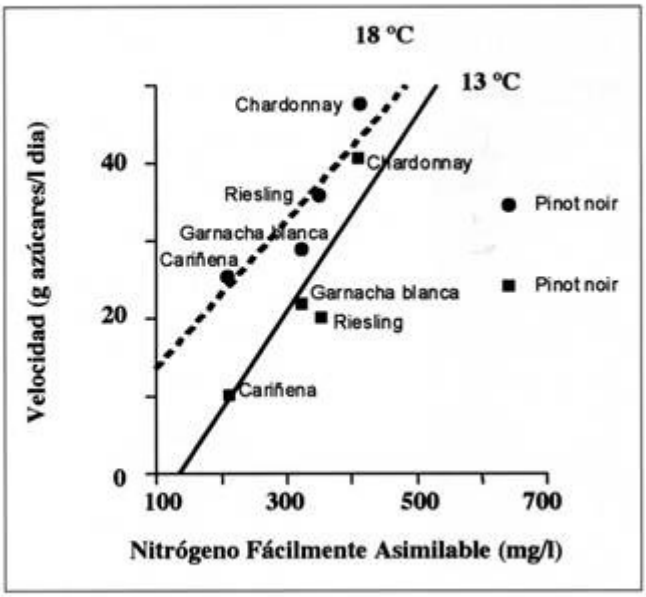

Figura 3. Relación entre el contenido en NFA de diferentes mostos blancos y rosados, la temperatura de fermentación y la velocidad máxima de fermentación [11].

No obstante, la concentración de NFA debe referirse a la concentración de azúcares en el medio, que es también un factor limitante.

Un efecto fundamental de las bajas temperaturas es el aumento del grado de insaturación de los ácidos grasos y la reducción de la síntesis de esteroles. La síntesis de todos estos compuestos requiere la presencia de oxígeno. Así que para evitar las paradas de fermentación, se hace necesaria la aireación de los mostos. El momento idóneo sería el inicio de la fase estacionaria (máximo de población), en donde haríamos un pequeño remontado y añadiríamos los nutrientes. De esta manera, se favorecería el consumo de azúcares y se disminuiría la duración total de la fermentación [16].

Quizá el punto clave en el desarrollo de las fermentaciones a baja temperatura es la fase de latencia inicial, en donde la levadura se está adaptando al medio después de la rehidratación. Posiblemente, el propio proceso de rehidratación sea trascendental en la adaptación a las bajas temperaturas. En general, se recomienda utilizar agua tibia para la rehidratación (35-40°C) durante un período de 30 minutos o en una mezcla de agua y mosto. En nuestro laboratorio hemos comprobado que el choque térmico que supone la inoculación directa de la levadura tras su rehidratación en un mosto refrigerado en torno a 13°C supone una mortalidad de hasta el 90% del cultivo (datos no publicados). Para evitar este choque térmico, se debería evitar que esta diferencia de temperatura fuese tan grande. Una solución ensayada por nuestro grupo durante la vendimia 2002 fue reducir progresivamente la temperatura del mosto una vez inoculada. También atemperar las levaduras después de la rehidratación podía ser eficaz para disminuir esta mortalidad inicial.

No obstante, y en relación con el proceso de rehidratación,

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hemos estudiado la utilización de diferentes medios de rehidratación para conseguir una mayor actividad metabólica de la levadura antes de su inoculación. Como indicador de esa activación metabólica hemos seguido la concentración intracelular de dos carbohidratos de reserva como son la trehalosa y el glucógeno. Especialmente la trehalosa, está relacionada con la movilización de reservas intracelulares previa a la utilización de azúcares del medio, siendo crucial para la viabilidad, crecimiento y adaptación a condiciones de estrés de la levadura [8 y 1]. Hemos observado que el medio usado en la rehidratación condiciona la activación metabólica de la levadura, medida por el consumo de la trehalosa intracelular [12]. La presencia de una fuente de carbono fermentable en el medio de adaptación reduce rápidamente los niveles de trehalosa. En nuestro estudio, la rehidratación con agua y posterior adición de mosto sintético (2% del volumen de la tina) redujo significativamente la fase de latencia y mejoró la cinética fermentativa.

En conclusión, teniendo presente la investigación actual, para el éxito de fermentaciones a bajas temperaturas se deben tener en consideración los siguientes procesos:

- Elección de la cepa de levadura adecuada al tipo de mosto a fermentar.
- Rehidratación de las levaduras según las recomendaciones del proveedor previendo el choque térmico que se puede producir.
- Bajar lentamente la temperatura de fermentación.
- Controlar y rectificar la concentración en nitrógeno y practicar una aireación al final de la fase de crecimiento de las levaduras o a la mitad de la fermentación.

7. Perspectivas futuras

Como hemos visto, la realización de la fermentación alcohólica a bajas temperaturas condiciona en gran medida el comportamiento y el desarrollo de las levaduras vínicas.

Es necesario, pues, identificar los parámetros genéticos que condicionan la adaptación de la levadura a las bajas temperaturas. Sólo de esta manera podremos intervenir de una forma efectiva y controlada en la mejora de estas fermentaciones. Las nuevas tecnologías en biología molecular son las herramientas que permitirán conocer el sistema metabólico global de la levadura durante los procesos enológicos.

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