YEAST STRESS RESPONSES DURING ACCLIMATION FOR SPARKLING WINE PRODUCTION

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Yeast stress responses to acclimation for sparkling wine production

Doctoral Thesis

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Universitat Rovira i Virgili

Departament of Biochemistry and Biotechnology

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This doctoral thesis was carried out between 2009-2016 within the Food Biotechnology Microbiology research group (Department of Biochemistry and Biotechnology, Faculty of Oenology) at the University Rovira i Virgili. The doctoral thesis was supervised by Professors Nicolas Rozès and Montse Poblet.

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We STATE that the present study, entitled "Yeast stress responses to acclimation for sparkling wine production", presented by Anna Borrull Riera for the award of the degree of Doctor, has been carried out under our supervision at the Department of Biochemistry and Biotechnology of University Rovira i Virgili.

Tarragona, May 2016

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Al meu pare.

CONTENTS

OBJECTIVES

The second fermentation for sparkling wines production, due to an accumulation of stressful factors, requires an extra effort for yeasts to survive and carry on with fermentation. Some stress factors are the same as those found in first fermentation, although with a different intensity: ethanol level, low pH, low nitrogen content, accumulation of toxic sub-products such as medium-chain fatty acids (MCFA) and organic acids. Others are found in the second fermentation: low temperature, low oxygen avaibaility, CO_2 overpressure, presence of SO_2 and total acidity (Juroszek *et al.* 1987). Some of these stress factors have a synergic effect between themselves.

Progressive acclimation of *Saccharomyces cerevisiae* is required before being inoculated into the base wine. Acclimation process defined by Tai *et al.* (2007) permits yeast to activate stress metabolism response to ensure the successful of *prise de mousse*. The main features of this method were first described by Françot and Geoffroy (1957) and the effects of ethanol (major stress factor to acclimate to) on yeast during its acclimation were analysed by Juroszek *et al*. (1987). Some variations of the yeast acclimation process and its subsequent use, known as the *Champenoise* method, have mainly been described by Valade and Moulin (1983), and Laurent and Valade (1994, 1998, 2007).

Although winemakers apply procedures in order to adapt yeast, there is always a lag phase after inoculation followed by a short cell proliferation, indicating that the cells are still not completely adapted. However, without this acclimation process, cells are highly susceptible to death.

The effect of ethanol on yeast has been widely studied (Aguilera *et al*. 2006; Bauer and Pretorius 2000). It has been reported that the alteration of membrane fluidity is one of the key factors in terms of their tolerance (D'Amore and Stewart 1987; Alexandre *et al.* 1994; You *et al.* 2003). However, yeast response to stress during fermentation, secondary fermentation or even acclimation process, remains not deeply studied due to the multifactorial effect of many stress factors. Within this framework, the working hypothesis of this thesis is:

The characterisation of yeasts during acclimation to ethanol and other stress, can improve second fementation success.

In order to validate this hypothesis the following objectives were attained:

1. Testing ethanol effect on certain commercial wine yeast strains in a defined growth media at low pH (3.5) in the presence of weak acids and classify them in function of their ethanol tolerance. Then, evaluate the yeast growth parameters in the presence of certain factors can affect yeast viability (pH, presence of glycerol, cysteine and MCFA). The yeast's behavioural response to these factors should enable an improvement in the acclimation of commercial wine yeasts to ethanol in order to achieve the secondary fermentation properly for sparkling winemaking.

Results are reported and discussed in **Chapter III**.

2. Physiological study of yeast cells during acclimation to relate it with to their performance during the secondary fermentation. To this end, the effect of aeration and external sugar content during the cell acclimation process on certain physiological parameters was analysed. These parameters were in relation to the carbohydrate reserve (glycogen and trehalose), the activity of vacuoles, Reactive Oxygen Species (ROS) intracellular accumulation and the lipid metabolism, trying to correlate the achievement of secondary fermentation and some physiological parameters analysed during the cell acclimation process.

Results are reported and discussed in **Chapter IV**.

3. The characterisation of cells during acclimation process by the way of omics studies such as transcriptomic, lipidomic and metabolomic were executed to permit us to specify the yeast behavior in these conditions.

Results are reported and discussed in **Chapter IV**.

4. Improve and design new methods for the omics analysis by optimising (i) a GC-MS method to cover major yeast lipids in a single run by analysing a lipid extract and (ii) an extraction protocol to get the major intracellular metabolites from cell yeasts in a single run by using a GC-MS method.

Results are reported and discussed in **Chapter II**.

CHAPTER I

Research and literature review

Chapter I

1. A brief history of sparkling wine production

1.1. The history of sparkling wine

The journey began in the cellars of Limoux in southwestern France. This place was further inland and at a higher altitude than may be expected for this region, decreasing the Mediterranean influence and accentuating the Continental one. Cooler nights and colder winters would be decisive factors in the direction that wine would go. When the Romans occupied Limoux, the Mauzac grape variety used to elaborate white wines was already famous.

In 1530, the Benedictine monks of the Abbey of St. Hilaire decided to use cork to store their finished wine from that year inside individual glass bottles, as they had a lot of this material from Catalonia. In the spring of 1531, the first sparkling wine appeared, full of their bubble up, and fascinate people from Limoux. They named it *Vin de Blanquette* (Fig. 1) or the small white. The same thing happened to the barrels of white wine, stored in cellars over the winter, but these were not popular.

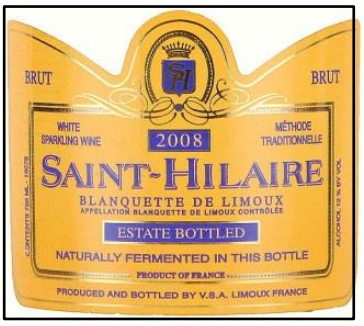

Figure 1. Modern-day label of Saint Hilaire

What was happening in both the Limoux and Champagne regions was a second fermentation, for two different reasons. In Limoux, the Mauzac grape was so late budding and ripening that harvest took place in late autumn. Fermentation was slow under winter conditions, so when the wine was bottled, sugar and live yeast still remained because the fermentation had not finished yet. In Champagne, being much further north, barrels were too cold for yeasts to perform fermentation and it completely stopped, starting again in the spring when the wine was stored in bottles.

The first recorded case of intentionally adding sugar and yeast into bottles to create a sparkling wine was the Englishman Christopher Merrett in 1662. He was a physician and

scientist interested in the glass-making business. English people enjoyed sparkling wines imported from Limoux and Champagne. However, in the course of experiments, glass at the time was not so sturdy and would break easily under pressure. In his own factories, Merret claimed that he could produce bottles up to the task, making it possible for sparkling wine producers to keep moving forward and for Champagne eventually to take the lead.

At first, and for a very long time, the winemakers of Champagne disliked the naturally occurring carbonation situation and saw it as an unwanted flaw. In 1718, as an attempt to stop it, the region adopted rules set by Dom Pérignon (Fig. 2): (i) fine wine can only be made from Pinot Noir, as red wine is less likely to re-ferment, (ii) vines must be pruned so they do not grow higher than three feet tall, (iii) harvest must be done in cool, damp conditions and before 10 o'clock in the morning, (iv) harvest picking must be done very carefully to avoid bruising the grape or breaking the skin and (v) rotten or super-large grapes must be tossed.

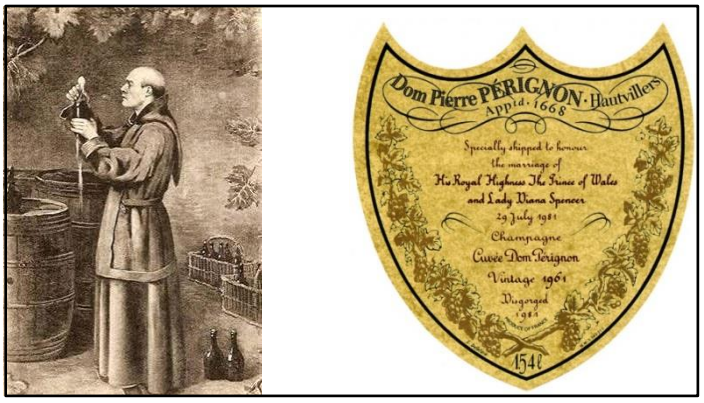

Figure 2. The Benedictine monk, Dom Pérignon and label of the famous champagne

In the late 18th Century, the producers in the region shifted away from the natural second fermentation and invented their own process of creating it in the bottle. Known as the *Champenoise* method in the Champagne region, and the Traditional method in all over the world, it involves the following steps:

1) A **base wine** high in acidity and low in alcohol is fermented to completion from carefully handled grapes.

2) The next step is *assemblage*. The winemaker blends their base wines to get the desired results, such as the classic house style or the new targeted character.

3) The wine is bottled with a mixture of yeast and sugar called the *"liqueur de tirage*" and enclosed with a cap. This starts a secondary fermentation and traps CO_2, making the wine bubbly. André François is credited with figuring out the formula in the 1830s in terms of the amount of sugar and yeast that should be added to get the desired amount of carbonation. Once fermentation is completed, the yeasts die in a process called autolysis, forming the lees. These lees are important for flavour, giving the wine a toasty, biscuity character.

4) The lees are now stuck to the sides and bottom of the bottle so it goes through a slow and meticulous process called *riddling* or *remuage* (Fig. 3) to collect all of the lees in one place. Bottles are put on a riddling rack (*pupitres*) at a slight angle (neck down) and are often shaken slightly, twisted an eighth of a turn, and put back at a slightly steeper angle. Eventually all of the sediment gravitates to the neck of the bottle.

Riddling was conceived by an employee of Barbe-Nicole Clicquot in 1816. Madame Clicquot had inherited her husband many businesses when she was only 27, becoming the first woman to run a champagne house. Veuve Clicquot (Widow Clicquot) is still one of the premier houses producing of Champagne. Until recently, each bottle had to be riddled by hand and it took one *remueur* eight weeks to riddle five-thousand bottles. Today, the process is mostly performed with machines called *gyropalattes* and it takes just one week.

Figure 3. Riddling rack

5) Madame Clicquot noticed that riddling was time-consuming and costly, and so the process of *dégorgement* or disgorging was more effective. There, the sediment of lees was removed. Before the invention of riddling, the wine would still be cloudy after dégorgement because much of the lees would remain. It would also lose

pressure. Now, with all of the lees having moved down into the neck of an upside-down bottle, it is all settled in one target area. To disgorge the lees, the bottle neck is dipped into a freezing solution and then the cap is cracked off, ejecting the frozen sediment. The result is a crystal clear, sparkling wine that retains its pressure of six atmospheres.

6) While the bottle is still open, it has to be topped up to replace the volume lost from the bottle during dégorgement, and this is the point at which a *dosage* of wine and sugar called the *liqueur d'expédition* is added. The amount of sugar depends on the desired level of sweetness. Originally, these wines were pretty sweet and, as they evolved to become drier than the previous driest level, new names were coined for the new maximum levels of dryness. This is why there are levels such as *Brut* that are drier than Extra-Dry. The term *Natural* means that no sugar was added at all in the dosage. Sometimes, the last three steps are skipped, with the *transversage* or the transfer method being used instead. In this case, after the wine goes through the secondary fermentation in the bottle, it is all emptied out into an enormous tank, where it is filtered (rather than being riddled and disgorged individually). The sugar dosage is added and then it is rebottled. This is used for Champagne and other wined produced using the traditional method when bottling bottles that smaller or larger than the usual 750 mL. It is less time-consuming and thus less expensive. If the label states that the wine is “fermented in bottle”, this is not the same as being “fermented in the bottle”, which means that the wine really was fermented in the same bottle that reaches the consumer.

7) Finally, the bottles are corked and then left for a few months to allow the dosage to spread out and settle. The muselet, or the wire cork cage that must be untwisted, was invented by Adolphe Jaqueson in 1844. Not much information is available about this mysterious man. Before his invention, wooden plugs wrapped with cloth or covered in wax were used to seal the bottle and withstand the pressure.

In the 1860s, a Catalan named Josep Raventos ambitiously travelled around Europe and was making his way back through France. He was the head of his family’s winery, named Codorniu, and he was looking to sell his wines. At the time, France was in the midst of a terrible time for wine, and needed to find other sources. Phylloxera, an insect that eats

the roots of *Vitis vinifera*, had just begun to strike and nobody knew what it was or how to cure it.

The Champagne region, which not yet fallen victim to phylloxera, was one of the stops on his route and what he saw and tasted there made him consider the possibility of recreating it back home. Sparkling wine was certainly already being produced in Catalonia at the time but not of this quality. After his tour of France, he returned to Sant Sadurní d'Anoia, in the Penedès region of Catalonia. In 1872, using champagne winemaking equipment that he had borrowed, he released his first sparkling wine made using the *méthode champenoise* and it was a great success.

Raventós named it *Champán*. He brought together a group of fellow winemakers and they began to shape the category together. It did not take long for them to decide that a different name was needed for their new specialty wine: Cava, from the Catalan word for cave or cellar.

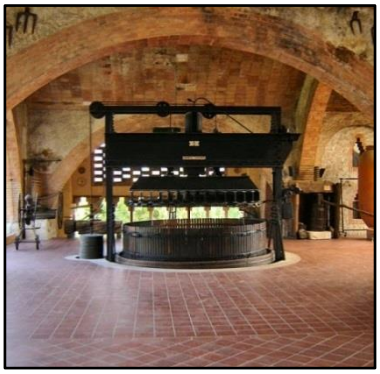

Figure 4. An old press at the Codorniu winery

Phylloxera would not strike the Champagne region until 1890 but it reached Penedès in 1887. Many grape varieties that could not withstand the plague, particularly reds, were simply uprooted and replaced by native ones that were more resistant, especially whites. The Raventós family chose three of the hardier white grape varieties for the future generations of their sparkling wine and, from that day forth, these varieties would be used to make Cava: Macabeo, Xarel·lo and Parellada.

Much like many places that traditionally produce blends, such as Bordeaux, it is always a good idea for the different varieties naturally to bud and ripen at different times. This way, if anything should happen at any stage to one variety, less or none of the affected variety can be used. The primary purpose of Macabeo is that its buds late and so will easily withstand a spring frost, unlike Xarel·lo and Parellada. It is neutral in flavour so

it projects the flavours from the traditional method and normally accounts for about half of the blend. Most of the other flavour characteristics of Cava, and what really distinguishes it from Champagne, come from the vocal aromatics and earthy flavours of Xarel·lo. Parellada is acidic and crispy. Chardonnay is also now used, as well as the red grapes Grenache, Monastrell, Pinot Noir and Trepat.

After Josep's death, his son Miguel took over operations. In 1889, at Freixenet, Pedro Ferrer started making Cava using the same method and grapes as Codorniu. The category started to spread from the Penedès region throughout Catalonia and to Spain.

Champagne is still the king of sparkling wines, despite being much more expensive than the other sparkling options. Champagnes remain on top because of their class, luxury and tradition and also due to large investments in marketing.

Sparkling wines are also produced in other countries and regions, such as *Blanquette de Limoux* in Southern France, *Espumante* in Portugal, Cava in Spain, *Franciacorta*, *Prosecco* and *Asti* in Italy (Fig. 5), *Cap Classique* in South Africa or Australian sparkling *Shiraz* in Australia.

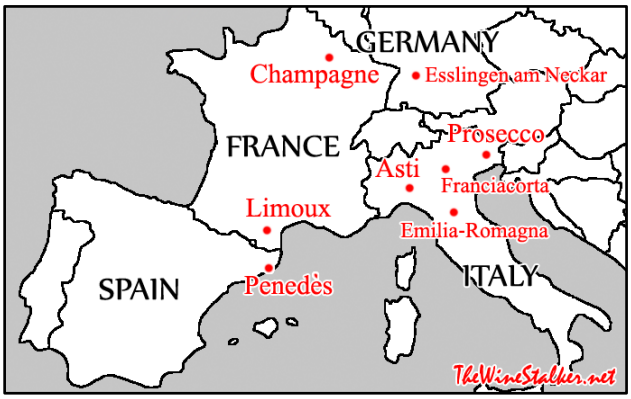

Figure 5. Principal regions with sparkling wine production

1.2. Sparkling wine production

1.2.1. Description and classification

Sparkling wine is a wine with significant levels of carbon dioxide (CO_2) in it, making it fizzy. The CO_2 may result from natural fermentation, either in a bottle, as with the *traditional method*, in a large tank designed to withstand the pressures involved (as in the *Charmat* process), or as a result of carbon dioxide injection. When served, sparkling

wines produce a persistent mousse and then gradually release bubbles. Traditional-method sparkling wines, which are made using particular varieties of grapes, contain CO_2 as a natural consequence of the sugar metabolism of yeasts. This gas is a by-product of the secondary fermentation of natural or added sugars in the base wine. The fermentation takes place in closed vessels and the resulting wine has a minimum pressure of 4 atm at 20ºC. Depending on the winemaking method used, sparkling wines are classified (see Table 1).

Table 1. Classification of sparkling wines depending on the winemaking method used to produce it.

	Method	Secondary fermentation	Ageing	Quality	Quantity
Bottle-fermented wines	Traditional (*Champenois*)	Fermentation takes place in **bottles**	Aged on lees in bottles for months or even years depending on the quality required	++	--
	Transfer	- **Bottles** generally magnums from 1.5-2 L to minimise storage requirements - Wine is cooled, filtered and sometimes transferred to another tank where dosage is added - Disgorging is not required	Aged on lees for at least 2 months in bottles and transferred to a tank to maintain gas under isobaric conditions	+	-
Tank-fermented wines	Continuous	- Must is pasteurised (to accelerate sucrose hydrolysis), cooled and filtered before inoculation - Fermentation occurs in **tanks** under isobaric conditions - Using base wine with 50-72 g/L sugar added	Large **tanks** are used to produce yeast autolysis simulating traditional method (Flanzy 2000)	-	+
	Charmat	- Large metal **tank** under isobaric conditions - Bottled after clarification	- Remains in contact with lees for a minimum of 21 days in **tanks.** - Pasteurisation induces autolysis with the aim of improving sensory quality (Pozo-Bayón *et al*. 2003)	--	++

The production of these wines involves two main stages: primary fermentation and secondary fermentation. The former converts the grape must into base wine and the latter creates the final product. Secondary fermentation is also known as *prise de mousse* or *toma de espuma* as it generates the carbon dioxide that gives the frothy mousse in the glass as the wine is served.

1.2.2. The traditional method

Depending on whether white or rosé sparkling wines are being produced, white or red grapes are harvested when the grape must reaches a natural level of alcohol of at least 8.5% and at most 9.5% (v/v). The grapes are then gently pressed and the must is transferred to a tank in the presence of sulphur dioxide. The clarification then takes place with or without the use of pectolytic enzymes at a low or room temperature. The clear must is then pumped off to a new tank for fermentation.

The primary alcoholic fermentation is the performance of the base wine step, which is generally associated with white winemaking. The limiting factors of white winemaking, namely the low fermentation temperature (15 ºC to 18ºC), low pH and low availability of oxygen, are often minor because of the low sugar content of these grape musts. Under these conditions, the *Saccharomyces cerevisiae* wine yeast strain is inoculated at approximately 10^6 cells/mL to ensure fermentation (Bidan *et al.* 1987). These yeasts are commercialized as Active Dry Yeasts (ADY) and the same yeast can be used for producing both the base wine and sparkling wine. At the end of the alcoholic fermentation, the sediment and lees are removed from the wine, and it is then generally cold stabilised. The next stage consists of blending wines made from different varieties of grape or the same varieties from different years. This is known as *coupage*. The level of sulphites must be kept under 15 mg/L. The final step is tartrate stabilisation, to prevent the precipitation of potassium bitartrate in the bottle as a result of low temperatures and high level of ethanol. Wine is usually stored at -4ºC to create potassium bitartrate crystals and a filtration step is performed to remove it. Wine produced during the first fermentation, known as the base wine, is not very pleasant by itself due to its acidity.

Chapter I

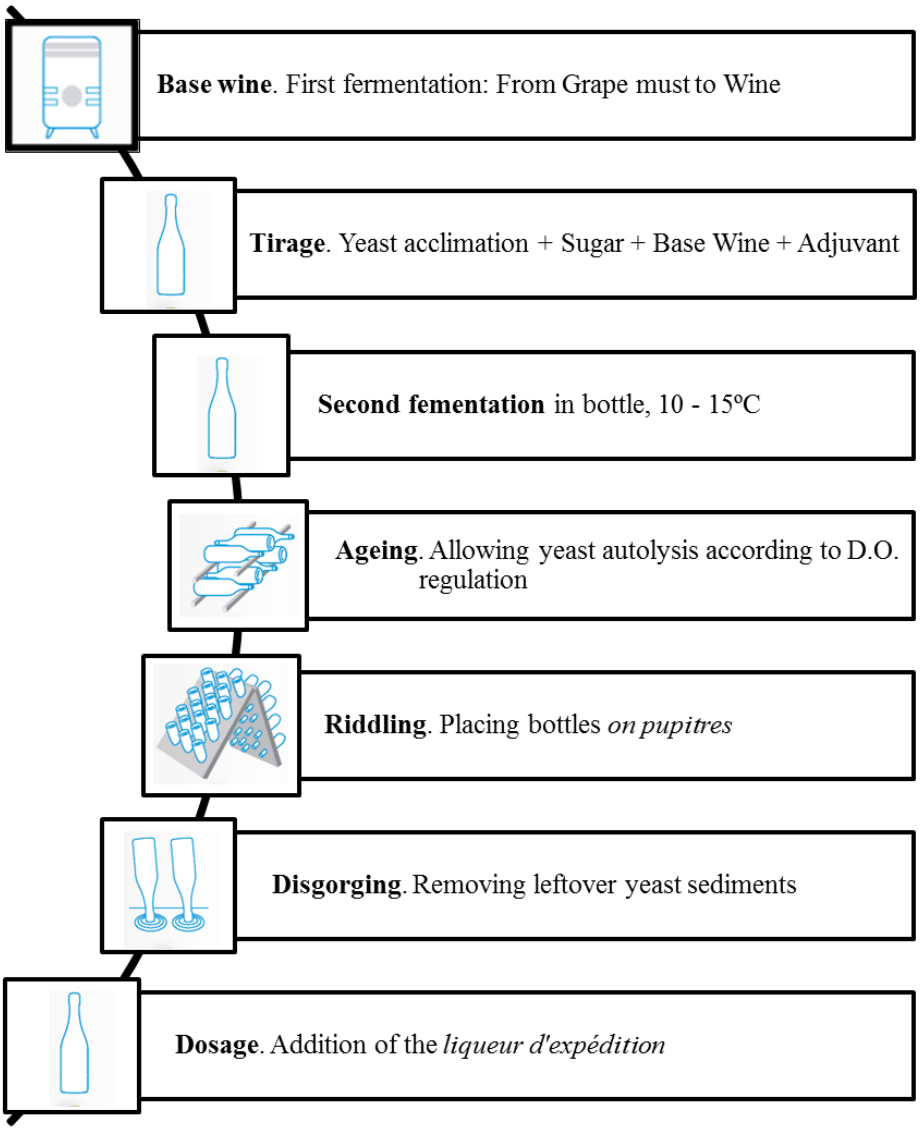

Figure 6. Synoptic description of different steps of the *Champenoise* method for Cava production.

The ***prise de mousse*** consists of the secondary fermentation, yeast autolysis and possibly the malolactic fermentation. Sparkling wines must remain in the bottle for some months before being sold. The main operations that take place during this period are:

- (i) *Tirage*, which consists of filling the bottle with the base wine, the acclimated yeast preparation, the *liqueur de tirage* (suspension of sucrose 20-25 g/L) and a small quantity of bentonite to aid flocculation and the subsequent removal of yeast cells.
- (ii) Bottles are then stacked horizontally in special ageing rooms.
- (iii) They are left to age.

- (iv) Wines are riddled, which involves gently shaking the bottles to direct the sediment (lees) formed by yeasts

The next stage is disgorging, which consists of removing the lees (dead yeasts) settled in the neck of the bottle. The lees are frozen and the crown cap is removed. Some liquid may be lost during disgorging, and this is compensated by the addition of the *dosage*. This liquid may be pure sparkling wine, or may contain sucrose, grape must, partially fermented grape must, etc. The dosage allows winemakers to give their sparkling wines a distinctive finish. The secondary fermentation really begins after *tirage*. During this process, CO_2 is trapped and forms the typical bubbles in sparkling wines. This starts with the inoculation of the base wine and ends when all of the fermentable sugars have been consumed (> 2g/L) and the pressure inside the bottle is 6 bars. The amount of added sugar determines the pressure of the bottle, which is caused by the gas produced, and in this case, it would be around 18 g. Champagne must be aged on the lees in the bottle for 15 months (non-vintage wines) to develop completely, but it could take much longer depending on the product desired, and up to over 3 years (vintage wines).

After ageing, the lees must be removal. The bottles are riddled (*remuage* in French), whereby they are placed on special racks to hold them at an angle and the sediments are coaxed to the neck of the bottle. The angle is gradually increased and the bottle is shaken slightly until (after 8-10 weeks) the position of bottle is vertical. The removal process is called disgorging (*dégorgement* in French). Traditionally, this involved a skilled manual process in which the crown cap and lees were removed without losing much liquid. Nowadays, the disgorgement is performed by freezing a small amount of the liquid in the neck and removing this plug of ice containing the lees.

Immediately after disgorging, the level of liquid is topped up with the *liqueur d'expédition*, commonly with a little sugar, in a practice known as *dosage*. The *liqueur d'expédition* is a mixture of the base wine and sucrose, plus 0.02 to 0.03 g of sulphur dioxide as a preservative. Some *maisons de Champagne* claim to have secret recipes for this, adding ingredients that make their wines unique. Sugar is added to balance out the acidity of the wine, except in *Champagne Nature*, which has no sugar added. A cork is then inserted, with a capsule and wire cage securing it. The sweetness level is known as *doux* (sweet) and, as the dryness increases, there are the levels shown in Table 2.

Table 2. Levels of sweetness (*doux*) depending on the grams of residual sugar per litre in Cava.

Sweetness levels: *doux*	Residual sugar g/L
Doux	50
Demi-sec	32-50
Sec	17-32
Extra sec	12-17
Brut	< 12
Extra brut	< 6
Brut nature	0-3

1.3. The sparkling wine market

The sparkling wine market has expanded in recent years, boosted by high global demand:

- Production has increased significantly: + 40% in 10 years (from 2000 to 2013), while still wine production only increased by 7% over the same period.
- Although EU countries are still the leaders in this market segment, sparkling wine production and consumption is on the rise in the rest of the world.
- Consumption of this type of product is changing: there is a progression from mainly celebratory consumption to more regular consumption, especially as an aperitif.

1.3.1. The producers

17.6 million hectolitres (Mhl) of sparkling wine were produced in 2013, according to a report drafted the OIV (International Organization of Vine and Wine) (Fig. 7). This amount has increased 11% compared to 2012 and +40% in the last 10 years. Nowadays, sparkling wine production accounts for 7% of the entire world production of wine, compared to just 4% in 2000. The following top five sparkling wine producers account for 74% of this wine production:

1. France: 3.5 Mhl (20% of total sparkling wine production)
2. Italy: 3.2 Mhl (18 %)
3. Germany: 2.8 Mhl (16%)
4. Spain: around 1.8 Mhl (10%)

5. Russia: around 1.8 Mhl (10%)

France, Italy and Spain come as no surprise, as they are also the world's top wine-producing countries. In contrast, Germany in third place and Russia in fifth may perhaps not have been expected. However, both Germans and Russians traditionally have a fondness for sparkling wine. In particular, Russians appreciate the sweetest versions of sparkling wine. Nowadays, France, Italy, Spain and Germany are losing market share in terms of world production, and now only produce just over 63% of the total world production. Ten years ago, their share was larger.

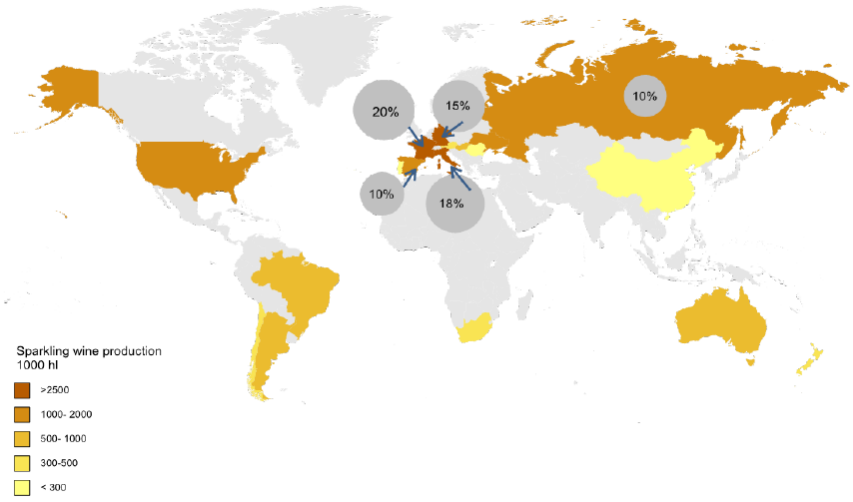

Figure 7. World sparkling wine production in 2013 and production of the top five countries, reported by the OIV (International Organization of Vine and Wine)

France remains the biggest producer, with 3.5 Mhl produced in 2013. Champagne alone accounts for more than 15% of all sparkling wine produced globally. Italy is in second place, with 3.2 Mhl. Its production has increased significantly over the past 10 years (+22% compared to 2002). Curiously, around 30% of all wine produced in Germany is sparkling, this country ranks as the third largest producer. Spain produces 1.8 Mhl of sparkling wines using the classic traditional method. Russia, however, is increasing its share of the world production of sparkling wine. The country now makes almost 1.8 Mhl, compared to barely 0.8 Mhl in 2002.

Production is increasing and diversifying. Although production of this type of wine remains concentrated in Western Europe, Eastern European countries are becoming

increasingly important, as well as Australia and the United States. In these countries, sparkling wine production has greatly increased over the past 10 years: +29% in Australia, +25% in the United States, +198% in Argentina and +248% in Brazil.

In terms of the countries with the greatest potential in terms of vineyards, Spain, and specifically Catalonia, is the top of the list of the major grape producers, followed by France, Italy, China and Turkey. Together, these 5 countries account for 50% of world's vineyards (Fig. 8). The global area cultivated for wine production in 2013 was 7,519 million hectares.

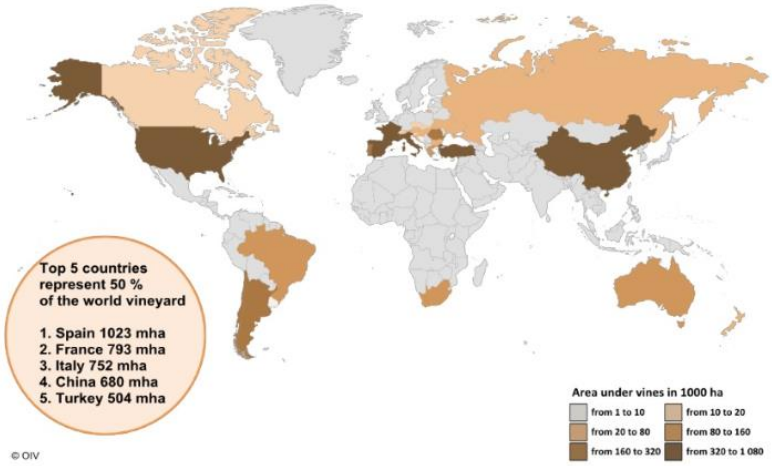

Figure 8. Vitivinicultural production potential of vineyards in the world

1.3.2. The consumers

The seasonality of sparkling wine consumption is its main characteristic and is generally linked to New Year celebrations. However, we are seeing a trend towards a levelling out in consumption. Sparkling wines have come to be consumed in a more regular and less specific manner. They are thus associated with all celebrations, as well as being consumed as an aperitif and used to make cocktails.

Despite the fact that world wine consumption has increased by 4% in the last 10 years, a 30% rise in sparkling wines (which represent 6% of total wine consumption) was recorded. After a decrease due to the economic crisis, consumption has risen again in the past three years to reach 15.4 Mhl, which is an increase of 4% compared to the previous year.

The top six markets for sparkling wine are:

- Germany: 3.1 Mhl
- France: 2.1 Mhl
- Russia: 2 Mhl
- United States
- Italy
- United Kingdom

The German and French sparkling wine markets remain the largest, with 3.1 and 2.1 Mhl consumed in 2014, respectively. There is, however, growing interest in these wines outside the EU, particularly in the United States, Australia, Russia and Eastern European countries, where consumption has significantly increased over the past 10 years. The growth in Australian consumption of this type of product (+7% in 5 years and +42% in 10 years) is due to Australia's strong level of purchasing power and a favourable exchange rate. The consumption of *Champagne* and, more generally, *bulles* (fine sparkling) wines is also on the rise in China. This country is now the fifth biggest *Champagne*-importing country in the world, after the EU and just behind the United States, Japan and Australia.

There is an obvious gap between the production and consumption of sparkling wine: 2.2 Mhl more was made in 2013 than was drunk. Some of these sparkling wines are being stored for ageing.

1.3.3. The international market

The sparkling wine trade has increased even more dramatically since the end of the last century, in terms of volume and value. Since 2000, world exports of sparkling wines have more than doubled (from 3.2 Mhl in 2000 to 8.7 Mhl in 2013), with an average annual growth rate of 9%. Almost 50% of all sparkling wine produced (in volume) is today sold and exported. The growth in terms of value has been relatively slower. The average annual growth rate since 2000 stands at 5%, going from 2.3 billion euros in 2000 to 4.3 billion in 2013. The 2008 economic crisis and its effects on world wine consumption,

particularly with regard to high-end products, significantly affected the change in terms of value.

After the shock produced by the economic crisis, prices have increased more rapidly than volumes. It is interesting to compare volume and value compared to world total wine exports:

- Sparkling wine represents 9% of the total volume of world exports
- Sparkling wine represents 18% of the total value of world exports

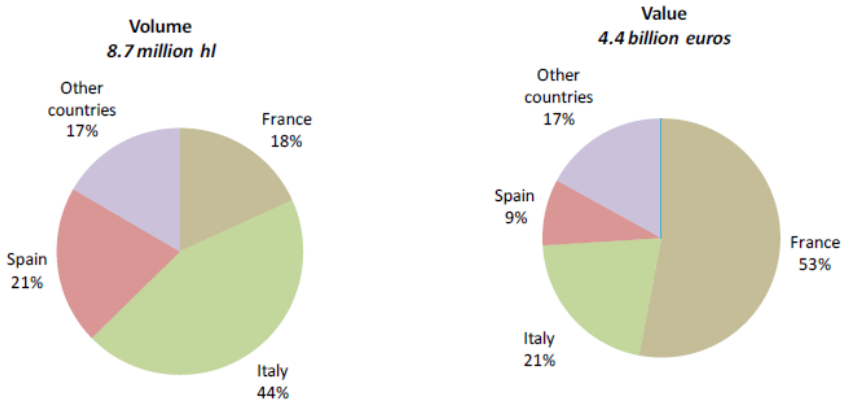

Figure 9. World sparkling wine exports, market shares, volumes and values during 2013. Source: Report by the OIV '*Le marché des vins effervescents*', published in the autumn of 2014.

The three biggest exporters of sparkling wines represent more than 80% of world exports in terms of volume and value, with France alone accounting for 53% of the total exports in terms of value (but only 18% in volume, which translates to fairly expensive bottles).

The market shares of the biggest exporters have grown significantly in the last 10 years. The economic crisis has favoured low-cost products such as Italian *Prosecco* and Spanish *Cava*, in contrast to *Champagne* from France. Nevertheless, it is important to note that Italian and Spanish exports are still worth less than French exports, driven by *Champagne* (which accounts for over half of the value despite less volume being exported). France has lost 8% of the market, in terms of value, while Italy has gained 7% compared with 2008. In terms of volume, Italy has gained 4% while France and Spain have lost 6% and 2% of the market share, respectively, compared with 2008.

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The role of certain countries with low production or producers of only sparkling wines has increased in recent years. Latvia is a good example (its sparkling wine exports have increased 5.5% per year in value and 11.8% in terms of volume since 2000). Singapore is the country that has experienced the most rapid growth in the sparkling wine trade. It has become the 4th biggest exporter of sparkling wine in the world since 2000 (8.7 million litres sold in 2013, equating to over 245 million euros).

The top five importers in the world account for nearly 50% of the total sparkling wine imports, with the United Kingdom (over 658 Million euros) and United States (616 million euros) each making up 15% of the total, followed by Germany with 9% (381 million euros), Japan with 7% (305 million euros) and Belgium with 6% (268 million euros).

2. Yeast stress responses

Yeast inoculated to develop secondary fermentation should: (i) have high resistance to ethanol (10-12%, v/v)); (ii) display fermentation activity at low temperatures, on occasions lower than 12ºC; (iii) be resistant to the pressure of carbon dioxide; (iv) be able to flocculate, as this facilitates the subsequent elimination of lees; and (v) not produce unpleasant aromas (Bidan *et al.* 1986). The measurement of the autolytic and foaming capacity of yeast might also be a valuable tool in terms of strain selection (Martínez-Rodríguez *et al.* 2001).

Although thousands of yeast species have been identified, only 15 correspond to wine yeasts (Ribéreau-Gayon *et al.* 2006). Yeasts present in must during the first few hours after filling the tanks belong to the same genera as those found on the grapes, especially *Hanseniaspora* and *Klockera*. Under these spontaneous alcoholic fermentation conditions, *Saccharomyces* yeasts (mainly *S. cerevisiae*) predominate after 3 or 4 days and are ultimately responsible for the alcoholic fermentation (Ribéreau-Gayon *et al.* 2006). This change in yeast population is linked to the increasing ethanol concentration, anaerobic conditions and the use of sulphites during harvesting and in the must, concentration of sugar and the greater tolerance of high temperatures displayed by *S. cerevisiae* compared with other yeasts (Fleet and Heard 1993; Fleet 2007).

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Currently, the usual strategy employed in winemaking involves inoculation of the must with selected yeasts in the form of Active Dry Yeasts (ADY). This practice shortens the lag phase, ensures rapid and complete fermentation of the must, and helps to create a more reproducible product (Bauer and Pretorius 2000; Fleet and Heard 1993). The selection of wine yeast with specific genetic and physiological markers provides a system for the precise monitoring of the growth of particular strains during fermentation. Fermentation is mainly driven by inoculated yeasts (Delteil and Aizac 1988). Given that the growth of the natural microbiota is not completely suppressed during the initial days of alcoholic fermentation, these strains can make substantial contributions to certain properties of the wine (Querol *et al.* 1992). The inoculated yeast strain must obviously be very carefully selected on the basis of certain necessary characteristics (Degré 1993; Fleet 2008). Mainly, it must produce vigorous fermentation with short lag phases and little residual sugar, be reproducible and tolerant of high pressure, ethanol and low temperatures. They can have other valuable properties, such as having glycerol and β-galactosidases (Darriet *et al.* 1988) in adequate quantities to achieve a good aroma or be resistant to the adverse growth conditions during winemaking (Zuzuárregui and Del Olmo 2004). Sometimes, native strains that meet desired criteria have been used for inoculation fermentations in an effort to maintain sensory characteristics associated with specific regions (Lafon-Lafourcade 1983).

Following the inoculation of base wine, there is a lag period in which the yeast adapts to the new conditions, although the yeasts have been selected to overcome wine stresses. Growth is slower than in the primary fermentation due to fewer sources of carbon and nitrogen being available. An inoculum of a 10^6 cell/mL would produce a population of a 10^7 cell/mL according to Martínez-Rodriguez *et al.* (2002) and tend to take 15-20 days, after which cell viability decreases slowly and no live cells would be detectable after 60-90 days, in line with results reported by Feuillat and Charpentier (1982).

In order to reduce this lag phase for yeasts and avoid sluggish fermentation due to the stressful conditions found during the second fermentation, a process known as acclimation is conducted to prepare yeasts to overcome wine stresses and, particularly the most problematic factor, namely high ethanol content. This process consists of taking yeasts from lower ethanol concentrations to higher ones in different stages. Acclimation is discussed later on in this introduction and is studied in-depth in the present doctoral thesis. Results nicely show its importance for cell survival and fermentation development.

Table 3. Comparison between optimal parameters for yeast and the conditions encountered in wine to develop second fermentation for Cava production (Duteurtre, 2011).

Parameters	Wine	Optimal yeast media
Temperature (ºC)	11-15	25-30
Free SO_2 (mg/L)	5-15	0
Alcohol (%, v/v)	11-11.5	0
pH	3.0-3.2	5-6

The successful performance of alcoholic fermentations depends on the ability of the yeast to cope with a number of stress factors occurring during the process (Van Uden *et al.* 1985). Basically, it depends on their capacity to tolerate ethanol and have high fermentation activity at low temperatures. The synergic effect of the two main factors affecting yeast (ethanol and low temperature) was demonstrated by Juroszek *et al.* (1987).

Industrial yeasts must sense and respond to stress conditions rapidly and adapt to these adverse environment factors by adjusting their metabolic activities to avoid substantial viability loss. Commercial wine yeasts have been selected in all parts of the world for use as starters due to their ability to ferment grape musts with high amounts of sugar and, therefore, to tolerate high levels of ethanol.

2.1. Secondary fermentation

To produce sparkling wines by the traditional (*Champenoise*) method, two consecutive fermentations are needed. The secondary fermentation, induced by the addition of yeast and sucrose to the product of the primary fermentation, is a hard challenge for yeast, due to an accumulation of stressful factors and it requires an extra effort for cells trying to survive and ferment. Some stress factors are the same as those encountered in first fermentation step, although with a different intensity: high ethanol concentration around 10%-12% (v/v), low pH (2.9 to 3.2), nitrogen starvation, low nutrient content, accumulation of toxic fermentation sub-products, such as medium-chain fatty acids (MCFA, C6-C12) and organic acids. However, others are typically only found in the second fermentation: low temperature (10ºC-15ºC), CO_2 overpressure, high SO_2 concentration (50-80 mg L^{-1}) and high total acidity (5-7 g L^{-1}) due to H_2SO_4.

Despite the large amount of information available about the most significant stress factors, the high ethanol level, little information is currently available, or at least it is not clarified, in relation to the behaviour of these strains growing in different levels of ethanol under restrictive conditions, such as low pH, and the presence of glycerol, cysteine or medium-chain fatty acids. The synergic effect of this alcohol with other factors makes results difficult to interpret.

2.1.1. Base wine composition

Base wine compounds are described separely and, therefore, the stress caused by the synergetic effect by combining some of them will be discussed.

2.1.1.1. Ethanol

The effect of ethanol on yeast has been widely studied, particularly during alcoholic fermentation (Aguilera *et al*. 2006; Bauer and Pretorius 2000). Ethanol is the main factor affecting the ability of yeast strains to conduct fermentation to produce wine or even in secondary fermentation for *Cava* production, in which ethanol levels are higher at the very beginning of the process, particularly with respect to its viability. Numerous works reviewed by Ding *et al.* (2009) have documented the effect of different factors related to the ethanol tolerance of *S. cerevisiae* strains during alcoholic fermentation or other industrial processes, such as biofuel production.

According to Kumar *et al.* (2008), there are two basic types of screening for sensitivity to ethanol: ethanol challenge and ethanol production ability. The former involves long exposure to different levels of ethanol and the strain's capacity to continue growing is evaluated (Bisson and Block 2002). The latter, ethanol production assay, enables the yeast cell to continue producing ethanol from sugar. For improving the preparation of ethanol-acclimated yeast cells for the production of sparkling wines, both of these aspects have to be studied. Li *et al.* (2012) suggest that relatively high ethanol stress prolongs the cell cycle and allows yeast cells to enter their exponential and stationary growth phases later than cells under control and low ethanol conditions.

The alteration of membrane fluidity is one of the key factors in terms of the tolerance and, more specifically, the ability of cells to modify their composition in fatty

acids, phospholipids and sterols (D'Amore and Stewart 1987; Alexandre *et al.* 1994; You *et al.* 2003). Generally, ethanol tolerance is associated with a higher degree of membrane unsaturation which implies that oxygen must be a factor in this tolerance because of its involvement in the synthesis of unsaturated fatty acids and sterols (Arneborg *et al.* 1995; Valero *et al.* 2001).

The yeast lipid bilayer membrane is mainly made up of phospholipids (PL) which have unsaturated fatty acids (UFA), oleic acid (C18:1) and palmitoleic acid (C16:1), and the saturated fatty acids (SFA), palmitic acid (C16:0) and stearic acid (C18:0), as the principal fatty acids (FA). Palmitoleic and oleic acids, which are formed by the oxygen and NADH-dependent desaturation of C16 and C18, are the predominant UFAs in *S. cerevisiae* (Ding *et al.* 2009; You *et al.* 2003). Under normal physiological conditions, the concentration of C16:1 is much higher than that of C18:1. *S. cerevisiae* does not synthetise polyunsaturated fatty acids (PUFAs) (Kajiwara *et al.* 2000). Sterols are also really important, especially ergosterol for yeast and fungi. MCFAs are found in lower proportions, but their concentration increases with the anaerobic growth (white winemaking), such as the fermentation processes.

Changes in either of these membrane features can significantly disturb the membrane functions and alter the activity of membrane associated enzymes and transporters. Consequently, many organisms have developed mechanisms to maintain the appropriate fluidity of the membrane lipids, regardless of the stress conditions (high ethanol, low temperature, acid pH, etc.).

Because fatty acids are major components of phospholipids of the cytoplasmic membrane of cells, they play a prominent role in defining its properties. Thus, they have an effect on membrane fluidity (Alexandre *et al.* 1994, 1996) and influence the activity of membrane-associated enzymes (Stadtlander *et al.* 1982; Alexandre *et al.* 1996), morphogenesis (Haslam *et al.* 1973), transport mechanisms (Prasad and Rose 1986, Szolderits *et al.* 1989), and, in the case of yeasts, can affect the viability and fermentation capacity (Rozès *et al.* 1988; Mauricio *et al.* 1991) and tolerance to ethanol (Thomas *et al.* 1978; Mishra *et al.* 1989; del Castillo-Agudo 1992).

Ethanol affects fluidity in the plasma membrane and the structure becomes loose under ethanol stress. To overcome this effect, the plasma membrane has to retain its structural integrity as much as possible to counteract the effect of ethanol. On the basis of

this hypothesis, the factors that make the plasma membrane more rigid are important for cells to maintain membrane stability and survive under conditions of ethanol stress. In terms of lipids, ergosterol increases membrane rigidity to antagonise the increased fluidity that is induced by high concentrations of ethanol (Ding *et al.* 2009), and some unsaturated fatty acids are also considered as the protective metabolites for *S. cerevisiae* cells under ethanol stress (Ding *et al.* 2010).

While the fact that ergosterol increases is clearly due to their effect on membrane rigidification, the increase in the UFAs may be contradictory with the hypothesis that a more rigid membrane could counteract the increase of fluidity by ethanol in the yeast membrane.

A previous review discussing the ethanol tolerance of *S. cerevisiae* indicates that the role of unsaturated fatty acids (UFAs) is complex and the function is not straightforward (Ding *et al.* 2009). It was suggested by Aguilera *et al.* (2006) that lower levels of C16:1 and higher levels of C16 and C18 help the membrane to overcome the stress, regulating its integrity. Yeast cells change the levels of these fatty acids to maintain the integrity of their plasma membrane through decreasing membrane fluidity in the medium containing ethanol. Some studies have shown that C18:1 is the main UFA that can overcome the inhibitory effect caused by ethanol in yeasts (Ding *et al.* 2009; You *et al.* 2003). However, another report indicates the absence of a relationship between the increase in the content of C16:1 and C18:1 and enhancement of *S. cerevisiae* cell viability, and found that the dienoic fatty acids were more important for the ethanol tolerance of *S. cerevisiae* (Kajiwara *et al.* 2000). Furthermore, a change in C16:1 acid level might not occur in a manner similar to C18:1, and both levels are not necessarily increased under ethanol stress. In fact, Aguilera *et al.* (2006) reported that the ethanol added to culture medium promotes an increase in C18:1 content, but a decrease in C16:1 content in yeasts cells. The ethanol-induced increase in C16:1 level shown there was consistent with results of Li *et al.* (2012). As no change was observed here in the levels of C18:1, which is regarded as more important for conferring ethanol tolerance to *S. cerevisiae* under ethanol stress, the increased levels of C16 and C18, and the decreased levels of C16:1 caused by ethanol might provide the self-protection of yeast to maintain the integrity of its plasma membrane by decreasing membrane fluidity.

Ethanol content can also inhibit the activity of crucial glycolytic enzymes and damage mitochondrial DNA (Casey and Ingledew 1986; Ibeas and Jiménez 1997; Ingram and Butke 1984; Salmon *et al.* 1993; Arroyo-López *et al.* 2010). The inhibitory effect of ethanol on specific growth rates of *S. cerevisiae* strains has been modelled mathematically (Pamment 1989; Arroyo-López *et al.* 2010; Borrull *et al.* 2015a) with the estimation of the Non-Inhibitory Concentration (NIC) and Minimum Inhibitory Concentrations (MIC) values of *S. cerevisiae* with mathematic modelling.

The cellular response to high ethanol content is strikingly similar to the heat stress response, and both responses may be designed to reduce the effects of water stress (Piper 1995; Hallsworth 1998). As in the case of other stresses, the cellular response includes the synthesis of trehalose and the induction of stress protection or heat shock proteins (Piper 1997), apart from the membrane composition modifications explained above. Ethanol is toxic for membrane proteins, leading to cell growth inhibition and even death. Yeast cells have several mechanisms associated to this process, including changes in gene expression, in membrane composition and increases in chaperone proteins that help stabilise other denatured proteins. Taking advantage of the omics technology, this stress response and the defence mechanisms of yeast cells during ethanol fermentation has been further explored. Genome shuffling and global transcription machinery engineering have been applied to breed stress-resistant yeasts.

During the first fermentation, the concentration of this alcohol increases gradually and yeast can activate all of its stress mechanisms, particularly when ethanol starts affecting the yeast membrane. However, in the second fermentation, the ethanol concentration is really high at the very beginning of the process. For this reason, the complex networking associated with ethanol stress must be activated as soon as possible to avoid cell death. The selection of resistant yeast strains to deal with second fermentation process is a big issue for winemakers and oenology researchers, and it may have a huge impact on the industry sector. In the present thesis, the effect of ethanol on commercial wine yeast is evaluated. Although these yeasts have been selected to overcome high alcohol concentrations to deal with hard industrial processes for microorganisms, many different tolerance levels have been found between them, and they are classified depending on their tolerance.

Moreover, various factors have been evaluated in the presence of different ethanol levels, namely low pH and the presence of glycerol, cysteine or MCFA, and these factors are known to have a synergic effect with high ethanol content.

2.1.1.2. Low nutrient content

With respect to nutrient availability, the stressful situation would occur at the end of fermentation (both primary and secondary), when almost all of the sugar and nitrogen have been consumed by yeasts.

Glucose and fructose are the main **s**ugar**s** present in wine. Monosaccharides, some disaccharides, such as sucrose (generally dissociated because of the low pH) and polysaccharides (not metabolised by yeasts) can also be found. The total sugar concentration should be between 170 and 220 g/L (Ribéreau-Gayon *et al.* 2006). These concentrations may inhibit yeast growth as a result of the high osmotic pressure, together with the elevated intracellular concentration of ethanol (Nishino *et al.* 1985).

Both external sugar content and oxygen control the switch from respiration to fermentation metabolism in *S. cerevisiae*. When the external sugar content exceeds 0.8 mM, cells have a mixed respiro-fermentative metabolism conducive to ethanol production (Verduyn *et al.* 1987). The amount of **s**ugar**s** in the medium completely changes the acclimation yeast cell metabolism, which may have an effect on their later performance during fermentation (Boulton *et al.* 1996).

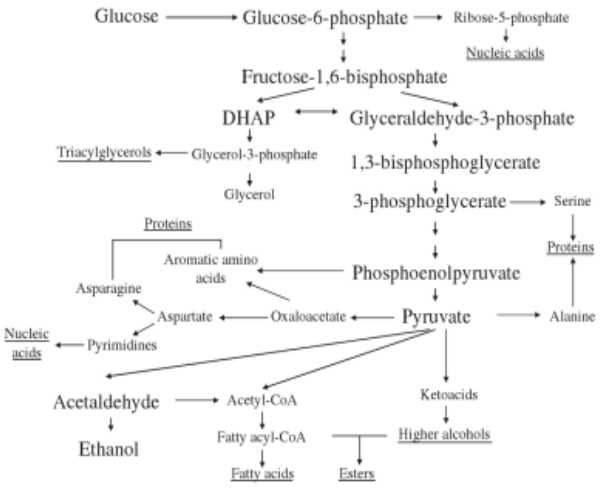

Figure 10. Biosynthetic precursors derived from alcoholic fermentation. DHAP=dihydroxyacetone phosphate. Figure adapted from Henschke and Jiranek (1993) by Aranda *et al.* (2011).

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Nitrogenous compounds are one cause of stuck fermentation because they tend to be limiting for yeast growth (Ingledew and Kunkee 1985; Bisson, 1999). Variations of nitrogen content in the must may influence yeast growth, fermentation rate and ethanol tolerance. The main compounds include ammonia (3-10%), amino acids (25-30%) and proteins (5-10%). Yeasts essentially depend on the concentrations of ammonia and amino acids, their preferred nitrogen sources (Ough and Amerine 1988). Yeasts can synthetize their own amino acids, so supplementation with diammonium sulphate or phosphate is enough to resolve nitrogen deficiencies (Ribéreau-Gayon *et al.* 2006).

S. cerevisiae is equally able to use amino acids, ammonia, uracil, proline derivative and urea as nitrogen sources (for a review, see Cooper 1982). Of the nitrogenous components that can be found in must, amino acids make the largest contribution to nitrogen provision for the synthesis of structural and functional proteins, and the production of enzymes and transporters. Most nitrogenous compounds are incorporated in the cell via active transport systems, specifically symport with ions, usually protons (Fig. 11) (Cooper 1982).

In *S. cerevisiae* a general amino acid permease (Gap1p) has been identified and also specific permeates for different amino acids (Horak 1986). Ammonia undergoes active transport of the protonated species that requires the presence of glucose (Roon *et al.* 1977) and three systems have been identified involving the proteins Mep1p, Mep2p and Mep3p (Marini *et al.* 1994, 1997).

Nitrogenous compounds are assimilated during the first few hours of fermentation (Monteiro and Bisson 1991) and degraded in a specific order that depends on factors such as (i) the requirement for each compound in biosynthetic process, (ii) efficiency of transport and (iii) possible conversion into ammonia or glutamate without releasing compounds that are toxic to the cell (Cooper 1982).

During yeast growth, more than half of the intracellular reserves of amino acids are in the vacuoles (Wiemken and Durr 1974). This compartmentalisation contributes to the regulation of the activity of various enzymes involved in their degradation (Sumrada and Cooper 1982).

Ammonia and glutamate are central to nitrogen metabolism in yeast. Ammoniacal nitrogen is rapidly incorporated in the biosynthesis pathways through the activity of

$NADHP^+$, dependent glutamate dehydrogenase. In addition, it represents the end product of the catabolic pathways for nitrogenous compounds. The metabolism of nitrogenous compounds also contributes to the formation of products that play an important role in the final quality of the wine by affecting sensory properties.

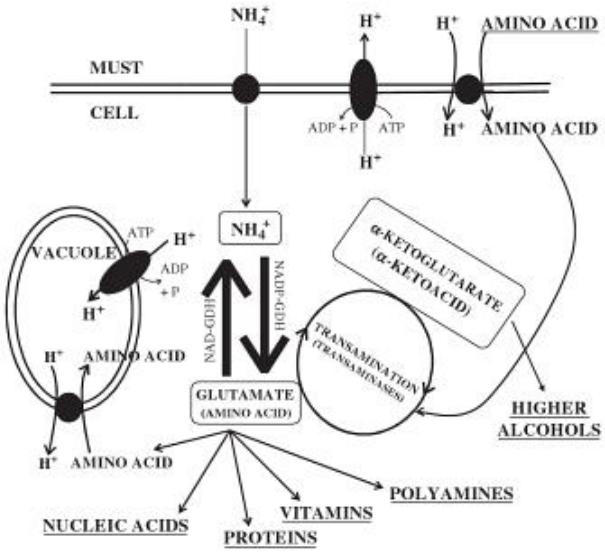

Figure 11. Schematic diagram of the uptake and metabolism of nitrogenous compounds (Llauradó and Reverchon 2002).

2.1.1.3. Accumulation of by-products

Accumulation of by-products during fermentation may activate stress mechanisms by the end of the process, even in primary or secondary fermentation for *Cava* production.

Yeast usually accumulates carbon and energy reserves to cope with starvation conditions. The accumulation of **glycogen and trehalose**, two main intracellular energy storage polymers, is sometimes crucial for overcoming general stresses.

Both glycogen and trehalose degradation yields glucose. Glycogen is a high molecular weight polymer, while trehalose is only a disaccharide. Glycogen accumulation occurs when growth is limited by a nutrient other than carbon (Lillie and Pringle 1980). Yeast stores function as a reserve of carbon and energy used during periods of starvation or when the cells are undergoing adaptation to a new growth phase (François *et al.* 2001). Furthermore, glycogen dissimilation provides carbon for sterol synthesis when necessary. Trehalose, in turn, may also function as a reserve material. However, it seems more likely that it serves as a stress protectant. Thus, it has the ability to increase the thermotolerance

of proteins and is capable of stabilising cellular membranes, thereby increasing the tolerance of cells to applied stresses, such as desiccation, reduced water activity and elevated temperature (Wiemken 1990). Glycogen accumulation commences when growth begins to be limited by the disappearance of a nutrient other than carbon. The onset of significant trehalose accumulation is delayed until there is little or no exogenous supply of carbon. Mobilisation of trehalose only occurs after prolonged starvation when the glycogen pool is relatively depleted. The disappearance of trehalose correlates with a rapid loss of viability.

Mutants with no glycogen or trehalose storage are able to grow and divide, indicating that sugars are not essential for cell cycle progression. However, wild-types can enter a cell cycle faster (Silijé *et al.* 1999). Trehalose does not primarily function as a reserve but as a highly efficient protecting agent to maintain the structural integrity of cytoplasm under environmental stress conditions (Wiemken 1990). D'Amore *et al.* (1987) correlate a high accumulation of this polysaccharide with a high survival rate in the face of stresses such as nutrient starvation and high ethanol content, making them perfect for developing difficult fermentations. Glycogen in yeast is formed upon limitations of carbon, nitrogen, phosphorous and sulphur (Lillie and Pringle 1980).

Yeast that can accumulate glycogen has a growth advantages (Anderson and Tatchell 2001). Vacuole has a key role to play in glycogen storage (Wilson 2002). There is a strong link between the ability to acidify vacuole appropriately (especially by multiprotein complex V-ATPase) and glycogen storage (Kane *et al.* 2006).

Besides water and ethanol, **glycerol** is the chemical compound with the highest concentration, around 5-20 g L^{-1}, depending on environmental factors, such as temperature, aeration, sulphite level and yeast strains in wine. Glycerol is a by-product of glyceropyruvic fermentation, which allows *S. cerevisiae* to oxidise the NADH via the glycerol-3-phosphate dehydrogenase at the beginning of alcoholic fermentation. The role of NADH-consuming glycerol formation is to maintain the cytosolic redox balance, particularly under anaerobic conditions (Van Dijken and Scheffers 1986). Apart from balancing redox, glycerol has the function in cell metabolism of osmoregulating yeast cells when extracellular water activity decreases in the growth medium, such as grape must (Blomberg and Radler 1992).

Another stress factor that can influence the achievement of secondary fermentation is the presence of **medium-chain fatty acids** in the base wine. These MCFAs are produced during alcoholic fermentation in hypoxic conditions as by-products of lipid synthesis (Taylor and Kirsop 1977a) and are toxic for them (Lafon- Lafourcade *et al.* 1984), with C10 being the most toxic because it is more liposoluble. Overcoming this stress is more difficult than expected due to the interaction between various stress factors, such as ethanol content and low pH. Growth inhibition caused by octanoic (C8) and decanoic (C10) acids has been evaluated, in particular by Viegas *et al.* (1989, 1997). Concentrations up to 16 $mg \cdot L^{-1}$ (for C8) and 8 $mg \cdot L^{-1}$ (for C10) decreased the maximum specific growth rate and the biomass yield exponentially, and increased the duration of growth latency. These toxic effects increased when the pH decreased to 3.0, indicating that the dissociated form of these molecules is the more toxic. MCFA in acid media (e.g. wine) can pass across the membrane and dissociate in the neutral cytoplasm by passive diffusion, causing a decrease of the intracellular pH which can induce cell death (Viegas *et al.* 1997).

Ethanol has a synergic effect with MCFA. C8 and C10 are secondary products of alcoholic fermentation, increasing the rate of loss of *S. cerevisiae* viability caused by high concentrations of ethanol, with the specific death rate being an exponential function of the acid concentration (Viegas *et al.* 1997). C10 is more toxic than C8 due to higher liposolubility. Both fatty acids may be esterified by the ethanol present during fermentation and became volatile and associated with flavours.

Another by-product that may affect fermentation development is **cysteine**. With its free sulphydryl group, it is one of twenty naturally occurring amino acids. Its free thiol group is involved in the formation of disulphide bonds, which are essential for protein stability, and it is also a catalytic and redox centre in various enzymes, cofactors and regulatory proteins. Although cysteine can play a role in detoxification and the stress response of the cells, an increase in the cysteine level from 0.25 mM to 5 mM has been shown to be toxic to cells (Kumar *et al.* 2006). The cysteine content in base wines (around 0.16 mM, Desportes *et al.* 2000) is not higher than other essential amino acids but its presence prevents the oxidation of aromatic compounds in wine. Meanwhile, reactive oxygen and sulphur species (ROS and RSS, respectively) are produced during normal cell function and in response to various stimuli (Paulsen and Carroll 2013). Oxidative stress results in an imbalance in the metabolism of these 139 molecules (cysteine, glutathione, superoxide anion (O^{2-}), hydrogen peroxide, etc.) and, consequently, can induce irreversible

damage in the cells and eventually cell death. Moreover, cysteine can induce serious growth defects without inducing ROS accumulation inside cells (Kumar *et al.* 2006).

2.1.2. Physicochemical parameters

Yeast cell surviving and fermentation performance depends on many physicochemical parameters.

2.1.2.1. Oxygen

Oxygen is known for being both essential and dangerous for life. This factor may be problematic in the primary fermentation, but not in the secondary, which occurs inside bottles. However, the present thesis shows how preparing yeasts during acclimation to second fermentation with correct oxygen availability is crucial for overcoming this process.

Weak aeration was found to lead to a 23% increase in the viable cell mass for ethanol fermentation (Alfenore *et al.* 2004), especially for fermentations at low temperatures (Redón *et al.* 2009) and with high ethanol content (Alexandre *et al.* 1994; Ding *et al.* 2009). Oxygen is known to be necessary for cells to produce biomass (Valero *et al.* 2001). More specifically, it is needed to synthesise lipids for its comfort; unsaturated fatty acids (Andreasen and Stier 1954) and sterols (Andreasen and Stier 1953). Kirsop (1982) found a correlation between the quantities of oxygen supplied to yeast growing in fermentation. However, there is no similar correlation between yeast growth and sterol concentration. This is unsurprising in view of the fact that only 10% of oxygen in wort is used for sterol synthesis and 15% for the synthesis of unsaturated fatty acids, while the remaining 75% has no known role. This is why Boulton *et al.* (2000) found that yeast forcibly exposed to oxygen will accumulate sterol to a similar concentration to that which is synthesised by yeasts during the aerobic phase of a normal oxygenated fermentation.

A more oxygenated environment may be helpful in the case of must with nitrogen deficiencies (Ingledew and Kunkee 1985). However, oxygen also causes ROS compound accumulation in cells, which can cause yeast death and may lead to undesirable production of acetaldehyde and hydrogen sulphide, as well as reduced production of aromatic esters (Nykänen 1986).

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Yeast membranes, mainly lipidic, have several functions including (i) acting as a diffusion barrier cell-environment, (ii) harbouring proteins that catalyse selective transport and act as enzymes, and (iii) harbouring receptors for cell recognition. The main components are phospholipids (PLs) and sterols. Yeasts are unable to grow in the complete absence of oxygen as it is necessary for synthetising unsaturated FA from PL and sterols (Alexandre *et al.* 1994; Ding *et al.* 2009). For that reason, for many years, winemakers have supplemented their fermentations with ergosterol and oleic acid to prevent a lack of these essential lipids, thereby ensuring the healthy growth of their yeasts and preventing sluggish fermentation (Andreasen and Stier 1954). FA and sterol composition in yeast have been shown to be responsible for an important physical characterisation of membranes, regulating membrane permeability and fluidity (Daum *et al.* 1998).

Unsaturated fatty acids (UFAs) and sterols are poorly synthesised in hypoxic conditions, while the levels of MCFA, squalene and lanosterol increase (Andreasen and Stier 1953, 1954; Parks 1978; Ratledge and Evans 1989). Oxygen has an influence on biosynthesis of cellular fatty acids, sterols and phospholipids during alcoholic fermentation by yeasts. Hypoxic conditions are precisely what can be found in the secondary fermentation for Cava production. Based on numerous works (Thomas *et al.* 1978; Mauricio *et al.* 1991, 1995; Alexandre *et al.* 1994, 1996), it has been well established that the ethanol tolerance of yeast cells is related to an increase in UFA and the maintenance of ergosterol, the main sterol of *S. cerevisiae*, and phospholipid contents. That is the reason that some winemakers add ergosterol to their fermentation in order to avoid a sluggish process. Nevertheless, anaerobic conditions *S. cerevisiae* produces a relatively high percentage of these fatty acids in order to achieve good cell yield and fermentation activity.

It has been proven that yeasts can directly incorporate sterols and unsaturated fatty acids from grape must during alcoholic fermentations (Chen 1980). Furthermore, the addition of ergosterol (25 mg/L) and oleic acid (31 mg/L) to fermentation medium by winemakers makes cells improve their cell yield and fermentation activity (Ortega *et al.* 1998). This effect is not so notable for other yeast strains, such as *Torulaspora delbrueckii*, which was tested by Ortega *et al.* (1998). Moreover, a short period of aeration for a 48h culture in anaerobiosis led to a total recovery of the cellular growth and fermentation activity in both yeasts. Likewise, the effect of a short aeration period on unsaturated fatty acids biosynthesis was similar for yeasts.

Meanwhile, aerobic metabolism, although very efficient in energy terms, is intimately associated with the generation of reactive oxygen species (ROS) (Moradas-Ferreira *et al.* 1996). These compounds react with DNA, lipids and proteins, alternating their functions. Superoxide anion (O^{2-}) is a reactive radical formed by an electron reduction of oxygen. It is mainly generated during respiration in mitochondria (Bouveris and Cadenas 1982). The toxic effect of superoxide anion results from their conversion to an extremely reactive (OH^{-}) species. This hydroxyl radical indiscriminately reacts with sugar, amino acids, phospholipids, nucleotides and organic acids (Halliwell 1995). Yeast cells have a range of responses to ROS that depend on the dose. At very low doses, cells can adapt to become more resistant to subsequent lethal exposure (Borrull *et al.* 2015b). At higher doses, cells activate various antioxidant functions, including cell-division cycle delay. At even higher doses, death of a proportion of cells occurs by apoptosis (Perrone *et al.* 2008).

2.1.2.2. pH

The second main factor that may cause some problems during the secondary fermentation, apart from high ethanol level, is low pH. The typical pH of must is between 2.8 and 4.2 (Heard and Fleet, 1988). The pH values have no negative effect on the growth of *Saccharomyces* yeasts, and problems only begin at pH levels below 2.8. In fact, the toxic effects of pH are due to the increased effects of ethanol (Pampulha and Loureiro-Dias 1989a) and sulphite (Farakas 1988). Potassium ions in the must can increase acidic pH tolerance (Kudo *et al.* 1998). Low pH favours the hydrolysis of disaccharides, and, therefore, fermentation, as well as preventing spoilage microorganisms.

At pH levels around 2.9-3.2, weak organic acids (tartaric, malic, succinic, acetic acids) present in the growth medium may enter cells in the undissociated form because their corresponding pKa are higher than the external pH. Consequently, they acidify the cytosol and can lead to sluggish or stuck fermentation. For example, acetic acid together with ethanol inhibits alcoholic fermentation in a synergistic way by decreasing the internal pH, fermentation rate and enolase activity (Pampulha and Loureiro 1989a; Pampulha and Loureiro-Dias 1989, 1990).

This is also the case of MCFAs. These acids may undergo dissociation because of the low pH causing problems for yeasts. This was reviewed by Borrull *et al.* (2015b). The

undissociated form of MCFAs and weak acids in general, which can be found in a free form in acid media (e.g. wine), can pass across the plasma membrane and dissociate in the neutral cytoplasm by means of passive diffusion, causing a decrease in intracellular pH (Viegas 1997) and possibly lead to cell death. This fact does not happen at physiological pH, at which fatty acids dissociate and require a carrier to cross the yeast membrane. Borrull *et al.* (2015b) clearly show how differences in the number of carbons for these acids can be crucial for yeast toxicity. Being more liposoluble than C8, C10 can enter the cell easily and is more toxic at lower concentrations. Both MCFAs are commonly present in wine media.

2.1.2.3. Temperature

Temperature is one of the main relevant environmental variables that yeasts have to cope with. Temperature is a key factor in some industrial processes involving microorganisms. *S. cerevisiae* has an optimal growth temperature of around 32ºC. Nevertheless, it can adapt to a wide range of temperatures up to a maximum of 40ºC, at which point it begins to decline drastically (Watson 1987). This does not mean that higher temperatures are most appropriate for fermentation of the must.

Low temperatures (10-15ºC) are used in wine fermentations to enhance production and to retain flavour volatiles. In this way, white and *rosé* wines of greater aromatic complexity can be achieved (Torija *et al.* 2003b). Thus, low temperature, in turn, has its disadvantages, such as an increased lag phase and a reduced growth rate, producing stuck and sluggish fermentations (Bisson 1999). Therefore, the quality of wines produced at low temperatures depends on the ability of yeast to adapt to cold.

Ethanol toxicity increases with temperature, which may evaporate ethanol and other volatile compounds essential to the organoleptic properties of wine (Torija *et al.* 2003b), particularly in white wine. Excessively low temperatures can cause stuck fermentation when yeast membrane fluidity begins to be affected (Bisson 1999). Several studies have confirmed that lipid membranes of yeast are stress sensors, modulating their composition to reach the optimal proportion (Vigh *et al.* 1998; Shobayashi *et al.* 2005). Several strategies have been proven to improve yeast viability. For instance, in Redón *et al.* (2009), cells incubated with C16:1 not only showed higher viability but also a significant reduction in fermentation time at 13ºC. These cells displayed higher levels of C16:1 and ergosterol, a

shorter chain length of fatty acids and higher sterol/phospholipid ratio. Another work by our group (Redón *et al.* 2012) used mutants to elucidate another strategy based on phospholipids to change membrane composition to cope with fermentation stresses. The laboratory *S. cerevisiae* strain BY4742 was tested to ascertain whether the suppression of certain genes could improve fermentation vitality at low temperatures. HMN1 mutant improved vitality at 13ºC (without being affected at optimal temperature, 25ºC). HMN1 mutant showed lower concentrations of phosphatidylcholine (PC). This is in line with the fact that the common trait of mutants with vitality affected was lower concentrations of PC, and also phosphatidylethanolamine (PE).

2.1.2.4. Carbon dioxide

A by-product of fermentation, carbon dioxide contributes to dissipation of some heat and produces convection currents that aid the diffusion of nutrients. Moreover, its evaporation favours the loss of ethanol and volatile compounds (Jackson 1994). In still wines, it is dispersed into the atmosphere, but this is not the case of sparkling wines, where it is retained inside the bottle. Its solubility is greater at low temperatures. It may also be lost from the solution by the formation of complexes with sugars. The size and frequency of the bubbles are referred to as the mousse. Furthermore, produced in excess, it affects viability, mainly due to membrane damage. One factor to take into account when selecting yeast for sparkling wine production is the capacity to be resistant to pressure, caused by carbon dioxide formation.

At pressure above 700 kPa (around 7 atm), yeast growth ceases, although pressure-related effects have been reported at pressures as low as 30 kPa (0.30 atm). Low pH and high alcohol content increase yeast sensitivity to CO_2 pressure (Kunkee and Ough 1966). This has its most significant influence during sparkling wine production. Even pressures of 20 kPa (0.20 atm), which are easily found in young *cuvée* wines, can have a minor but noticeable effect on slowing fermentation and cell division (Jackson 2014). Pressure upward of 600 kPa (5.92 atm) is typically reached by the end of second fermentation in bottle. Nevertheless, the fermentative ability of yeasts may not be completely inhibited until about 3,000 kPa (29.6 atm). In addition, carbon dioxide accumulation may affect metabolism by influencing the balance between carboxylation and decarboxylation

reactions. The effect of pressure on the synthesis of aromatic compounds during the process appears not to have been investigated.

Some of the consequences of high pressure on cell growth and metabolism may accrue from a decrease in water viscosity (Bett and Cappi 1965). This may disrupt the intramolecular hydrogen bonding that is vital to protein structure and function. In addition, critical changes appear to involve damage to cellular organelles (Iwahashi *et al.* 2003). Synthesis of heat shock proteins (such as Hsp104) and trehalose can limit protein denaturation (Hottiger *et al.* 1994) and stabilise membrane fluidity (Iwahashi *et al.* 1995).

The pressure problem during fermentation has occasionally been used to encourage a more constant rate of fermentation velocity. It has also been used to induce the premature stop of fermentation, leaving the wine with a sweet flavour.

2.1.3. Combination of stress factors

During wine production, the hostile environment can affect the efficiency of yeast fermentation. This effect is even worse in *Cava* production. Although it is essential to study the response of wine yeasts during real industrial processes, analysis of their behaviour in response to individual stresses allows the assessment of correlations with their adaptation to unfavourable environmental changes. A realistic scenario should be studied but, sometimes, it is not fully understood without studying all of the variables separately. The main problem is that some of these stresses have a synergic effect which may make it difficult to gain a full understanding of the metabolic scenario.

2.1.3.1. Physiological state

The organelle organisation (mitochondria and vacuoles) may give us an idea of (i) which type of metabolism (respiration/fermentation) cells are undergoing and (ii) in which vacuole state cells have to be maintained. This can give us information about which cells have the best diagnostic depending on the acclimation condition.

Mitochondria are dynamic organelles (Chan 2006), a classic favourite for studies into respiration metabolism. During respiration, mitochondrion is organised, with the fusion of all cell mitochondria making them work as if they were a single organelle. This is

used to locate them as close to the membrane as possible, as this facilitates the acquisition of oxygen for producing energy in the electron chain. When mitochondrion is organised, there is a signal for the cell to respire. Cells lacking mitochondrial fusion show cellular defects, such as growing slowly and having reduced activity for respiratory complexes in the presence of oxygen. The opposite process, mitochondrial fission is necessary due to the segregation of mitochondria for dividing cells. However, recent studies also relate mitochondrial fission, when this process is not expected, with an induction of apoptosis. During a fermentation process, mitochondrial fusion is not needed because energy is not produced in this organelle, so it is common to find mitochondria occupying all of the cytoplasm in a disorganised form. This is known as fission form.

Vacuole physiology is also important for making cell diagnostics. A cell with (i) an active vacuole (with low pH) indicates an active metabolism and (ii) a high level of microvacuolation, which might be an evidence of a cell being close to division, may indicate that cells are ready to overcome a stress with a general stress response (Li and Kane 2009). In a study into the genome-wide identification of *S. cerevisiae* genes required for maximal tolerance to ethanol, conducted by Teixeira *et al.* (2009), evidence was obtained that vacuolar acidification and increased resistance to cell wall lytic enzyme β-glucase occur in response to ethanol-induced stress. This was suggested by the identification of certain genes specifically involved in the targeting of the major amino acid permease. Meaden *et al.* (1999) corroborate the idea that intracellular protein trafficking through the endosome and vacuole is important for ethanol stress tolerance. Vacuolar morphology and endocytosis have been shown to be altered in yeasts cells exposed to ethanol stress. For both ethanol stress and heat shock, vacuolar morphology altered from segregated structures to a single, large one. The findings of this study reinforce previous observations that ethanol stress and heat shock induce similar response in yeast. In addition Martínez-Rodríguez *et al.* (2001) while simulating autolysis in yeast incubating cells during 24 h in base wine; observe an increase in size of vacuole, which ocupated practically the whole of the cytoplasm.

2.1.3.2. Omics

As described in the present thesis, the successful performance of alcoholic fermentations depends on the ability of the yeast strains used to cope with a number of

stress factors occurring during the process. These include osmotic pressure imposed by initial high sugar concentration and stress induced by fermentation end products or by-products such as ethanol and acetate (Teixeira *et al*. 2009). However, the stress induced by increasing amounts of ethanol is the major factor responsible for eventually stuck fermentations (Gibson *et al*. 2007). Thus, yeast strains that can endure stress imposed by high ethanol concentrations are highly desirable.

Over the years, great efforts have been made to characterise the mechanisms underlying fermentative stresses tolerance and especially ethanol tolerance (Alper *et al*. 2006; Hirasawa *et al*. 2007; Van Uden 1985, You *et al*. 2003). The successful engineering of yeast transcription machinery for this purpose has been reported (Alper *et al*. 2006). A number of studies based on detailed physiological and molecular analyses have contributed to increasing the understanding of the process underlying ethanol toxicity and yeast tolerance of stress induced by this metabolite (Rosa *et al*. 1991, 1996; Salgueiro *et al*. 1988). Few studies have reported the molecular mechanism activated by yeasts during a real fermentative procedure, due to the huge number of factors contributing to stress cells being very difficult to understand and extract conclusions from (Penacho *et al*. 2012).

2.1.3.2.1. Genomic and transcriptomic

The present chapter aims to give a brief overview of the molecular changes occurring during the fermentation process and particularly in relation to ethanol stress as a major factor.

The reported studies indicate that ethanol interferes with membrane lipid organisation (Ogawa *et al*. 2000), affecting its function as a matrix for enzymes, perturbing the conformation and function of membrane permeability, and leading to the dissipation of transmembrane electrochemical potential (Salgueiro *et al*. 1988; Van Uden 1985), including changes in levels and composition of membrane phospholipids and ergosterol (Aguilera *et al*. 2006; Chi *et al*. 1999; You *et al*. 2003).

Due to its effect at the level of the plasma membrane, ethanol produces intracellular acidification (Meaden *et al*. 1999; Rosa *et al*. 1991, 1996; Salgueiro *et al*. 1988). Yeast exhibits increased plasma membrane H^+-ATPase activity, important for maintaining the intracellular pH, and the secondary transport mechanism depends on the proton gradient

across the plasma membrane (Aguilera *et al*. 2006; Monteiro *et al*. 1998; Ogawa *et al*. 2000, Rosa *et al*. 1991). Ethanol also inhibits crucial glycolytic enzymes (Casey *et al*. 1986) and induces the generation of ROS (Costa *et al*. 1993; Du *et al*. 2007). Yeast tolerance depends on the stability of the mitochondrial genome (Jimenez *et al.* 1988) and on the activity of the mitochondrial superoxide dismutase encoded by *SOD2*.

In recent years, many studies that have tried to describe genome-wide surveys have been used to identify the genes involved in the yeast response and resistance to ethanol stress (Alexandre *et al.* 2001; Fujita *et al.* 2006; Hirasawa *et al.* 2007; Kubota *et al.* 2004; van Voorst *et al*. 2006; Yoshikawa *et al.* 2009). The studies used slightly different genetic backgrounds and most of them work with cells grown in YPD media. Despite their efforts, only 4 genes have been reported to confer ethanol resistance to yeasts beyond the strain or the condition growth, showing a high dependence of gene variability with respect to these two factors.

Teixeira *et al.* (2009) have identified genes required for maximal tolerance to ethanol in a really exhaustive study using laboratory strain BY4742 to create mutants, using haploid yeast for non-essential genes and diploid yeast deleting one of the copies for essential genes. They identified over 250 genes conferring ethanol tolerance to yeasts, 121 of which had not previously been described. Most of them encode proteins involved in intracellular trafficking, transcription, pH homeostasis and peroxisome biogenesis. The effects of ethanol on peroxisome proliferation, vacuolar acidification and cell wall resistance were assessed.

Focusing on the **ethanol stress** factor, a great deal of literature can be found. The following tables show the genes found to be determinants for ethanol tolerance, organised by function and organelle. In addition, the authors citing the genes are shown, using the following key: Kumar *et al.* 2008 (Km); Kubota *et al.* 2004 (Kb); Fujita *et al.* 2006 (F); Teixeira *et al.* 2009 (T); Yoshikawa *et al.* 2009 (Y); van Voorst *et al.* 2006 (V); Expinazo-Romet *et al.* 2008 (E); Gorsich *et al.* 2006 (G) and Du and Takagi 2007 (DT).

Table 4. Genes conferring ethanol tolerance with functions related to vacuole, concretely; to V-ATPase activity, vacuole organisation, and biogenesis cited by reviewed authors.

VACUOLE. *V-ATPase, organisation and biogenesis*	
Km, F and T	*CUP5*
T	*PPA1, RAV1, TFP3, THP3, VMA7, VMA9, VMA13, VM21*
Km, Kb, F, Y and T	*VMA2*
Km, F, Y, V and T	*VMA4*
F, Y, V and T	*VMA5*
Km, Kb, F and T	*VMA8*
Km, Kb and T	*VMA22*
Km	*AVT3, VMA6*
E	*BTN2*

V-ATPase function is involved in yeast resistance to ethanol induced-stress. The important role of V-ATPase has been highlighted in many related studies. It has long been established that exposure to ethanol stress leads to intracellular acidification (Rosa *et al.* 1996). In the work by Teixera *et al.* (2009), it was demonstrated that pH_v decreases in a dose-dependent manner in cells exposed for 1 h to ethanol concentrations, leading to moderate growth inhibition. The reduction of pH_v in response to ethanol is hypostatised to be an adaptive response that helps the cell control ethanol-induced cytosolic acidification at more physiological values. In addition, proteomic studies have described the main interaction network maps with proteins that determine ethanol tolerance, including both the vacuolar protein sorting and V-ATPase complex assembly. In this thesis, vacuole morphology changes analysed during ethanol acclimation for doing second fermentation reveals the importance of vacuole function to complete the process without problems.

Table 5. Genes conferring ethanol tolerance with functions related to peroxisome, concretely; to peroxisome organisation and biogenesis cited by reviewed authors.

PEROXISOME. *Peroxisome organisation and biogenesis*	
T	*PEX1, PEX2, PEX3, PEX4, PEX5, PEX8, PEX10, PEX12, PEX14, PEX19, PEX22*

It is suggested in Teixeira *et al.* (2009) that the function of peroxisomes may be involved in yeast resistance to ethanol-induced stress due to the expression of some genes (Table 5), encoding proteins of peroxisome transport machinery and/or the membrane of peroxisome import machinery that are required for peroxisome organisation and

biogenesis. This is quite surprising; taking into account that glucose represses the function of peroxisomes.

The individual deletion of genes encoding fatty acid β-oxidation enzymes or proteins involved in lysine biosynthesis, the major metabolic processes taking place in peroxisomes, did not result in increased susceptibility to ethanol stress. No other authors who have screened to find genes responsible for ethanol resistance have highlighted peroxisome genes. However, Penacho *et al.* (2012) found *PEX14* to be up-regulated in a transcriptomic study during all secondary fermentation. In addition, in the transcriptome analysis of acclimation included in the present thesis, *PEX5* is up-regulated under our optimal conditions in comparison to other acclimation conditions studies, suggesting it may confer resistance for yeast trying to overcome a stress process such as fermentation.

Table 6. Genes conferring ethanol tolerance with functions related to mitochondria, concretely; to protein synthesis, oxidative phosphorylation, ATP synthesis, protein transport and DNA maintenance and repair cited by reviewed authors.

MITOCHONDRIA.	
Protein synthesis	
V and T	*IMG2*
T	*MRP2, MRPL21, MRPS16, PET112, RSM19, SLM5, SWS2, YNR036C, HTS1*
Km and T	*MRPL4*
Km	*MRPL6, MRPL15, MPRL25, MPRL31, MPRL32, MPRL33, MPRL35, MPRL37, MPRS28, PET123, RSM18, RSM22, RSM27, MHR1, OCT1, PIF1, SLS1*
Km and Kb	*MSR1, MDJ1*
Km and T	*MTG1*
Km, Kb and V	*TOM5*
Km, Kb, V and F	*TOM37*
Km and F	*YME1*
Oxidative phosphorylation	
Km	*COX6, COX9, COX18*
T	*COX19, NDE1, OAR1, SOD2*
ATP synthesis	
V	*ATP1*
Km	*ATP2, APT7, ATP17*
Km and Kb	*ATP11*
T	*ATP5, ATP12, ATP5, FMC1*
Km, F and T	*ATP15*

Continue	
Protein transport	
T	*SAM37, TOM5, TOM40*
DNA maintenance and repair	
Km, F and T	*FZO1*
Km and T	*MIP1*
Km, Kb and T	*MSH1*
Km	*MTF1*
V	*HMI1*
Others	
T	*POR1, SSQ1*
V	*MSK1, MTF2*
Km	*MDM12, AFG3*

Mitochondria-related genes, together with other categories of genes such as those involved in the oxidation-reduction process and cellular response to oxidative stress, are clearly related to aerobic respiration. On first impression, it seems that all of this large amount of genes shown in Table 6 would not be relevant in a context such as primary or secondary fermentation in Cava production, because there would not be a respiratory process. Mitochondria must be affected by ethanol taking into account the large number of authors citing those genes. Surprisingly, in their transcriptomic study into secondary fermentation, Penacho *et al.* (2009) found the genes *COX6*, *SOD2* and *ATP17* up-regulated. All of these genes were described the previous year by Kumar *et al.* (2009) as being related to high ethanol resistance. It is interesting to note that mitochondria-related genes found to increase ethanol resistance can be divided in two groups: (i) protein synthesis and (ii) oxidative phosphorylation. In the second group, some genes related to both oxygen and ethanol toxicity can be found: *COX6, COX9, COX18, COX19, NDE1, OAR1* and *SOD2*. The last of these is a mitochondrial superoxide dismutase, which protects against both toxics, and would be related to genes classified in the oxidative stress response category. All *COX* genes are related to cytochrome c oxidase.

Table 7. Genes conferring ethanol tolerance with functions related to metabolic reactions occurring in cytosol, concretely; to vesicular transport, transcription machinery, chromation remodelling and RNA/DNA processing, cytoskeleton organisation and biogenesis/cell morphology, cell wall biosynthesis, phospholipid, fatty acid and ergosterol metabolism, arbohydrate metabolism, aminoacids biosynthesis and transport, riboflavin biosynthesis and transport, nucleotide biosynthesis, oxidative stress response, chaperone activity, phosphate metabolism, ribosome biogenesis, protein ubiquitination, meiosis, mitosis and cell cycle, and finally N-acetyl transferases; all them cited by reviewed authors.

CYTOSOL	
Vesicular transport	
T	*ARF1, ATG11, BRO1, DID4, DOA4, END3, FES1, GCS1, GET1, GLO3, MON2, PEP12, RIC1, SEC66, SHE4, SLA1, SNF7, SNF8, STP22, VPS20, VPS27, VPS33, VPS41, VPS54, VPS66, SEC12, TRS33*
Km, Kb and T	*PEP3*
Km and T	*PEP5*
F, Y, V and T	*VPS4*
Km, Kb, F, Y, V and T	*VPS15*
F, Y, V and T	*VPS16*
F, Y and V	*VPS28, VPS30, VPH1*
Km, F, Y, V and Kb	*VPS34*
Km, F, Y, V, Kb and T	*VPS36*
F, Y and V	*VPS39*
Km	*COY1, AVL9, SAC1*
Km, Kb and F	*CLC1*
Transcription machinery, chromatin remodelling and RNA/DNA processing	
T	*ARP8, BDP1, CAF16, CSL4, CSE2, CTR9, CWC25, DBP7, DHH1, FHL1, HPR1, HDA2, IRR1, LDB7, MAF1, MAK32, MED2, MED8, MET18, MPE1, NCL1, NUP84, NUP170, PGD1, PRP11, POL32, REF2, RPB4, RTF1, RRN10, RRP3, RXT2, SIN4, SNT309, SOH1, SPP381, SSN8, TFB5, TFC1, SRB5*
Km	*CAF120, RLR1, HTA3, SGF29, ELP4, GIS1, RPA49, TUP1, CPB1, CPB2, DSC1, LSM1, MSS51, NPL3, PSY4*
Km, Kb, V, F and T	*GCN5*
Km, Kb and F	*POL2, POL3*
Km, Kb, V and F	*RPB9, HTL1, RPB9, GIM4, GIM5*
V and T	*SGF73, SPT3*
Km and Kb	*SNF6, SPT10, SPT20, NOT5, ROX3, MOT2*
Km and V	*SPT7*
Km, Kb, V, F and T	*SRB2*
Km and F	*IES6, ANC1*
V	*MSN2, MSN4*

Chapter I

Continue	
Cytoskeleton organisation and biogenesis/Cell morphology	
T	*ARC35, AKR1, BEM1, BEM4, BUD27, BUD31, CIN2, RVS161, SAC6, SHS1, SIT4, SSD1*
V	*SMI1*
V, Km, Kb and T	*BEM2*
Km and T	*TPD3, VRP1*
Km	*BIM1, SLA2, BUB32, ALF1*
Km and Kb	*YKE2*
Cell wall biosynthesis	
Km and T	*ANP1*
T	*HOC1, KRE6, MNN10, SLT2*
Km	*KRE28, GON7, CAX4, FUR1, FUR4*
V and T	*SLG1*
V	*ROM2*
Km and F	*OCHI1*
Phospholipid, fatty acid and ergosterol metabolism	
T	*ERG2, ERG6, ERG24, IDI1, KCS1, LIP5, OPI3*
Km	*ERG5, ACB1, STE1, SRV2*
V	*ERG10, FEN1, SUR4*
Km and T	*PDX3*
V and Km	*PLC1*
Carbohydrate metabolism	
T	*FPS1, GCR1, GPH1, HAP2, MIG1, PFK26, REG1, RTG3*
Km	*PFK2, ADH1, POP2*
V and T	*TPS1*
Km and Kb	*KGD2*
G	*ZWF1, GND1, RPE1, TKL1*
Amino acid biosynthesis and transport	
T	*AGP2, BNA1, GCN4, GLY1, GTR1, LST8, PTR3, SLM4,*
Y	*ARO1, ARO2, ARO7*
Km	*MET17, YPK1*
Km and Kb	*PRO1*
Riboflavin biosynthesis and transport	
T	*MCH5, RIB4*
Nucleotide biosynthesis	
T	*ADE1, ADO1, PRS3, RNR2*
Km	*RNR1, KAP120*
Km and T	*RNR4*
N-acetyl transferases	
T	*ARD1, NAT1, NAT2, NAT3*

Continue	
Oxidative stress response	
T	*CCS1, SKN7*
V and T	*SOD1*
Km	*GHS1*
DT	*MPR1*
Chaperone activity	
T	*SIS1*
Phosphate metabolism	
T	*PHO85*
Ribosome biogenesis	
T	*RPL13B, SFP1*
Km and T	*RPL1B*
Km	*RPL16B, PRL23B, EAP1, HCR1, MEF1, MEF2, RSA1, UBX2*
Kma and Kb	*MSM1*
Protein Ubiquitination	
T	*BRE5, DEF1, RAD6, RCY1, SWM1, STS1*
Km, Kb and F	*RAD27*
Meiosis, mitosis and cell cycle	
T	*CDH1, CIK1, SW16*
Km and T	*DOC1, MCK1*
F, Km, Kb and T	*UME6*
T	*PRM5, RRD1, STE50,*
Km	*MDS3, CTF8, PAC2, DBF2, SPO77*
Km and Kb	*CIN1*
Km, Kb and F	*BUB1, CTK3*
Km, Kb and V	*KAR3*

Many genes with a broad range of different functions are determinants of ethanol resistance in yeasts at a cytosol level (Table 7). The main functions of these genes are (i) vesicular transport, (ii) transcription machinery, chromatin remodelling and RNA/DNA processing, (iii) cytoskeleton organisation and biogenesis and cell morphology, (iv) cell wall biosynthesis, (v) phospholipids, fatty acids and ergosterol metabolism, (vi) carbohydrate metabolism, (vii) amino acid biosynthesis and transport, (viii) oxidative stress response, (ix) nucleotide biosynthesis, (x) ribosome biogenesis, (xi) protein ubiquitination and (xii) mitosis, meiosis and cell cycle.

Genes involved in the control of cell wall or membrane composition (iv and v), as determinants of resistance to ethanol, suggest that these structures undergo remodelling in response to ethanol stress, and thus increase cell resistance. Alteration of membrane

fluidity has been identified as key factor in terms of ethanol tolerance. The ability of yeasts to modify composition in fatty acids and sterols may be a determining factor in their survival (D'Amore and Stewart 1987). Ergosterol increases membrane rigidity to antagonize the increase of fluidity induced by ethanol (Ding *et al.* 2009), which corresponds to the high number of genes related to ergosterol biosynthesis that increase ethanol tolerance (*ERG2, ERG5, ERG6, ERG10, ERG24*).

Generally, ethanol tolerance is associated with a higher degree of membrane unsaturation (Arneborg *et al.* 1995; Valero *et al.* 2001), and some unsaturated fatty acids are also considered as protective metabolites for *S. cerevisiae* cells under ethanol stress (Ding *et al.* 2010). However, the determinant function of these acids is not clear and there is a certain level of controversy with respect to the degree of unsaturation required for better survival. *SLG1* and *SLG2* encode a sensor and a kinase belonging to the stress-activated cell wall integrity protein kinase C (PKC) signalling pathway. As such, they protect the membrane from stress.

The analysis includes genes (*HAP2, MIG1, RTG3* and *GCR1*) encoding transcription factors that act in carbohydrate metabolism. Even the presence of glucose; mitochondrial functions are essential for ethanol tolerance. Two of the authors found *TPS1* to be related to trehalose synthesis and *GPH1* to be related to glycogen degradation, key genes for ethanol resistance. Both are carbon energy reserves accumulated to overcome general stresses. In the case of glycogen accumulation, it occurs when growth is limited and yeast uses it during periods of starvation of nutrients. In the case of trehalose, it may function as a reserve material. However, it seems more likely that it is a stress protector that stabilises membranes, such as in the case of desiccation stress (Wiemken, 1990; Rodríguez-Porrata *et al.* 2012), which is in line with results found in the case of a gene to enhance its synthesis.

The genes related to amino acid metabolism (vii) mostly include those involved in sensing and regulatory processes. For instance, *SLM4, GTR1* and *LST8* encode proteins involved in protein trafficking and *AGP2* encodes a low affinity amino acid permease. The importance of amino acid permeases and sensors may be related to the effect of ethanol on yeast plasma membrane organisation and the activity of embedded transport systems.

Vesicular transport (i) also being altered may be due to changes in vacuole occurring to many genes related to this organelle and high ethanol resistance. As shown in

Figure 2, the vacuole is an organelle that determines ethanol tolerance due to the interaction network of proteins and the gene map.

The relationship between the oxidative stress responses (ROS compounds accumulation, iii) and stress response to other environmental stress responses was studied to ascertain whether stress responses are all distinct from one another or whether they are all manifestations of a global general stress response. A great deal work with bacteria and yeast has shown that many (if not all) of the different stress responses are indeed distinct, although there is significant overlap between many responses, and some genes are considered to be activated as a general stress response as cell protection (Jamieson 1998). What is known is that ethanol increases ROS accumulation, thereby activating genes related to both oxidative stress response and stress caused by the alcohol. In genome analyses, some authors have found *SOD1*, a cytosolic superoxide dismutase, to be essential in overcoming ethanol stress. In addition, Teixeira *et al*. (2009) cite *CCS1*, which is closely related to *SOD1*, as a copper chaperone for superoxide dismutase Sod1p involved in oxidative stress response. This would be well related to the activation of *SOD2* from the mitochondrial genes.

To sum up, the main interaction network maps of genes that determine ethanol tolerance can be divided into 3 categories and are represented in Figure 12: (i) vacuolar protein sorting; (ii) V-ATPase complex assembly; and (iii) peroxisome protein import machinery.

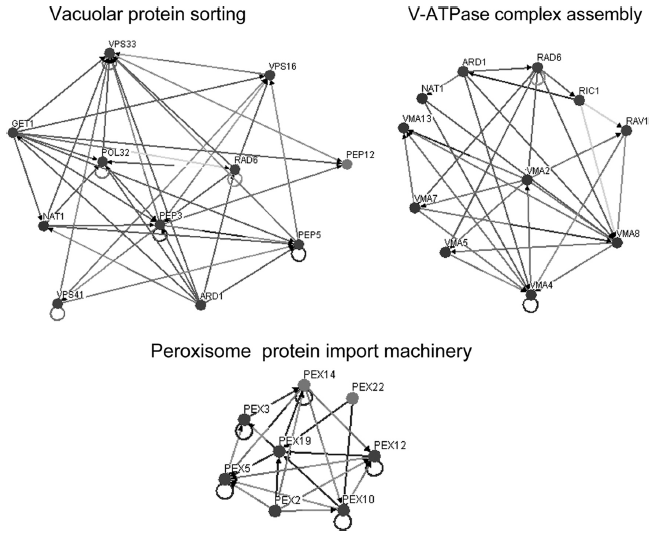

Figure 12. Main interaction network maps of the determinants of ethanol tolerance. The map shows protein and genetic interaction within three groups of genes. The existence of an interaction between two genes or proteins is represented by a connection between two nodes. (Teixeira *et al.* 2009).

Obviously, other stress factors have a synergic effect with ethanol during fermentation, making this process high complex. Some studies, such as Penacho *et al.* (2012), have described metabolic pathways that may be affected. During the second fermentation for sparkling wine production, transcriptomic analysis reveals that the main pathways affected are those related to aerobic respiration but also genes related to vacuolar and functions of peroxisomes are highlighted (Penacho *et al*. 2012).

In this work, different sample points were taken to be analysed 7, 15 and 19 days after the inoculation of the second fermentation, and they were named sample point (SP) 1, 2 and 3 respectively. In the list of categories of up- and down-regulated genes, categories in bold are those also found to have genes related to the ethanol stress response, with almost all categories being listed by Penacho *et al.* (2012). In addition, major interaction networking gene maps of determinants of ethanol resistance are represented in listed categories (Table 8).

Table 8. Genes over- and under- expressed in secondary fermentation at three diferent points (7, 15 and 19 days of fermentation) according to Penacho *et al.* 2012. Genes were organised by cataegories respresented the present table. Categories in bold are those also respresentative to ethanol stress.

Genes overexpressed in second fermentation (SP1-3) categories

- **mitochondrial electron transport**
- **oxidation-reduction process**
- **aerobic respiration, TCA cycle and electron transport chain**
- CVT pathway
- **autophagic vacuole assembly**
- **carbohydrate metabolic process**
- **cellular response to oxidative stress**
- **glycogen metabolic process**
- sporulation resulting in formation of a cellular spore
- oxidative phosphorylation
- **starch and sucrose metabolism**
- **peroxisome**

Continue

Genes underexpressed in second fermentation (SP1-3) categories

- protein import into nucleus *'de novo'* IMP biosynthetic process
- RNA metabolic process and transport into nucleus
- regulation of translation
- regulation on translational elongation
- purine nucleotide biosynthetic process
- ribosomal large subunit biogenesis and export from nucleus
- regulation of translational initiation

According to the results of Penacho *et al.* (2012), 348 genes was simultaneously up-regulated and 125 genes were down-regulated at different selected points throughout the second fermentation (the end of the lag phase, maximum ethanol production rate and first obvious inflection point after maximal fermentation rate), in comparison to the reference point. In general terms, transcripts encoding products involved in aerobic respiration, autophagy, peroxisomal and vacuolar function were significantly overrepresented throughout the second fermentation, while transcripts encoding products involved in protein synthesis and cell growth were underrepresented.

Several categories in Table 8 are clearly related to aerobic respiration in *S. cerevisiae*, such as mitochondrial electron transport, oxidation-reduction process, aerobic respiration and cellular response oxidative stress, or the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways oxidative phosphorylation and tricarboxylic acid cycle (TCA). These results are interesting, as yeast cannot respire during the second fermentation. However, a positive correlation between resistance to oxidative stress in several wine yeast strains and their fermentation performance has been reported. High expression levels of genes coding for mitochondrial proteins and respiration functions seem to be characteristic of *S. cerevisiae* in other industrial fermentation processes (James *et al*. 2003). Ethanol content is probably the main factor responsible for this transcriptomic pattern, as explained above. There is evidence of the involvement of genes related to mitochondrial function and oxidative metabolism in ethanol stress tolerance. Tolerance to ethanol has already been related to the mitochondrial properties by Jimenez and Benitez

(1988). Likewise, when analysing the whole genome of yeasts hardly resistant to high ethanol concentrations, Teixeira *et al*. (2010) found 30 genes encoding for mitochondrial proteins as determinants of resistance to ethanol. Those genes were mainly involved in mitochondrial protein synthesis, respiration and maintenance of mitochondrial genome.

In addition, genes evolved to peroxisome function are up-regulated during the second fermentation. Similar to the above case, the main determining factor affecting this would be ethanol content. This relation was confirmed by Yoshikawa *et al*. (2009) and Teixeira *et al*. (2009).

The categories CVT pathway and autophagic vacuole assembly are also overrepresented, showing autophagy takes place during the second fermentation of sparkling wines, even before the sugar has been used up (Cebollero *et al.* 2005).

Genes that are up- and down-regulated during the acclimation process for second fermentation performance were evaluated in the present thesis and will be discussed in another chapter.

2.1.3.2.2. Proteomics

Analysis of mRNA levels, while useful for assessing the effect of wine production stresses on yeasts, is not sufficient for a complete description of biological systems. This also requires an accurate measurement of the expression and activity of proteins. The correlation between genes and proteins may not be exact for all genes (Ideker *et al.* 2001). Changes at a proteome and transcriptome level generally occur in parallel. However, the post-transcriptional regulation justifies the need for proteomic studies (Griffin *et al.* 2002).

The yeast proteome is still largely undefined (Fey *et al.* 1997), and most studies are performed with laboratory strains (Washburn *et al*. 2001). Tabalzini *et al*. (2003) investigated the physiological response to fermentation stress and, in particular, ethanol stress. They found the main influence for changes in proteomics during wine production is related to increasing ethanol levels. Ethanol stress has been associated to oxidative damage and cytotoxic effects. Ethanol also induces the expression of heat shock proteins and proteins involved in trehalose metabolism, the purpose of which is to stabilise membranes and proteins and suppress protein aggregation.The importance of proteomic study,

additionally, is very important during auto-proteolysis (fermentation) and autolysis (ageing), which are responsible for the organoleptic properties of wine.

Two recent studies have compared the transcriptome and the proteome of wine yeasts. In the first of these, Zuzuárregui *et al.* (2006) compared different yeasts, concluding that those with fermentation difficulties had an excess proton uptake, a sign of ethanol intolerance and increased oxidative damage. Rossignol *et al.* (2009) compared proteomic changes in strains until they reached the stationary phase and during wine fermentation, finding major changes in the abundance of proteins related to (i) glycolysis, (ii) ethanol production and (iii) amino acid metabolism. Interestingly, these changes have not been observed in the transcriptome analysis, as least as far as the major changes occurring are concerned.

2.1.3.2.3. Metabolomics and Lipidomics

An approach in metabolomics aims to characterise the physiological state of a cell by identifying not just molecules but also metabolic pathways and fluxes. This may provide the best and most direct measurement of an organism's physiological activity and bring us a little closer to a rude approximation of its phenotype (Delneri *et al.* 2001).

The metabolic mechanism of *S. cerevisiae* cells in response to ethanol is not fully understood, even without the interaction of other fermentative stress factors. Metabolomic studies may improve our knowledge in this field and the area has been studied by several authors. For instance, Li *et al.* (2012) found 29 metabolites expressed significantly differently between cells growth with and without ethanol in YPD liquid medium (2% glucose, 1% yeast extract, and 2% peptone). The metabolic relevance of these compounds in the response of *S. cerevisiae* to ethanol stress was investigated, concluding that, under ethanol stress, glycolysis was inhibited and the use of carbon sources for fermentation was diminished, which might account for the growth inhibition of cells.

In a TCA with these 29 main metabolites (Table 3) Li *et al.* (2012) showed how samples with high ethanol concentration (10% and 15%, v/v) were well separated from the others (7.5%, 5%, 2.5% and control with 0%). Compared with the control cells, ethanol treatment induced metabolic changes in yeasts, especially in the metabolism of **carbohydrates, lipids** and **amino acids**.

On the other hand, ethanol promoted the intracellular accumulation of glucose and galactose, which indicates that glycolysis, was inhibited by ethanol and that the use of **carbon sources** for fermentation is diminished. Furthermore, ethanol stress caused an increase in the levels of glycine, serine, threonine, glycerol, alanine, leucine, isoleucine and valine. These metabolites could be converted from 3-P-glycerate and pyruvate, which are both metabolic intermediates in glycolysis and, to some extent; this also indicates the inhibition of this pathway by ethanol. This is also corroborated by a transcriptomic study conducted by Ma and Liu (2010), which showed that genes related to glycolysis in *S. cerevisiae* are repressed during long-term exposure of cells to sub-lethal alcohol concentration (8%, v/v).

Analysing these results in depth, it can be seen that almost all of the **amino acids** found increased with the ethanol, except lysine and tyrosine, which were not detected at higher ethanol concentrations. However, the maximum amino acid concentrations were at 10% (v/v) and not at 15% (v/v), (maximum ethanol rate). This may be due to the loss of membrane integrity or because their metabolism remains fairly static with this stress. The amino acids that increased most were: glycine (5.48 times higher at 10% (v/v) ethanol in comparison to the control, 0%), alanine (4.74 times higher), proline (4.39) and glutamine (3.35). The relation between different amino acids in *S. cerevisiae* is shown in Figure 13.

Supplementation with some amino acids promotes the ethanol tolerance of yeast cells by stabilising membrane structures (Ding *et al*. 2009). Describing metabolite variations under high ethanol conditions, Li *et al.* (2012) show that most amino acids, for instance isoleucine and proline, increase with the percentage of ethanol. However, there are some exceptions of amino acids decreasing in high ethanol media, such as lysine and tyrosine. A high concentration of intracellular L-proline also promotes ethanol tolerance in *S. cerevisiae* (Takagi *et al*. 2008). Based on this fact, proline synthesis genes *PRO1* and *PUT1* are also considered to be ethanol tolerance candidate genes (Ma and Liu 2010). In addition, isoleucine may confer protection against high ethanol content in media.

Chapter I

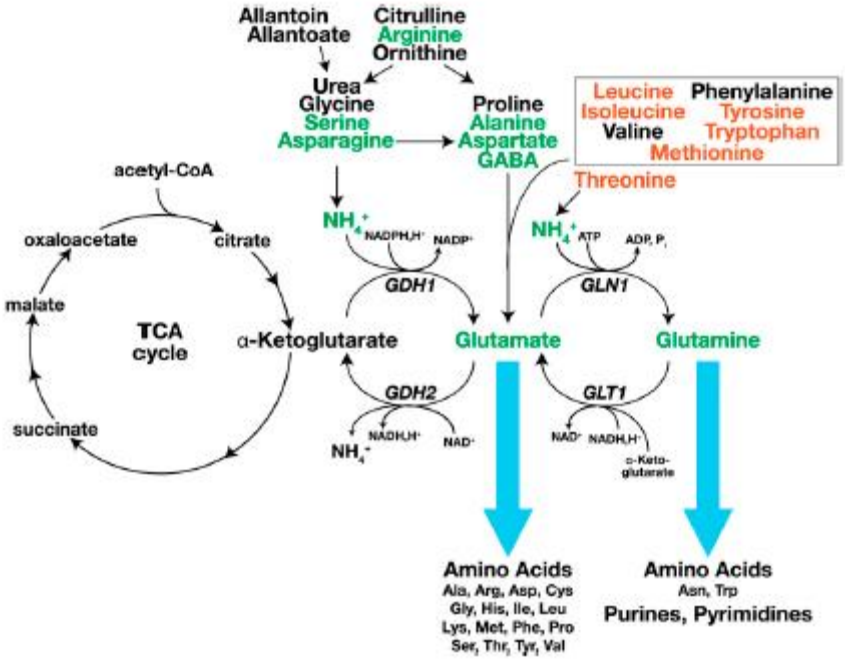

Figure 13. Schematic diagram of the main pathways of nitrogen metabolism. The entry routes of several nitrogen sources into the central core reactions are shown. The green routes are preferred by yeast, compared to non-preferred routes shown in red (from Ljungdahl *et al.* 2012).

Ethanol affects fluidity in the plasma membrane. The membrane structure becomes slack under ethanol stress. The plasma membrane should retain its structural integrity as far as possible in order to counteract the effect of ethanol. On the basis of this hypothesis, the factors that make the plasma membrane more rigid are important for cells to maintain membrane stability and survive under conditions of ethanol stress. In terms of **lipids**, for instance, ergosterol increases membrane rigidity to antagonise the increased fluidity that is induced by high concentrations of ethanol (Ding *et al*. 2009), and some saturated fatty acids (C16, C18) are also considered as the protective metabolites for *S. cerevisiae* cells under ethanol stress (Ding *et al*. 2010). A previous review discussing ethanol tolerance in *S. cerevisiae* indicates that the role of UFAs is complex and the function is not straightforward (Ding *et al*. 2009). It is suggested by Aguilera *et al.* (2006) that lower levels of C16:1 acid and higher levels of C16 and C18:1 helps membrane to overcome the stress regulating its integrity.

It is suggested that yeast cells change the levels of fatty acids (C16, C18 and C16:1) to maintain the integrity of their plasma membrane by decreasing membrane fluidity in the medium containing ethanol. These changes will be explained in-depth when describing

lipidomics in a later chapter. Moreover, the increased levels of some amino acids can also confer ethanol tolerance to *S. cerevisiae*. These results reveal that the metabolomic strategy is a powerful tool for gaining insight into the molecular mechanism of a microorganism's cellular response to environmental stress factor as ethanol.

2.2. Acclimation of yeasts for second fermentation

For all of these reasons, a progressive acclimation of yeast is particularly required before being inoculated into base wine, taking into account the synergic effect of some of these stress factors. The acclimation process was defined by Tai *et al*. (2007) as an adaptation over a period of several hours or days of yeast to one or various stressful factors. During this period, yeast could activate a stress metabolism response associated to high ethanol content and other stress factors to ensure the successful *prise de mousse* (i.e. the complete consumption of sugars added into base wine, containing ethanol and $CO_{2)}$. The main features of this method were first described by Françot and Geoffroy (1957) and the effects of ethanol on yeast during its acclimation were analysed by Juroszek *et al.* (1987). Some variations of the yeast acclimation process and its subsequent use, the *Champenoise* method, have principally been described by Valade and Moulin (1983) and Laurent and Valade (1994, 1998, 2007). During the secondary fermentation, yeasts still encountered unfavourable conditions despite their previous acclimation. In spite of procedures implemented by winemakers to adapt yeast and their years of experience, there is always a lag phase after inoculation followed by a short cell proliferation, indicating that cells are still not completely adapted to secondary fermentation media. However, without this acclimation process, cells are highly susceptible to death and, consequently, fermentation may be stopped before completion.

Martínez-Rodríguez *et al*. (2001) showed the structural changes in yeast cells during autolysis in a model wine system and in sparkling wines. Clearly, yeasts grown in synthetic medium during 24 hours have an appearance similar to those grown in a real fermentation for 20 days. The morphology of these cells is elongated and ovoid, and, within the cytoplasm, there is a large vacuole containing a number of spherical bodies around its edges. In contrast, cells after 24 hours of induced autolysis in a model wine medium are similar to those after 12 months ageing in the case of a natural sparkling wine. It is also possible to observe an increase in size of the vacuole, which occupied practically

the whole of the cytoplasm. Natural autolysis takes place during the ageing of sparkling wines in a slow process. However, yeasts in contact with a model wine medium undergo an induced autolysis caused by the stressful media conditions. This is the reason that cells acclimate progressively to stressful conditions, with a special enhancement to ethanol, the most toxic compound for cells. The main problem for winemakers is preparing a starter that is well-adapted to these stressful medium conditions and particularly to a high level of ethanol (Valade *et al*. 1983). Autolysis has been defined as the hydrolysis of intracellular biopolymers under the effect of hydrolytic enzymes associated with cell death, forming products with a low molecular weight (Babayan and Bezrukov 1985). Nevertheless, it has been reported that a gradual acclimation may keep almost all cells viable. Yeast in wine media with around 8% (v/v) at 20ºC maintains its population with 88% viability.

The second fermentation step usually lasts for 20 days. Afterwards, cell viability falls quickly, and almost no viable cells are detected after 60 days in a standard *Cava* production process on an industrial scale (Penacho *et al*. 2012; Feuillat and Charpentier 1982).

Fully understanding second fermentation and the acclimation mechanism will facilitate the development of methods to improve the ethanol tolerance of *S. cerevisiae* and contribute to the construction of industrial strains with high ethanol tolerance or even selecting strains that naturally have a high tolerance level for this alcohol, in order to achieve more effective formative capacities.

There are many industrial protocols available to carry on with acclimation process. Carrascosa *et al*. (2011) propose an acclimation procedure for ADY (Active Dry Yeast) prior to inoculation for second fermentation. Firstly, there is a rehydration phase for 20 min (500 g of yeast in 5 L of water with 250 g of sucrose) at between 35 ºC and 40ºC. When an agar slant culture of a selected strain is used, the strain should be grown in sterilised must or complete medium, with 10^8-10^9 CFU/mL being achieved. The next stage is the conditioning environment. In our example, 600 L of wine, 645 L of water, 120 Kg of sucrose, 500 g of yeast extract or 200 g of ammonium salts, 3 Kg of tartaric acid, and 5 L of active biomass are added to a 2000 L tank, which is kept at a temperature of 20ºC until a density of 0.994-0.998 Kg/cm^3 is reached (around 24 h). This culture can then be added to the base wine with 20 to 25 g/L of sugar and a fining agent in a proportion of between 8% and 10% (w/v). This produces a concentration of 8-12 x 10^6 CFU/mL and a sufficient

amount with which to prepare amounts to be used for other volumes. This operation is known as *tirage*.

Following the inoculation of the base wine, there is a lag phase, after which slow growth starts due to limited nutrients and stress factors from secondary fermentation. Martinez-Rodríguez *et al.* (2001) observed that a starter inoculum of approximately 10^6 cells/mL produced a population of close to 10^7 cells/mL during the secondary fermentation.

CHAPTER II

Material and Methods

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Chapter II

1. Strains, culture media and culture conditions

1.1.Strains and culture media from Article 3

A total of 45 yeast strains of *Saccharomyces cerevisiae* (44 commercial wine yeast strains + one laboratory yeast strain BY4742) were used in the present study. All commercial strains were analysed by mtDNA restriction analysis and delta sequence typing to ensure that they were different (see supporting information). Inocula were prepared by rehydrating active dry yeast (ADY) from commercial yeast strains for 30 min at 37ºC in water. After rehydration, yeast cells were pre-cultured in 5 mL of Yeast-Peptone-Dextrose (YPD) medium, containing 20 g L^{-1} Glucose (Panreac, Barcelona, Spain), 20 g L^{-1} Peptone (Panreac) and 10 g L^{-1} Yeast extract (Panreac). After overnight incubation at 25ºC with agitation of 150 rpm, the total number of cells was determined by flow cytometry using the CYFlow® instrument (Partec, Germany) to inoculate the different growth media described below at 2 10^6 cell mL^{-1}.

The basal growth medium (BGM) selected for all experiments was 6.7 g L^{-1} Yeast Nitrogen Base (YNB, Difco, Becton & Dickson & Company, Sparks, USA), 5 g L^{-1} tartaric acid (Panreac), 2 g L^{-1} malic acid (Sigma-Aldrich, Steinheim, Germany), 0.5 g L^{-1} citric acid (Panreac), 0.5 g L^{-1} succinic acid (Sigma-Aldrich), 12 g L^{-1} glucose (Panreac) and 12 g L^{-1} fructose (Panreac) at pH 3.5. The medium was sterilised by autoclaving except YNB by filtration. Increasing concentrations of ethanol (Panreac) were added to obtain the following concentrations: 0%, 5%, 10% and 15% (v/v) in order to test yeast tolerance to ethanol. For the fatty acid (FA) toxicity test, 3 mg L^{-1} of C8 and 6 mg L^{-1} of C10 were added to the BGM with 10% of ethanol. For cysteine addition, 50 mg L^{-1} was also added to the BGM, with 10% ethanol content. For the effect of the pH, the pH of the BGM was adjusted to 3.0 with 0%, 5%, 10% and 15% (v/v) of ethanol. For the glycerol effect, 0, 5 and 10 g L^{-1} of glycerol were added to the BGM at 0%, 10% and 15% (v/v) of ethanol. For the oxygen effect, yeast cells were pre-cultured overnight either with oxygen (growth culture under agitation) or without oxygen (static culture).

In order to estimate the NIC (Non-Inhibitory Concentration) and MIC (Minimum Inhibitory Concentration) parameters, growth media were constituted by the BGM with 0%, 4%, 8%, 10%, 12%, 14%, 16%, 18%, 20% and 22% (v/v) of ethanol.

1.2. Strains and inocula conditions for acclimation process from Article 4

The *S. cerevisiae* strain used in this study (EC1118, Lallemand S.A., Montreal, Canada) was rehydrated in accordance with the manufacturer instructions. After rehydration of 1 g of dry wine yeasts cells in 10 mL water ($2 \cdot 10^9$ cells mL^{-1}) according to manufacturer's recommendation, cells were then inoculated in basal medium 1 (9 mL of sucrose (150 g L^{-1}), 5 mL of $PO_4(NH_4)_2$ (4 g L^{-1}) and 6 mL of defined base wine) under agitation (150 rpm) for 24 hours which corresponds to phase 1 (P1, final volume 30 mL, 8.6 10^8 cells mL^{-1}). Cells followed the same activation process during the P1 for all conditions. Then this medium was filled to 500 mL with basal medium 2 (50 mL of sucrose (500 g L^{-1}) and 420 mL of defined base wine) to enable yeast cells acclimated to ethanol for three days which corresponds to phase 2 (P2). Based on P1, some changes were made in P2, depending on the condition studied: the OXI condition is constant aerobic yeast growth (medium is 1/3 of full volume of recipient) in the presence of sugars, the NOXI condition is semi-anaerobic yeast growth (medium is 4/5 of full volume of recipient in static) in the presence of sugars and the ETOXI condition represents constant aerobic yeast growth in the absence of sugars at the end of P2. The yeast cell acclimations were conducted at 25ºC. The composition of the defined base wine (DBW) was: 10% (v/v) of ethanol, 5 g L^{-1} tartaric acid, 0.5 g L^{-1} citric acid, 2 g L^{-1} malic acid, 0.5 g L^{-1} of succinic acid, 5 g L^{-1} glycerol, 24 g L^{-1} sucrose, octanoic and decanoic acids (MCFA), 6 and 3 mg L^{-1}, respectively, and 0.2 g L^{-1} of acetic acid at pH 3. All products were purchased from Panreac (Barcelona, Spain), except MCFA, which was supplied by Sigma-Aldrich (Madrid, Spain). Triplicate samples were taken at P0 (corresponding to the yeast rehydration in water), P1 and P2 for further analysis.

1.3. Secondary fermentation culture media

750 mL glass bottles were filled with synthetic wine and pre-culture media (from 3 different acclimations: OXI, NOXI and ETOXI) until an Optical Density (O.D.) of 0.2 ($2 \cdot 10^6$ cells mL^{-1}) was reached. For each set of conditions, two bottles were fitted with a manometer to monitor changes in pressure, while others were closed with a bottle shutter and a crown cork. Another assay was conducted with a direct inoculation of the active dry yeast (LSA-W) without rehydration. When two bottles under the same conditions reached the desired pressure (2, 4 or 6 bars), they were opened. Cells were collected for the

cytometer and physiological parameter was carried out analysis as described in subsequent paragraphs.

2. Physicochemical parameter determination

2.1. Sugar and ethanol determinations in acclimation medium and wine

The sugar content throughout the second fermentation was determined by enzymatic analysis with a Glucose/Fructose commercial kit (Roche, R-Biopharm, Darmstadt, Germany). The ethanol level was analysed by ebulliometry with a GAB electronic ebulliometer (GAB, Barcelona, Spain), in accordance with the method described by García-Barceló (1990).

2.2. Glycogen and trehalose determinations in yeasts

The glycogen of cells was determined in accordance with the method reported by Quain and Tubb (1983). To summarise, yeast samples of *ca* 2–4 mg (dry weight) were directly suspended in the iodine/potassium iodide reagent in a plastic cuvette with a path length of 1 cm. This reagent, a solution of iodine (1 mg mL^{-1}) in potassium iodide (10 mg mL^{-1}), was prepared freshly each time. Measurements were made immediately after mixing and optical density was determined at 660 nm. A blank sample of iodine solution on its own was made and subtracted from the sample values. From these corrected values, the glycogen concentration (x mg mL^{-1}) was calculated by substituting y (absorbance at 660 nm) in the following equation: $x = (y - 0.26) / 1.48$ (Quain and Tubb, 1983). The final results were expressed in μg of glycogen per mg of dry weight (D.W.).

The intracellular trehalose content of cells was measured by gas chromatography coupled to mass detector. Around 5 10^8 cells were immediately quenched by cold 70 % (v/v) of ethanol and pelleted. Then, after the addition of 400 μL of methanol/water (1/1), cells were broken in the presence of 0.5 mm acid-washed glass beads (BioSpec Products, USA) in a multitube bead-beater (BioSpec Products) using five cycles of 1 min per beat, followed by resting for 30 s in ice. After centrifugation, the supernatant was dried in a SC110 speed vacuum system SC110 (Savant Instruments, USA) for 4 hours. The dried residue was derivatised for 1 h at 40ºC in 50 μL of 20 mg mL^{-1} of methoxyamine

hydrochloride (Sigma-Aldrich) in pyridine (Sigma-Aldrich), followed by treatment at 40ºC with 70 μL of N-methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA, Sigma-Aldrich) in accordance with Roessner *et al*. (2000). Chromatographic conditions were the same as those described for metabolomic studies by López-Martínez *et al*. (2014), using a gas-chromatograph 6890N coupled to a mass elector detector 5975 (Agilent Technologies, Wilmington, DE, USA). To summarise, 2 μL of the intracellular extract was injected at a split ratio of 20:1 into a DB-5HT column (30 m x 0.25 mm x 0.10 μm, Agilent Technologies) with an automatic injector 7683B (Agilent Technologies). External calibration curves of trehalose were performed in the range of 25 to 500 μg under the same conditions described above for sample treatments. From the total trehalose areas, a good fit of R^2 = 0.980 was obtained using a power regression model. The trehalose (μg) content was calculated using the following equation: $x = (y / 1\ 10^8)^{1/\ 0.4207}$. Finally, the trehalose content of cells was expressed as μg of trehalose per mg of Dry Weight (D.W.).

2.3. Glutathione determination in yeasts

Yeast cells, *ca* 5 10^8 cells, were quenched by cold 70% (v/v) ethanol and then centrifuged at 5,000 x g for 5 min. At this stage, cell pellets can be stored at -80ºC or can be processed. To obtain cell extracts of total glutathione (GSH_{tot}), pellets were washed twice with PBS pH 7.4 and centrifuged at 5,000 x g for 5 min. Three volumes of 5% of 5-sulphosalicylic acid (SSA) in distilled water were then added and vortexed. The cell suspension was frozen twice using liquid nitrogen and thawed at 37ºC in a water bath, and left for 5 min at 8ºC. After centrifuging at 10,000 x g for 5 min, the supernatant was used for glutathione analyses. For the determination of oxidised (GSSG) glutathione, 20 μL of 20% (v/v) of 2-vinylpyridine (Sigma-Aldrich) in ethanol was added to the cell pellets before extraction. GSH_{tot} and GSSG were determined by a modified version of the method described by White *et al*. (2003). To summarise, 20 μL aliquots of supernatant were transferred to a 96-well plate designed for fluorescence detection (BMG Labtech, Offenburg, Germany). Then, 180 μL of 2, 3-naphthalenedicarboxaldehyde (NDA, Sigma-Aldrich) derivatisation solution (50 mM TRIS, pH 10, 0.5 N NaOH and 10 mM NDA in dimethylsulphoxide, v/v/v) was added to all of the wells. The plate was covered to protect the wells from the light and allowed to incubate at room temperature for 30 min. GSH_{tot} and GSSG determinations were performed on a fluorescence plate reader SPECTROstar

Omega instrument (BMG Labtech), using the 488 nm for excitation and 530 nm for collect fluorescence emission. Linear regression curves were performed from a reduced (GSH) glutathione standard solution made from a GSH standard stock solution of 10 mM diluted with 5% SSA solution. GSH was calculated from the difference between GSH_{tot} and GSSG and expressed as nM of glutathione per mg D.W.

3. Physiological parameter analysis

3.1 Growth curves (Article 3)

A total of 96 well plates were inoculated with yeast in the BGM with different ethanol concentrations according to Arroyo-López *et al* (2010a) with some modifications. Optical density measurements were carried out at 600 nm using a SPECTROstar Omega instrument (BMG Labtech, Offenburg, Germany) at 25ºC. Measurements were taken every 30 min after pre-shaking for 20 sec, and the growth was monitored until the yeast reached stationary phase. The wells of the microplate were filled with 250 μL of BGM medium and 10 μL of yeast inocula (OD of approximately 0.2, which corresponds to $2 \cdot 10^6$ cells mL^{-1}). Control wells were filled only with the corresponding medium (without yeast inoculum) to subtract the noise signal. All experiments were carried out in quadruplicate. In this way, a total of 720 growth curves (4 levels of ethanol x 45 strains x 4 biological replicates) were obtained and analysed.

Growth parameters were calculated under each set of conditions by directly fitting OD measurements versus time to the re-parameterised Gompertz equation, as proposed by Zwietering *et al*. (1990):

$$y = A * \exp(- \exp(((\mu_{max} * e)/A) * (\lambda * t)))$$

where $y = \ln (OD_t/OD_0)$, OD_0 is the initial OD and OD_t is the OD at time t; $A = \ln (OD_{max}/OD_0)$ is the asymptotic maximum, μ_{max} is the maximum specific growth rate (h^{-1}) and λ is the lag phase period (h). Growth data were obtained by a non-linear regression procedure using the GraphPad Prism 4.0 software package (GraphPad Software, Inc, La Jolla, CA, USA).

3.2.NIC and MIC estimation (Article 3)

The NIC and MIC parameters were estimated from the curves obtained by the cell growths in increasing levels of ethanol content in the BFM media. After subtracting the blank signal, the area under the OD = f(time) was calculated using the GraphPad Prism 4.0 software package. According to Arroyo-López *et al*. (2009), the fractional area (f_a) for each ethanol content was obtained using the ratios of the test area ($area_{test}$) to that of the control ($area_{cont}$). Therefore, the NIC and MIC parameters can be estimated using the equation of Lambert and Pearson (2000), which has the following expression:

$$f_a = A + C * \exp(-\exp((B(x - M))$$

where A is the lowest asymptote of f_a (around 0), B is the slope parameter, C is the distance between the upper and lower asymptote (around. 1) and M is the $\log_{10}$ (ethanol) of the inflexion point. Finally, the estimation of NIC and MIC parameters was deduced using the following equations from Lambert and Pearson (2000):

$$NIC = 10^{(M- 1.718 / B)} \text{ and } MIC = 10^{(M+ 1/ B)}$$

3.3. Reactive oxygen species determination

To observe the reactive oxygen species (ROS) with the microscope, cells were pretreated with dihydroethidium (DHE, Molecular Probes, USA), as described by López-Martínez *et al*. (2012). The pellets with a concentration of *ca*. $5 \cdot 10^6$ cells mL^{-1} were washed with a phosphate-buffered saline solution (PBS) at pH 7.4. After washing, pellets were re-suspended in 250 µL of DHE (2.5 mg mL^{-1} in PBS). After vortex agitation, samples were incubated for 10 min under darkness conditions. Subsequently, the cells were washed again with 250 µL of PBS pH 7.4 and placed on a slide. Stained ROS cells were examined and counted using a DM4000-B fluorescence microscope LEICA (Wetzlar, Germany) equipped with a digital camera (Leica DFC300FX). For the image acquisition, Leica IM50 software was used. Positive cells emitted red fluorescence and were counted. The percentage of ROS cells was calculated from the number of red cells to the number of total cells (1,000 cells counted for each condition).

3.4.Viability estimation

The viability of cells was estimated by flow cytometry using a CYFlow® space instrument (PARTEC GmbH, Germany) fitted with a 22 mW ion laser for excitation (488 nm) while a single emission channel (575-nm band-pass filter) was used for monitoring. FloMax software (Quantum Analysis GmbH, Germany) was used for instrument control, data acquisition and data analysis. The two-colour fluorescent probe LIVE/DEAD® Yeast Viability Kit (Molecular Probes Inc., Eugene, Oregon, USA) was used to label the cells in accordance with the manufacturer's instruction.

3.4 Mitocondrial organisation, microvacuolation and vacuolar activity determination

The fluorochrome 3, 3'-dihexyloxacarbocyanine iodide ($DiOC_6(3)$) was used to characterise mitochondrial cell activity. Vacuoles were observed with 7-amino-4-chloromethylcoumarin (CMAC) coloured in blue and vacuolar activity was highlighted with FUN1 (red for active vacuoles and green for cytoplasm), in accordance with López-Martínez *et al*. (2015). The cell samples were viewed with a Leica microscope (DM4000B, Germany) equipped with a digital camera (Leica DFC300FX), and Leica IM50 software was used for image acquisition. The percentage of microvacuolation and vacuolar activity cells was calculated from the number of blue and red cells to the number of total cells (1,000 cells counted) respectively.

4. Omics analysis

4.1.Typing of commercial winery yeast strains: mtDNA (Article 3)

Yeast mitochondrial DNA (mtDNA) restriction analysis and delta sequence elements were used for the detailed genotyping of selected commercial winery yeast strains in order to ensure that none was repeated.

Firstly, mtDNA restriction analysis was performed on all of the strains, as described by Querol *et al*. (1992). Firstly, the DNA should be extracted. Fresh pellet were washed with sterile water. Add 500 µL of buffer 1 (sorbitol 0.2M, EDTA 0.1M at pH 7.5). Add 30 µL of a zymoliase solution and incubate at 37ºC for 20 min. Centrifuge 2 min at 10,000 rpm and discart the supernatant. Cells were resuspended in 500 µL of buffer 2 (Tris 50 mM, EDTA 20 mM, pH 7.4). Add 15 µL of SDS (10%) and shake. Incubate at 65ºC

during 5 min. Add 300 µL of potassium acetate (5M) and shake. Incubate in ice for 5 min. Centrifuge in cold during 10 min at 12,000 rpm to precipitate the SDS. Transfer the supernatant to another eppendorf, add 700 µL of isopropanol and incubate at room temperature during 5 min. Centrifuge 10 min at 12,000 rpm and discart the supernatant. Add 500 µL of ethanol (70%). Centrifuge 5 min in cold at 12,000 rpm. Discart the supernatant. Dry it on vacuum. Resuspend pellets on 20 µL of sterile water. The agarose gel to visualize samples has a density of 0.8%. To do the restriction of DNA; 10 µL of DNA extracted was mixed with 6 µL of water, 2 µL of buffer H, 1 µL of RNase, 1 µL of enzyme *Hinf I* and left overnight at 37ºC.

For all strains, but especially in the case of strains with an identical mtDNA pattern, delta sequence typing was used to distinguish them, in line with the method developed by Schuller *et al*. (2004).

A very small amount of cells is need into PCR reaction mix (20 µL): 0.6 µL $MgCl_2$ (50Mm), 0.4 µL dNTPs (10mM), 0.25 µL delta element with primer (5' to 3') CAA AAT TCA CCT ATA TCT CA and 0.25 µL GTG GAT TTT TAT TCC AAC A, 0.2 µL taq polymerase, 2µL buffer, 0.2 µL BSA.

Table 9. PCR cycle profile.

x1	94ºC	8 min
x35	94ºC	1 min
	55-68ºC	45 sec
	72ºC	2 min
x1	72ºC	7 min
	4ºC	infinity

4.2.Lipidomic protocols

4.2.1. Fatty acid analysis

The fatty acid methyl ester analysis was performed using a modified version of the method reported by Borrull *et al*. (2014a). Specifically, from a yeast pellet of *ca* 5 10^8 cells, 500 µL of sodium methoxide 0.5 M (Sigma-Aldrich) in methanol (MeOH) and 10 µL of internal standards (IS, heptanoic acid (C7), 1 g L^{-1} and heptadecanoic acid (C17), 4 g L^{-1}

were added to glass tubes heated to 60ºC for 30 min. After cooling to room temperature, 500 μL of HCl (1.25 M in MeOH) was added and the tubes were heated to 60ºC for 45 min. After cooling, samples were extracted twice with 500 μL hexane. If necessary, tubes were centrifuged at 3,000 g for 5 min to allow better phase separation. Analytical GC was carried out on an Agilent 5890 connected to an HP Vectra computer with ChemStation software (Agilent Technologies). The extract (2 μL) was injected (splitless, 0.75 min) into an FFAP-HP column of 30 m x 250 mm x 0.25 μm phase thickness (Agilent Technologies) with an automatic injector (Agilent Technologies). The temperature programme was from 100ºC to 240ºC (5 min) at 4ºC min^{-1}. Injector and detector temperatures were 220ºC and 250ºC, respectively. The carrier gas was helium at a flow rate of 1.2 mL min^{-1}. Relative amounts of the given fatty acids were calculated from their respective chromatographic peak areas after normalisation with IS areas. The results are expressed as individual percentages of the total sum of identified fatty acids.

4.2.2. Total sterol analysis by GC-MS

Sterols and squalene were determined using the method reported by Quail and Kelly (1996). To summarise, sterols and squalene were saponified from around $5 \cdot 10^8$ cells re-suspended with 1.5 mL methanol (MeOH), 1 mL of pyrogallol (0.5% (w/v) in MeOH, 1 mL KOH solution (60% (w/v) KOH in distilled water) and 10 μL of α-cholestane (internal standard (IS), 1 mg mL^{-1} in hexane) into a glass tube. The tubes were then put in a dry bath at 90ºC for 2 hours. Finally, sterols and squalene were extracted three times with 500 μL hexane. Gas chromatography was performed using an Agilent GC system 6890N connected to an HP Vectra computer with ChemStation software (Agilent Technologies). From the collected organic phase, 2 μL were injected in pulsed splitless mode (70 psi, 0.10 min) into a DB-5HT column (30 m x 0.25 mm x 0.1 μm, Agilent Technologies) with an automatic injector (7683B, Agilent Technologies). Helium was used as the carrier gas at a constant flow of 1.2 mL min^{-1}. The injector temperature was 160ºC. The column oven temperature was initially held at 160ºC for 1 min and then increased first to 260ºC min^{-1} at a rate of 50ºC min^{-1} and then to 320ºC at a rate of 4ºC min^{-1}, where it was held for 1 min. Sterols and squalene were detected using an inert mass selective detector (MSD, model 5975, Agilent Technologies). The MSD transfer, MS Quad and MS Source temperatures were 300ºC, 150ºC and 280ºC, respectively. The MSD data were acquired in electronic ionisation scan mode at 70 eV within a range of 60 to 700 amu after a solvent delay of 2.60

min. Post-run analysis was performed with the enhanced Agilent MSD Chemstation (version 1989-2005, Agilent Technologies). The identification of each compound was carried out by comparing the mass fragmentation pattern of peak with those of the injection of available standard or described by Quail and Kelly (1996). The relative abundance of each identified compound was calculated according to the respective chromatographic peak areas corrected with respect to the internal standard peak area. The results are expressed as individual percentage of total sum of identified sterols and squalene.

4.3.Phospholipids determined by HPTLC

Lipid extraccion was carried out as descrived in Borrull et al. (2015a). The same extract is used to analyse neutral lipids (NLs) or even phospholipids (PLs). In the case of PL determination, they were separated and quantified by one-dimensional TLC on silica gel 60F254 plates (10 x 20 cm, 200 µm) (Merck, Germany), as described by Redón et al. (2009). The plate application of samples and lipid standards was carried out with a semi-automatic injector (Linomat 5, Camag, Switzerland). Three successive migrations were conducted with an semi-automatic developing chamber (ADC2, Camag): the first step with chloroform (13mL), acetone (3mL), methanol (2mL), acetic acid (2mL) and water (0.5mL), the second with hexane (8mL), MTBE (2mL) and acetic acid (0.2mL) and the third with hexane (10 ml). The timing of the three steps was 65min, 75min and 85min, respectively. The plate was dried for 15 min between each migration step. Visualisation was achieved by spraying with 10% (w/v) copper sulphate in 3% (v/v) of phosphoric acid and heating for 15 min at 150ºC on a TLC Plate Heater (Camag). The PLs were identified by comparison with a lipid standard solution spotted at different concentrations on the plate. The plate with identified spots was acquired with an Image Scanner (Amersham Biosciences). Each spot of the image was quantified in terms of integrated optical densities (IOD) with ImageJ software (a public domain, Java-based image processing program developed at the National Institute of Health). Calibration curves were constructed by plotting the IOD of the lipid standard against the amount of lipid loaded.

Chapter II

Article 1

Development of a new method for lipid profiling of yeast cells using GC-MS

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Chapter II

Abstract

A simple and suitable GC-MS method has been developed for the determination of neutral lipids in yeast. This method was compared to the conventional TLC method used in our laboratory. This new method enabled the measurement of molecular species of diacylglycerols (DAG), triacylglycerols (TAG) and sterol esters (SE). With a classic lipid extraction, samples can be injected directly into the GC-MS without any previous derivatisation procedure. However, the main characteristic of this new method is its versatility because GC parameters can be modified based on the biological samples analyzed: volume and injection modes, derivatisation of samples, etc. In order to validate the method, yeast lipid extracts from two distinct culture growth conditions were compared, the first in presence of oxygen and the second in its absence. Although the lipid profile of both yeast samples is qualitatively similar, the use of the GC-MS method rather than the TLC method enables the identification of a sterol, neoergosterol, in yeast cells grown in absence of oxygen. Moreover, this method enables phospholipid detection in samples but their identification is difficult.

Chapter II

Introductiory highlights

The recent rapid growth of lipidomics can mainly be attributed to technological advances in mass spectrometry, especially by the development of soft ionisation techniques; in combination with computational tools has created new method anlaysis (Shui et al. 2010). There are current techniques for the separation and determination of neutral lipids including thin-layer chromatography (TLC), high pressure liquid chromatography (HPLC) and gas chromatography (GC) with a flame ionisation detector (FID) or mass spectrometry detector (MSD).

TLC is very simple (Johnsson *et al.* 2007), however, procedure is time-consuming and does not determine molecular species of DAG, TAG and intermediates of sterol biosynthesis. Meanwhile, GC with FID or preferably MSD is a very suitable technique for the separation, identification and determination of different molecules from biological samples. In addition, GC does not involve the same drawbacks as TLC and HPLC techniques (Fagan *et al.* 2004).

Lipidomic profiling are constitute with (i) lipid species of cell membranes, protecting cell from environmental changes, and (ii) others which store metabolic energy and act as bioactive molecules with vital signalling functions (Jesch and Henry 2005; Daum *et al.* 1998). Lipidomes of several eukaryotic cell models remain relatively uncharacterized (Ejsing *et al.* 2009).

Saccharomyces cerevisiae is a powerful model for studying lipidomics in eukaryotic cells as it has a relatively simple network of lipid metabolic pathways. Deletion mutants have facilitated studies of the function of lipids (Daum *et al.* 1999).

Unfortunately, results of these studies can sometimes be difficult to interpret. The lipid composition of yeast cells is modulated by experimental growth conditions, for instance oxygen (Alexandre *et al*. 1996; Taylor and Kirsop 1977b; Lafon-Lafourcade *et al.* 1984).

The aim of this work was to optimize a GC-MS method to cover major yeast lipids in a single run by using a very simple lipid extract protocol. In order to validate the method, yeast grown under two different conditions (in the presence or absence of oxygen) were evaluated.

Material and methods

Reagents

The following lipid standards were purchased from Sigma-Aldrich (Madrid, Spain): squalene (SQ), α-cholestane (IS), cholesterol (CHL), dihydrocholesterol (DiCHL), ergosterol (ERG), stigmasterol (STIG), β-sitosterol (SITO), lanosterol (LST), cholesterol palmitate (ChlC16), cholesterol margarate (ChlC17), cholesterol oleate (ChlC18:1), 1-monooleyl-rac-glycerol (C18:1 MAG), 1,3-dioleyl-rac-glycerol (C18:1 DAG), glyceryl trioctanoate (C8 TAG), glyceryl tridecanoate (C10 TAG), glyceryl tridodecanoate (C12 TAG), glyceryl tritetradecanoate (C14 TAG), glyceryl trihexadecanoate (C16 TAG), glyceryl trioleate (C18:1 TAG), glyceryl trilinoleate (C18:2 TAG), stearyl stearate (C18-C18), phosphatidic acid (PA), phosphatidylcholine (PC), phosphatidylethanolamine (PE) and phosphatidylglycerol (PG). All lipid standards were of a purity > 95% and were dissolved in chloroform-methanol (2:1, v/v). All solvents (chloroform, methanol, hexane, methyl tert butyl ether (MTBE), glacial acetic acid), were Chromasolv grade and purchased from Panreac (Barcelona, Spain).

Strains and culture medium

The strain used in the present paper is a commercial wine strain (*Saccharomyces cerevisiae* 1118, Lallemand S.A., Canada). The yeast was presented as ADY (active dry yeast) which was rehydrated for 30 mins in deionised water at 37ºC in line with the manufacturer's instructions. The culture growth medium was YEPD (w/v), (1% Yeast Extract, 2% Peptone, 2% Dextrose, Cultimed, Barcelona, Spain). Yeast cultures were carried out in the presence of oxygen ($+O_2$) with an agitation of 150rpm or without oxygen ($-O_2$) in a static heater overnight, both at 28ºC.

Lipid extraction from yeast cells

Yeast cells were harvested in early stationary growth phase to obtain around $2 \cdot 10^8$ cells/ml (10-15 mg of Dry Weight). Lipid extraction was performed following the protocol described by Redón *et al*. (2009). To summarize, 20 µL of EDTA, 100 µL of cold methanol and 1 g of glass beads (0.5 mm, Biospec Products, Qiagen, Barcelona, Spain) were added to the cell pellet in an Eppendorf tube and then mixed for 5 minutes with an interval in ice of one minute in a mini-bead-beater-8 (Biospec Products). Four successive

extractions were then carried out with chloroform-methanol (v/v), (2:1), (2:1), (1:1) and (1:2), respectively. All fractions were collected and then washed twice with KCl (0.88%w/v) in deionised water. The volume used to clarify the sample had to be a quarter of the organic solvent volume. After the recuperation of the organic phase, extracts were evaporated under nitrogen. Extract samples were suspended with chloroform-methanol (2:1, v/v) and collected in a glass insert, which was put into a vial to be analysed by either TLC or GC-MS.

Thin-layer chromatography (TLC) procedure

Yeast neutral lipids (NL) were separated and quantified by one-dimensional TLC on silica gel $60F_{254}$ plates (10 x 20 cm, 250 μm) (Merck, Germany), as described by Redón *et al*. (2009). The plate application of samples and lipid standards was carried out with a semi-automatic injector (Linomat 5, Camag, Switzerland). Three successive migrations were conducted with a semi-automatic developing chamber (ADC2, Camag): the first step with hexane (5 ml), MTBE (5 ml) and glacial acetic acid (0.2 ml), the second with hexane (8 ml), MTBE (2 ml) and glacial acetic acid (0.1 ml) and the third with hexane (10 ml). The timing of the three steps was 35min, 60min and 80min, respectively. The plate was dried for 15 min between each migration step. Visualisation was achieved by spraying with 10% (w/v) copper sulphate in 3% (v/v) of phosphoric acid and heating for 15 min at 150ºC on a TLC Plate Heater (Camag). The neutral lipids were identified by comparison with a lipid standard solution spotted at different concentrations on the plate. The plate with identified spots was acquired with an Image Scanner (Amersham Biosciences). Each spot of the image was quantified in terms of integrated optical densities (IOD) with ImageJ software (a public domain, Java-based image processing program developed at the National Institute of Health). Calibration curves were constructed by plotting the IOD of the lipid standard against the amount of lipid loaded.

Gas chromatography mass spectrometry (GC-MS) procedure

GC was performed on a DB-5HT fused silica capillary column (30 m x 0.32 mm i.d., 0.1 μm film thickness) using a 6890N Agilent (Agilent Technologies, Germany) instrument fitted with a mass spectrometry detector (5975 Agilent). Using an autosampler (7683 Agilent), samples or standards (from 1 to 3 μl) were injected in pulsed split mode (80 psi, 0.20 min) at the split ratio of 2:1. The injector temperature was 320ºC. The column

oven temperature was initially held at 150ºC for one minute and then increased firstly to 320ºC at the rate of 15ºC/min and subsequently to 380ºC at the rate of 4ºC/min, where it was held for 8 min. Helium was used at the carrier gas at a constant flow of 1.0 ml/min (average velocity of 38 cm/s). The MSD transfer temperature was 350ºC. The MS quadrupole and source temperature were maintained at 150ºC and 280ºC, respectively. The MS acquired data in the electronic ionisation (EI) scan mode at 70 eV for the range of 60-900 amu after a solvent delay of 3 min. Post-run analysis was performed with the Agilent MSD Chemstation.

Results and discussion

1. *Thin-Layer Chromatography (TLC) analysis*

Figure 14 shows a traditional TLC development used in our laboratory. From the bottom to the top of the plate, the correct separation of all neutral lipid classes from external standards and yeast samples were observed as follows: ergosterol (Erg, 1), diacylglycerides (DAG, 2), lanosterol (Lst, 3) free fatty acids (4), triacylglycerides (TAG, 5), fatty acid ethyl esters (6), sterol esters (SE, 7) and squalene (SQ, 8). The phospholipids (0 in the Fig. 1) did not migrate in the solvent system used. To calculate the lipid content of samples, applications on the plate of different concentrations of neutral lipids were performed. After scanning the sprayed plate with copper sulphate, standard curves were carried out to determine the lipid content of samples. Although all steps were optimised with semi-automated application of samples, a semi-automatic developing chamber, spraying, heating and scanning of plates, the method was not suitable for the identification of molecular species of some neutral lipids. However, it should be noted that the quantification of each lipid class is generally accurate and reproducible as observed in Table 10 by the correlation coefficient (R^2) calculated from the average of five independent plates. The major drawback of this TLC method was the lack of information regarding some neutral lipid molecular species. Nevertheless, our TLC method gave a detection limit of *ca* 0.5 µg on plate for almost all of the neutral lipids studied in our experimental conditions. However, this detection limit could be decreased by the use of a fluorochrome reagent.

With respect to the yeast samples, as expected, cells grown in the presence of oxygen had more ergosterol, SE and TAG than cells grown in its absence (Beltran *et al.*

2008). In the latter case, more squalene and lanosterol was accumulated. While most neutral lipids could easily be detected on plate using the copper sulphate reagent, this was not, however, the case of some metabolites which had a certain degree of unsaturation, such as unsaturated fatty acids, DAG or TAG. It should be noted that the accurate quantification of these molecules by TLC was good but not optimal. For this reason, the use of TLC remained limited with respect to the absolute quantification of these molecules.

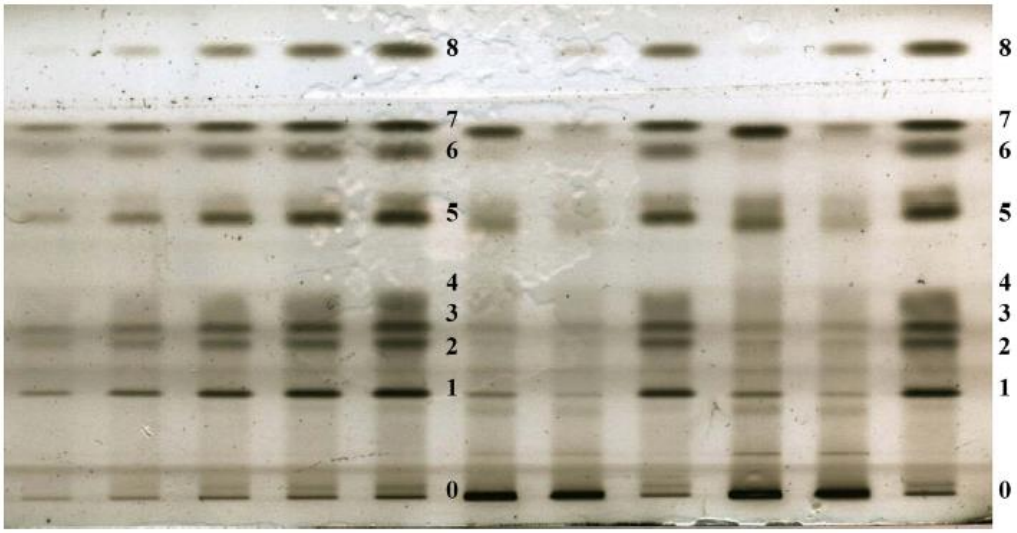

Figure 14. TLC analysis of yeast samples and neutral lipid standards. Tracks 1 to 5 of neutral lipid standards for establishing the calibration function (0.5. 1. 2. 3 and 5 µL); track 6 and 9 (3 and 5 µL of lipid extract of yeast grown in oxygen presence, respectively); track 7 and 10 (3 and 5 µL of lipid extract of yeast grown without oxygen, respectively); track 8 (3 µL of neutral lipid standards); track 11 (5 µL of neutral lipid standards).

2. Gas chromatography–mass spectrometry (GC-MS) analysis

With respect to the GC-MS method, Figure 2A shows a global chromatogram of the neutral lipid separation. Although it was decided only to point out the separation of major neutral lipids, such as sterols, DAG, SE and TAG, during the first eight minutes of the analysis, it was also possible to detect free and esterified fatty acids and monoacylglycerides (MAG). The initial time of analysis was determined to ensure the possible detection of medium-chain fatty acids which were produced under hypoxic conditions by the yeast.

By examining some parts of the GC analysis, neutral lipid classes had specific zones in which elution took place. In Figure 2B (time 8 to 14 minutes, zone x in Fig. 2A), squalene, cholesterol, ergosterol, stigmasterol, β-sitosterol and lanosterol were detected.

Although some of these sterols were not present in yeast cells, the ability to separate other sterols that are present in human or plant cells was useful for showing the versatility of the method. Between 14 and 32 minutes, fatty acids that had esterified between each other, DAG and phospholipids (zone y in Fig. 2A), and SE and TAG (zone z in Fig. 2A) could be detected (Fig. 2C). This part of chromatogram was very complex and basically depended on the nature of the fatty acid which esterified the lipid. With the column used that is characteristic of this method, unsaturated fatty acid eluted before the saturated version with the same number of carbon atoms. Similarly, longer fatty acids eluted after shorter ones.

Table 10. Correlation coefficient comparisons of neutral lipid standard calibrations between TLC and GC-MS methods. **[a]:** Average of 5 independent TLC plates based on application volumes in the range of 0.5-5 μg; **[b]**: Calculated from 43 column loadings in the range of 0.1-3 μg.

	TLC	**GS-MS**	
Neutral lipid	**Power regression [a]**	**Linear regression [b]**	**Slope [b]**
Squalene	0.978	0.973	$2.56\ 10^{8}$
Ergosterol	0.959	0.973	$6.43\ 10^{7}$
Lanosterol	0.965	0.985	$1.60\ 10^{8}$
C18:1 DAG	0.982	0.969	$1.71\ 10^{8}$
Cholesteryl oleate	0.969	0.958	$2.22\ 10^{7}$
C18:1 TAG	0.982	0.978	$4.38\ 10^{7}$

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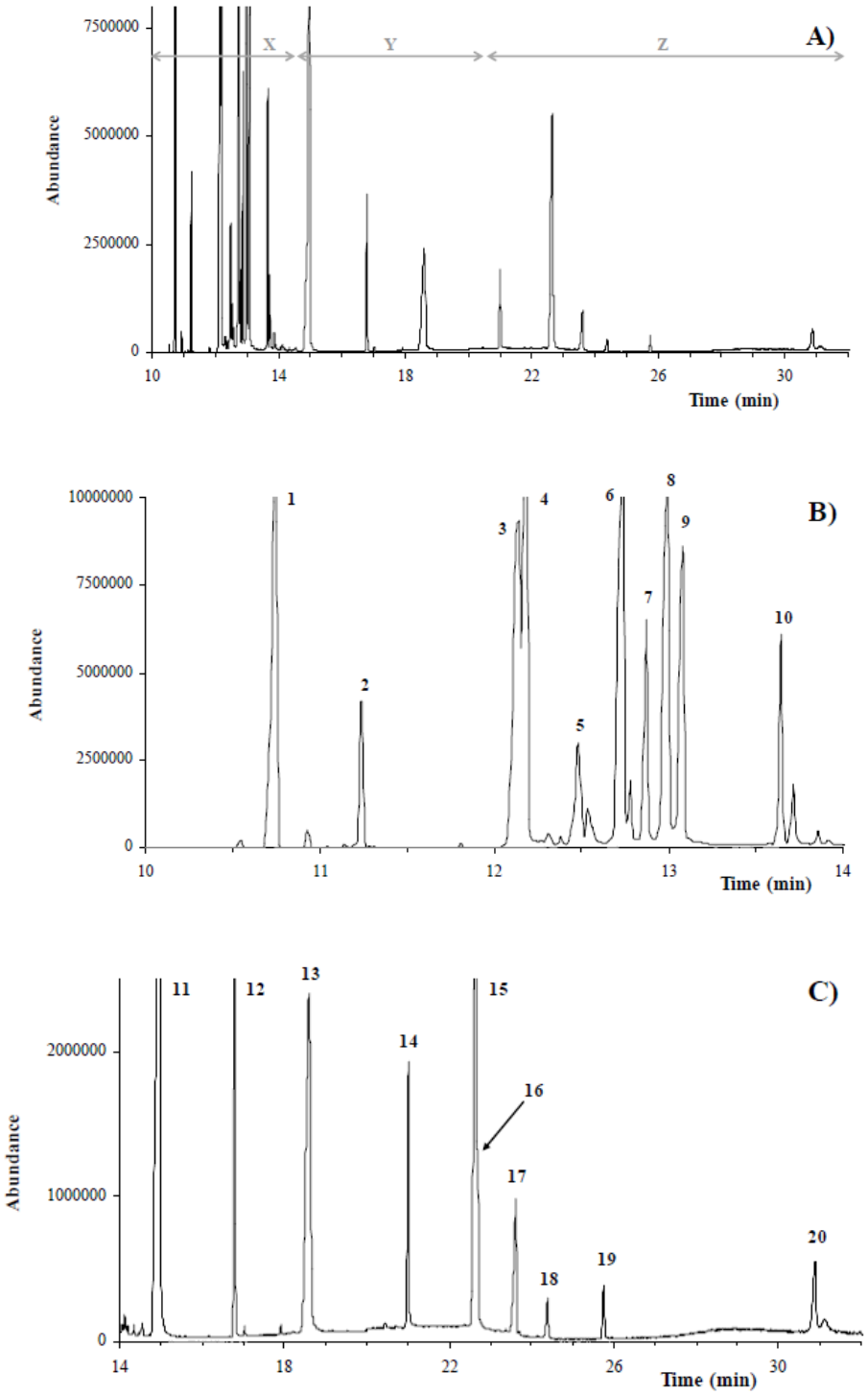

Figure 15. GC-MS total ion chromatogram of (A) full chromatogram of lipid standard (0.6 µg loaded), (B) chromatogram from 10 to 14 min of A and (C) chromatogram from 14 to 32 min of A. Peak identification: 1, squalene; 2, C8 TAG; 3, cholesterol; 4, dihydrocholesterol; 5, ergosterol; 6, stigmasterol; 7 dihydrolanosterol; 8, β-sitosterol; 9 lanosterol; 10, C10 TAG; 11, Stearyl stearate; 12, C12 TAG; 13, C18:1 DAG; 14, C14 TAG; 15, C14-C16 TAG; 16, C16 Cholesterol; 17, C17 Cholesterol; 18, C18:1 Cholesterol; 19, C16 TAG; 20, C18:1 TAG; 21, C18:2 TAG.

Interestingly enough, it was possible to detect phospholipids on the chromatogram, specifically in zone y (see Fig. 15A) (Fig. 16). Separated injections of phospholipid standards such as phosphatidic acid (PA), phosphatidylcholine (PC), phosphatidylethanolamine (PE) and phosphatidylglycerol (PG), all containing oleic acid as the esterified fatty acid in its moiety, indicated that not only could they be detected but also retention times were different according their polar head. Thus it would be conceivable to quantify the phospholipid content of lipid extracts on the same chromatogram as neutral lipids without any derivatisation procedure using a Selective Ion Monitoring (SIM) method choosing from two to four specific ions of some phospholipids. With the same fatty acid composition, anionic phospholipids (PA and PG) eluted before zwitterionic (neutral) phospholipids (PC and PE). It was also interesting to note that PC and PE eluted just before C18:1 DAG.

The presence of a phosphate group and a simple organic molecule, such as choline or ethanolamine, with respect to the native DAG did not substantially modify the retention times but it was surprising to observe that both phospholipids had two peaks. This was also true for PG and, to a lesser extent, for PA.

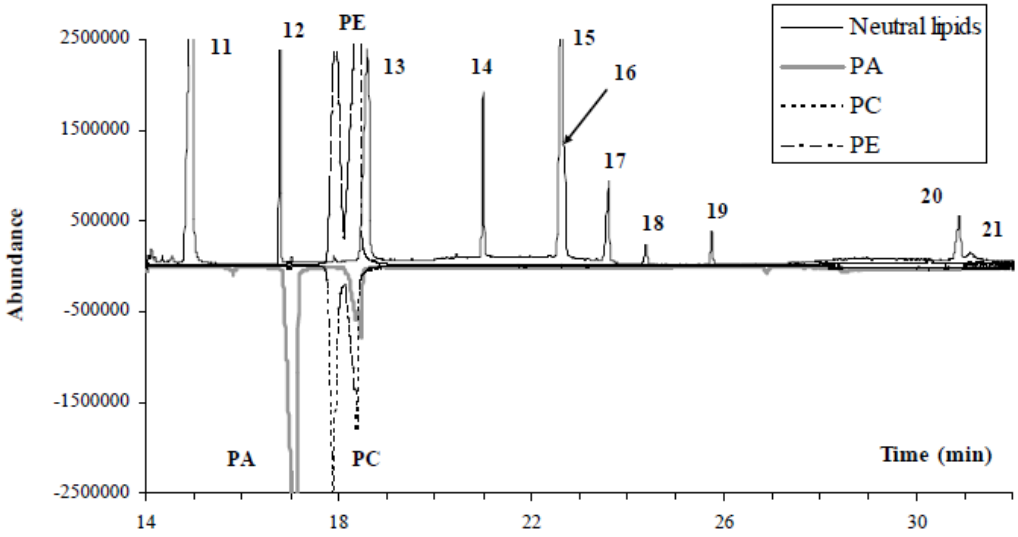

Figure 16. GC-MS total ion chromatogram of lipid standards compared with phosphatidic acid (PA), phosphatidylcholine (PC) and phosphatidylethanolamine (PE) injections. (See peak identification numbers in Figure 15).

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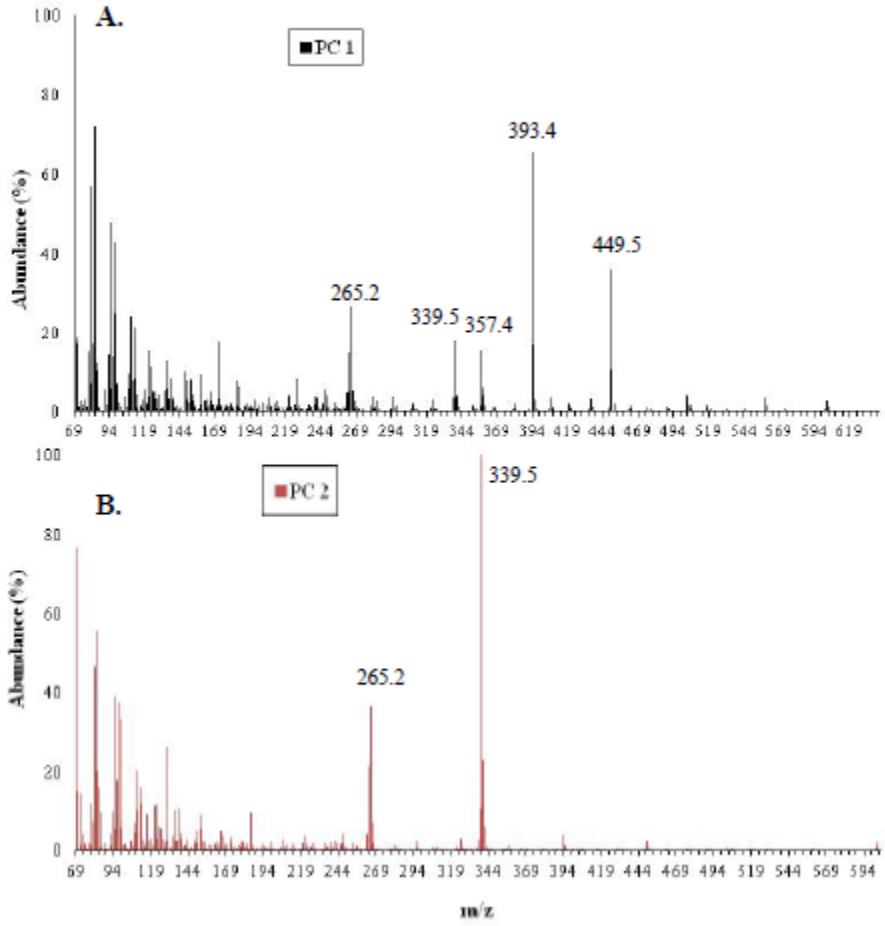

Figure 17. Fingerprinting of phosphatidylcholine (PC) standard. Panel A. A representative of EI mass spectrum of the first peak of PC (Retention Time of 17'897). This mass spectrum corresponds to the 1, 2-Dioleyl-PC (CAS nº 56648-95-4) of NIST05 library. Panel B. A representative of EI mass spectrum of the second peak of PC (Retention Time of 18'360). This mass spectrum corresponds to the 1, 3-Diolein (CAS nº 2465-32-9) of NIST05 library.

The GC responses shown in Fig. 15 were for column loadings of approximately 0.5-0.7 μg each lipid. The GC-MSD detection limits calculated for the main neutral lipids used in the TLC method (SQ, Erg, Lst, DAG, SE and TAG) were in the range of 0.1-0.2 μg for 1 or 2 μl injections (nine determinations). Although this detection limit was low, the precision of measurement (RSD) was low for each compound. For instance, the precision was 49% for squalene at 0.1 μg loaded on the column and in the range of 20-50% for 0.2 μg loaded (results not shown). Only at 0.3 μg did the RSD improve, registering in the range of 5-13% for each neutral lipid used as a reference (SQ, Erg, Lst, DAG, SE and TAG). As observed in Table 10, the regression coefficients obtained for each standard were good and the GC-MSD responses were linear for a range of 0.1-3 μg.

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3. Comparison of TLC and GC-MS methods with yeast samples

From a conventional lipid extraction, TLC methods were successfully used for lipidome profiling for age. As with most existing methods, TLC does not permit the evaluation of the lipid content (neutral lipids and phospholipids) of biological samples in a single run (*i.e*. on the same migration plate). Nevertheless, under our laboratory conditions, TLC is now considered to be a good method for the rapid determination of lipid contents of yeast. As observed in Table 11, the lipid composition of yeast cells that had been grown under two different sets of conditions was determined from the use of external standards in the same migration run. The results observed were as expected in terms of the lower content in Erg, SE and TAG in cells growing in the absence of oxygen, while squalene and lanosterol were accumulated in these cells (Redón *et al*. 2009; Beltran *et al.* 2008).

Table 11. Neutral lipid composition (μg/ mg of Dry Weight) of yeast cells grown in the presence ($+O_2$) or absence ($\pm O_2$) of oxygen determined by either TLC or GC-MS methods. The values represent the average of two determinations for each method: applications of 3 and 5 μl on plate, and column loadings of 3 and 5 μl. **[a]**: sum of identified peaks as sterol esters = a + b + c (see Fig. 18D); **[b]**: sum of identified peaks as TAG = d + e +f + g (see Fig. 18D).

	TLC		GC-MS	
	$+O_2$	$\pm O_2$	$+O_2$	$\pm O_2$
Squalene	1.54	3.81	1.07	1.65
Lanosterol	1.85	2.47	0.62	0.86
Ergosterol	5.95	2.71	8.92	5.40
Sterol esters	6.89	2.60	21.05 [a]	9.15
TAG	7.49	2.74	14.16 [b]	8.25

Meanwhile, the same lipid yeast extracts (3 μl loaded on the column) were analysed by GC-MS (Fig. 18). The positive abundance shown on the graph refer to the sample obtained from cells grown in the presence of oxygen while the negative values of abundance represent the lipid extract of cells grown in the absence of oxygen. Three differentiated zones can be described (fatty acid elution on the column was not shown) as follows: the first zone (x) corresponded to detection of squalene and sterol intermediates (see also Fig. 18B), the second zone (y), where fatty acids esterified between each other,

DAG and phospholipids were found (see also Fig. 18C), and the last zone (z), where sterol esters and TAG were detected (see Fig. 18D).

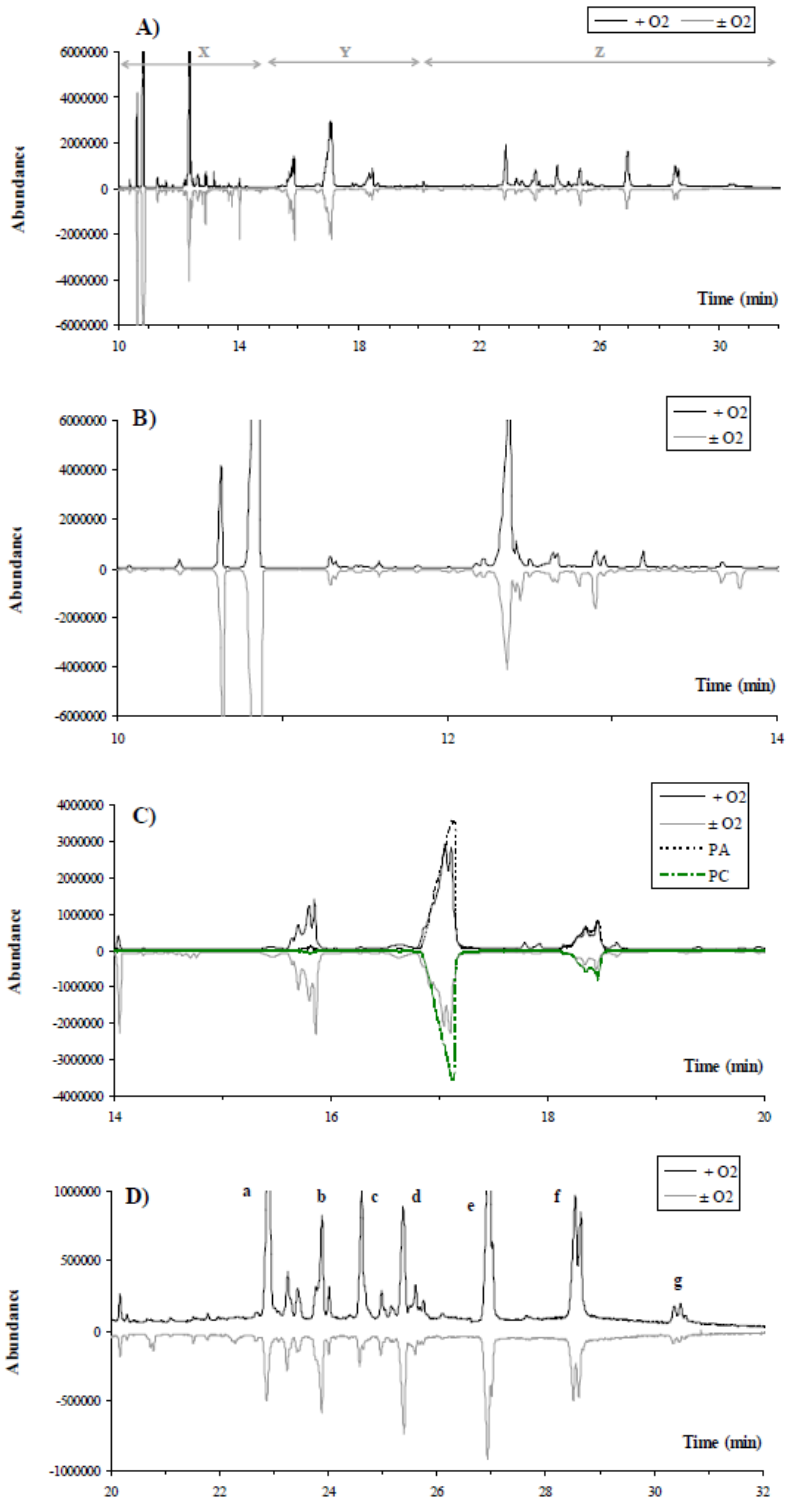

Figure 18. GC-MS total ion chromatogram of (A) full chromatogram of yeast lipid extracts (positive values represent cells grown in the presence of oxygen while negative values represent cells grown without oxygen), (B) chromatogram from 10 to 14 min of A, (C) chromatogram from 14 to 20 min of A and (D) chromatogram from 20 to 32 min of A. a, b and c identified as sterol esters; d, e, f and g identified as TAG.

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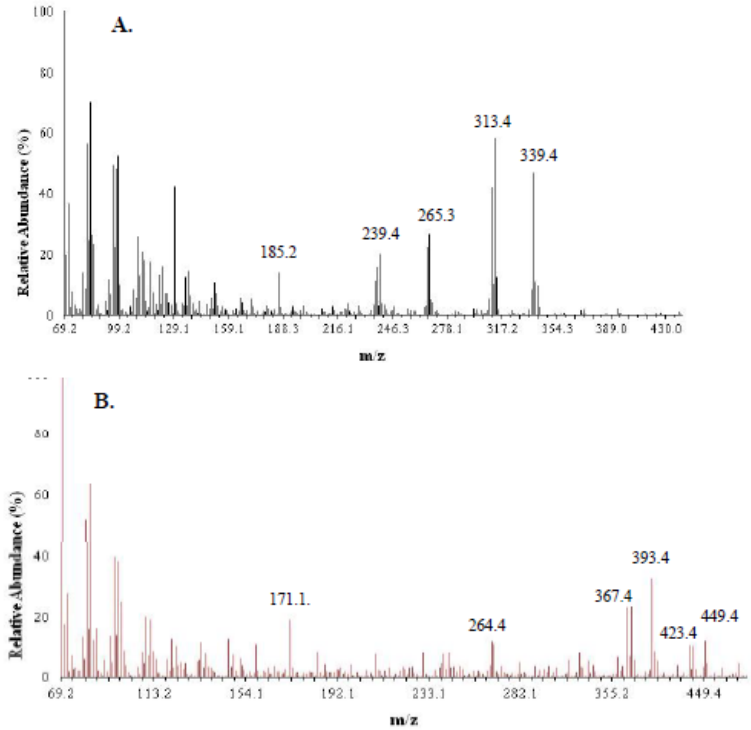

Figure 19. Fingerprinting of lipidic extract of yeast cell growing in presence of oxygen. Panel A. A representative of EI mass spectrum of the DAG C16-C18:1 (Retention Time of 16'850). Panel B. A representative of EI mass spectrum of PC C16-C18:1 (Retention Time of 16'620).

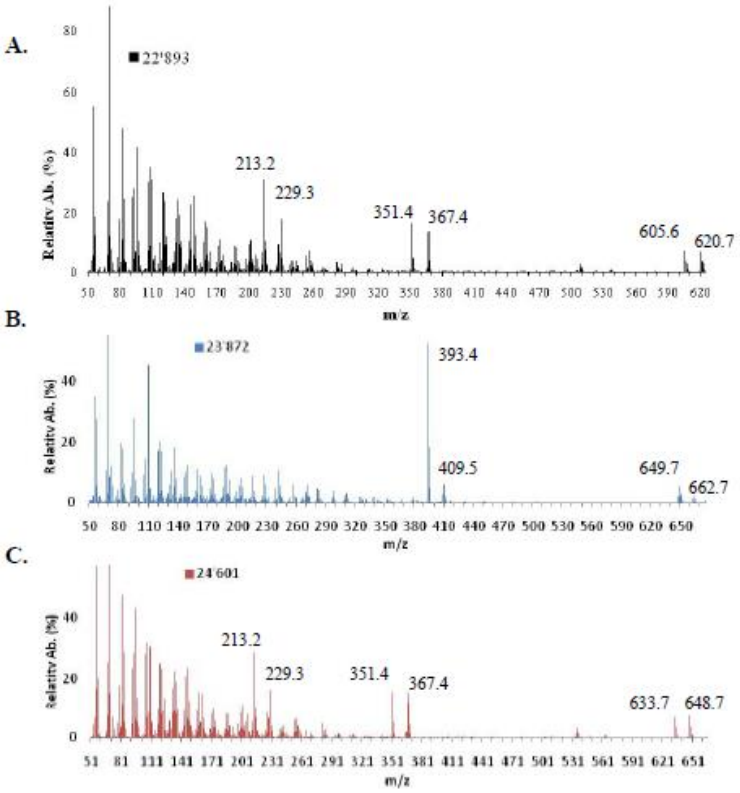

Figure 20. Fingerprinting of lipidic extract of yeast cell growing in presence of oxygen. Panel A. EI mass spectrum of the Zymosterol-C16:1 (Fig. 18D peak a), (Retention Time of 22'893). Panel B. A representative of EI mass spectrum of Lanosterol-C18:1 (Fig. 18D peak b), (Retention Time of 23'872). Panel C. A representative of EI mass spectrum of Zymosterol-C18:1(Fig. 18D peak c), (Retention Time of 24'601).

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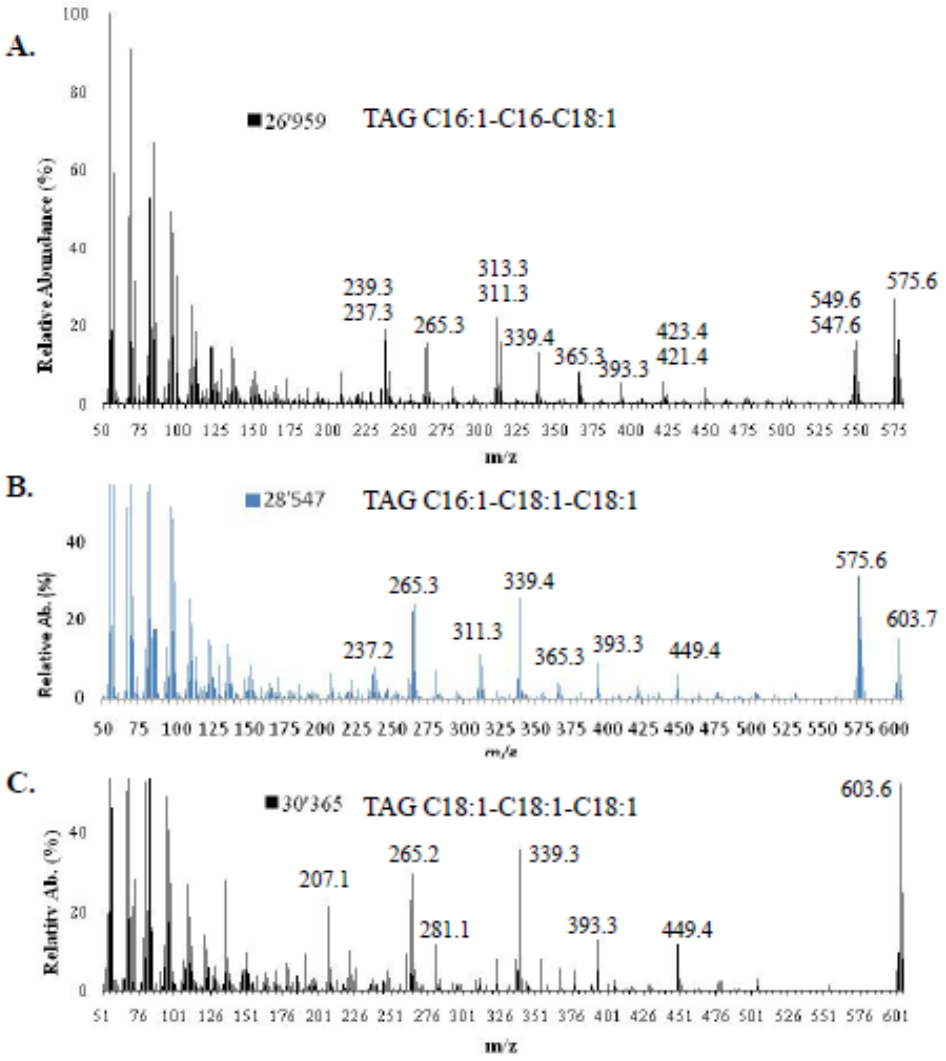

Figure 21. Fingerprinting of lipidic extract of yeast cell growing in presence of oxygen. Panel A. A representative of EI mass spectrum of the TAG C16:1-C16-C18:1 (Fig. 18D peak e), (Retention Time of 26'959). Panel B. A representative of EI mass spectrum of TAG C16:1C18:1-C18:1 (Fig. 18D peak f), (Retention Time of 28'547). Panel C. A representative of EI mass spectrum of TAG C18:1-C18:1C18:1 (Fig. 18D peak g), (Retention Time of 30'365).

According to the results shown in Table 11 for both cell lipid extracts, lower contents of squalene and lanosterol were found, while ergosterol, sterol esters and TAG levels obtained by GC-MS were higher than those obtained by TLC. This outcome highlighted two features: (i) the levels of squalene and lanosterol found by GC-MS were always lower than those obtained by TLC and (ii) those in Erg, SE and TAG were increased. For SQ, Erg and Lst contents, one explanation may be due to better sensitivity in the detection of these molecules by GC-MS, which led to a more accurate quantification. However, the higher levels detected for Lst by TLC could surely be due to a common migration of structurally neighbouring molecules, such as intermediates of sterol

biosynthesis. Meanwhile, in terms of SE and TAG contents, it should be noted that SE levels obtained by GC-MS for cells grown with or without oxygen respectively, were two- and three-fold higher than those determined by TLC. These results may be explained by (i) higher accuracy in the detection of these molecules by GC-MS and (ii) a lack of information for cells grown in the absence of oxygen. Indeed, yeast produces more saturated than unsaturated fatty acids under these conditions that are likely not to be detected by TLC analysis, or only slightly so. Further work was needed to clarify whether the spots detected by TLC corresponded to peaks identified as sterol esters by GC-MS. The same was true in the case of TAG.

As regards the detection and identification of intermediates in the sterol biosynthesis, the GC-MS method was undoubtedly a remarkable advance (Table 12). While with TLC, only Erg and Lst were detected, more than ten intermediates of sterol biosynthesis were identified by GC-MS. Observing the results in Table 3, intermediates were present in both yeast cell extracts. Interestingly enough, the identification of two intermediates, neoergosterol (Barrero *et al.* 1998, 2002), and ergosta-5, 8-dien-3-ol, only occurred in cells grown in the absence of oxygen. Although the matching of sample peaks with the reference peak (NIST05 library) were 99% and 95% respectively, it would be useful to confirm the mass patterns of these molecules using mutant strains of wine yeast. Indeed, according to the literature (Quail and Kelly 1996), some of intermediates were found and there was ergosterol, ergosta-7-en-3-ol, lanosterol and cholesta-8, 14-dien-3-ol-4, 4-dimethyl.

Comparison between our results with those mentioned above proved to be extremely difficult because of the differences between yeasts, with a laboratory strain as opposed to a wine yeast strain. For instance, confirmation of these results may be performed using derivatisation by the way of TMS-derivative formation and/or combining with NMR techniques to analyse spectra.

Table 12. Identification and mass fragmentation patterns of non-derivatized sterols obtained from yeast cells grown in the presence or absence of oxygen using GC-MS (peak identification in increasing order according their retention time). [a]: only found in cells grown in absence of oxygen.

RT	Identification	Purity (%)	Mass fragmentation patterns
12.179	Arthroergosta-5, 7, 9, 22-tetraen-3-ol	84	251, 341, 361, 376, 394, 429
12.225	Ergosta-5, 8, 22-trien-3-ol	83	363, 396, 253, 271, 337
12.346	Ergosterol	96	363, 396, 253, 337, 271
12.410	5, 6-dihydroergosterol	90	271, 255, 383 = 398, 213, 300
12.420	Ergosta-7, 22-dien-3-ol	97	271, 255, 398, 229, 246, 383
12.447	Neoergosterol [a]	99	237, 380, 213, 183, 195, 253
12.503	Cholesta-8, 24-dien-3-ol-4-methyl	90	383, 398, 227, 285, 247
12.605	Ergosta-5, 8-dien-3-ol [a]	83	365, 398, 339, 380, 271, 255
12.642	Cholesta-5, 24-dien-3-ol	52	271, 365, 314, 383, 398, 300
12.669	Ergosta-7-en-3-ol	95	255, 400, 229, 213, 392, 271
12.901	Lanosterol	86	411, 426, 393, 259
12.956	Cholesta-8, 14-dien-3-ol-4, 4-dimethyl	90	412, 379, 397, 299, 285 = 351

Conclusions

The proposed method for the separation and quantification of neutral lipid cells in yeast using GC-MS is very simple and suitable for small laboratories. It is designed to be used routinely for any kind of extracts, such as human, plant or microbial cells, from conventional lipid extraction. It is also a flexible method which enables researchers to change gas chromatographic parameters when necessary. In other words, the injection mode can be changed and oven temperature conditions can be modified depending on the purpose of lipid class studied. Furthermore, one internal standard (α-cholestane, cholesterol margarate, for example) or more can be added before or after the lipid extraction to improve the quantification.

With respect to TLC analysis, the GC-MS method is suitable for the simple separation and identification of molecular species of MAG, DAG, TAG, intermediates of sterol pathway and sterol esters in a single injection. Although this method seems to have potential, additional work is necessary, especially for identifying the intermediates of sterol biosynthesis and all of the molecular species produced by *S. cerevisiae*. In addition, it is also clear that the method must be validated with more precision, particularly in terms of the metabolites for which there are no standards.

4.3. Metabolome protocols

In this study both untargeted global metabolomic analysis determined by ultra-performance liquid chromatography quadrupole time of flight mass spectrometry (UHPLC-qTOF-MS) using the electrospray ionisation (ESI) technique with positive and negative ion polarity, and untargeted profiling determined by GC-MS (see article 2) were conducted to compare the effect of acclimation condition on the intracellular metabolites.

4.3.1. Metabolite extraction

The extraction of intracellular metabolites of yeast cells were performaed according to López-Martínez *et al.* (2014, article 2 in this manuscript). Cells were collected at different time of acclimation process according to the experimental design shown in Figure 22.

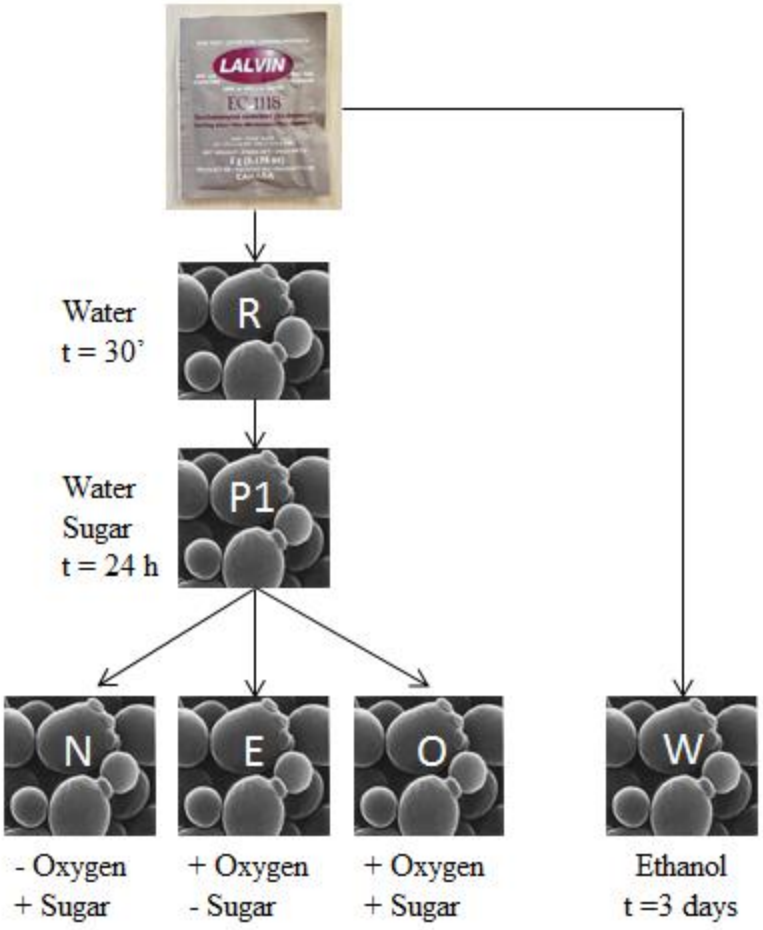

Figure 22. Exprimental design for the metabolomic study. R, LSA-R; W, LSA-W; P1, yeast cells in phase 1 of acclimation process; N, NOXI condition; O, OXI condition and E, ETOXI condition.

4.3.2. Untargeted global metabolomic analysis determined by UPC-ESI-qTOF

The analysis of samples (n = 5 for each condition) was carried out at the Centre for Omic Sciences (COS, Reus, Spain).

Sample preparation

The samples (kept at -80 ° C until their analysis as a dry residue) were resuspended in 150 µL of water / methanol (50:50, v/v) in 0.2% (v/v) of acetic acid.

Instrument. UHPLC-(ESI)-Q-TOF (Agilent Technologies, Sta. Clara, CA, USA).

Materials. Guard column: Zorbax SB-C8 (30 x 2.1 mm., 1.8 µm) (Agilent Technologies, USA). Analytical column: Zorbax SB-Aq RR (50 x 2.1 mm, 1.8 µm) (Agilent Technologies, USA).

Instrumental analysis.

The samples were analysed by UHPLC-QTOF using a method consisting of a gradient elution using Milli-Q water (Solvent A) and 0.2% (v/v) of acetic acid in methanol (Solvent B) 0.2% CH3COOH as mobile phase.

The injection volume was 2 µL (4ºC), temperature 60 ° C and the column flow 0.6 mL /min. Quality controls (QCS) were included by injecting sample controls every six samples in order to monitor the adequacy of the system throughout all chromatographic analysis. Samples were analysed at random.

Detection modes.

In the positive mode (ESI^+), the analyte was ionised using an electrospray source according to the conditions described in Table 13. The ions observed by mass spectrometry may be quasimolecular ions created by the addition of a hydrogen cation and denoted $[M+H]^+$,In negative ion mode (ESI^-), the analysis is normally carried out well above a molecules isoelectric point to deprotonate the molecule.The instrument was calibrated before use and reference masses have been used throughout the analysis. The acquisition and data processing was done by the team's own software (Agilent Mass Hunter B.06.01).

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Table 13. Analytical parameters del qTOF

Parameter	**ESI+**	**ESI-**
Gas Temp.	200ºC	200ºC
Drying Gas Flow	14 L/min	14 L/min
Shealth Gas Temp.	350ºC	350ºC
Shealth Gas Flow	11 L/min	11 L/min
Capillary Voltage	4000 V	3500 V
Nozzie Voltage	1000 V	1000 V
Fragmentor	140 V	140 V
Skimmer	65 V	65 V
OCT 1RF Voltage	750 V	750 V
Scan Range	100-1200 m/z	100-1200 m/z
Ref. Mass	121.050873	119.03632
	922.009798	980.016375

Stadistical analysis

In order to carry out further statistical analysis, we must first pre-processing of the acquired data. This pre-processing is deconvolución chromatograms obtained in order to find the signals corresponding to potential metabolites (entities) in each of the chromatograms acquired and which are an indication only according to their retention times and mass neutral. This was carried out by deconvolution algorithm find by Molecular Feature own computer software (Agilent MassHunter) that seeks possible “entities” and groups throughout the chromatogram possible ions originating from the same molecule (eg., distribution isotopic adduct sodium, ammonium, etc., or neutral losses of water, among others) allowing calculate its mass neutral through these relationships.

Once this processing is performed aligned data from different samples using the software Mass Profiler Professional (v. 12.6, Agilent Technologies) to find them "entities" between different samples and then look for recursively these "entities" in all samples analysed by the algorithm "Find by formula" (Agilent MassHunter) that takes into account mass and retention time of "entities". This second algorithm produces results more refined, resulting at last in stronger statistical results.

The statistical analysis consists of the following comparisons (Table 14) that have been made according to the experimental design (Figure 1).

Table 14. Applied statistical test to compare the different groups

Grups	Test	Tipus	Correcció	p-value
R-F-N	Friedman	Non-parametric, paired	Benjamini-Hochberg	<0.05
N-E-O-V	Kruskall-Wallis	Non-parametric, unpaired	Benjamini-Hochberg	<0.01

4.3.3. Metabolomic analysis determined by GC-MS

The same samples used for untargeted global metabolomic were used for the untargeted metabolomic analysis performed by GC-MS.

Samples (n = 4 for each condition) were dried before the derivatisation process as described below in the article 2.

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Article 2

Metabolomic characterisation of yeast cells after dehydration stress

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International Microbiology 17 (2014) 131-139

[¶] These authors contributed equally to the study

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Abstract

In this study, we analyzed the metabolite features of the yeasts *Saccharomyces cerevisiae*, *Naumovia castellii*, and *Saccharomyces mikatae*. The three species are closely related genetically but differ in their tolerance of desiccation stress. Specifically, we determined whether certain metabolites correlated with cell viability after stress imposition. The metabolomics profiles of these strains were compared before cell desiccation and after cell rehydration. In *S. mikatae*, the presence of lysine or glutamine during rehydration led to a 20% increase in survival whereas during dehydration the levels of both amino acids in this yeast were drastically reduced.

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Introductory highlights

In yeast populations, intracellular metabolome analysis has improved our understanding of cell metabolism and thus of cellular responses to external physiological conditions (van Ravenzwaay *et al.* 2007).

The metabolomic profile of a selected yeast strain with respect to its response to dehydration is of commercial interest. In the food industry, dry yeast (active dry yeast) formulations, having greater genetic stability at room temperature can save storage and transport costs. However, there is a lost of cell viability during the industrial drying processes and the resulting lower activity (Rodríguez-Porrata *et al.* 2011).

Thus, identifying the changes in metabolites that occur in response to desiccation stress would facilitate efforts aimed at optimising the drying process. During desiccation, the yeast's metabolic processes are in a suspended state (Crowe *et al*. 1992; Rapoport *et al.* 1995).

The methodological steps required for metabolite detection and data analysis are well established (Castrillo *et al.* 2003; Kell 2004; Mashego *et al.* 2003). Villas-Bôas *et al.* (2005) described methods to improve yeast sample preparation, especially with respect to (i) cold methanol quenching, (ii) metabolic extraction by chemical treatment, and (iii) sample concentration through solvent evaporation.

The quenching step consists of a metabolic arrest, to obtain a "snapshot" of the cellular response to environmental conditions. Most commonly applied cell quenching method involves immersion of the sample in a cold aqueous methanol solution (Koning *et al.* 1992). An advantage of this method compared to environmental pH variations is that it allows the subsequent separation of extra- from intra-cellular fractions, which reduces contamination between the two (Gonzalez *et al.* 1997). Other widely used extraction methods in yeast research include methanol-chloroform, pH-dependent, and boiling ethanol treatments. However, problems have been identified by Castrill *et al*. (2003). In the case of metabolite extraction from *Escherichia coli* and *S. cerevisiae*, these effects can be mitigated by extracting in 50% (v/v) methanol after a –80ºC cycle (Maharjan *et al.* 2003; Villas-Bôas *et al.* 2005).

Most of the methods for metabolite extraction have been optimised for laboratory strain batch cultures, which are more easily manipulated than industrial strains or cells

from non-growing conditions, in which cell wall becomes a problematic barrier (Sasidharan *et al*. 2012).

The majortiy of metabolite extraction protocols require a final sample concentration step, typically a lyophilisation procedure (Ding *et al.* 2010).

The aim of the present article is to study main metabolic differences between three species of industrial yeasts, Saccharomyces cerevisiae, Naumovia castellii, and Saccharomyces mikatae that differ in their dehydration tolerance. Through metabolomic characterization, the aim of this study was to identify the putative cellular compounds involved in overcoming dehydration/rehydration stress by using an optimised gas chromatography-mass spectrometry (GC-MS) metabolomic method.

Chapter II

Materials and methods

Strains, growth conditions, and desiccation-rehydration.

Overnight liquid cultures of the yeast species *Saccharomyces cerevisiae* (CECT-1477, from Burdeos sparkling wine), *Naumovia castellii* (CECT-11356, from Finland soil), and *Saccharomyces mikatae* (CECT-11823, Japan monosporic culture) at an initial OD600 of 0.5 were used as the inoculants. All three strains were grown in shake flasks (170 rpm) in YPD [1% (w/v) yeast extract, 2% (w/v) bacto-peptone, and 2% (w/v) glucose (Cultimed, Barcelona, Spain)] at 28ºC for 24 h. The effects of lysine (1, 2, 5, and 10%), Na_2HPO_4 (2, 5 and 10%), and glutamine (2, 5 and 10%) on cell viability during rehydration were studied by adding each compound individually to the pure water basal condition. Desiccation-rehydration, and cell viability were determined by flow cytometry, performed as previously described (Rodríguez-Porrata *et al.* 2012).

Metabolite extraction

Each step of the extraction was optimised based on the methods of Roessner *et al.* (2000). Five × 10^8 live cells were immediately frozen in cold 70% (v/v) of ethanol and stored at –20ºC until further analysis. After cell quenching, three methods for intracellular metabolite extraction were evaluated by assessing the percentage of unbroken cells. The mean number of unbroken cells per ml was calculated according to the CFU (colony-forming units) of treated cells after taking into account the CFU per ml before cell breakage. Frozen cells were pelleted and resuspended in methanol-water (1:1, v/v) to a volume of 400 µl, as described by Villas-Bôas *et al.* (2005), with 10 µl of ribitol at 2 mg/ml (Sigma, Switzerland) added as an internal standard (IS). To optimise this step, samples were incubated at 90ºC either for 10 min, or for 5 min in the presence of 0.2-mm glass beads (BioSpec Products, USA) in a sonication bath (J.P. Selecta, Spain), or for 5 min in the presence of 0.5-mm acid-washed glass beads (~300 µl). The samples were then disrupted in a multitube bead-beater (BioSpec Products, USA) using five cycles of 1 min/beat, followed by 30-s rest for cooling. After centrifugation, the supernatant was dried in a SC110 speed vacuum system SC110 (Savant Instruments, USA) for 4 h. The dried residue was redissolved and derivatised for 1 h at 40ºC in 50 µl of 20 mg/ml methoxyamine hydrochloride in pyridine (Sigma, Japan; Fluka, India), followed by a 90-

min treatment at 40ºC with 70µl of *N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide (Sigma, USA).

Gas chromatography-mass spectrometry analysis.

Gas chromatography (GC) was performed using an Agilent Technologies Network GC system 6890N connected to an HP computer with the ChemStation software (Agilent Technologies). Compounds were detected using an inert mass selective detector (MSD, model 5975, Agilent Technologies). Two µl of the cell extract was injected at a split ratio of 20:1 into a DB-5HT column (30 m × 0.25 mm × 0.1 µm; Agilent Technologies) with an automatic injector (7683B, Agilent Technologies). Helium was used as the carrier gas at a constant flow of 1.0 ml/min. The injector temperature was 200ºC. The column oven temperature was initially held at 80ºC for 4 min and then increased fi rst to 200ºC at a rate of 5ºC/min and then to 300ºC at a rate of 25ºC/min, where it was held for 7 min. The MSD transfer temperature was 300ºC. The MSD quadrapole and source temperatures were maintained at 180ºC and 280ºC, respectively. The MSD data were acquired in electronic ionisation scan mode at 70 eV within the range of 35–650 amu after a solvent delay of 4 min. Postrun analysis was performed with the Agilent MSD Chemstation. The relative abundance of each identified compound was calculated according to the respective chromatographic peak areas corrected with respect to the IS peak. These values were relativised to the 1×10^9 CFU, according to the cells plated and counted after 24 h of culture and desiccation/rehydration, and expressed as percentages.

Flow cytometry analysis.

Flow cytometry was carried out using a CYFlow space instrument (PARTEC, Germany) fi tted with a 22-mW ion laser for excitation (488 nm); a single emission channel (575-nm band-pass fi lter) was used for monitoring. Instrument control, data acquisition and data analysis were performed using FloMax software (Quantum Analysis, Germany). The two-color fluorescent probe from the LIVE/DEAD yeast viability kit was used to label the cells. In this system, plasma-membrane integrity and fungal metabolic function are required to convert the intracellular yellow-green fluorescence of FUN1 into red-orange intravacuolar structures, Calcofl uor White M2R labels cell-wall chitin with blue fluorescence regardless of metabolic state (Life Technologies). An overnight YPD

culture of a *Saccharomyces* sp. strain was taken as a control of full viability (99% by FUN1 red-orange/Calcofluor white stain).

Microscopy.

The cells were viewed with a Leica microscope (DM4000B, Germany) and a digital camera (Leica DFC300FX). The Leica IM50 software was used for image acquisition.

Statistical analysis

The data was analysed by principal component analysis (PCA) using the SPSS 20.0 statistical software package. For further analysis, one-way ANOVA and the Tukey test were used with the same program. Statistical significance was set at $P \leq 0.05$.

Results and Discussion

Yeast cell viability after dehydration stress.

Differences in the desiccation tolerance of the three closely related yeast species were assessed by flow cytometry FUN1/Calcofluor co-staining was performed to quantify cell metabolic activity and cell wall integrity (Fig. 23A). This staining protocol allowed metabolically active cells (live cells) to be distinguished from damaged or non-active cells (dead cells). The mean number of viable cells after rehydration was calculated relative to cell viability before drying. After drying, the cells of each species were resuspended in either 10% (w/v) of trehalose or, as the reference condition, pure water to evaluate viability. The viability of cells in the deionised water condition was lower for *S. mikatae* and *N. castellii* than for *S. cerevisiae* (20%, 40% and 84%, respectively) whereas the viability of yeast cells dried in the presence of trehalose was increased by approximately 20% for all three species (Fig. 23A). Trehalose was previously shown to act as a membrane protector by reducing the membrane phase-transition temperature during the desiccation-rehydration process, without having a significant impact on cell metabolites (Eleutherio *et al.* 1993; Rodríguez-Porrata *et al.* 2012).

Metabolite extraction.

The metabolite fractions of the three strains were investigated to determine their dehydration stress tolerance metabolite profiles of the yeasts. The extraction process was

optimised for these strains based on the protocol of Roessner *et al.* (2000). After the cells were frozen to stop their metabolism, extracts were prepared using three different methods: thermal shock, sonication, and vortexing. Fig. 1B shows the cells treated according to these methods and then evaluated by microscopy and flow cytometry to quantify the unbroken cell fraction, both before and after treatment. Resuspending the cells in the presence of 0.2 mm glass beads after shaking with a mini-bead beater for 5×1 min with a 1-min rest between treatments resulted in 98% broken cells whereas thermal shock and sonication resulted in 30% and 20% un broken cells, respectively.

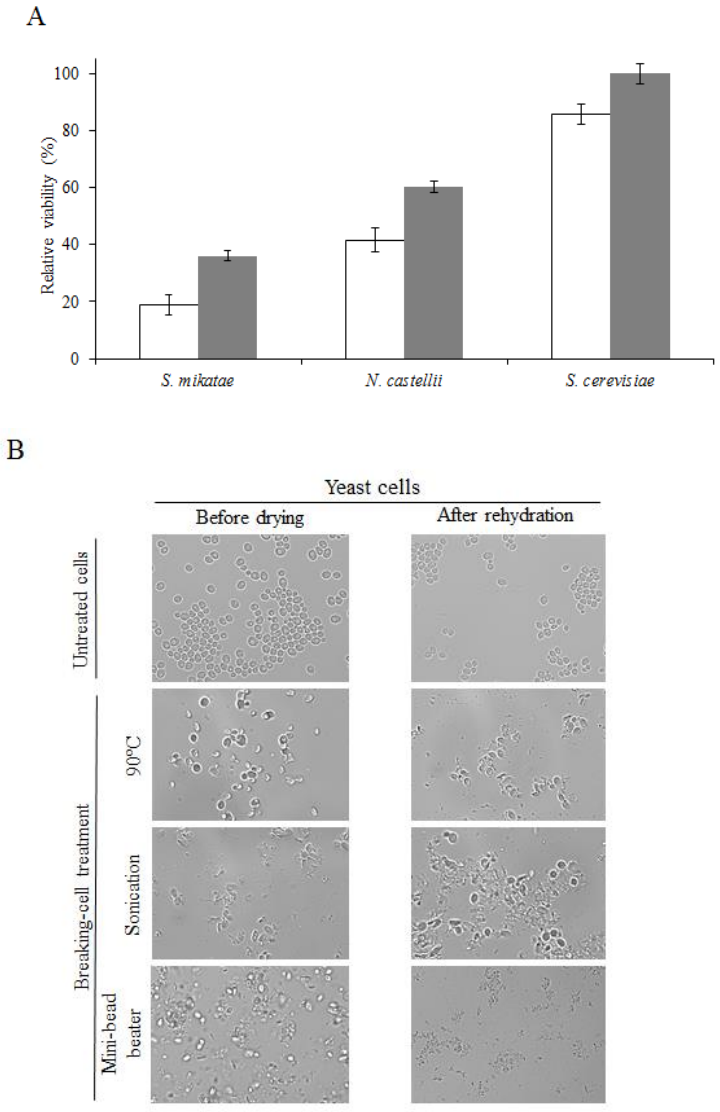

Figure. 23. Yeast cell viability following air drying and rehydration. (**A**) Cells were dried to a moisture content of 5% at 28ºC. The scale of relative viability (%) indicates the percentage of experimental values for the different yeasts relative to the highest viability for *Saccharomyces cerevisiae*. The values shown represent the means of five independent samples ± SD, as evaluated by fl ow cytometry. The viability of cells dried in the presence (gray bars) and absence (white bars) of trehalose was evaluated. (**B**) Microscopy images show the broken *S. cerevisiae* cells after treatment, and the predominance of large cell fragments. White spots are the cell walls of empty cells, and dark spots are intact cells.

Chapter II

Metabolomic profiling of yeast cells during the desiccation process.

To understand the metabolic differences between *S. mikatae, N. castellii*, and *S. cerevisiae* cells before the imposition of stress, their metabolomic profiles before dehydration (BD) and after rehydration (AR) were compared. The cellular metabolites were analyzed by GC-MS, with 44 peaks identified and quantified for each strain and condition (BD and AR). These metabolites included amino acids, glycolytic compounds, sugars, fatty acids and organic acids. Multivariate data analysis was performed using PCA to examine the variations among metabolites between the BD and AR steps in *S. cerevisiae*, *N. castellii*, and *S. mikatae*. The relative amounts of fatty acids, organic acids, and compounds resulting from glycolysis differed significantly in the three species, both BD and AR. By contrast, there were no significant differences in the BD amounts of amino acids and sugars. The largest differences occurred in *N. castellii*, whose metabolite profile during dehydration was the opposite of that of *S. cerevisiae*, which also had a 40% greater viability AR (Fig. 23A).

Endogenous trehalose increased by 8.7-fold, 5.5-fold, and 3-fold during stress imposition in *S. mikatae, N. castellii* and *S. cerevisiae,* respectively due to the addition of this protective sugar during dehydration process. However, intracellular trehalose did not correlate with the desiccation tolerance of the three species, as previously reported by Rodriguez-Porrata *et al.* (2011).

Although phosphoric acid is toxic to yeast cells, the BD concentration in *N. castellii* was 4-fold higher than in the two other yeast strains and increased 1.5-fold during stress, whereas in *S. mikatae* and *S. cerevisiae* the concentration decreased by 3-fold. These results suggested that the viability of *N. castellii* would be negatively affected during stress due to inefficient detoxification and/or a poor capacity for metabolic esterification of phosphoric acid into nucleic acids, proteins, lipids, and sugars (Ogawa *et al.* 2000). Yeast cells store phosphate in vacuoles as polyphosphate, which normally increases during the stationary growth phase (Ogawa *et al.* 2000). No significant differences among the yeast species were recorded BD in terms of phosphate content (Fig. 24). However, between the BD and AR steps, the phosphate content of *S. mikatae* and *S. cerevisiae* decreased by 70% and 30%, respectively, while it doubled in *N. castellii* (Fig. 24). Nonetheless, this greater accumulation of phosphate by *N. castellii* than by *S. cerevisiae* during AR did not enhance its dehydration tolerance, as reflected in the differences in viability: 60% vs. 95%,

respectively (Fig. 23A). *S. mikatae* had a significantly lower content of phosphate after drying and rehydration, consistent with its low viability (36%).

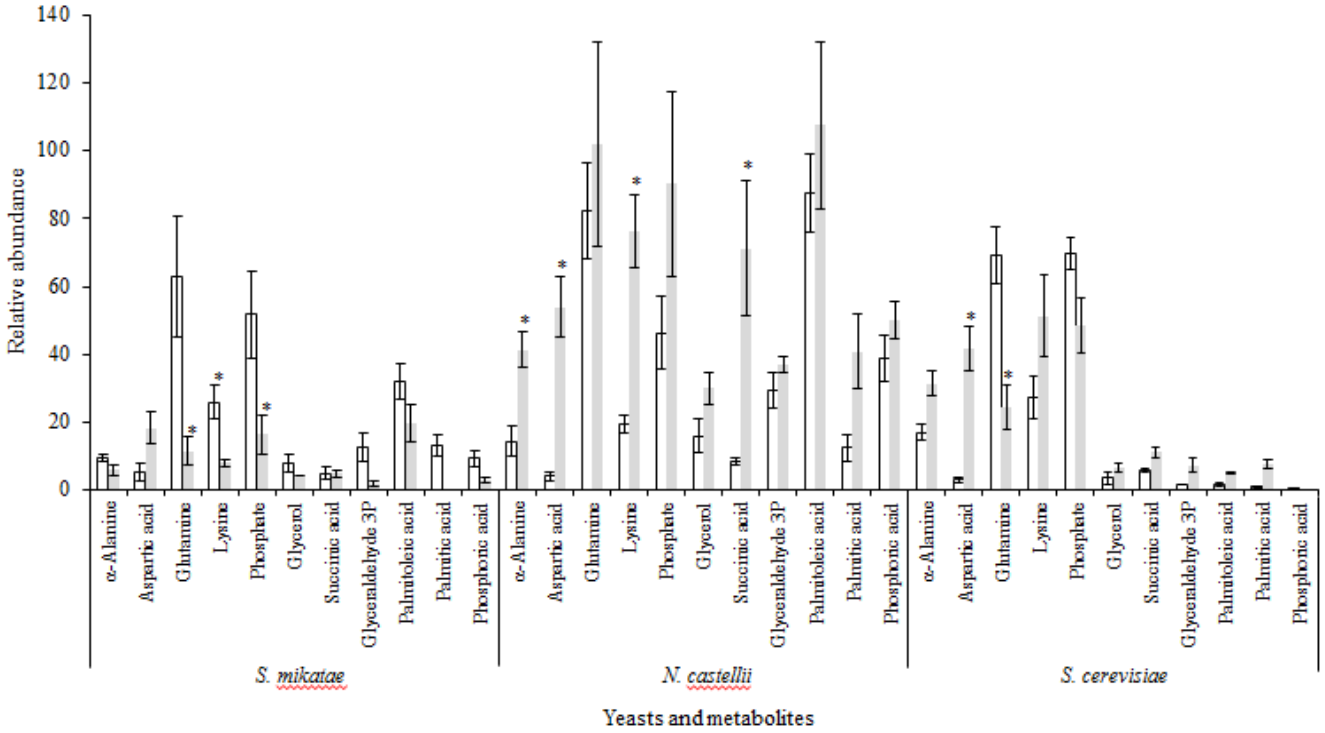

Figure. 24. Relative abundance of significant and stress-related compounds BD (white bars) and AR (grey bars) in *Saccharomyces cerevisiae, N. castellii* and *S. mikatae*. The values represent the means of three independent experiments. Results for each compound with statistically significant differences ($P < 0.05$) compared to the BD condition.

The main metabolic response of *S. cerevisiae* to osmotic pressure, which fluctuates during dehydration and rehydration, is the cytoplasmic retention of glycerol (Larson *et al.* 1993; Simonin *et al.* 2007). In fact, in *N. castellii* and *S. cerevisiae* intracellular glycerol accumulation during stress imposition increased by 50%, whereas in *S. mikatae* it decreased by 50%, which further explains the low viability of these species (Fig. 24). Our findings suggest a relationship between glycerol accumulation and desiccation tolerance, whereby a reduction in glycerol content during stress induction reduced cell viability. Glycerol biosynthesis is an important side-reaction of the glycolytic pathway, as it provides the NAD^+ necessary for cellular glycolysis activity (Barnett *et al.* 2005). Thus, according to our results, cells that synthesized glycerol during dehydration also had a backup supply of NAD^+ that allowed rapid glycolytic activation when cell activity was resumed. The NAD^+ generated is oxidised to maintain redox balance by the conversion of glyceraldehyde-3-phosphate to 1, 3-bisphosphoglycerate by the enzyme glyceraldehyde-3-

phosphate dehydrogenase (GAPDH). In the GAPDH glycolytic pathway, under stress conditions, glycolytic flux is strongly directed to glycerol accumulation, depending on the kinetics of GAPDH (Norbeck *et al.* 1996). *S. cerevisiae* is unusual in that three genes (*TDH1-3*) encode three different GAPDH isoenzymes, whereas other yeasts, such as *C. albicans* and *K. marxianus*, contain only one gene (Almeida *et al.* 2003; McAlister-Henn *et al.* 1997; Villamón *et al.* 1999). The *TDH1* gene encodes a less efficient GAPDH isoenzyme but it is the only gene that is expressed under conditions of cell stress (Delgado *et al.* 2001; Norbeck *et al.* 1996). Additional experiments are required to determine whether *TDH1* activation is responsible for glycerol accumulation during dehydration.

Glycolysis is used by almost 800 yeast species in a sequence of enzymatic reactions that convert glucose to pyruvate, which, under aerobic conditions, is channelled into the mitochondria, where it enters the tricarboxylic acid cycle (TCA) and is eventually converted into acetyl-CoA. Alternatively, pyruvate acts as a precursor for a side-reaction in α-alanine synthesis (Pronk *et al.* 1996). Intracellular α-alanine increased by 3-fold and 2-fold in *N. castelli* and *S. cerevisiae*, respectively, while in *S. mikatae* there was a 2-fold decrease between the BD and AR steps (Fig. 24). Acetyl-Co-A branchpoint metabolites can be converted into citric acid or lipids and fatty acids, such as palmitoleic acid, which during dehydration increased in *N. castellii* and *S. cerevisiae* whereas in *S. mikatae* a 30% decrease was recorded. These results concerning palmitoleic acid and α-alanine indirectly suggest a greater reduction of pyruvate availability from the glycolytic pathway in *S. mikatae* than in the other two yeasts during stress induction.

The only TCA cycle intermediate that showed significant variations during stress was succinic acid, whose levels increased by 9-fold and 2-fold in *N. castellii* and *S. cerevisiae*, respectively, while in *S. mikatae* the levels remained the same (Fig. 24). This accumulation of succinic acid in *S. cerevisiae* does not agree with previous results shown by Raab *et al.* (2010). The opposite is true in *S. mikatae* and could explain the insignificant differences between the BD and AR values while the high level of accumulation in *N. castellii* highlights its metabolic difference compared to the other two yeast species. In the TCA cycle, after three successive enzymatic steps, succinic acid is converted to oxaloacetate, another branch-point metabolite because it can be transaminated to form aspartate. The BD abundances of aspartic acid were similar in the three yeasts. However, in *N. castellii* and *S. cerevisiae* the AR levels of aspartic acid increased by 13-fold, but only by 3.5-fold in *S. mikatae* (Fig. 24). The changes in the abundances of aspartic acid

and succinic acid suggested that *S. mikatae* cells should lack TCA activity during dehydration and rehydration, in contrast to *N. castellii* and *S. cerevisiae*. The TCA intermediate 2-oxoglutarate is also a branch-point metabolite; it is converted through transamination to lysine and glutamate, which can be used to synthesise other amino acids, such as glutamine, proline, and arginine.

In our study, the BD contents of lysine and glutamine were similar in the three yeast species, whereas during stress imposition the changes in the abundances of these amino acids did not correlate with putative changes in yeast TCA activities. Thus, *S. mikatae* contained 3-fold less lysine after stress induction, while in *N. castellii* and *S. cerevisiae* lysine increased by 3-fold and 2-fold, respectively. Glutamine decreased significantly in *S. mikatae* and *S. cerevisiae*, by 6-fold and 3-fold, respectively. In fact, one of the two anaplerotic pathways responsible for replenishing the TCA cycle with intermediates, thereby maintaining its function, is the glyoxylate cycle, in which isocitrate dehydrogenase converts isocitrate into 2-oxoglutarate. The participation of both the TCA and glyoxylate cycles in providing 2-oxoglutarate to cells is not consistent which the changes in glutamine and lysine concentrations during stress induction. These variations also did not correlate with the dehydration tolerance profiles of any of the yeast species.

Effect of compound supplementation on yeast cell viability.

We next sought to ascertain whether the low viability of *S. mikatae* after rehydration was due to low levels of lysine, Na_2HPO_4, or glutamine. Therefore, we tested several rehydration media in an attempt to overcome the drop in yeast viability during this process. In this experiment, all additives were resuspended in deionised water and pure water served as the reference condition for cell viability evaluation by flow cytometry. A statistically significant increase of 20% in cell survival driven by 1, 2, and 5% lysine or 10% glutamine supplementation was observed in *S. mikatae* (Fig. 25) but not in *S. cerevisiae*. Unexpectedly, phosphate supplementation was detrimental and increased cell death by 30% compared to the pure water reference condition (data not shown).

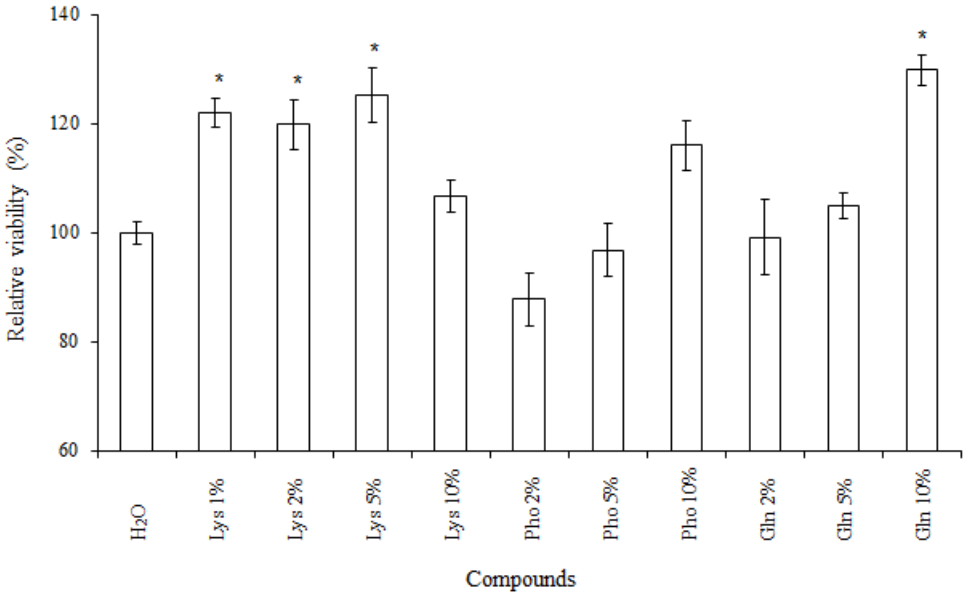

Fig. 25. Effect of rehydration on cell viability. *Saccharomyces mikatae* yeast cells were incubated at 37ºC for 30' in pure water or in the presence of lysine (Lys), phosphate (Pho), or glutamine (Gln). The scale of relative viability (%) indicates the percentage of experimental values for the different yeasts relative to the non-complemented condition (H_2O). The values are the means of three independent experiments. Results with statistically significant differences ($P < 0.05$) compared to the H_2O condition are presented.

In conclusion, the changes that take place in cells that allowed them to overcome dehydration stress were investigated at the metabolomic level in *S. cerevisiae*, *N. castellii*, and *S. mikatae*. The extraction method for GC-MS analysis was optimised using a mini bead beater system, which enhanced cell breakage by ~20%. After data analysis and PCA, the observed variations in intracellular metabolites could be related to the form of central carbon metabolism, which offered a better understanding of the cellular metabolic differences between yeasts with respect to desiccation tolerance. Nevertheless, the poor survival of *S. mikatae* and *N. castellii* could not be ascribed to a single metabolite; rather, at least in *N. castellii*, the accumulation of both succinic acid and phosphoric acid by stress imposition might account for its low viability (60%).

The metabolite profiles suggested the activity of the glycolytic pathway and TCA cycle in *N. castellii* and *S. cerevisiae*, but not in *S. mikatae*, which in the latter species could have led to a reduction in the levels of secondary metabolites required for viability following rehydration. Our results also demonstrated that the presence of lysine or glutamine during rehydration had a positive effect on the recovery of *S. mikatae* cell activity, although the benefits achieved by the supplementation of these compounds might be yeast dependent. Further investigations extending to other aspects such as global gene

expression and proteomics will provide a better understanding of the cellular mechanisms involved in overcoming desiccation stress.

The present paper try to explain the poor viability of some commercial yeast after rehydration according to metabolome analyse. The optimisation of the method was a key point due to the fact that, the process of metaoblits extractions from yeasts was not optimised for commercial strains. Some of them have a really rigid membrane, difficult to broken. The completely, or at least almost completely broken of all membrane cells guarantee results are comparable between strains; proving that, when working with cells not as well characterised as laboratory strains we must optimise the protocol to get value results.

4.4.Transcriptome analysis

At the end point of acclimation for all conditions analysed (OXI, NOXI and ETOXI) samples were collected for ARN purification. Each sample has a biological replicate.

The total RNA purification from yeast samples; the RNeasy Mini Kit (Qiagen, Hilden, Germany) was used. The protocol was detailed in the RNeasy Mini Handbook; basically the extraction follows the steps: (i) enzymatic lysis of freshly harvested cells (*ca.* $5 \cdot 10^7$ cells); harvest the cells in 12mL centrifuge tube by centrifuging at 1000x g for 5 min at 4ºC. Decant the supernatant and carefully remove any remaining media by aspiration. Resuspend the cells in 100 µL freshly prepared Buffer Y1 containing zymolyase. Incubate for 30 min at 30ºC with gentle shaking to generate spheroplasts. Add 350 µL Buffer RLT and vortex vigorously to lyse the spheroplasts. Add 250 µL ethanol to homogenised lysate and mix. (ii) Transfer the sample including any precipitate to an RNeasy spin column placed in a 2 mL collection tube. Close the lid and centrifuge for 15 s at 8000 x g. Discart the flow-throught. (iii) Add 700 µL Buffer RW1 to the RNeasy spin column and centrifuge and discart the flow-throught as step (ii). (iv) Add 500 µL Buffer RPE to RNeasy spin column and repeat the procedure of centrifuge and discart. (v) Repeat previous step with a centrifuge time of 2 min. (vi) Place the RNeasy spin column in a new 1.5 mL collection tube. Add 30 µL RNase-free water directly to the spin column membrane. Close the lid, centrifuge for 1 min at 800 x g to elute the RNA.

The microarray analysis to determine differential genetic expression between acclimations was done by *GeneChip Yeast Genome 2.0* en el *Institute for Research in Biomedecine* (IRB, Barcelona).

5. Statistics

5.1.Statistics from Article 3

The data were statistically processed using the SPSS 19 software package. The differences between the various conditions were determined by analysing variance (One-factor and Multivariate factor ANOVA, Tukey test). Discriminant factor analysis was

performed from growth parameter estimations (μ_{max}, λ and OD_{max}) for the 45 yeast strains growing at 0%, 5%, 10% and 15% (v/v) of ethanol in the BGM medium at pH 3.

5.2. Statistics Article from Article 4

The data were statistically processed using the SPSS 19 software package. The differences between the various conditions were determined by analysing variance (One-factor and Multivariate factor ANOVA, Tukey test). Meanwhile, two optimisation indexes were calculated from data from the secondary fermentation (OI_{Ferm}) and physiological parameters ($OI_{phypara}$) values determined on acclimated cells. After certain statistical treatments (correlation between physiological parameters), a linear regression was then performed between OI_{Ferm} and $OI_{phypara}$. The secondary fermentation parameters were calculated as follows: the maximum value for one parameter was considered to be 1 (x/x), if this had a positive effect on the performance of a secondary fermentation, or 0 (1 – x/x) if it had a negative effect. For instance, in the case of residual sugar content in the sparkling wines, the maximum concentration considered to be an uncompleted fermentation was observed under OXI conditions at 5.44 g L^{-1} and for the minimal content under NOXI conditions at 3.51 g L^{-1}. Consequently, OXI and NOXI conditions had an O.I. for final sugar content in sparkling wines of 0, (1-(5.44/5.44)) and 0.59, (1-(3.51/5.44)), respectively.

CHAPTER III

Yeast ethanol stress during fermentation

Article 3

New insights into the capacity of commercial wine yeasts to grow on sparkling wine media. Factor screening for improving wine yeast selection

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Chapter III

Abstract

During the production of sparkling wine, wine yeasts are subjected to many stress factors apart from ethanol (the main one), which lead to the need to achieve their acclimation in line with various industrial protocols. In the present work, 44 commercial wine *Saccharomyces cerevisiae* strains and one laboratory strain (BY4742) were firstly subjected to increasing concentrations of ethanol to cluster the yeasts using discriminant function analysis. Afterwards, non-inhibitory concentration (NIC) and minimum inhibitory concentration (MIC) were estimated, revealing some differences between 24 of these strains. This study confirms the negative synergistic effect of low pH with ethanol on the maximum specific growth rate (μ_{max}) and lag phase time. Moreover, a negative effect of increasing levels of glycerol in the growth medium was observed. Interestingly enough, an interactive positive effect was found between cysteine and medium-chain fatty acids (MCFA). While cysteine did not have a really significant effect in comparison to the control, it was able to restore the damage caused by MCFA, making the growth rate of cells recover and even reducing the formation of reactive oxygen species (ROS). Few differences were observed between NIC and MIC estimation depending on if the culture aeration; however, it can be shown the importance of oxygen availability for the composition of cell fatty acids.

Chapter III

Introductory highlights

Ethanol is the main factor which affects yeast strain viability during secondary fermentation. Commercial wine yeasts have been selected to be used as starters due to their ability to ferment high sugar amounts of grape must and, subsequently tolerate high ethanol levels. Little information is available in relation to the behaviour of these strains growing in different levels of ethanol in restrictive conditions also found in wine media; such as low pH and the presence of glycerol, cysteine or MCFA. These factors may have synergic effects with ethanol.

Parameters analysed in the present article are described briefly below:

- At low **pH** (2.9-3.2) of wine, weak organic acids may enter cells in the undissociated form causing the cytosol acidification.
- **Glycerol** presence allows *S. cerevisiae* to oxidise the NADH. The role of NADH-consuming glycerol formation is to maintain the cytosolic redox balance, particularly under anaerobic conditions (Van Dijken and Scheffers 1986). Apart from balancing redox, glycerol has the function of osmoregulating yeast cells when extracellular water activity decreases in the growth medium (Blomberg and Radler 1992).
- Specific concentrations of **MCFA** (C8 and C10) decreased the maximum specific growth rate and the biomass yield exponentially, and increased the duration of growth latency. These toxic effects increased with the pH decreasing, indicating that the dissociated form is the more toxic. Ethanol leads to a more fluid membrane which can be cross easily for these acids.
- **Cysteine** is related to protein stability; play a role in detoxification and the stress response of the cells; however, an increase in the cysteine level may be toxic (Kumar *et al*. 2006). On the other hand prevents oxidation of aromatic compounds in wine. Moreover, cysteine can induce serious growth defects without inducing ROS accumulation inside cells (Kumar *et al*. 2006).

Chapter III

The aim of this work is (i) to classify certain commercial wine yeasts in terms of their ethanol tolerance in a defined growth medium at low pH (3.5) in the presence of weak acids and (ii) to evaluate the yeast growth parameters in the presence of certain factors (pH and presence of glycerol, cysteine and MCFA). The yeast's behavioural response to these factors should enable an improvement in the acclimation of commercial wine yeasts to ethanol in order to achieve the secondary fermentation properly for sparkling winemaking.

Chapter III

Results and Discussion

Ethanol classification

The first objective of this study was to observe the behaviour of 44 commercial wine yeasts, pre-cultured in YPD, growing in a defined medium with three increasing ethanol concentrations: 5%, 10% and 15% (v/v). A laboratory strain, BY4742, was added because of its low ethanol tolerance. Before this experiment, the difference between strains was shown by analysing mtDNA restriction fragments and delta elements (see supporting information). After the determination of the main growth parameters (OD max, maximum specific growth rate and lag phase) using the modified Gompertz equation, discriminant function analysis was built to classify yeast strains (45) in four predicted groups defined by the four ethanol contents used in this study: Group I (0%), Group II (5%), Group III (10%) and Group IV (15%, v/v of ethanol). Thus, the total number of yeast strains for each group would be 45 if every strain was equally affected by the ethanol content. The classification results (Table 15) reveal that 72.2% (130/180, total number of classified strains located on the diagonal of the table/number of cases, 45 strains x 4 ethanol levels) of original grouped cases were correctly classified into the four groups. However, some strains displayed bad growth in the defined medium without ethanol, because 9, 2 and 1 of them were situated in predicted group II, III and IV, respectively. The reasons why these strains were misclassified are that they had either a very long lag phase (λ) or a low maximum specific growth rate (μ_{max}), or both. In contrast, 11 strains were classified in the predicted group I, although they grew in 5% of ethanol content. Examining the other results in Table 15, it can be seen that some of these commercial yeast strains, a total of 17, can be pre-cultured in the presence of 15% of ethanol and had similar behaviour to pre-cultured cells in only 10% of ethanol (Group III). However, interestingly, only four strains could be classified in the 5% ethanol group (II), although they grew in 10% ethanol content. Moreover, it should be noted that the laboratory strain was still classified in the fourth group because of its very low rate of growth in the defined medium, which drastically decreased with ethanol content. With this new classification, the four groups, 0% (I), 5% (II), 10% (III) and 15% (IV) ethanol-tolerance, had a new composition: 44 strains for group I (sum of cases in the group I column), 42 strains for group II, 63 strains for group III and 31 for group IV. Some strains belonging to groups I, II and III are repeated because of the good growth displayed by these strains in the presence of 0% and 5%, 5% and 10%, 10% and 15% of ethanol,

respectively. Of these strains, four in particular will be used for evaluating the effects of MCFA, oxygen and glycerol on growth parameters.

Table 15. Classification results of discriminant analysis according to the growth parameter estimation of 45 yeast strains (44 commercial wine yeast + 1 BY4742) in BGM medium (pH 3.5, temperature, 25ºC) with different ethanol contents (0%, 5% 10% and 15%, v/v). Growth parameters were maximum specific growth rate, lag phase and OD maximum obtained from 720 growth curves.

Classification Results **(**72.2% of original grouped cases correctly classified)						
			Predicted Group Membership			
			Group I 0%	Group II 5%	Group III 10%	Group IV 15%	Total
Original	Count	0%	33	9	2	1	45
		5%	11	29	4	1	45
		10%	0	4	40	1	45
		15%	0	0	17	28	45
	%	0%	73.3	20.0	4.4	2.2	100.0
		5%	24.4	64.4	8.9	2.2	100.0
		10%	0	8.9	88.9	2.2	100.0
		15%	0	0	37.8	62.2	100.0

Finally, based on the new composition of the groups, the growth parameters are plotted with μ_{max}, λ and maximum OD achieved (Fig. 26 A, B and C). The decreasing μmax was linearly proportional to the increasing level of ethanol content in the defined medium with a regression coefficient of 0.9949 (Fig. 26A). The lag phase was only significantly different in the case of 15% ethanol content (v/v), (Fig. 26B). The standard deviation calculated from the estimation curves (around 180) was very large because of the strain variability at this ethanol level. It was interesting to note the maximum OD reached by strains during the growth in the presence of ethanol in the BGM medium (Fig. 26C).

While the effect of 5% (v/v) of ethanol was no different from the control, the presence of 10% and 15% (v/v) of ethanol in the growth medium induced a significant decrease in the maximum OD. All these results were in agreement with those obtained by Salvadó *et al.* (2011) and Arroyo-López *et al.* (2010a), though with one new aspect: that the growth medium mimics the conditions to which wine yeasts may be subjected: low pH (3.5) and presence of organic acids (tartaric, malic and citric acids).

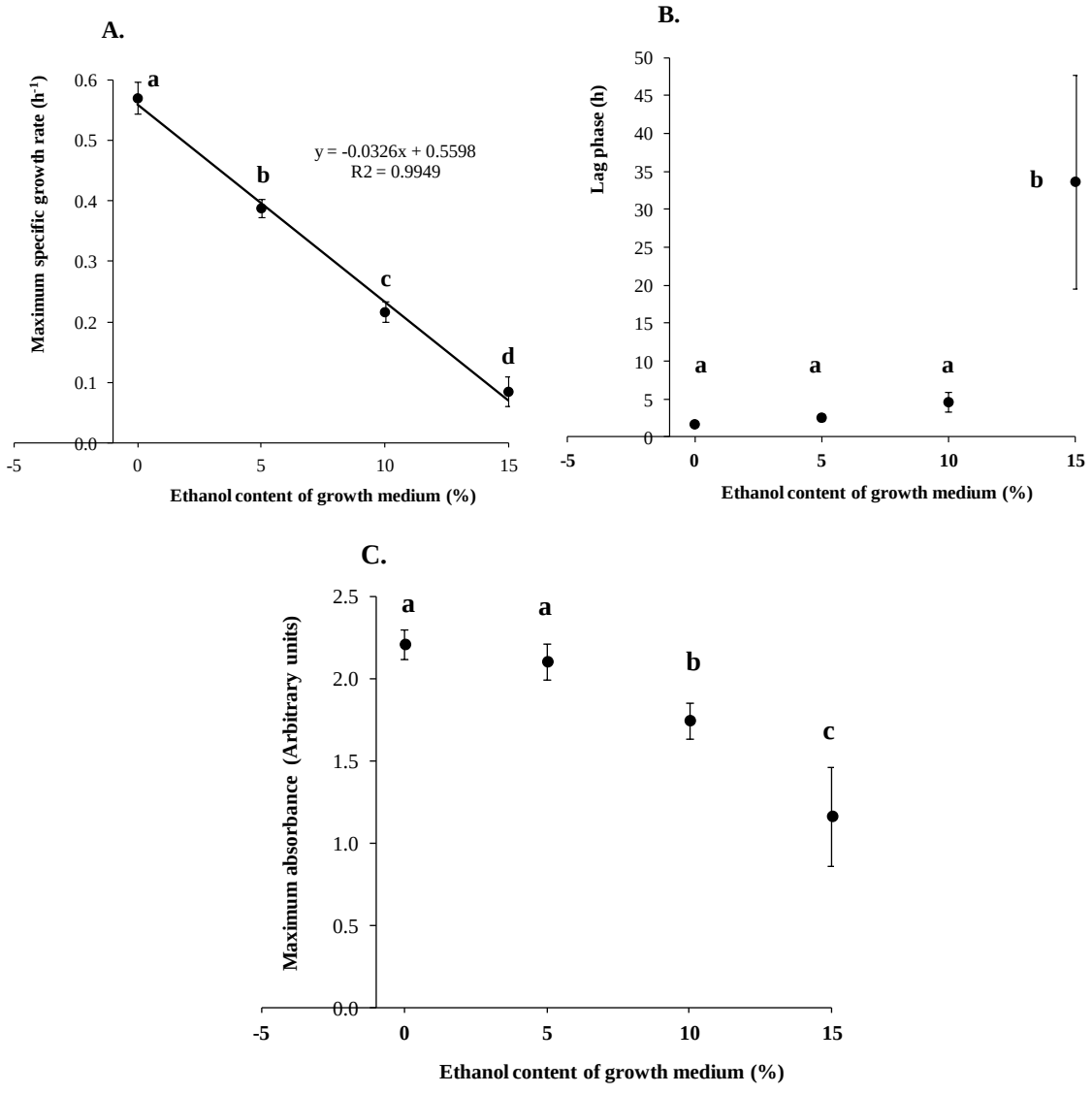

Figure 26. Effect of ethanol content (%, v/v) on the maximum specific growth rate (h^{-1}), (panel A), the lag phase (h), (panel B) and the maximum absorbance (panel C). Number of strains classified for each ethanol content by discriminant analysis: 0% = 44; 5% = 42; 10% = 63 and 15% = 31. Bars are standard deviations; different letters indicate a significant difference at $p < 0.05$, according to a Tukey post-hoc comparison test.

NIC and MIC estimation

The determination of NIC and MIC was only carried out on 24 commercial wine yeast strains in relation to their classification in the first cluster by discriminant function analysis. It should be noted that only a pre-culture in YPD medium of these strains was carried out before their evaluation in the BGM in the presence of ethanol (from 0% to 22%, increment of 2%, v/v). Under these conditions, the results observed in Figure 27 showed that eleven strains reached 15.5% (v/v) of ethanol for the estimated MIC. Of these 24 strains, six had an estimated NIC less than 10.5% (v/v) of ethanol, which could mean that these strains were ethanol-sensitive and may generate some problems to ferment high sugar grape must. The response is not easy because, for instance, one of these strains has a high estimated MIC (around 16%, v/v of ethanol). On the other hand, the progressive inhibitory region (PIR) was different for various *Saccharomyces* species, as described by Arroyo-López *et al*. (2010a). As shown in Figure 27, PIR ranged from 3.09% to 7.40% (v/v) and a significant positive correlation was observed, with a level of + 0.842 between PIR and MIC values (data not shown). This fact may indicate that commercial wine yeast strains with the highest MIC values progressively perform their acclimation to the growth conditions by increasing the PIR phase.

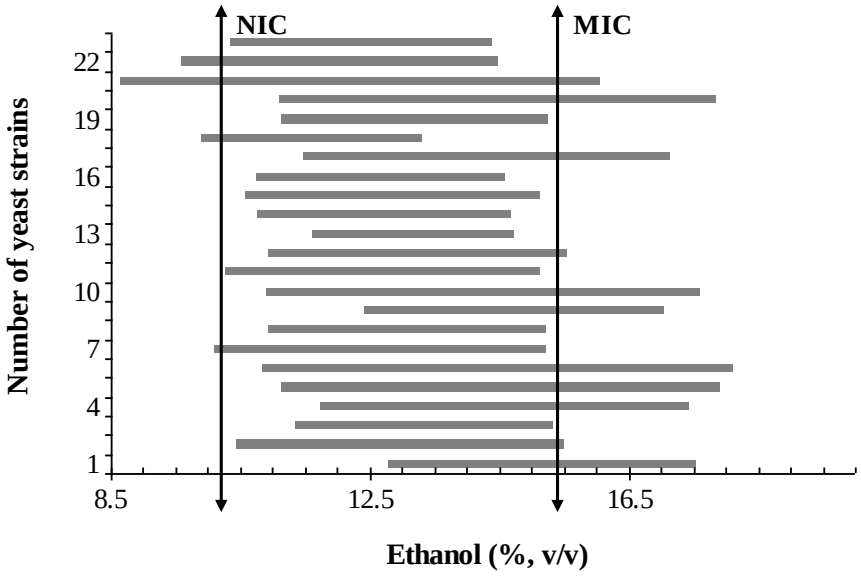

Figure 27. NIC and MIC estimations for 24 commercial wine yeasts in BGM medium. Values of NIC and MIC were averages from quadruplicate determinations according to the corresponding material and method section.

pH effect

Modifying the pH of the BGM medium from 3.5 to 3.0, the effect of increasing ethanol content is more remarkable. Whatever the ethanol content in the growth medium, there was a significant decrease of μ_{max} of the yeast strains (total of 25 - Fig. 28). The same effect was observed in the case of the time of lag phase (not shown). Moreover, the low pH reduced the μ_{max} of these strains in the absence of ethanol. These results confirm previous studies showing the drastic effect of the presence of organic acids and MCFA in the growth medium (Viegas *et al*. 1989, 1997; Pampulha and Loureiro, 1989b). However, the effect of sulphur dioxide was not taken into account in our study. Knowing the important role it plays in low-pH wines, *i.e*. high proportion of molecular sulphur dioxide, it is reasonable to assume that its contribution with other stress factors on yeast growth would be even more drastic.

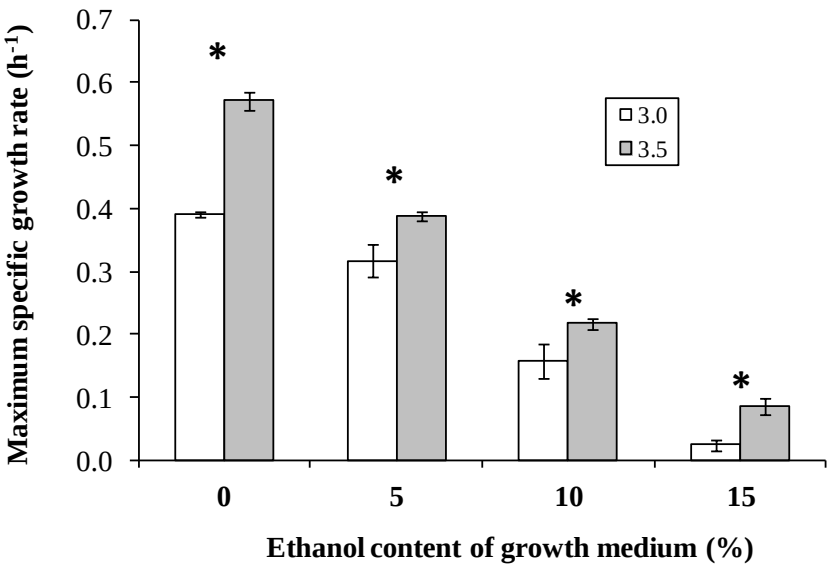

Figure 28. Effect of pH on the maximum specific growth rate (h^{-1}) with respect to ethanol content (0%, 5%, 10% and 15% (v/v)). White bars, pH 3.0 and grey bars, pH 3.5. Number of strains classified for each ethanol content by discriminant analysis: 0% = 44; 5% = 42; 10% = 63 and 15% = 31. Bars are standard deviations; star symbol indicates a significant difference between pH for the same ethanol content at $p < 0.05$, according to a Tukey post-hoc comparison test.

Glycerol effect

Glycerol is a by-product of alcoholic fermentation and its concentration can vary according to mainly the vinification conditions and yeast strains. The objective of this experiment was to determine the effect of two glycerol levels, 5 and 10 g L^{-1} with the

presence of 10% or 15% (v/v) of ethanol in the BGM medium on the estimation of growth parameters of four representative strains (7, 17, 44 and 56). These strains were chosen because of the good growth displayed in the presence of ethanol in these cases. In Table 15, they are classified in the lower neighbouring group of ethanol content used. The results showed that the μ_{max} was strongly and significantly affected by 10 g L^{-1} of glycerol, regardless of the ethanol level found in the growth medium (Fig. 29A). Meanwhile, the presence of 5 g L^{-1} of glycerol did not seem to modify the behaviour of yeast strains in the presence or absence of ethanol. In relation to the estimation of lag phase (Fig. 29B); the same comments could be made. Only in the presence of 10 g L^{-1} of glycerol in the BGM medium, the time to initiate the growth phase was significantly longer than under other conditions, and in the case of the control and 5 g L^{-1} of glycerol. A high glycerol level in the base wine could delay the performance of the secondary fermentation and, depending on the chemical composition of wine and yeast acclimation to ethanol, could lead to stuck fermentation. Consequently, the choice of wine yeast to conduct the first fermentation, *i.e.* grape must fermentation, may be important.

High glycerol-producing wine yeasts, such as *Saccharomyces kudriavzevii* (Arroyo-López *et al*. 2010b), may prevent good secondary fermentation. A more complete study could determine whether the yeast pair for first and second fermentations must be identical, whether glycerol production is limited or not, and if any wine yeast is available from the moment that ethanol acclimation is successful. Alternatively, cell exposure to high glycerol content may mimic a similar effect to the deletion of the FPS1 gene aquaglyceroporin channel involved in the efflux and influx of glycerol, of the BY4741 strain, as observed by Lourenço *et al.* (2013). Indeed, the *fps1Δ* deletion mutant exhibited lower μ_{max} and longer λ compared to the parental strain, being comparable to the effect of the highest glycerol content under our conditions, regardless of the ethanol level. Moreover, an increase in glycerol intracellular accumulation and a decrease in NAD^+ content were observed, suggesting a permanent state of redox imbalance. In other words, the membrane-fluidising effect of ethanol may increase the passive diffusion of glycerol, inducing its accumulation in the cells and causing redox imbalance. Another hypothetical explanation of the effect of high glycerol content relates to the different hygroscopic properties of glycerol and ethanol. Indeed, glycerol has better ability than ethanol in terms of attracting and holding water molecules. Thus, the surrounding environment effect created by the presence of glycerol may limit cellular transport and induce a long lag phase followed by low growth.

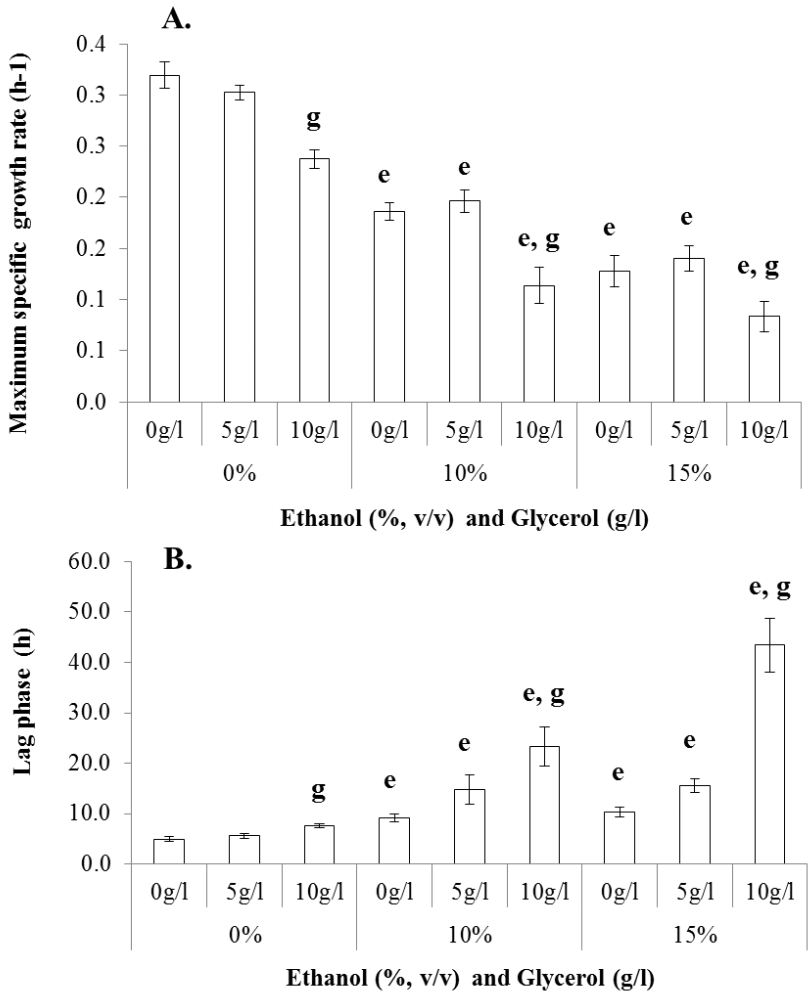

Figure 29. Effects of ethanol content (%, v/v) and glycerol concentration (g L^{-1}) on the maximum specific growth rate (h^{-1}), (panel A) and the lag phase (h), (panel B). Number of strains examined = 4. Bars are standard error of means; different letters indicate a significant difference at $p < 0.05$, according to a Tukey post-hoc comparison test: letter **e**, differences between ethanol contents for the same glycerol concentration and letter **g**, differences between glycerol concentrations for the same ethanol content.

MCFA and cysteine effects

The production of base wine, according to oenological practices of white wine production, generally induces the synthesis and excretion from yeast cells of medium-chain fatty acid (MCFA, from C6 to C12) into wine. These fatty acids are toxic in high concentrations depending on the yeast strains, and can often lead to stuck alcoholic fermentation or a delay in the trigger of secondary fermentation. For the production of sparkling wine, it is generally appropriate to use another *S. cerevisiae* strain to ensure the complete secondary fermentation and to increase the organoleptic properties of these

products. Consequently, the presence of MCFA in base wine can cause some difficulties with respect to re-fermenting it. The goal of this study was to determine the effect of MCFA addition, specifically octanoic (C8) and decanoic (C10) acids, to the BGM medium in the presence of 10% (v/v) of ethanol on the estimation of the main growth parameters.

Moreover, cysteine, a thiol amino acid which has an intracellular antioxidant property, was evaluated. Although some *Chardonnay*-based wines have a weak cysteine level (around 20 mg L^{-1}, Desportes *et al*. 2000), its presence can enhance the antioxidant capacity of yeast. The results showed that the presence of C8 and C10 significantly decreased the μ_{max} for all of the 44 commercial yeast strains studied (Table 16). In the presence of 50 mg L^{-1} of cysteine in the BGM medium, a significant reduction in μ_{max} (around 30%) was also observed with respect to the control. However, while cysteine at this concentration had an effect on μ_{max}, its presence in the BGM medium supplemented with MCFA enabled μ_{max} to increase significantly. The average μ_{max} calculated from the curves of 44 strains was significantly higher with cysteine than in its absence, with levels of 0.09 h^{-1} to 0.06 h^{-1}, respectively (Table 16). In relation to the estimation of the lag phase, under these conditions, no significant changes were observed between the control and cysteine-supplemented BM medium (Table 16). As expected, the addition of MCFA to the growth medium leads to a significant increase in the length of the lag phase (48.89 h compared to 5.89 h for the control). However, this time was significantly reduced (to 38.21 h) when cysteine was supplemented.

Table 16. Effects of addition of MCFA (C8, 3 mg/L and C10, 6 mg/L), cysteine (50 mg/L) and both at 10% (v/v) of ethanol on the maximum specific growth rate (μ_{max}, h^{-1}) and the lag phase (λ, h) in the BGM medium. Control: 10% (v/v) of ethanol. Number of strains examined = 44. Mean ± Standard Error of Mean (SEM); different letters in the same column indicate a significant difference at $p < 0.05$, according to a Tukey post-hoc comparison test.

	μ_{max} (h^{-1})	λ (h)
Control	0.211 ± 0.011^{a}	5.89 ± 1.60^{c}
+ MCFA	0.060 ± 0.005^{d}	48.89 ± 2.43^{a}
+ Cysteine	0.148 ± 0.014^{b}	11.07 ± 2.01^{c}
+ MCFA + Cysteine	0.090 ± 0.007^{c}	38.21 ± 2.07^{b}

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To complete the experiment, the determination of intracellular ROS was performed on three strains (7, 44 and 56). The results presented in Figure 30 show that, throughout the first two days of yeast growth, values of ROS significantly changed for all strains, whatever the conditions under study, except in the case of cysteine addition, when cells maintained the lowest ROS percentage, at around 25%. Furthermore, the ROS level of cells fell after two days of growth under all of the conditions, with the exception of the cysteine condition, in which case it remained similar. The average lag phase of the three strains seemed to indicate a relation between the chemical composition of the growth medium and the ROS level. The longer the lag phase, the higher the ROS. It is worth noting that the addition of cysteine to the BGM medium allowed cells to have a lower ROS level.

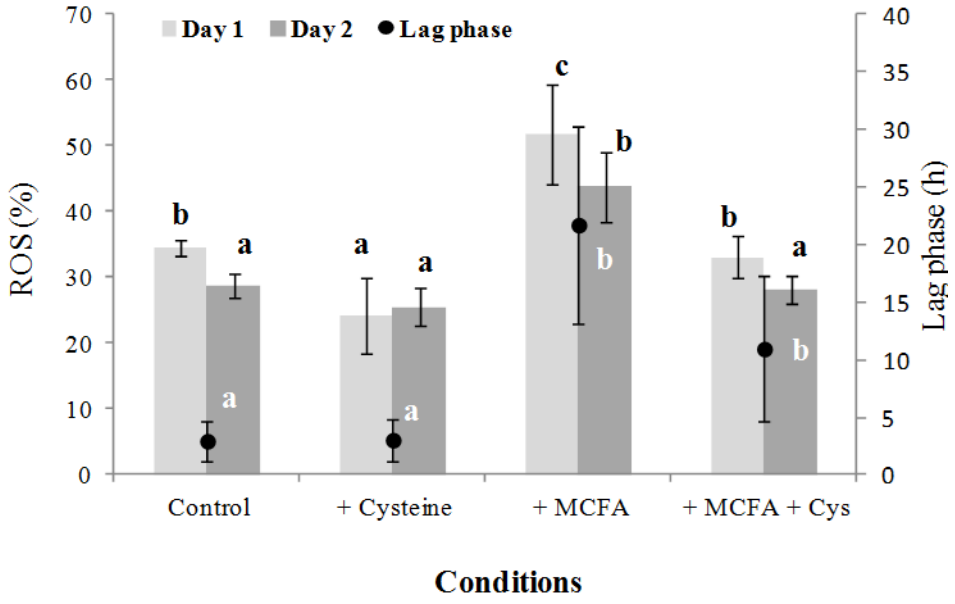

Figure 30. Effect of addition of MCFA (C8, 3 mg/L and C10, 6 mg/L), cysteine (50 mg/L) and both at 10% (v/v) of ethanol on the ROS (%) and lag phase (h) in the BGM medium. Control: 10% (v/v) of ethanol. Number of strains examined = 3. Bars are standard error of means; different letters indicate a significant difference at $p < 0.05$, according to a Tukey post-hoc comparison test.

When cells were compared growing in the presence of MCFA and in the presence and absence of cysteine, ROS levels were significantly lower, around 17% in cells in the presence of both MCFA and cysteine compared to cells with only MCFA on all of the days observed. Besides, yeast strains displayed different behaviour with respect to ROS formation, whatever conditions were used (data not shown). For instance, the average production of ROS under any conditions between strains 56 and 7 were 27.1% (± 4.7%)

and 52.6% (± 19.1%), respectively. This result may mean that wine yeast strains have (i) a different response to the MCFA presence and/or (ii) an active sulphur metabolism. Indeed, strain 56 is a high hydrogen sulphide (H_2S) and volatile sulphur producer in sparkling wines (Pecero *et al.* 2013) which could be related to an attenuated cell response to oxidative stress caused by MCFA in comparison to the other strains studied. These findings are in agreement with Pan *et al.* (2006), in which H_2S-mediated inhibition of cytochrome c oxidase decreased the cellular metabolic rate and O_2^- production. Moreover, cysteine generally reacts with carboxyl and carbonyl molecules in wine (Marchand *et al.* 2000), which may play an anti-oxidant role, thus limiting the formation of such molecules into cells.

Oxygen effect

Oxygen availability in the growth medium enables yeast cells to synthesise unsaturated fatty acids and sterols. To date, cells which have good ethanol tolerance increase their membrane fluidity by increasing the UFA level (Alexandre *et al*. 1994, 1996; Arroyo-López *et al*. 2010a). In this last experiment, the fatty acid composition of three commercial strains growing in YPD medium were determined in the presence and absence of oxygen. The aim was to compare NICs and MICs from cells growing under non-oxygen and aerated conditions in low-sugar content growth media. The results presented in Table 17 (average of three strains - 7, 44 and 56) showed that no significant differences were found between cells cultured in the presence or absence of oxygen for the estimations of NIC and MIC, although there were significant differences in the MCFA, SFA and UFA compositions between the yeast strains in our growth conditions which mimic the industrial acclimation conditions of yeast for producing sparkling wines. Moreover, it was noted that cells with apparently different membrane fluidity had a similar response to the effect of the ethanol level on NIC and MIC values. In other words, the increase in MCFA content at the expense of UFA due to hypoxic conditions allowed yeast cells to maintain appropriate membrane fluidity. Under our growth conditions (25ºC, low pH and presence of organic acids), contrary to the results obtained by Arroyo-López *et al*. (2010a), a lower UFA to SFA ratio may also enable good tolerance to increasing ethanol levels. However, these results agree with those of del Castillo-Agudo (1992). For two of the three studied strains, the estimated MIC was even higher than for cells cultured in the presence of oxygen (data not shown). The theoretical transition phase temperature of MCFA and SFA

is higher than that of UFA, which, under hypoxic conditions, may explain the ability of strains to obtain lower fluidity in its membranes and, consequently, limit the membrane-fluidising effect of ethanol. To support and develop these findings, other studies should be conducted to determine whether an ergosterol/squalene ratio counteracts this different fatty acid composition between the two growth conditions, or whether a higher phosphatidylcholine/phosphatidylethanolamine ratio confers better ethanol tolerance to cells, as described by Chi and Arneborg (1999).

Table 17. Effect of aeration conditions on the NIC, PIR and MIC estimations and the cell fatty acid composition. Number of strains examined = 3. PIR, progressive inhibitory region calculated by subtracting NIC from MIC. MCFA, medium-chain fatty acids (C6-C12); SFA, saturated fatty acids (C14 + C16 + C18); UFA, unsaturated fatty acids (C14:1 + C16:1 + C18:1). + Oxygen, constant agitation of cell growth culture; ± Oxygen, static cell growth culture. All strains, result averages of the three strains (x4 replications).

	Culture conditions		ANOVA
	± Oxygen	+ Oxygen	P value
NIC (%, v/v) [a]	12.33 ± 1.21	11.69 ± 0.68	0.127
PIR (%, v/v) [a]	5.28 ± 1.60	5.18 ± 0.89	0.852
MIC (%, v/v) [a]	17.53 ± 1.17	16.87 ± 1.21	0.192
MCFA (%) [b]	10.85 ± 7.24	2.27 ± 0.49	0.000
SFA (%) [b]	26.48 ± 5.65	15.18 ± 3.14	0.000
UFA (%) [b]	62.67 ± 12.76	82.55 ± 3.53	0.000

[a]: expressed in % (v/v) of ethanol; [b]: expressed in % of fatty acid total areas after normalisation with internal standard.

Conclusion

In conclusion, commercial wine yeasts display different behaviour when subjected to a range of abiotic stress factors, such as pH, glycerol, MCFA, oxygen availability during its culture. However, the level of ethanol remains the truly differential factor between them. Some wine strains could be used for any types of winemaking because of their high adaptive capacity to any ethanol range, while others seem to be limited. Interestingly enough, some strains showed similar growth parameters in the presence of a higher ethanol content to those displayed at a lower neighbouring level. These strains would be good

candidates for conducting the secondary fermentation or for restarting stuck fermentation due to their acclimation capacity with respect to ethanol.

It was interesting to note the unique effect of glycerol on the growth parameters of wine yeasts. To the best of our knowledge, this is the first time that a negative synergistic effect between ethanol and glycerol is described. High extracellular glycerol content in the presence of ethanol seems to mimic the effect of the absence of the aquaglyceroporin channel (FPS1 gene) on the growth parameters. Further work is necessary to confirm if intracellular glycerol accumulation really occurs, which may modify the redox balance and, consequently, the ethanol stress resistance characteristics of strains. However, this by-product of alcoholic fermentation may have negative synergistic effects on, for instance, pH, MCFA and nutrient requirements in terms of achieving proper secondary fermentation.

Meanwhile, changes in the fatty acid composition of cells (only three strains were tested) show that the NIC and MIC parameters can be increased, even with a lower UFA level in the cells. It was interesting to note the increase of cell ROS species in the presence of MCFA, which induces a longer lag phase in low-pH medium.

Finally, further studies have to be conducted to ascertain the real effect of cysteine, concentration dependence and analogue molecules, such as glutathione, on yeast acclimation to ethanol or on the performance of alcoholic fermentation.

CHAPTER IV

Yeast acclimation for *Cava* production

Article 4

New insights into the physiological state of *Saccharomyces cerevisiae* ethanol acclimation for producing sparkling wines

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Abstract

The production of sparkling wines is the result of two consecutive alcoholic fermentations. The first is the fermentation of the grape juice, through which the base wine is obtained, which is fermented again by yeast to enable the effervescence process in bottles. As the use of active dry yeast after rehydration is usually direct and simple, the inoculation of base wine by yeast requires certain preparations to ensure good yeast growth under these conditions: 10% (v/v) of ethanol, low pH, low temperature, etc. Although there are industrial protocols that are widely used to adapt wine yeast to these conditions, information is scarce with respect to the physiological state of strains during the acclimation process. In this study, a commercial wine yeast was acclimated according to different conditions in terms of aeration and sugar content, and used to inoculate a defined base wine to ferment at 25ºC in the bottle. The results showed that some physiological parameters played a decisive role with respect to reaching the maximum pressure of 6 bars in bottles. These parameters were higher glycogen and trehalose contents, lower ROS accumulation, better vacuolar activity and lower ratios of ergosterol/squalene and oleic acid/stearic acid.

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Introductory highlights

The second fermentation for sparkling wines production, due to an accumulation of stressful factors, it requires an extra effort for yeasts to carry on with fermentation. Some stress factors are the same as those found in first fermentation, although with a different intensity: high ethanol, low pH, low nitrogen, accumulation of toxic sub-products such as MCFA and organic acids. Others are found in the second fermentation: low temperature, CO_2 overpressure and high SO_2 and total acidity (Juroszek *et al.* 1987). Some of these stress factors have a synergic effect.

Progressive acclimation of *S. cerevisiae* is required before being inoculated into the base wine. Acclimation process was defined by Tai *et al*. (2007); permits yeast to activate stress metabolism response to ensure the successful *prise de mousse*. The main features of this method were first described by Françot and Geoffroy (1957) and the effects of ethanol on yeast during its acclimation were analysed by Juroszek *et al*. (1987). Some variations of the yeast acclimation process and its subsequent use, known as the *Champenoise* method, have mainly been described by Valade and Moulin (1983), and Laurent and Valade (1994, 1998, 2007).

Although winemakers apply procedures in order to adapt yeast, there is always a lag phase after inoculation followed by a short cell proliferation, indicating that the cells are still not completely adapted. However, without this acclimation process, cells are highly susceptible to death.

The effect of ethanol on yeast has been widely studied (Aguilera *et al*. 2006; Bauer and Pretorius 2000). It has been reported that the alteration of membrane fluidity is one of the key factors in terms of their tolerance (D'Amore and Stewart 1987; Alexandre *et al.* 1994; You *et al.* 2003). Generally, ethanol tolerance is associated with a higher degree of membrane unsaturation, which implies that oxygen must be a major factor in this tolerance because of its involvement in the synthesis of unsaturated fatty acids and sterols (Arneborg *et al.* 1995; Valero *et al.* 2001). On the other hand, the cellular response to high ethanol content is strikingly similar to the heat stress response (Piper 1995; Hallsworth 1998). As in the case of other stresses, the cellular response includes the synthesis of trehalose and the induction of stress protection or heat shock proteins (Piper 1997; Landolfo *et al*. 2008).

Other factors play an important role in the acclimation process of yeasts:

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- **Oxygen** is required for the synthesis of specific lipids (Andreasen and Stier 1954, 1953), which allows an increase in biomass and better fermentation (Valero *et al.* 2001; Redón *et al.* 2009; Alexandre *et al.* 1994; Ding *et al.* 2009; Alfenore *et al.* 2004). However, the presence of oxygen in the cell may cause excessive accumulation of Reactive Oxygen Species **(ROS)** (Moradas-Ferreira *et al.* 1996; Halliwell 1995).
- The amount of **sugars** in the medium completely changes the acclimation yeast cell metabolism, which may have an effect on their subsequent performance during fermentation (Boulton *et al*. 1996). Both oxygen and external sugar content control the switch from respiration to the fermentation metabolism in *S. cerevisiae* (Verduyn *et al*. 1987). **Organisation of organelles** may give an insight into (i) which type of metabolism (repiration/fermentation) cells are undergoing defined by the organisation of **mitochondria** into yeasts (Kitagaki and Shimoi 2007; Carmona-Gutierrez *et al.* 2012); and (ii) in which **vacuole** statre cells have to be maintained to get great acclimated cells; taking a look into vacuole activity and microvacuolation process (Li and Kane 2009).

The goal of the present study was to relate the acclimation of S. cerevisiae cells to their performance during the secondary fermentations carried out in bottles at 25ºC. To this end, the effect of aeration and external sugar content during the cell acclimation process on certain physiological parameters was analysed. These parameters were in relation to the carbohydrate reserve (glycogen and trehalose), the activity of vacuoles, ROS accumulation and the lipid metabolism. The results showed that a correlation can be established between the achievement of secondary fermentation and certain physiological parameters analysed during the cell acclimation process.

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Results and Discussion

1. *Acclimation process*

The yeast acclimation process according to the traditional *Champenoise* method to produce sparkling wines was adapted to laboratory scale in order to study the importance of the metabolism and physiology of yeast cells to improve the onset and accomplishment of secondary fermentation in bottle. A representative shceme were developed in Figure 31.

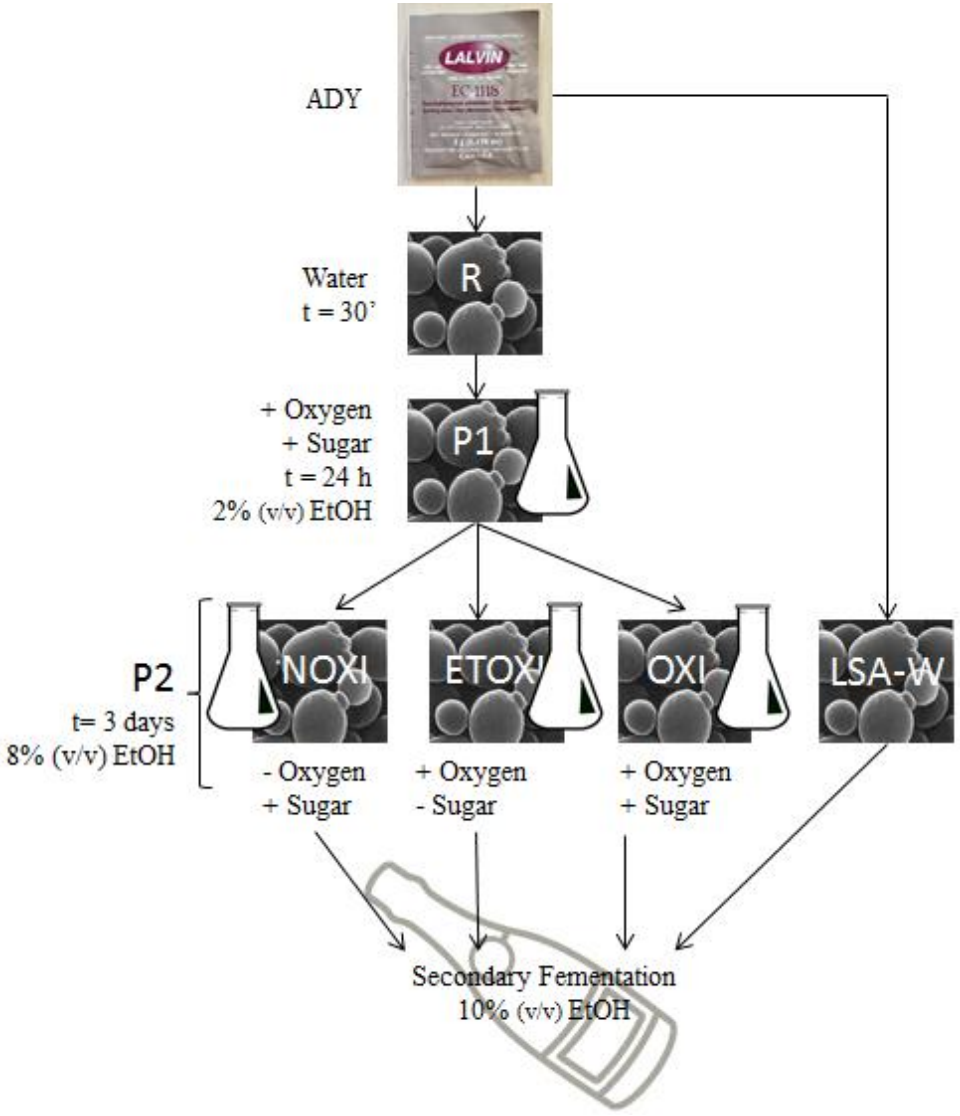

Figure 31. Scheme of the acclimation process adapted at laboratory scale from traditional *Champenoise* method. R= rehydrated yeast cells; P1= phase 1 accliamtion; P2= phase 2 acclimation; NOXI, ETOXI, OXI and LSA-W were different conditions applied at P2 descrived below.

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The 4-day process was performed on a defined wine base medium to enable us to establish the main physiological parameters that could play an essential role in this respect, such as glycogen and trehalose contents, cell lipid composition, vacuolar activity and ROS intracellular accumulation.

As observed in Figure 32, yeast cells consumed all disposable sucrose during the first 24 hours, producing an increase in the ethanol content of the acclimation medium. After this activation phase (P1), cells placed under different conditions developed a different response in relation to the availability of a carbon source and oxygen. Specifically, cells were placed in unlimited oxygen cultures in which sugar content was or was not added during the second phase of acclimation (P2), to establish OXI and ETOXI conditions, respectively. This arrangement allowed us to measure the behaviour of yeast cells under respiro-fermentative (OXI) and respiratory (ETOXI) conditions. Another set of condition was developed to determine the incidence of fermentative behaviour (NOXI), in which cells were acclimated in the presence of sugar in a limited oxygen culture. In the presence of sugar, OXI and NOXI conditions displayed a different production curve of ethanol, rising to 13.61 ± 0.15 % (v/v) for OXI and 14.78 ± 0.19% (v/v) for NOXI, over the course of the 3 days of P2 (Fig. 32). The difference between the two sets of conditions was the consumption intensity of sugar at the end of the acclimation process. For cells grown under OXI condition, the final sugar content was significantly different (1.86 g L^{-1}) compared to 5 g L^{-1} under NOXI condition.

Based on this, it can be assumed that, in the case of OXI conditions, cells began a respiro-fermentative metabolism because the *Crabtree* effect was decreased. In contrast, cells under NOXI condition may be subject to the *Crabtree* effect and, consequently, had a fermentative metabolism. Meanwhile, cells under ETOXI conditions completely consumed the sugar within 60 hours (P2) and then developed a respiratory metabolism, using a non-fermentable carbon source, ethanol, as observed in Figure 32, resulting in an ethanol content of 6.40 ± 0.15 % (v/v) at the end of acclimation process. Finally, in this way, three types of yeast acclimated cells were obtained enabling the identification of three adaptive features.

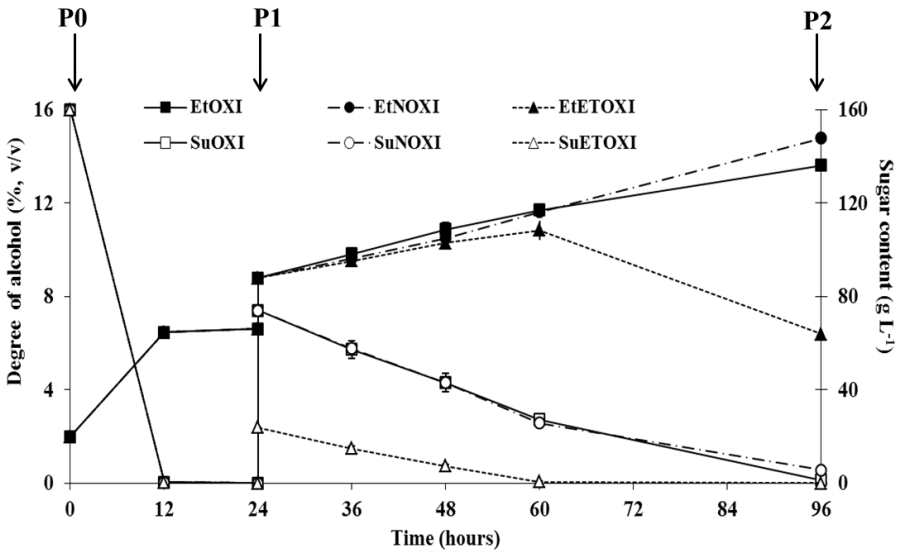

Figure 32. Evolution of sugar (open symbols) and ethanol (closed symbols) contents during the acclimation process of *S. cerevisiae* EC1118 at 25ºC. The conditions applied were: OXI, Aerobiosis + sugar addition (square symbols); NOXI, Semi-anaerobiosis + sugar addition (circle symbols); ETOXI, Aerobiosis without sugar addition (triangle symbols). Et, Ethanol; Su, Sugar. P0, corresponding to LSA rehydrated in water; P1, corresponding to yeast cells in phase 1; P2, corresponding to yeast cells in phase 2.

2. Secondary fermentation in the bottle

To observe the features induced during the acclimation process, secondary fermentations in bottles were conducted at 25ºC in a base wine with OXI, NOXI and ETOXI cells at a concentration of 2 10^6 cells mL^{-1}. Moreover, to complete this study, a direct inoculation with the active dry yeast was used without rehydration in water (LSA-W conditions), with the same initial population level. The time taken for the *prise de mousse* to reach 6 bars, corresponding to the addition of 24 g L^{-1} of sugar in the *tirage* operation, was different depending on the acclimated cells used. The secondary fermentation was only completed with NOXI cells, in 35 days, while, in the other cases, the fermentation stuck (experiment stopped after 40 days) (Table 18). If the time taken to reach 4 bars is analysed, some differences can be observed due the type of acclimation used for cells. In this case, the fastest was NOXI and the slowest was LSA-W, with a difference of 11 days between the two. With respect to the final sugar and ethanol contents, NOXI conditions showed the highest degree of alcohol with 10.90% (v/v) and the lowest sugar level with 2.24 g L^{-1}. Interestingly, some results showed that ethanol production was not correlated to sugar consumption. For example, under OXI conditions, wine had 10.30 % (v/v) of ethanol

with a residual sugar content of 5.41 g L^{-1}, the highest of whole experiment, whereas, in the LSA-W assay, the lowest ethanol level was found, 10% (v/v) with 3.52 g L^{-1} of sugar not consumed. In other words, regardless of the acclimation process or yeast cell preparation, the yield of sugar transformation in ethanol was not the same during the secondary fermentation. In this way, under our conditions, it was observed that these yields (Sugar consumed/ Ethanol produced) were 11.45, 13.22, 14.3 and 20.98 for NOXI, ETOXI, OXI and LSA-W, respectively (data not shown).

However, the cell viability determined at a pressure of 4 bars was the same for all conditions, except in the case of LSA-W, when a low viable population was found (Table 18). It should be pointed out that cell viability was almost the same whatever acclimated cells were used up to a pressure of 4 bars. From 4 bars upwards, damaged cells began to be observed in all secondary fermentations, but live cells were only found under the NOXI condition at 6 bars. For all of the other conditions, no living cells were detected when the experiment was terminated (results not shown).

In the end, the most suitable arrangement for cell acclimation was to place cells in a fermentable environment, corresponding to the NOXI conditions in our experiment. Furthermore, under our conditions, the secondary fermentation of the defined base wine was conducted without the addition of nitrogen. This nutritional factor seemed to have little effect on yeast fermentation kinetics during the *prise de mousse* because real base wines had a normal level of residual nitrogen, *ca* 30 mg N L^{-1} (Martí-Raga *et al*. 2015). However, according to the results of these researchers, nitrogen intake during the yeast acclimation process was crucial for the onset of secondary fermentation, and could be the key factor, in association with the yeast strain and fermentation temperature, for correctly ensuring the secondary fermentation. Our results showed that not only these factors have to be taken into account but also the type of yeast metabolism during the acclimation process. Copying the industrial protocol, the yeast strain synthesised and excreted in the medium amino acids from ammonium phosphate during the four days of acclimation (See Table 23 in Supplementary information).

Table 18. Evolution of some parameters during the secondary fermentation of bottled base wine at 25 ºC with acclimated *Saccharomyces cerevisiae* EC11118. The conditions used were: OXI, Aerobiosis + sugar addition; NOXI, Semi-anaerobiosis + sugar addition; ETOXI, Aerobiosis without sugar addition; LSA-W, Active Dry Yeast directly added in base wine during 30 min. Numbers were an average ± standard deviation of two independent replicates. Different superscripts indicate significant differences within a ROW ($P<0.05$).

	LSA-W	**OXI**	**NOXI**	**ETOXI**
Final sugar content (g l^{-1})	3.52 ± 0.08^{b}	5.41 ± 0.15^{c}	2.24 ± 0.24^{a}	3.51 ± 0.53^{b}
Alcoholic degree (%, v/v)	10.00 ± 0.00^{a}	10.30 ± 0.00^{a}	10.90 ± 0.28^{b}	$10.55 \pm 0.07^{a,b}$
Days to reach 4 bars	21	16	10	13
Days to reach 6 bars	$> 40^{*}$	> 40	35	> 40
Cell viability at 4 bars (cell ml^{-1})	$5.42\ 10^{5}$	$1.18\ 10^{6}$	$1.34\ 10^{6}$	$1.30\ 10^{6}$

***:** > 40 days because fermentation was uncompleted.

3. Physiological state of acclimated cells

Mitochondria, microvacuolation and vacuolar activity

Visualising the organisation of yeast organelles during the acclimation process was our first objective, in order to see the effect of different conditions used. To achieve this, specific fluorochromes were used that are able to stain distinct compartments within the cells, such as mitochondria and vacuoles, thereby giving us valuable information on the health and activity of the cells. Generally, the mitochondrion is well known to be an organelle related to energy metabolism, cell signalling and pathways of programmed cell death. 3, 3'-dihexyloxacarbocyanine iodide ($DiOC_6(3)$) is an indicator of mitochondrial membrane potential that allows the energetic state of mitochondria to be observed.

The organisation of mitochondria is related to the yeast respiration metabolism (Okamoto and Shaw 2005). In the present work, different metabolisms are represented with conditions tested, and differences were expected between them. If mitochondria are disorganised in the cytoplasm, as observed in the case of the NOXI conditions, it means that cells are fermenting (Fig. 33A). However, respiring cells have mitochondria remaining organised as near to the cell membrane as possible in order to capture the oxygen to produce energy, as in the case of OXI conditions (Fig. 33A), or at least cells are prepared to do so. The ETOXI conditions have a completely different kinetic profile to other

conditions, as sugars are consumed and yeasts metabolise ethanol. Under OXI conditions, even though there is constant aeration of the culture, there is no respiration due to the high amount of sugar, responsible for the Crabtree effect. At the end of the acclimation under OXI conditions, the sugars are practically consumed (Table 18).It was uncertain before testing whether cells would be respiring or still fermenting. Even though there is little organisation in some of the cells, it can be seen that, on the whole, mitochondria are not organised. This means that, even though cells have the conditions to respire, they are not able to, perhaps because the cells are not well adapted to culture media.

Analysing the results of cytological parameters, such as vacuole morphology and activity, the best state of cells could be observed in those acclimated under NOXI conditions (Fig. 33B, 33C and Table 19). By a significant margin, they displayed the best microvacuolation (fragments into multiple small vesicles, Fig. 33B), the highest vacuolar activity (Fig. 33C) and the lowest ROS accumulation. Vacuoles are involved in many processes, including the homeostasis of cell pH and modified their morphology depending on the growth conditions (Li and Kane 2009). Under our conditions of acclimation to ethanol, cells placed under NOXI conditions seemed to be better prepared for the secondary fermentation challenge because of their high degree of vacuole fragmentation and their capacity to maintain an acid pH in the vacuole. Under osmotic or ionic stress, vacuoles must be able to fuse and fragment at will to adjust to different cellular challenges (Li and Kane 2009). When yeast is dividing, there is a microvacuolation process and part of these vacuoles is transferred to the new cell. In addition, when there is microvacuolation, cells are ready to overcome certain general stresses. NOXI conditions displayed around 4 times more microvacuolation than OXI and around 2.5 times more than ETOXI condition (Table 18). This fact also corroborates that theory that NOXI conditions seems to have the best diagnostic for inoculation and triggering the second fermentation.

Cells rehydrated in water for 30 min (LSA-R) were fermenting because of the bad mitochondria organisation (Fig. 33A), which is to be expected just after the rehydration. Both microvacuolation results of LSA-W and P1 conditions were interesting (Table 19). Both conditions showed that mitochondria were well organised, which means the cells were prepared to respire. It was logical to observe this phenomenon under P1 conditions because of the presence of oxygen and the rapid exhaustion of sugars in the growth medium. However, it was surprising to find a microvacuolation value so low in yeast cells rehydrated in the defined base wine.

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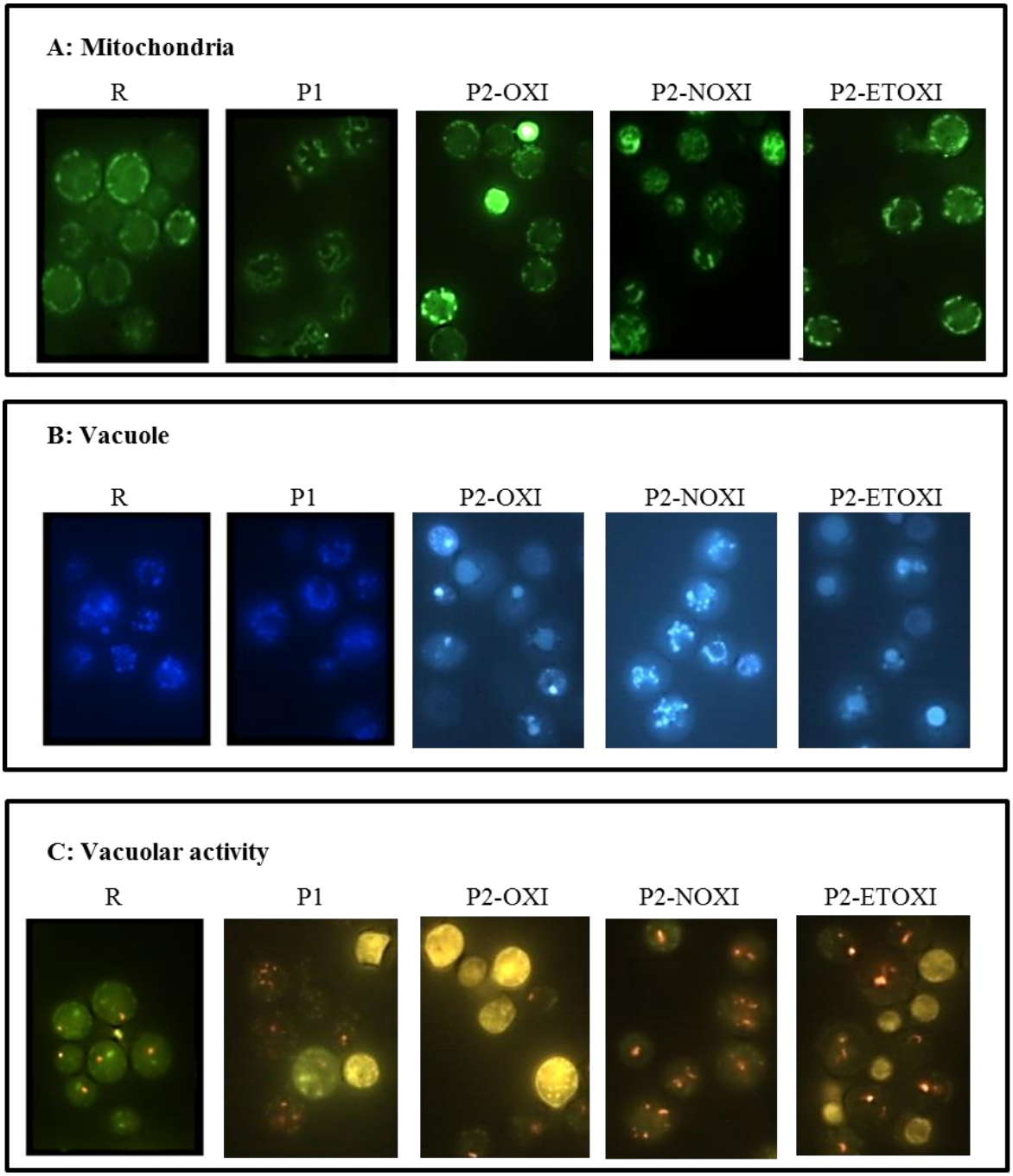

Figure 33. Yeast cells stained with fluorochromes to make a cell diagnostic and evaluate respiratory metabolism. For observing the mitochondrial organisation (green coloured mitochondria), 3, 3'-dihexyloxacarbocyanine iodide (DIOC) was used (panel A); vacuoles can be observed in blue by staining with 7-amino-4-chloromethylcoumarin (CMAC) (panel B) and vacuole activity was observed by FUN1 staining (red active vacuoles) (panel C). Damaged yellow cells can be observed when fluorochrome has entered the cell in excess. LSA-R, Active Dry Yeast Rehydrated in water for 30 min; P1, activation phase (24h); OXI, Aerobiosis + sugar addition; NOXI, Semi-anaerobiosis + sugar addition; ETOXI, Aerobiosis without sugar addition.

Table 19. Microvacuolation, vacuolar activity and ROS determinations of *Saccharomyces cerevisiae* EC1118 cells through the acclimated process at 25 ºC. The conditions used were: LSA-R, Active Dry Yeast Rehydrated in water during 30 min; LSA-W, Active Dry Yeast directly added in base wine during 30 min; P1, activation phase (24h); OXI, Aerobiosis + sugar addition; NOXI, Semi-anaerobiosis + sugar addition; ETOXI, Aerobiosis without sugar addition. Different superscripts indicate significant differences within a column ($P<0.05$). Three independent replicates were analysed.

Conditions	Microvacuolation (%)	Vacuolar activity (%)	ROS (%)
LSA-R	78.0 ± 1.1^{f}	80.0 ± 8.0^{d}	19.4 ± 1.7^{a}
LSA-W	19.9 ± 0.3^{b}	13.6 ± 1^{a}	100.0 ± 0.0^{b}
P1	33.4 ± 0.5^{d}	32.6 ± 3.3^{b}	51.9 ± 6.1^{d}
OXI	16.8 ± 0.2^{a}	46.8 ± 4.7^{c}	78.5 ± 7.7^{d}
NOXI	65.7 ± 0.9^{e}	84.2 ± 8.4^{d}	36.8 ± 4.3^{b}
ETOXI	25.2 ± 0.4^{c}	54.3 ± 1.4^{c}	55.1 ± 3.8^{c}

With respect to the vacuolar activity found in yeast cells rehydrated in either water or base wine, it was found that the former (LSA-R) had a similar value to NOXI cells, and the latter (LSA-W) had the lowest of all values observed (Table 19). These results show that the commercial active dry cells have good fitness when they are rehydrated in water and ready to use to ferment grape musts.

In contrast, when these yeast cells are directly placed in a medium other than water, such as wine, they suffered shock, probable due to the presence of ethanol and organic acids, which at pH 3.0 can disturb the vitality of yeast cells. For instance, tartaric acid, with a pK_{a1} of 3.01 (Ribéreau-Gayon *et al*. 2006), can easily enter in undissociated form and cause excessive acidification. This organic acid was not assimilated by *S. cerevisiae*. Our efforts were focused on observing a physiological diagnostic after cell acclimation, taking into account different conditions. It seemed that the results found for acclimated cells under NOXI conditions appeared to be critical with regards to the end of the secondary fermentation under our conditions. In short, disorganised mitochondria with a high fragmentation of active vacuoles gave cells an advantage in terms of reaching the final pressure of 6 bars in a defined base wine.

Chapter IV

Intracellular Reactive Oxygen Species (ROS) accumulation and Glutathione

During the acclimation process, the cells were subjected to the presence of oxygen, which may have some harmful effects on their cellular fitness, for example, inducing the accumulation of reactive oxygen species (ROS).

Aerobic growth conditions induce an oxidative stress caused by partially reduced forms of molecular oxygen, known as reactive oxygen species (ROS) (Jamieson 1998). These compounds, superoxide anion radical ($O2^{\bullet-}$), hydrogen peroxide (H_2O_2), alkylhydroperoxides (ROOH) and hydroxyl radical ($HO^{\bullet}$), react with DNA, lipids and proteins, altering their functions (Wolff *et al*. 1986). To detect the total ROS in the cells throughout the acclimation process, cells were pretreated with dihydroethidium and counted using fluorescence microscopy. ROS accumulation was increased under constant aeration, OXI and ETOXI conditions, compared to NOXI conditions. Besides, a significant decrease in the presence of non-fermentable carbon source (ETOXI) was observed with respect to OXI conditions (Table 19). In addition, ROS compounds accumulated in active dry yeasts directly inoculated in wine (LSA-W) were determined after 1, 3, 5 and 24 hours of incubation (data not shown). At all timing points, 100% of cells were accumulating ROS compounds. This fact suggests that acclimation is a necessary process for cells before performing the secondary fermentation. Interestingly enough, the lowest ROS accumulation found in LSA-R samples, for which water was used during the cell rehydration for 30 min at 37ºC, did not induce oxidative stress.

Meanwhile, to counteract the effect of increasing accumulation of ROS in some cells, it was decided to ascertain whether the glutathione content of cells was different. In other words, the aim was to identify whether cells could respond to this excessive content of molecular oxygen. Glutathione (γ-L-glutamyl-L-cysteinylglycine; GSH) is a tripeptide, the biological significance of which is mostly related to its free sulphydryl moiety of the cysteine residue, which confers unique redox and nucleophilic properties (Penninckx 2000). The major functions of GSH play a role in protection against oxidative stress, endogenous toxic metabolite detoxification and sulphur and nitrogen metabolism (Penninckx 2002). More than 90% of GSH is present in the reduced form in the cell (Li *et al*. 2004). GSH is regarded as an important defence molecule against oxidative damage in *S. cerevisiae* and is a non-enzymatic scavenger of ROS (Anderson 1998). Due to this fact, GSH is oxidised to GSSG by direct interaction with free radicals, thereby removing the

reactive oxidants from inside the cells. With respect to the reduced glutathione (GSH_{red}) content, some significant differences were observed depending on the acclimation process (Table 20). The cells that contained the highest level of GSH_{red} were LSA-R and NOXI, with 15.11 ± 1.08 and 13.24 ± 0.60 nM mg^{-1} D.W., respectively.

Table 20. Reduced glutathione (mM mg^{-1} Dry Weight) and ratio of reduced GSH and oxidised (GSSG) glutathione of *Saccharomyces cerevisiae* EC1118 cells through the acclimated process at 25 ºC. The conditions used were: LSA-R, Active Dry Yeast Rehydrated in water during 30 min; LSA-W, Active Dry Yeast directly added in base wine during 30 min; P1, activation phase (24h); OXI, Aerobiosis + sugar addition; NOXI, Semi-anaerobiosis + sugar addition; ETOXI, Aerobiosis without sugar addition. Different superscripts indicate significant differences within a column ($P<0.05$). Three independent replicates were analysed.

	GSH (nM mg^{-1} D. W.)	GSH/GSSG
LSA-R	15.11 ± 1.08^{c}	7.97 ± 0.64^{b}
LSA-W	10.32 ± 0.98^{a}	8.08 ± 2.49^{b}
P1	10.84 ± 0.69^{a}	8.23 ± 2.41^{b}
OXI	9.50 ± 0.25^{a}	5.96 ± 1.05^{ab}
NOXI	13.24 ± 0.60^{b}	7.58 ± 0.42^{b}
ETOXI	10.72 ± 0.40^{a}	3.36 ± 0.52^{a}

Under the other conditions, cells had a similar content, ranging 9.50 ± 0.25 to 10.84 ± 0.69 nM mg^{-1} D.W. (OXI, the lowest; P1, the highest). Another interest feature was the decrease of GSH_{red} content in yeast directly rehydrated in wine (LSA-W), compared to cells rehydrated in water (LSA-R). A relationship could be established between this decrease and the 100% accumulation of ROS found in LSA-W cells. Indeed, one part of GSH_{red} had to be used by cells to respond rapidly to the oxidative stress generated by the shock of ethanol and perhaps also by the presence of organic acids at pH 3.0. Unfortunately, the ratio between GSH_{red} and GSSG for both conditions was similar and, consequently, cells placed under LSA-W conditions were unable to use GSH to counteract this harmful stress. The non-utilisation of GSHred by LSA-W cells could be explained by the fact that these cells presented low vacuolar activity (see Table 19). The GSH pool in cells is equally distributed between the cytosol (synthesis) and vacuoles (degradation) in a medium containing nitrogen (Mehdi and Penninckx 1997). Moreover, some enzymes

related to the biosynthesis of GSH were repressed by ammonium ions as nitrogen sources (Jaspers *et al*. 1985). For these reasons, cells directly rehydrated in wine had some difficulties to recover their vitality. However, cells placed under OXI and ETOXI conditions had a lower GSH content than NOXI cells. Furthermore, these cells presented a significant decrease in the GSH/GSSG ratio with respect to NOXI cells.

Glycogen and trehalose estimations of acclimated cells

Glycogen and trehalose are the two glucose carbohydrate reserves of yeast cells. Their presence confers survival and reproductive advantages to the cell (François and Parrou 2001). The purpose of this section was to ascertain whether the acclimation conditions affected the glycogen and trehalose content of cells. Glycogen determination was performed as described by Quain and Tubb (1983). This convenient procedure is routinely used in the beer industry to monitor changes in yeast composition during storage and to compensate for variations in the glycogen content during the yeast pitching.

Depending on the acclimation process used, a high glycogen content was generally observed in the case of LSA-R, P1, NOXI and ETOXI conditions, with an average of *ca* 118 μg mg^{-1} D.W. Of these conditions, cells taken at P1 state showed the most remarkably significant accumulation of glycogen at 140.50 μg mg^{-1} D.W. At the other end of the scale, cells under LSA-W and OXI conditions had the lowest contents with 15.3 and 78.8 μg mg^{-1} D.W., respectively. As commented by François and Parrou (2001), in the case nutrient starvation, the lack of one essential nutrient causing an arrest of growth in G_0/G_1, yeast cells synthesised and accumulated more glycogen. Under P1 and ETOXI conditions, cells were really placed in this situation, although they could use a non-fermentable carbon source such as ethanol. No cell multiplication was observed under these conditions (data not shown). However, the trehalose content in cells significantly varied depending the acclimation process, which enabled the classification of the yeast cells into three groups: cells with a lower content, at around 20 μg mg^{-1} D.W. (LSA-W, OXI and ETOXI); cells with an intermediate content of 36 μg mg^{-1} D.W. (P1); and cells with the highest content, at *ca* 60 μg mg^{-1} D.W. (LSA-R and NOXI). Our results, obtained using a new GC/MS analysis for measurement of intracellular trehalose content, were in agreement with those recorded by Moonsamy *et al*. (1995), which ranged between 30 and 130 mg for beer yeast cells. In the end, by comparing the medium of rehydration for the active dry yeast (water *vs*

ethanol), a rapid depletion of the two storage carbohydrates was observed in the presence of ethanol. The consumption was around 70% and 86% for trehalose and glycogen, respectively (Figure 34). Similar results were reported by Novo *et al.* (2003), who observed an 80% depletion of the initial trehalose content after 60 minutes, when yeast cells were rehydrated in synthetic grape must. However the glycogen content remained unchanged.

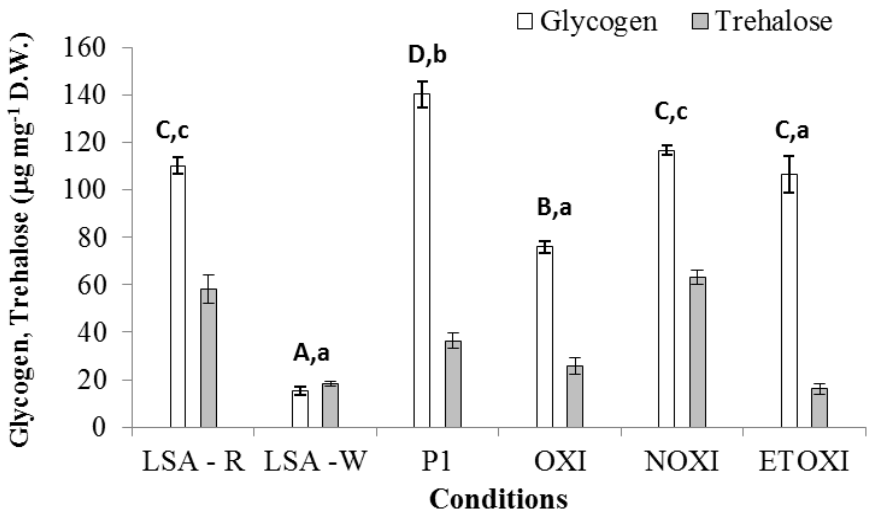

Figure 34. Glycogen and trehalose contents of *S.cerevisiae* EC1118 cells through the acclimation process at 25ºC. The conditions applied were: LSA-R, Active Dry Yeast Rehydrated in water for 30 min; LSA-W, Active Dry Yeast directly added to base wine for 30 min; P1, activation phase (24h); OXI, Aerobiosis + sugar addition; NOXI, Semi-anaerobiosis + sugar addition; ETOXI, Aerobiosis without sugar addition. Different letters indicate a significant difference within glycogen (upper case) and trehalose (lower case) ($P<0.05$). Three independent replicates were analysed.

Lipid composition of acclimated cells

It is well known that ethanol increases the membrane fluidity of cells by increasing the level of unsaturation in fatty acids (D'Amore and Stewart 1987; Alexandre *et al*. 1994; You *et al*. 2003), which also increases its ethanol tolerance in the growth medium (Arroyo-López *et al*., 2010). However, to synthesise unsaturated fatty acids and ergosterol in *S. cerevisiae* cells, oxygen is indispensable (Andreasen and Stier 1953, 1954). Under our conditions, the presence or absence of oxygen during the acclimation process of yeast cells (minimal presence of 10% (v/v) of ethanol) was evaluated to ascertain whether the lipid composition was really another important factor to consider.

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The relation between unsaturated fatty acids (UFA) and saturated fatty acids (SFA) was significantly different depending on the acclimation process (Table 21). In the presence of oxygen with a carbon source, cells had a UFA/SFA ratio of 2.58 for fermentable carbon source and 2.84 for non-fermentable sources, under OXI and ETOXI conditions, respectively. In the case of NOXI acclimation, in the absence of oxygen and presence of a fermentable carbon source, cells only reached a UFA/SFA ratio of 1.60. It was interesting to note that the differences observed in these UFA/SFA ratios were mainly due to the relation between oleic and stearic acids (data not shown).

For Mannazzu *et al*. (2008) an increase of C16/ total fatty acids increased ethanol tolerance and yeast cell viability. The higher the ratio of these acids, the higher the UFA/SFA. However, the medium of cell rehydration seemed to play a role in the UFA/SFA values. In water, rehydrated cells (LSA-R) had a ratio of 2.09, while cells directly rehydrated in wine (LSA-W) had a ratio of 1.73. It is very difficult to explain this curious result by the fact that the 10% ethanol content could partially deplete the UFA level in the cells in 30 minutes. Lastly, in the case of cells reactivated, under P1 conditions, a decrease of UFA/SFA was observed even though oxygen was present. Not only does oxygen have a real effect on the lipid biosynthesis but so does the type of carbon source and its level in the growth medium.

With respect to the sterol composition, it was interesting to note that no changes occurred in the ergosterol percentage regardless of the conditions applied during the acclimation (data not shown). In the case of NOXI or P1 conditions, the ergosterol/squalene ratio was significantly lower than the other conditions, demonstrating that either an absence of oxygen or the presence of a high sugar content in the growth medium lead to an increase in squalene. However, in the case of catabolite derepression (exhaustion of fermentable sugars, ETOXI condition), the results were in agreement with those of Quain and Haslam (1979). Indeed, a decrease in the ergosterol/zymosterol ratio was observed for one ergosterol content slightly higher in ETOXI than under both OXI and NOXI conditions. As described by Quain and Haslam (1979), steryl esters increase when yeast cells grow in ethanol (ETOXI). Under our conditions, this fact was observed with the ratio ergosterol/zymosterol. As mentioned earlier with respect to the UFA/SFA ratio, cells directly inoculated in wine (LSA-W) had a significantly lower ergosterol/squalene ration than in the case of the water-rehydrated cells (Table 21).

Table 21. Lipid composition of *Saccharomyces cerevisiae* EC1118 cells through the acclimated process at 25 ºC. The conditions used were: LSA-R, Active Dry Yeast Rehydrated in water during 30 min; LSA-W, Active Dry Yeast directly added in base wine during 30 min; P1, activation phase (24h); OXI, Aerobiosis + sugar addition; NOXI, Semi-anaerobiosis + sugar addition; ETOXI, Aerobiosis without sugar addition. SFA, Saturated Fatty Acids; UFA, Unsaturated Fatty Acids. Different superscripts indicate significant differences within a column ($P<0.05$). Three independent replicates were analysed.

	UFA/SFA	Ergosterol/Squalene	Ergosterol/Zymosterol
LSA-R	2.09 ± 0.05^{b}	10.71 ± 0.50^{d}	2.11 ± 0.17^{a}
LSA-W	1.73 ± 0.09^{a}	7.80 ± 0.31^{bc}	2.79 ± 0.03^{c}
P1	1.58 ± 0.04^{a}	6.74 ± 0.63^{ab}	2.48 ± 0.13^{b}
OXI	2.58 ± 0.11^{c}	8.70 ± 0.45^{c}	3.37 ± 0.06^{d}
NOXI	1.60 ± 0.02^{a}	6.40 ± 0.53^{a}	3.30 ± 0.09^{d}
ETOXI	2.84 ± 0.14^{d}	9.99 ± 0.69^{d}	3.00 ± 0.06^{c}

Optimisation index of the cell acclimation process.

To perform a better acclimation process of yeast cells for the production of sparkling wines, optimisation indexes were calculated based on observed physiological parameters and the secondary fermentation performance of cells. For the latter, results obtained in Table 18 were normalised in a range from 0 to 1 and summed. In other words, for the five secondary fermentation parameters analysed, the worst and best optimisation indexes (O.I.) should be 0 and 5, respectively. In decreasing order, the best acclimated cell conditions for ensuring the secondary fermentation was NOXI, with an O.I. of 3.76, followed by ETOXI (2.67), OXI (2.06) and LSA-W (1.67). Cells acclimated under the NOXI conditions enabled the secondary fermentation to be fully accomplished in bottle at 25ºC, the lowest sugar content, the highest degree of alcohol, 6-bar pressure and the best viability when the pressure reached 4 bars.

However, when seven physiological parameters are used to determine the O.I. of the acclimation conditions (Table 22), the ranking was as follows: NOXI (5.31), ETOXI (2.64), OXI (2.28) and LSA-W (1.32). The choice of only seven parameters was based on the correlation results between the different parameters (data not shown). Some of these results showed a good correlation between the parameters and they could be eliminated to

reduce the number of parameters involved in calculating the O.I., while others were not important in terms of the estimation of the O.I. By determining both optimisation indexes, for the secondary fermentation and physiological parameters, a relationship was found (Fig. 35) with a good coefficient of determination, R^2 = 0.9065. This correlation was improved by changing the preference order in relation to the two lipid ratios, ergosterol/squalene and C18:1/C18. In fact, it was assumed that a slight rigidity in terms of the cell membrane should be an advantage for cells in an ethanol-growing medium. The numbers and our hypothesis support this assumption.

Table 22. Overview of optimisation index of both secondary fermentation and acclimation process at 25ºC. LSA-W, Active Dry Yeast directly added in base wine during 30 min; OXI, Aerobiosis + sugar addition; NOXI, Semi-anaerobiosis + sugar addition; ETOXI, Aerobiosis without sugar addition.

Secondary fermentation	**Conditions**			
	LSA-W	**OXI**	**NOXI**	**ETOXI**
Final sugar content	0.35	0.00	0.59	0.35
Alcoholic degree	0.92	0.94	1.00	0.97
Days to reach 6 bars	0.00	0.00	0.65	0.00
Days to reach 4 bars	0.00	0.24	0.52	0.38
Living cells at 4 bars	0.40	0.88	1.00	0.97
Optimisation Index 1	**1.67**	**2.06**	**3.76**	**2.67**
Acclimation process				
Glycogen	0.13	0.65	1.00	0.91
Trehalose	0.29	0.41	1.00	0.26
Mirovacuolation	0.30	0.26	1.00	0.38
Vacuolar activity	0.16	0.56	1.00	0.64
ROS	0.00	0.22	0.63	0.45
Ergosterol/Squalene	0.22	0.13	0.36	0.00
C18:1/C18	0.22	0.07	0.32	0.00
Optimisation Index 2	**1.32**	**2.28**	**5.31**	**2.64**

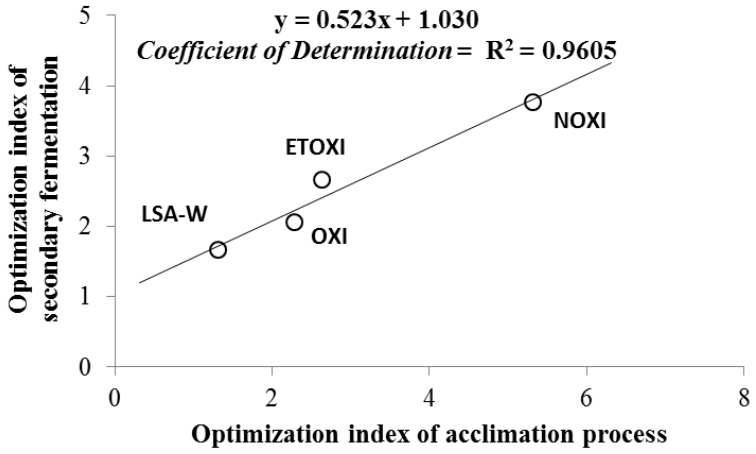

Figure 35. Relationship between optimisation index of acclimation process and secondary fermentation parameters at 25ºC. LSA-W, Active Dry Yeast directly added to base wine for 30 min; OXI, Aerobiosis + sugar addition; NOXI, Semi-anaerobiosis + sugar addition; ETOXI, Aerobiosis without sugar addition. Three independent replicates were analysed.

4. Conclusions

In conclusion, the cells acclimated under NOXI conditions, i.e. fermentative metabolism without an excess of aeration, had an advantage with respect to the other conditions because of the secondary fermentation accomplished under our conditions of a defined base wine and 25ºC. In other words, cells have to have a good carbohydrate reserve, a high vacuolar activity and a slightly rigid membrane in terms of the presence of higher level of squalene and stearic acid. Further research should be carried out to confirm these results at low temperatures and using industrial base wine. However, although the commercial yeast cells being directly inoculated in the defined base wine is the worst way of conducting the secondary fermentation; it is interesting to note that the same cells, when rehydrated in water, had a similar O.I. to NOXI cells. An unknown phenomenon occurs when cells are put into a low pH medium, containing ethanol.

Supplementary information

Table 23. Amino acid composition of medium during the acclimation process. P1, activation phase (24h); OXI, Aerobiosis + sugar addition; NOXI, Semi-anaerobiosis + sugar addition; ETOXI, Aerobiosis without sugar addition. Others amino acids: aspartic acid, glutamic acid, serine, histidine, glycine, threonine, arginine, alanine, γ-aminobutyric acid, proline and tyrosine. Numbers were an average of two independent replicates.

	Acclimation condition			
	P1	**OXI**	**NOXI**	**ETOXI**
Total nitrogen (mg L^{-1})	**512.90**	**10.15**	**15.73**	**73.41**
Main amino acids (% of Total Nitrogen)				
Ammonium	**36.6**	**7.6**	**4.3**	**1.0**
Glutamine	**24.9**	**0.0**	**0.0**	**89.0**
Lysine	**0.5**	**13.9**	**12.5**	**0.1**
Ornithine	**1.0**	**8.6**	**12.2**	**0.8**
Tryptophan	**1.9**	**11.9**	**6.1**	**1.1**
Valine	**1.4**	**10.6**	**7.0**	**0.4**
Phenylalanine	**3.8**	**11.9**	**7.1**	**1.6**
Methionine	**0.8**	**5.8**	**27.2**	**0.0**
Cysteine	**0.6**	**9.4**	**8.5**	**4.3**
Leucine	**4.1**	**8.8**	**6.5**	**0.8**
Isoleucine	**0.1**	**1.3**	**2.4**	**0.0**
Others	**24.4**	**10.3**	**6.3**	**0.6**

Amino acid analysis

Fermentation samples were taken every 24 h and derivatized with diethyl ethoxymethylenemalonate (DEEMM) to identify and quantify the individual amino acids and the presence of ammonium ions (Gómez-Alonso et al., 2007). They were then analysed by high performance liquid chromatography (HPLC) using a device (Agilent 1100 Series) equipped with a low gradient quaternary pump, UV detector (Agilent Technologies, Germany) and a Hypersil ODS column (Agilent Technologies, Germany) measuring 4.6 x 250 mm x 5 μm. The mobile phase consisted of solvent A (25 mM acetate buffer at pH 5.8 with 0.02 (w/v) sodic acid) and solvent B (80:20 acetonitrile/methanol). The flow rate was 0.9 mL min^{-1}, the oven temperature was 20 ºC, and the self-sampler temperature was 4 ºC. The identification of each amino acid was carried out using standards. The purities of all the standards used ranged between 99 and 100%, all of which were purchased from Sigma-Aldrich. All analyses were run with the Agilent ChemStation Plus software program (Agilent Technologies, Germany).

Chapter IV

Article 5

Transcriptomic analysis of acclimation of *Saccharomyces cerevisiae* to ethanol for sparkling wine production

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Abstract

Secondary fermentation with the traditional method *Champenoise* is a hard challenge for yeasts trying to survive and ferment. Even though it has been done for ages by winemakers, yeast acclimation to ethanol and other stresses involved in this process, it is not completly understood or even controlled. Some industrial protocols are available but the effect of oxygen and sugar content on the gene expression (transcriptoma) of *S. cerevsiae* is not very well known under these conditions. In the present chapter, it is shown that, depending on these two factors, cells give different response based on the differential study of genes expression. Our results show a slight overexpression of genes related under lipid metabolism (*ERG5, ERG10, ERG13, OLE1, YEH1, EEB1*) in the strain acclimated fermentative conditions (NOXI). In contrast, some genes, such as *GUT1* or *MDH1*, are preferentially activated in respiratory and fermentative-respiratory conditions, respectively. Only yeast undergoing fermentative metabolism during acclimation procedure achieves 6 bars, thereby completing the fermentation.

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Introductory highlights

A number of industrial protocols are available for acclimating cells to secondary fermentation, but sometimes they ae not suitable proceadures to follow. At the beginning of the secondary fermentation, yeasts still have a long lag phase, even though they have been acclimated before being inoculated.

Not a lot of literature is available regarding secondary fermentation and acclimation procedures. However, the genetic machinary responsible for ethanol tolerance, the main stress in alcoholic fermentations, has been described in depth in the literature and is reviewed in the present thesis.

Teixeira *et al.* (2009) have identified over 250 genes required for maximal tolerance to ethanol in a comprehensive study using mutants of the BY4742 laboratory strain. Most encoding proteins involved in intracellular trafficking, transcription, pH homeostasis and peroxisome biogènesis. The effects of ethanol on peroxisome proliferation, vacuolar acidification and cell wall resistance were assessed.

Genes confering ethanol resistance unfortunatelly, have been shown to be high dependent on the strain and the growth conditions.

The main interaction networks maps of proteins that determine ethanol tolerance are (i) Vacuolar protein sorting; (ii) V-ATPase complex assembly and (iii) Peroxisome protein import machinary.

The aim of the present work is to compare the transcriptomic profiles of cells acclimated under different conditions to ascertain which genes are up- or down- regulated in order to relate it to our phenotype. Thus, a better cell preparation can be developed to fully ensure complete secondary fermentation.

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Results

As shown in previous chapter, there are three sets of conditions with different metabolic scenarios:

(i) ETOXI conditions represent a respiratory metabolism for cells that consume ethanol.

(ii) OXI conditions indicate that cells are undergoing fermentative-respiratory metabolism in the presence of oxygen because the concentration of sugar by the end of acclimation is below the sugar level of the *Crabtree* effect. However, it is not clear whether cells respire based on results obtained by the mitochondria analysis (previous chapter). Cells seem to start respiring by the end of acclimation process when *Crabtree* effect is decreased.

(iii) NOXI conditions represent cells with a fermentative metabolism in the absence of oxygen.

Cells acclimated under the last of these conditions are the only ones to reach the pressure of 6 bars in bottles and, thus, the only set of conditions with which secondary fermentation is completed.

The transcritpome of the most favorable set of conditions, NOXI, was compared with other conditions, OXI and ETOXI. Genes up-regulated at least two times in comparison to other conditions (as shown in Figure 36) are represented in Figure 37 and genes down-regulated under NOXI conditions from other specified conditions are shown in Figure 38. All of them are presented in Figure 36, showing an overall view of transcriptomic analysis.

Focusing firstly on Figure 36, it can be obseved than many genes are up regulated under the NOXI conditions in comparison to the others. These genes may hold, the key to explaining why NOXI is the only set of conditions that achieved secondary fermentation, reaching the pressure of 6 atm. In addition, cells acclimated under these conditions share many genes up- or down-regulated with cells of each of the other two sets of conditions. This can particularly, be seen in the lower part of Figure 36. For instance, NOXI and OXI conditions share *HXT3, HST17, HSP30* and *RPS20* and *ASC1* for up- and down-regulated genes; while, with ETOXI conditions, the shared up- and down-regulated genes were *INO1, MDH1* and *ENO2* and *CWP1*, for example.

Not many genes have significative variations, but, some metabolic pathways were affected differently depending on the conditions. These metabolic pathways include over-expression of genes related to lipid metabolism, such as *ERG5*, *ERG10*, *CYB5*, *OLE1*, *EEB1* and *YEH1* for NOXI, with respect to OXI and ETOXI conditions.

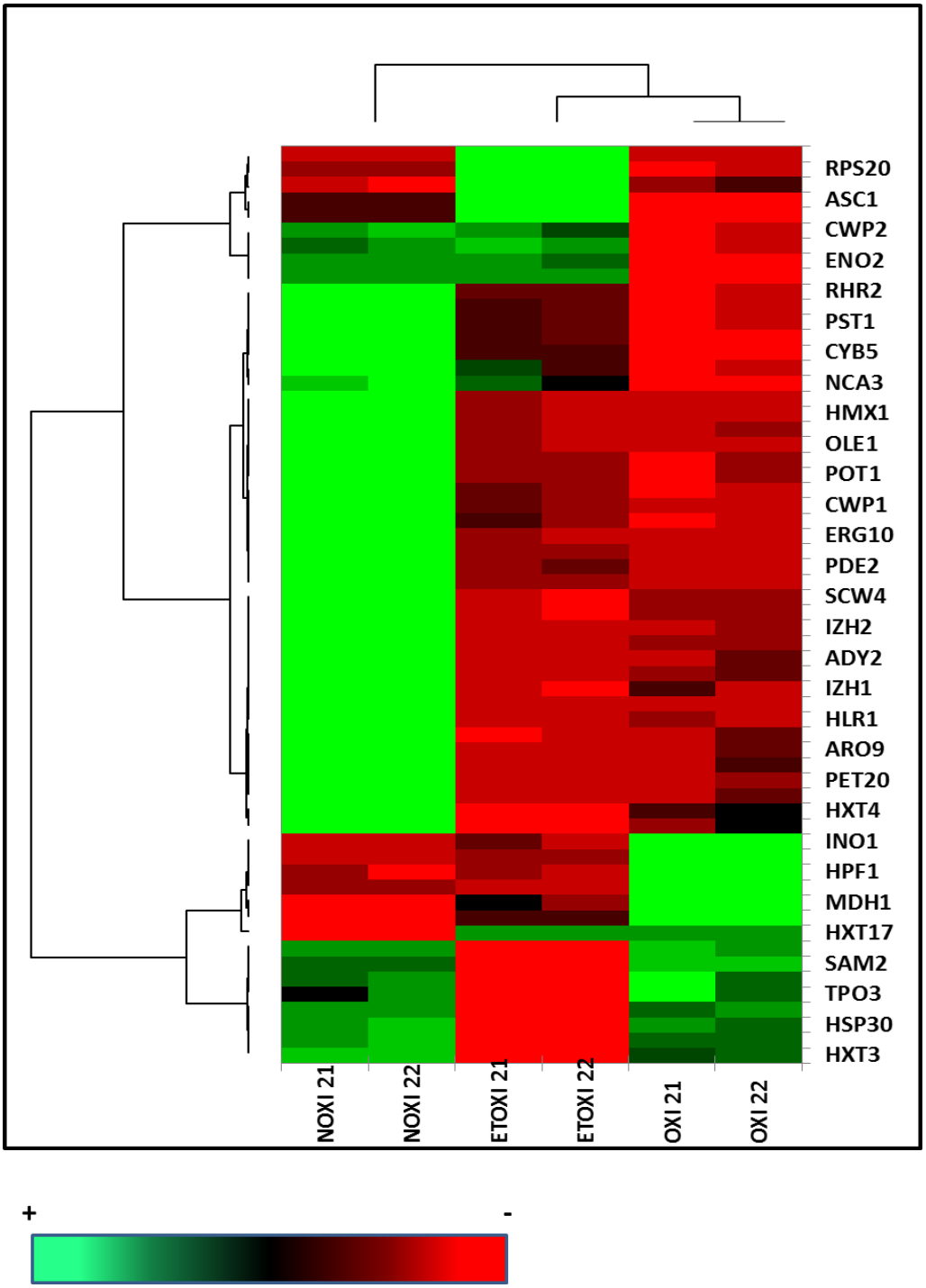

Figura 36. Representation of genes down-regulated and up-regulated in NOXI in comparison with other conditions; OXI and ETOXI in *Saccharomyces cerevisiae* EC1118 at 25 ºC during acclimaton process. OXI, Aerobiosis + sugar; NOXI, Semi-anaerobiosis + sugar; ETOXI, Aerobiosis - sugar.

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Meanwhile, other interesting genes were up-regulated, such as *TIR4*, which is related to the mannoproteins of cellular wall and which was overexpressed under anaerobic conditions (NOXI). In addition, the same occurred in the case of *HLR1*, which is related to the preservation of the integrity of the cell membrane. Induction of transcritption for genes *RHR2* (*GPP1*) and *HOR2* (*GPP2*) is the response to hypoxic conditions and is related to glycerol formation under NOXI in comparison to OXI conditions (Figure 37). Apart from balancing redox, glycerol has the function of osmoregulating in yeast cells metabolism when extracellular water activity decreases in the growth medium (Blomberg and Radler 1992).

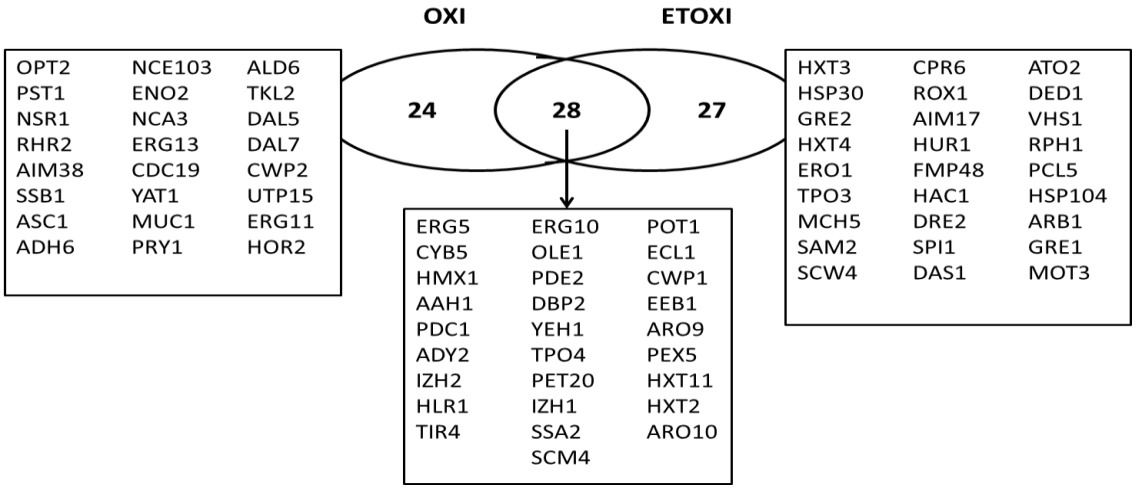

Figura 37. Up-regulated genes under NOXI conditions in comparison with OXI and ETOXI conditions in *S. cerevisiae* EC1118 at 25ºC at the end of acclimation process. OXI, Aerobiosis + sugar; NOXI, Semi-anaerobiosis + sugar; ETOXI, Aerobiosis - sugar.

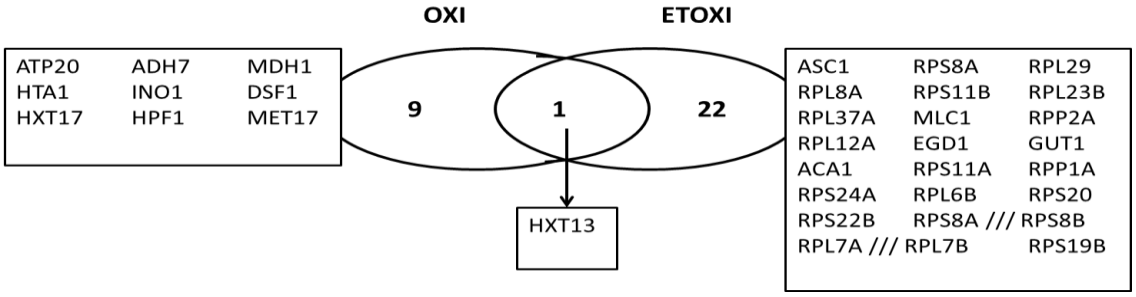

Figura 38. Down-regulated genes under NOXI conditions in comparison with OXI and ETOXI conditions in *S. cerevisiae* EC1118 at 25ºC at the end of acclimation process. OXI, Aerobiosis + sugar; NOXI, Semi-anaerobiosis + sugar; ETOXI, Aerobiosis - sugar.

In Figure 38, it is interesting to observe that one of the activated genes under OXI compared to NOXI conditions is *HPF1*, which is related to excretion of mannoproteins. The fact could limit the formation of protiens agregations in white wines (Brown *et al.* 2007). On the other hand, condition ETOXI showed anoverexpression of *GUT1* caused by the presence of a non-fermentable carbon source.

Many genes have been reported to be related to ethanol stress, which is considered to be the main stress affecting cell viability during the fermentation process. A great number of theses experiments have been performed using genomics and deletion mutants. Based on our limited review (see introduction), comparing genomics with transcriptomics in this field, there is support for our work. As can be seen in Figure 36, many genes are up-regulated under NOXI conditions in comparison to others.

Ergosterol biosynthesis is important for overcoming ethanol stress. In our study, not only *ERG5*, but also *ERG10* were up-regulated under our optimal set of conditions compared to the other conditions, as described by Kumar *et al.* (2008) for *ERG5* and Voorst *et al.* (2006) for *ERG10*. Moreover, Teixeira *et al.* (2012) found that other related genes from the same pathway, such as *ERG2*, *ERG6* and *ERG24* confer ethanol resistance to yeast cells.

In our experiment, *DBP2* was up-regulated under NOXI conditions but no work published to date support this fact. However; Teixeira *et al.* (2012) cited *DBP7* as being a determining factor in conferring ethanol resistance. These genes are related to transcritpome machinery; specifically, are involved in ribosomal biogenesis.

Our transcritpome study also showed that *ARO9* and *ARO10* were up-regulated for under NOXI conditions. Yoshikawa *et al.* (2009) have found *ARO1*, *ARO2* and *ARO7*. All are involved in aromatic amino acids catabolism.

PEX5 have been reported as an up-regulated gene under NOXI conditions as described by Teixeira *et al.* (2012), who; also found relation between ethanol resistance and *PEX1*, *PEX2*, *PEX3*, *PEX4*, *PEX 8*, *PEX10*, *PEX12*, *PEX14*, *PEX19* and *PEX22*. All of these are related to peroxisomal organisation and biogenesis.

A gene related to protein syntheisis in mitochondria, *PET20* was found in transcritpomic analysis to be up-regulated under NOXI conditions. This gene has not been

cited by other authors before. However, Teixeira *et al.* (2012) identified the gene *PET112* as conferring ethanol resistance and Kumar *et al.* (2008) reported *PET123*.

Conclusions

Different conditons in terms of sugar and oxygen availability were tested during the yeast acclimation process for sparkling wine production. The sampling point was at the end of the acclimation process and transcriptomic comparative analyse were carried out. Although few significant differences were found, some interesting findings were gathered.

Many genes have been up-regulated under NOXI conditions, which have the best phenotype. In other words, it is the only set of conditions which enables cells to reach the pressure of 6 bars in bottles during the secondary fermentation.

Some of those genes are supported by other authors to confer ethanol resitance to yeasts. Or at least it has been found genes related to metabolic pathways envolving those genes. Genes affected came from a sort of categories confirming ethanol has a complex and damage effect on yeast cells. Also it is pointed out that this effect is depending on cell culture and yeast strain. Further work should be done to completely understand this complex metabolic scenario of acclimation and secondary fermentation.

(i) Genes related to cell wall integrity, such as *HLR1*; required for the correct integrity of cell membrane and *TIR4*; needed to synthetise mannoproteins from the cell wall.
(ii) Genes related to the lipid metabolism, such as *ERG5, ERG10, CYB5, OLE1* and *EEB1*.
(iii) Genes related to transcriptomic machinery, such as *DBP2*.
(iv) Genes related to amino acid catabolism, such as *ARO9* and *ARO10*.
(v) Genes related to peroxisome, such as *PEX*.
(vi) Genes related to protein synthesis in mitochondria, such as *PET2*.
(vii) Genes related to glycerol formation, such as *RHR2* and *HOR2*.

Some of these genes are supported by other authors, who observe that they confer ethanol resistance to yeasts, and others have at least been found to be related to metabolic pathways involved in these genes. The genes affected came from a sort of category,

confirming that ethanol has a harsh and complex effect on yeast cells. It was also observed that this effect depends on the cell culture and yeast strain. Further work should be carried out to gain a full understanding of this complex metabolic scenario of the acclimation process and secondary fermentation.

Chapter IV

Article 6

Lipidomic approach to ethanol acclimation of *Saccharomyces cerevisiae* for producing sparkling wines

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In preparation for submission

Chapter IV

Abstract

The production of sparkling wines is the result of two consecutive alcoholic fermentations. The first is the fermentation of the grape juice, through which the base wine is obtained. Base wine is fermented again by yeast to enable the effervescence process in bottles. As the use of active dry yeast after rehydration is usually direct and simple, the inoculation of base wine by yeast requires certain preparations to ensure good yeast growth under hard conditions: 10% (v/v) of ethanol, low pH, low temperature, etc.

The lipid bilayer is the first barrier that yeasts have to overcome in terms of stress coming from the media. This is why its composition is so important when trying to survive and ferment in order to maintain its fluidity and permeability. Analysing lipid cell composition, the aim is to ascertain which of them corresponds to a better cell state in that scenario. Although there are industrial protocols that are widely used to adapt wine yeast to these conditions, information is scarce with respect to lipid composition or even the physiological state of strains during the acclimation process. In this study, a commercial wine yeast was acclimated according to different conditions in terms of aeration and sugar content, and used to inoculate a defined base wine to ferment at 25ºC in the bottle. A thorough lipid analysis of yeasts cells during the process was carried out.

In this chapter, the lipid composition of yeast cells during the process of acclimation is studied by means of different methodological approaches towards the main lipid classes. Thus, the use of chromatography techniques (GC and TLC) with different types of detection (FID and MS) enabled us to perform this study.

Chapter IV

Introductory highlights

As seen in previous chapters, the proceadure of sparkling wines by the traditional *Champenoise* method needs two consecutives fermentations. Many stresses accumulate during the second fermentation and the synergic effect of some of these make fully successful fermentation a hard challenge for *Saccharomyces cerevisiae*.

A progressive acclimation of *S. cerevisiae* is required before being inoculated in the base wine, taking into account the synergistic effect of some of these stress factors. The acclimation process was defined by Tai *et al*. (2007). During this period, the yeast can activate its stress metabolism response associated to especially high ethanol content. The main features of this method were first described by Françot and Geoffroy (1957) and the effects of ethanol on yeast during its acclimation were analysed by Juroszek *et al*. (1987). Some variations of the yeast acclimation process and its subsequent use, known as the *Champenoise* method, have been described by Valade and Moulin (1983), and Laurent and Valade (1994, 1998, 2007).

Ethanol affects fluidity in the plasma membrane. Generally, ethanol tolerance is associated with a high degree of fatty acid (FA) unsaturation, for which oxygen is required (Arneborg *et al.* 1995; Valero *et al.* 2001). The membrane structure becomes slacker under ethanol stress. The plasma membrane should retain its structural integrity as far as possible in order to counteract the effect of ethanol. On the basis of this hypothesis, the factors that make the plasma membrane more rigid are important for cells to maintain membrane stability and survive under conditions of ethanol stress. In terms of lipids, for instance, ergosterol increases membrane rigidity to antagonize the increased fluidity that is induced by high concentrations of ethanol (Ding *et al*. 2009), and some unsaturated fatty acids are also considered as the protective metabolites for *S. cerevisiae* cells under ethanol stress, as C16 and C18 (Ding *et al*. 2010). A previous review discussing ethanol tolerance in *S. cerevisiae* indicates that the role of UFAs is complex and the function is not straightforward (Ding *et al*. 2009). It is suggested by Aguilera *et al.* (2006) that lower levels of C16:1 acid and higher level of C16 and C18:1 help the membrane to overcome the stress, regulating its integrity. The role of FAs in cell membranes in a medium with ethanol should be researched further.

In this chapter, the lipid composition of yeast cells during the process of acclimation is studied by means of different methodological approaches towards the main

lipid classes. Thus, the use of chromatography techniques (GC and TLC) with different types of detection (FID and MS) enabled us to perform this study.

In a previous paper by this group, the acclimation of *S. cerevisiae* cells was reported with respect to their performance during the secondary fermentations carried out in bottles at 25ºC. To this end, the effect of aeration and external sugar content during the cell acclimation process on certain physiological parameters was analysed. These parameters were related to the carbohydrate reserve (glycogen and trehalose), the activity of vacuoles, ROS accumulation and the lipid metabolism. The results showed that a correlation can be established between the achievement of secondary fermentation and certain physiological parameters analysed during the cell acclimation process.

In the present paper, the objective is to acquire more in-depth knowledge of the lipid composition of cells exposed to a situation as stressful as the acclimation process of yeast for sparkling wine production, and ascertain the best composition for cells to survive, avoiding sluggish fermentation.

Chapter IV

Results and discussion

1. *Fatty acid composition*

It is well known that ethanol increases the membrane fluidity of cells by increasing the level of unsaturation in fatty acids (D'Amore and Stewart 1987; Alexandre *et al* 1994; You *et al*. 2003), which also increases its ethanol tolerance in the growth medium (Arroyo-López *et al*. 2010). However, to synthesise unsaturated fatty acids and ergosterol in *S. cerevisiae* cells, oxygen is indispensable (Andreasen and Stier 1953, 1954). Under our conditions, the presence or absence of oxygen during the acclimation process of yeast cells (minimal presence of 10% (v/v) of ethanol) was evaluated to ascertain whether the lipid composition was really another important factor to consider.

The relation between UFAs and SFAs was significantly different depending on the acclimation process (Borrull *et al.* 2016). In the presence of oxygen with a carbon source, cells had a UFA/SFA ratio of 2.58 for fermentable carbon source and 2.84 for non-fermentable sources, under OXI and ETOXI conditions, respectively. In the case of NOXI acclimation, in the absence of oxygen and presence of a fermentable carbon source, cells only reached a UFA/SFA ratio of 1.60. It was interesting to note that the differences observed in these UFA/SFA ratios were mainly due to the relation between oleic and stearic acids (data not shown). For Mannazzu *et al*. (2008), an increase in the ratio of C16/ total fatty acids increased ethanol tolerance and yeast cell viability. The higher the ratio of these acids, the higher the UFA/SFA. However, the medium of cell rehydration seemed to play a role in the UFA/SFA values. In water, rehydrated cells (LSA-R) had a ratio of 2.09, while cells directly rehydrated in wine (LSA-W) had a ratio of 1.73.

It is very difficult to explain this curious result by the fact that the 10% ethanol content could partially deplete the UFA level in the cells in 30 minutes. Lastly, in the case of reactivated cells, under P1 conditions, a decrease of UFA/SFA was observed even though oxygen was present. Not only does oxygen have a real effect on the lipid biosynthesis but so does the type of carbon source and its level in the growth medium.

Chapter IV

Table 24. Fatty acid composition of *S. cerevisiae* EC1118 cells throughout the acclimated process at 25 ºC. The conditions used were: LSA-R, Active Dry Yeast rehydrated in water during 30 min; LSA-W, Active Dry Yeast directly added to base wine for 30 min; P1, activation phase (24h); OXI, Aerobiosis + sugar addition; NOXI, Semi-anaerobiosis + sugar addition; ETOXI, Aerobiosis without sugar addition. SFA, Saturated Fatty Acids; UFA, Unsaturated Fatty Acids. Different superscripts indicate significant differences within a column ($P<0.05$). Three independent replicates were analysed.

	C16:1/C16	C18:1/C18	Average chain length
LSA-R	1.25 ± 0.00^{b}	3.48 ± 0.10^{a}	17.10 ± 0.02^{a}
LSA-W	1.18 ± 0.05^{b}	2.62 ± 0.17^{c}	17.02 ± 0.02^{bc}
P1	1.13 ± 0.04^{b}	2.30 ± 0.06^{d}	16.99 ± 0.01^{c}
OXI	2.16 ± 0.12^{a}	3.11 ± 0.11^{b}	17.03 ± 0.01^{b}
NOXI	1.16 ± 0.01^{b}	2.29 ± 0.03^{d}	16.99 ± 0.00^{c}
ETOXI	2.45 ± 0.33^{a}	3.35 ± 0.08^{ab}	17.05 ± 0.01^{b}

As showed in Table 24, oxygen has a direct effect on the relation between the main UFA of *S. cerevisiae*, C16:1 and C18:1, and their corresponding SFA, C16 and C18. Cells under NOXI conditions, as well as LSA-R, LSA-W and P1 cells, had the lowest C16:1/C16 ratio. In contrast, cells under OXI and ETOXI conditions had the highest. For the ratio between C18:1 and C18, the same trait was observed but with a noticeable difference between cell conditions. The lowest levels were found in P1 and NOXI preparations, whilst the highest were under LSA-R, OXI and ETOXI conditions. Cells in direct contact with the base wine (30 min., LSA-W) had an intermediate value of 2.62. All of these results indicated, in relation to the optimization index (previous article), that an excessive presence of UFA could be a disadvantage for cells growing in the presence of ethanol or performing second fermentation at 25ºC. In other words, decreasing the theoretical fluidity by decreasing the unsaturation degree enables cells to finish second fermentation better under our experimental conditions.

Another interesting aspect was the correlation between C16:1 (and C18:1) and saturated fatty acids (from C8 to C18) shown in Figure 39 for all conditions used in this work.

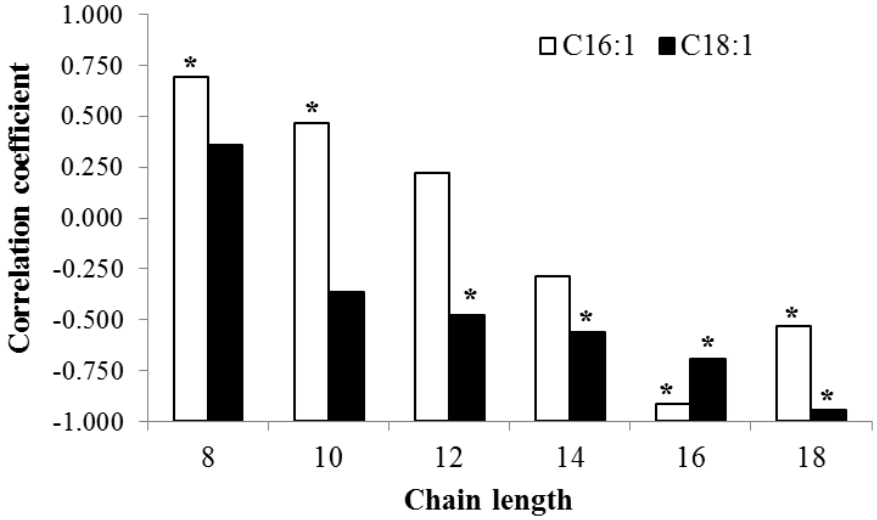

Figure 39. Evolution of correlation coefficient in accordance with the Pearson calculation between the fatty acid chain length and C16:1, and chain length and C18:1. *,, p-value < 0.05.

For both UFA, C16:1 and C18:1, there was a clear and significant decrease in the correlation index depending on the chain length of the saturated fatty acid. In other words, UFAs were largely correlated with C8 and C10, and less so with C16 and C18. This feature may indicate that, in wine, for *S. cerevisiae* strains, the fatty acid metabolism is modulated to preserve good integrity and functionality of cell membranes, regardless of the availability of oxygen, as observed by Rozès (1992) and Redón *et al.* (2007). In addition, this characteristic may be more related to the type of consuming sugar (*Crabtree* effect) than with the availability of oxygen (Johnston and Brown 1972).

2. Total sterol composition

With respect to the sterol composition, it was interesting to note that no significant changes occurred in the ergosterol percentage regardless of the conditions applied during the acclimation (Figure 25). In the case of NOXI or P1 conditions, the ergosterol/squalene ratio was significantly lower than the other conditions, demonstrating that either an absence of oxygen or the presence of a high sugar content in the growth medium led to an increase in squalene. However, in the case of catabolite derepression (exhaustion of fermentable sugars, ETOXI condition), the results were in agreement with those of Quain and Haslam (1979). Indeed, a decrease in the ergosterol/zymosterol ratio was observed for one ergosterol content slightly higher in ETOXI than under both OXI and NOXI

conditions. As described by Quain and Haslam (1979), steryl esters increase when yeast cells grow in ethanol (ETOXI). Under our conditions, this fact was observed with the ergosterol/zymosterol ratio. As mentioned earlier with respect to the UFA/SFA ratio, cells directly inoculated in wine (LSA-W) had a significantly lower ergosterol/squalene ratio than in the case of the water-rehydrated cells (Table 25).

Otherwise, squalene distribution in cells depended on the experimental procedure. The lowest percentages were found in cells where oxygen is available, such as LSA-R (last step preparation of active dry yeast), ETOXI and OXI (in increasing order), while the highest was found in cells under NOXI conditions. Interestingly, cells in the activation phase (P1) also had a high proportion of squalene even though oxygen was always present in the culture medium. Finally, the highest level of episterol and fecosterol was found in cells cultivated under NOXI conditions.

Table 25. Total sterol composition (%) of *S. cerevisiae* EC1118 cells throughout the acclimated process at 25ºC. The conditions used were: LSA-R, Active Dry Yeast rehydrated in water for 30 min; LSA-W, Active Dry Yeast directly added to base wine for 30 min; P1, activation phase (24 h); OXI, Aerobiosis + sugar addition; NOXI, Semi-anaerobiosis + sugar addition; ETOXI, Aerobiosis without sugar addition. Different superscripts indicate significant differences within a column ($P<0.05$). Three independent replicates were analysed.

	Squalene	Zymosterol	Ergosterol	Episterol + Fecosterol	Lanosterol
LSA-R	4.79 ± 0.20^{a}	23.69 ± 1.89^{c}	51.28 ± 2.52	14.50 ± 0.52^{d}	5.74 ± 0.32^{a}
LSA-W	6.97 ± 0.37^{bc}	19.68 ± 0.15^{b}	55.18 ± 0.65	12.74 ± 0.18^{c}	5.42 ± 0.39^{a}
P1	7.54 ± 0.78^{cd}	20.38 ± 0.61^{b}	50.51 ± 1.37	14.42 ± 0.46^{d}	7.15 ± 0.87^{a}
OXI	6.48 ± 0.63^{bc}	16.70 ± 0.84^{a}	52.74 ± 2.92	11.21 ± 0.40^{b}	12.87 ± 1.09^{b}
NOXI	8.48 ± 0.62^{d}	16.35 ± 0.33^{a}	53.98 ± 0.53	15.65 ± 0.19^{e}	5.55 ± 0.39^{a}
ETOXI	5.56 ± 0.37^{ab}	18.49 ± 0.30^{ab}	54.80 ± 1.34	8.98 ± 0.24^{a}	12.17 ± 0.75^{b}

3. Neutral lipid composition

The neutral lipid composition of cells determined by the GC-MS method developed in the corresponding previous article is shown in Figure 40.

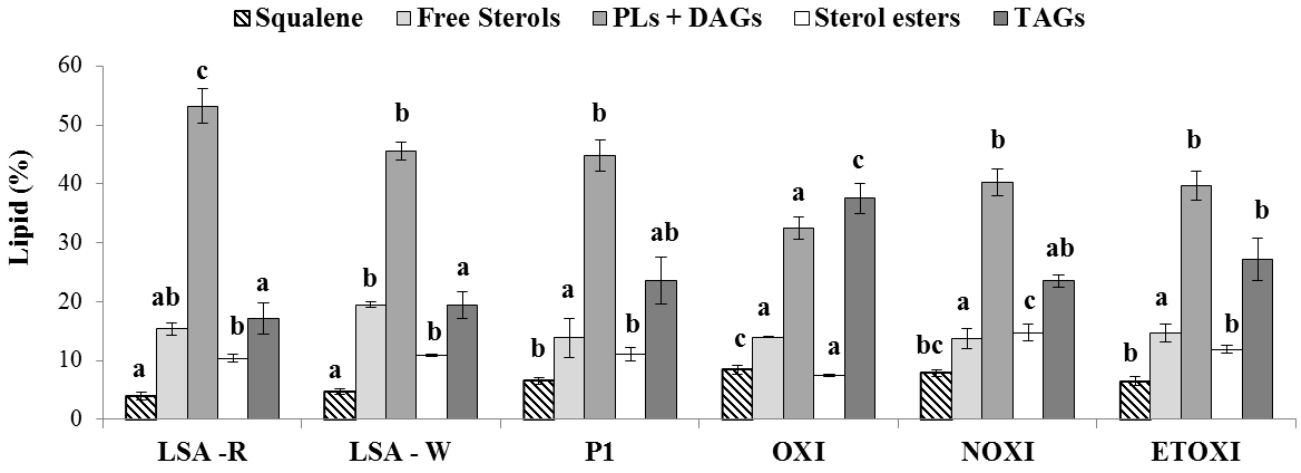

Figure 40. Lipid composition of *S. cerevisiae* EC1118 cells throughout the acclimated process at 25ºC determined by GC-MS. The conditions used were: LSA-R, Active Dry Yeast (ADY) rehydrated in water for 30 min; LSA-W, ADY directly added to base wine for 30 min; P1, activation phase (24 h); OXI, Aerobiosis + sugar addition; NOXI, Semi-anaerobiosis + sugar addition; ETOXI, Aerobiosis without sugar addition. PLs + DAGs, Sum of phospholipids and diacylglycerides; TAGs, Sum of triacylglycerides. Different superscripts indicate significant differences within a lipid family ($P<0.05$). Three independent replicates were analised.

With respect to free sterols (sum of ergosterol, episterol and lanosterol), no significant changes were observed between the sets of conditions. In contrast, the sum of phospholipids and diacylglycerides (PL + DAG) showed significant differences, with the highest proportion being found in LSA-R, and the lowest in all of the acclimation conditions, OXI, NOXI and ETOXI. It seemed that the continual presence of ethanol played a determining role in the synthesis of these compounds. The more ethanol there was in the culture medium, the less phospholipid there was in cells.

The level of sterol esters, basically represented by fatty acid esters of ergosterol, zymosterol and lanosterol, were significantly higher in the case of the NOXI conditions than for the other conditions. In terms of triacylglycerides (TAGs), higher contents were observed in cells of LSA-R, LSA-W, P1 and OXI. However, a decreasing trend was observed for all cells cultivated in the presence of ethanol.

4. Phospholipid composition

As shown in Figure 41, the total phospholipid content differed significantly depending on the conditions used. The highest level was found in rehydrated active yeast cells (LSA-R), while the lowest were found in the case of all conditions in which ethanol

was present throughout the time. This characteristic was in line with the results of Cot *et al.* (2007), who found a similar pattern when yeast cells were cultivated with high ethanol concentration. Meanwhile, the difference observed between LSA-R and LSA-W was significant. Depending on the rehydration medium, cells rapidly lost phospholipids. The 10% (v/v) of ethanol was sufficient to dissolve cellular phospholipid in just 30 minutes.

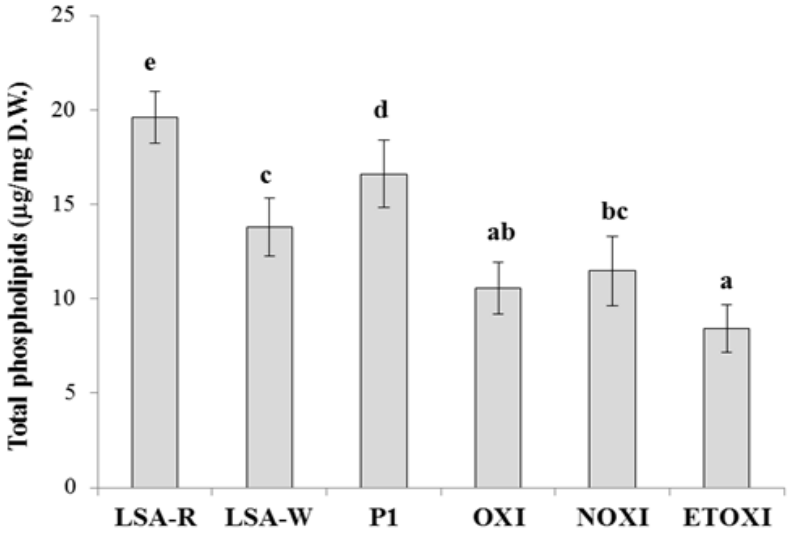

Figure 41. Total phospholipids (µg/mg D.W.) of *S. cerevisiae* EC1118 cells throughout the acclimated process at 25ºC determined by HPTLC. The conditions used were: LSA-R, Active Dry Yeast rehydrated in water for 30 min; LSA-W, Active Dry Yeast directly added to base wine for 30 min; P1, activation phase (24 h); OXI, Aerobiosis + sugar addition; NOXI, Semi-anaerobiosis + sugar addition; ETOXI, Aerobiosis without sugar addition. Different superscripts indicate significant differences ($P<0.05$). Six independent replicates were analysed.

Another interesting aspect, based on the results presented in Figure 42, was the theoretical relation between anionic and neutral phospholipids (PLs). The anionic PLs were phosphatidic acid, cardiolipin, phosphatidylinositol and phosphatidylserine, while the neutral PLs were phosphatidylcholine (PC, major PL in *S. cerevisiae*) and phosphatidylethanolamine (PE). A clear division was observed between the conditions. Thus, cells in LSA-R, LSA-W and P1 had a significantly higher ratio of anionic PLs than cells in OXI, ETOXI and NOXI cultures.

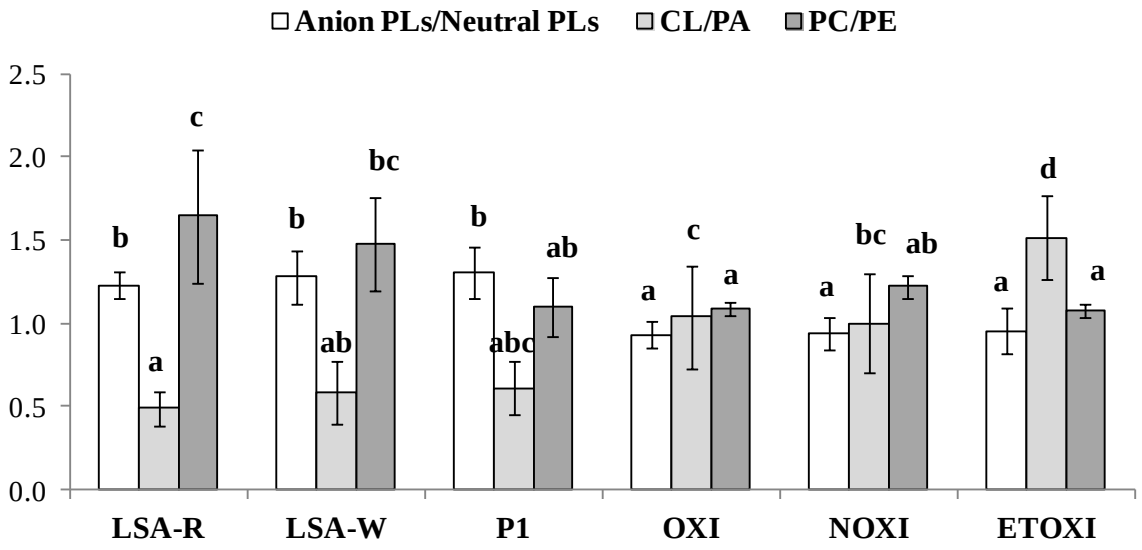

Figure 42. Theoretical relation between anionic and neutral phospholipids, CL and PA and PC and PE of *S. cerevisiae* EC1118 cells throughout the acclimated process at 25ºC determined by HPTLC. The conditions used were: LSA-R, Active Dry Yeast rehydrated in water for 30 min; LSA-W, Active Dry Yeast directly added to base wine for 30 min; P1, activation phase (24 h); OXI, Aerobiosis + sugar addition; NOXI, Semi-anaerobiosis + sugar addition; ETOXI, Aerobiosis without sugar addition. CL, cardiolipin; PA, acid phosphatidic; PC, phosphatidylcholine; PE, phosphatidylethanolamine. Different superscripts indicate significant differences ($P<0.05$). Six independent replicates were analysed.

With respect to the relation between cardiolipin (CL, normally only found in mitochondria membranes) and phosphatidic acid (PA, first step of PL biosynthesis), it was observed that cells in presence of ethanol had a higher ratio than cells of LSA-R. Moreover, the highest ratio was found in cells which had a respiration metabolism, i.e. ETOXI conditions.

Finally, the relation between the main PLs of yeast cells, PC and PE, showed a significant decrease in cells cultivated through the acclimation process compared to rehydrated cells. This trait was mainly due to the decreasing PC content of in the first of the aforementioned cells.

Conclusion

With respect to lipid composition, the relation between unsaturated fatty acids (UFA) and saturated fatty acids (SFA) was significantly different depending on the acclimation process. In the presence of oxygen, cells had a UFA/SFA ratio much higher

than in the case of NOXI. Differences observed were mainly due to the relation between oleic and stearic acids. Not only does oxygen have a real effect on the lipid biosynthesis, but so does the type of carbon source and its level in the growth medium. The membrane structure becomes slack under ethanol stress and, to retain its structural integrity, the membrane should counteract the effect as much as possible by producing more SFA, or less UFA with a concomitant increase in MCFA, depending on oxygen availability and increasing ergosterol (Ding *et al.* 2010). SFA concentration is higher in NOXI condition in comparison to others; however, C18:1 is also increased. Concretely C18:1 was reported to confer ethanol stress tolerance (Ding *et al.* 2010). The role of UFAs is complex and their function is not straightforward.

With respect to the sterol composition, it was interesting to note that no changes occurred in the ergosterol percentage regardless of the conditions. In the case of NOXI conditions, the ergosterol/squalene ratio was significantly lower than P1, demonstrating that either an absence of oxygen or the presence of a high sugar content in the growth medium lead to an increase in squalene. Steryl esters increase when yeast cells grow in nutrient depletion (ETOXI). This fact was observed by the ergosterol/zymosterol ratio. Cells directly inoculated in wine (LSA-W) had a significantly lower ergosterol/squalene ratio than in the case of the water-rehydrated cells.

Chapter IV

Article 7

Metabolomic approach to ethanol acclimation of *Saccharomyces cerevisiae* for producing sparkling wines

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Chapter IV

Abstract

The production of sparkling wines is the result of two consecutive alcoholic fermentations. The first is the fermentation of the grape juice, through which the base wine is obtained. Base wine is fermented again by yeast to enable the effervescence process in bottles. Although the use of Active Dry Yeast (ADY) after rehydration is usually direct and simple, the inoculation of base wine by yeast requires acclimation to 10% (v/v) ethanol, low pH, low temperature, among other conditions.

In this study, a commercial wine yeast was acclimated according to differnet conditions in terms of aerationand sugar content, and used to inoculate a defined base wine to ferment at 25ºC in the bottle. An exhaustive untargeted metabolic analysis of yeast cells during the process was carried out. The characterisation of the metabolic pathways occurring in such a complex scenario may bring us a little closer to understanding what happens in terms of behaviour of cells when they are in contact with ethanol for a long time.

Many changes were found in the metabolism of carbohydrates, lipids and amino acids. The use of carbon sources for fermentation was diminished (Li *et al.* 2012), most amino acids increase its concentration under high ethanol stress (Ding *et al*. 2009; Li *et al.* 2012) and changes in fatty acids make cells preserve their membrane integrity. Even though many amino acids increased in all studies the presence of ethanol: their regulation in their concentration depended on culture media and conditons. Other pathways have been affected by alcohol effect as β-oxidation of fatty acids or energetic metabolism (ATP synthesis pathway). Organic acids were also important regulating intracellular pH and many of them are decreasing its concentration when exposed to ethanol except tartaric acid (Torija *et al*. 2003a).

Chapter IV

Introductory highlights

Sparkling wine production requires two consecutive fermentations. The first converts must into base wine and the second consists of fermenting the wine, with sugar being added to bottles to get the typical CO_2 bubbles. Ethanol is the main stress factor to overcome for yeast cells. What is more, the synergistic effects with other factors, such as acidic pH and medium-chain fatty acids (MCFA) accumulation, makes it even more toxic for yeast and consequently the metabolic scenario becomes complicated (Borrull *et al.* 2015b). Therefore, an acclimation process of yeast to ethanol and other stresses is required prior.

The characterisation of the metabolic pathways and fluxes occurring in such a complex scenario may bring us a little closer to understanding what happens in terms of the phenotype of cells when they are in contact with ethanol. The metabolic characterisation of yeast under the stress conditions could help us to design strategies to overcome ethanol stress when cells have fermentative probleems and to select best prepared strains to optimise fermentations. To the data, the mechanism of *Saccharomyces cerevisiae* in response to ethanol are not fully understood, even without the interaction of other fermentative stress factors.

Previous studies demonstrated that, in the presence of ethanol in the culture media, 29 intracellular metabolites increase significantly by yeast cells in comparison to control cells (Li *et al.* 2012). The main changes were found in the metabolism of carbohydrates, lipids and amino acids.

Carbon sources

Under ethanol stress, the glycolysis is inhibited and the use of carbon sources for fermentation is diminished, with them being accumulated inside cells. Ethanol promotes the intracellular accumulation of glucose and galactose, which might account for the growth inhibition of cells (Li *et al.* 2012). The main carbon reserves, glycogen and trehalose, are accumulated during ethanol stress, thereby improving cell tolerance (Borrull *et al.* 2015a). Trehalose is a critical disaccharide stress protectant in *S. cerevisiae* (Attfield 1987; Kaino *et al.* 2009), increasing its tolerance to high ethanol, freezing and dehydration. The hypothesis was that the chemical stability of the cellular components involved in water

replacement and the glass-forming ability of trehalose limit cell damage (Buitink *et al.* 2004). In their transcriptomic study, Ma and Liu (2010) corroborate that genes related to glycolysis in *S. cerevisiae* are repressed during long-term exposure of cells to sub-lethal alcohol concentration (8%, v/v).

Lipids

S. cerevisiae changes levels of C16, C18, C16:1 and C18:1 to maintain cell wall integrity due to its fluidisation caused by ethanol. It has been suggested that an increase in saturated fatty acids (SFA, C16 and C18) antagonize the excessive fluidity caused by ethanol in the cell membrane. However, the function of fatty acids in counteracting the ethanol effect is complex and not fully understood. Ding *et al.* (2009) demonstrated that oleic acid supplies in cultures with mutants of *OLE1* (mutant yeast cells were not able to synthetise unsaturated fatty acids, UFA) increase their viability, but it does not increase with the supplementation of palmitoleic acid. These results do not agree with the need of building a more rigid membrane. This fact was corroborated by You *et al.* (2003) and by Ingram in bacteria (1990). A possible explanation could be that an increase of some UFA, such as C18:1, in a medium containing ethanol, protects cells against the stereo-chemical effect caused by the quite lipophilic ethanol on the head-groups of the phospholipid bilayer (Weber and de Bond 1996).

Amino acids

Almost all the amino acids found increased in the presence of ethanol (Martinez-Rodríguez *et al.* 2012; Hu *et al.* 2005; Ma and Liu 2010; Ding *et al.* 2009; Li *et al*. 2012). Hu *et al.* (2005) pointed out isoleucine, methionine and phenylalanine, whilst Li *et al.* (2012) observed an increase in glycine, alanine, proline and glutamine concentration in cells grown under ethanol stress, and a decrease of lysine and tyrosine; this lasts amino acids were not detected at higher ethanol concentrations. However, the maximum amino acid concentrations were at 10% (v/v) and not at 15% (v/v), possibly due to the loss of membrane integrity at this high alcohol concentration. For instance, during dehydration stress, cells lose up to 30% of soluble cell compounds, including amino acids and nucleotides, due to the non-functionality of the membrane (López-Martínez *et al*. 2013). Another hypothesis is that general metabolism of *S. cerevisiae*, remains fairly static with

this stress, including amino acids pathways. Amino acids may be converted from 3-P-glycerate and pyruvate, which are both metabolic intermediates in glycolysis and, to some extent; the increasing concentration of amino acids in fermentative media also indicates the inhibition of this pathway by ethanol (Li *et al.* 2012).

Supplementation with certain amino acids promotes the ethanol tolerance of yeast cells by stabilising membrane structures (Ding *et al*. 2009, Takagi *et al.* 2005). The analysis of amino acid composition of plasma membrane proteins and the determination of plasma membrane fluidity through fluorescence anisotropy using diphenylhexatriene, revealed that the significantly increased ethanol tolerance in yeast cells was due to the incorporation of the supplementary amino acids into the plasma membranes that subsequently led to the enhanced ability of plasma membranes to counteract the fluidising effect of ethanol efficiently (Ding *et al.* 2009). A high concentration of intracellular L-proline promotes ethanol tolerance in *S. cerevisiae* (Takagi *et al*. 2008) by stabilising the membrane and scavenging reactive oxygen species (ROS). Based on this fact, proline synthesis genes *PRO1* and *PUT1* are also considered to be candidate genes for ethanol tolerance. *PRO1* is responsible for γ-glutamyl kinase, which promotes proline synthesis while *PUT1* is a great candidate for mutant construction because its deletion stops proline feedback inhibition. In addition, isoleucine may confer protection against high ethanol content in media (Ma and Liu 2010).

Organic acids

Organic acids are not only important due to their organoleptic contribution to wine but also because these acids are in equilibrium with their salts, acting as a buffer and, thus keeping the pH of wine within a range of 2.9 to 4.0 (Dartiguenave *et al.* 2000). In Torija *et al*. (2003a), an addition of tartaric acid ensured the completion of fermentation regardless of the initial pH (3.5-5.5). Tartaric was acid accumulated inside the cells throughout the fermentation and reached maximal concentration at the end of the process. Contrastingly, all of the other main organic acids (citric, malic, succinic, acetic and lactic) decreased over the course of the process. Tartaric acid is the most stable organic acid in wine because a few microorganisms can metabolise it completely during winemaking.

Other compounds

Many other compounds participate in such a complex scenario, for e. g. glycerol increases its concentration (Li *et al*. 2012) under ethanol stress. Glycerol is considered the most important by-product in yeast ethanol fermentation and it helps yeast cells in terms of adapting cells to changing environmental conditions (Blomberg and Adler 1989). The major role of glycerol means that it has been considered a compatible solute that can adjust the intracellular osmotic pressure (Blomberg and Adler 1992; Brewster *et al.* 1993; Hohmann 2002).

Finally, glutathione and cysteine also act as antioxidants against cellular stress (Tirelli *et al.* 2010; Borrull *et al.* 2015a). According to Tirelli *et al*. (2010), compounds containing cysteine residues, such as, glutathione can affect the redox potential of must and wine by reduction of oxidising compounds. The oenological yeast cell wall fraction contains cysteine residues in its protein structure. Glycerol is also considered to be an ethanol protectant by Li *et al.* (2012), an increase in concentration can be observed when cells are exposed to stress.

The main objective of the present chapter is to describe the modification of intracellular metabolite accumulation of yeast cells throughout the acclimation process. To that end, two strategies were performed to understand the response of cells to ethanol. The first was the comparison of the production or accumulation of metabolites during the acclimation period and under LSA-R, P1 and NOXI conditions. The second was the comparison of yeast cells at the point previous to being inoculated to base wine (OXI, NOXI and ETOXI) between them and with the control LSA-W (directly inoculated in base wine).

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Results and discussion

1. *UPLC analysis of intracellular metabolites during acclimation process*

In the Ultra-Performance Liquid Chromatography (UPLC) analysis, two set of conditions were studied. The first consisted of observing the changes in the intracellular metabolite content of yeast cells acclimated to ethanol over the course of the four days, from the rehydrated ADY, EC 1118 (LSA-R conditions), to the acclimated cells in THE presence of sugar without oxygen (NOXI set of conditions), and with an intermediary step at 24 hours of the activation of yeast cells (P1 conditions). This approach is represented in Figure 43 with R, P1 and N; respectively; and cited thereafter as RFN.

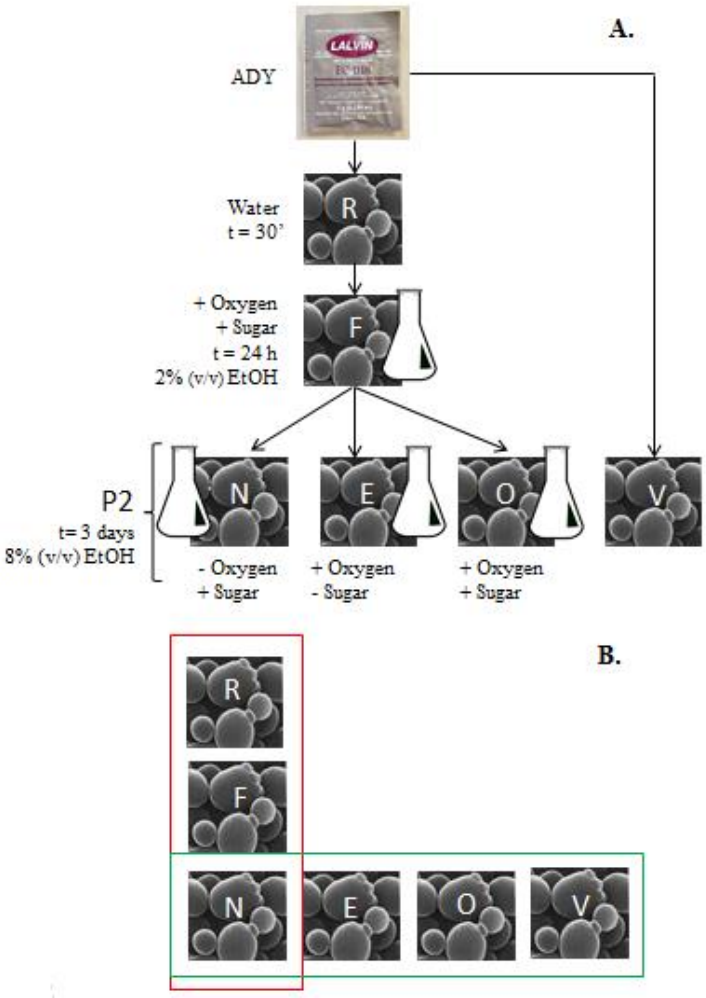

Figure 43. (A) Diagram of *S. cerevisiae* acclimation to ethanol. (B) In red, kinetically evaluated in the study of RFN and, in green,in the analysis of NEOV (R, ADY rehydrated cell in water 30 min, LSA-R; F, phase 1 acclimation, P1; N, NOXI conditions; E, ETOXI conditions; O, OXI conditions; V, ADY rehydrated directly into wine, LSA-W).

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The second set of conditions consisted of comparing the intracellular content after four days of the acclimation process, with cells rehydrated in base wine medium for 30 min. The conditions were: LSA-W, Active Dry Yeast directly added to base wine for 30 min; OXI, aerobiosis + sugar addition; NOXI, Semi-anaerobiosis + sugar addition; and ETOXI, aerobiosis without sugar addition. It is respresented in Figure 44 by N, E, O and V (NEOV).

1.1. Intracellular metabolites accumulated through the acclimation process (RFN)

The Ultra-Performance Liquid Chromatography (UPLC) analysis of samples R, F and N aimed to evaluate how intracellular metabolites could change throughout the kinetic process. Three points were analysed at the beginning, medium and end of acclimation. N conditions were selected as cells under these conditions have a better phenotype performing secondary fermentation. A large amount of metabolites were detected and, after some selections some of them could be identified. The number of compounds found in the UPLC analysis can be seen in Table 26.

Table 26. Number of entities found with the analysis of R, F and N by UPLC. The analysis were carried out in positive (ESI+) and negative (ESI-) ionisation.

Study	Nº of initial entities	Nº of filtered entities	Nº of entities after statistical filtration	Database Hits
RFN ESI+	7350	1982	1204	340
RFN ESI-	1888	1124	691	204

With respect to the kinetic RFN process, 7,350 metabolites were detected with ESI+ and 1,888 with ESI- (Table 26). After the first filter, only those metabolites detected in all of the replicates were selected, which limited our search to 1,982 and 1,124 entities for ESI+ and ESI-, respectively. Subsequenlty, only the metabolites displaying differences between the conditions analysed were kept, which totalled 1,204 in the case of the positive ionisation mode and 691 for the negative one. A whole overview of these metabolites that change depending on conditions (R, F or N) is represented in Figures 45 (ESI+) and 46 (ESI-). Looking at both diagrams, some metabolites from the same metabolic pathway that

change depending on the conditions studied. With the Principal Component Analysis (PCA) carried out with these results it can be seen how conditions R (rehydrated cells), F (phase 1 cells) and N (NOXI conditions, semi-aerated culture with sugar) were well differentiated, especially for ESI+ analysis (see Figure 58 in Supplementary information). Finally, only those entities matching the database were identified. The number of identified compounds is shown in Database Hits column in Table 26.

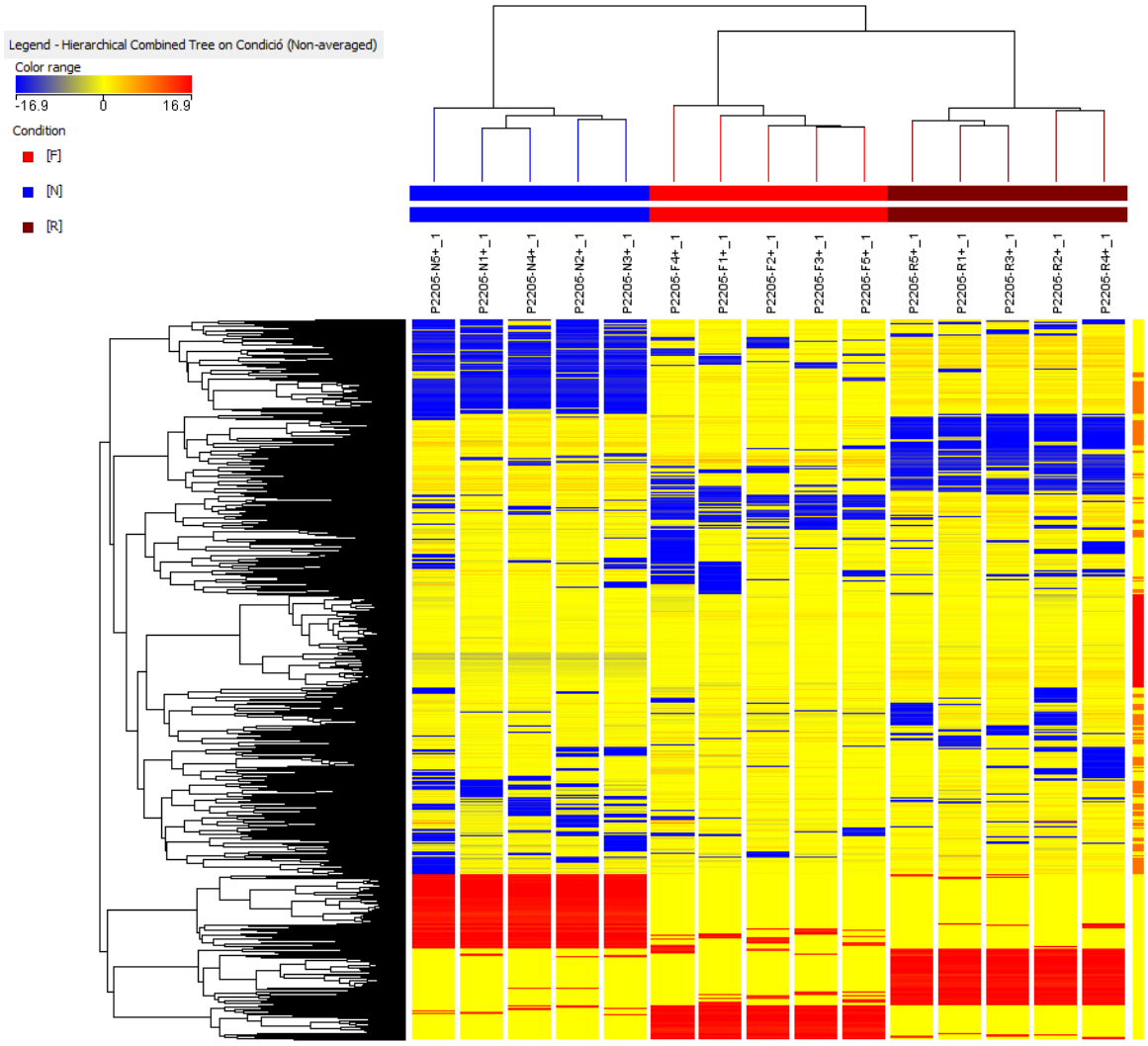

Figure 44. Clustering by normalised intensity values of intracellular metabolite content (n = 1204) of acclimated yeast cells after using the Friedman statistical test ($P < 0.05$). Positive ionisation mode (ESI+). R, LSA-R, Active Dry Yeast Rehydrated in water for 30 min; F; P1, activation phase (24h); N, NOXI, Semi-anaerobiosis + sugar addition.

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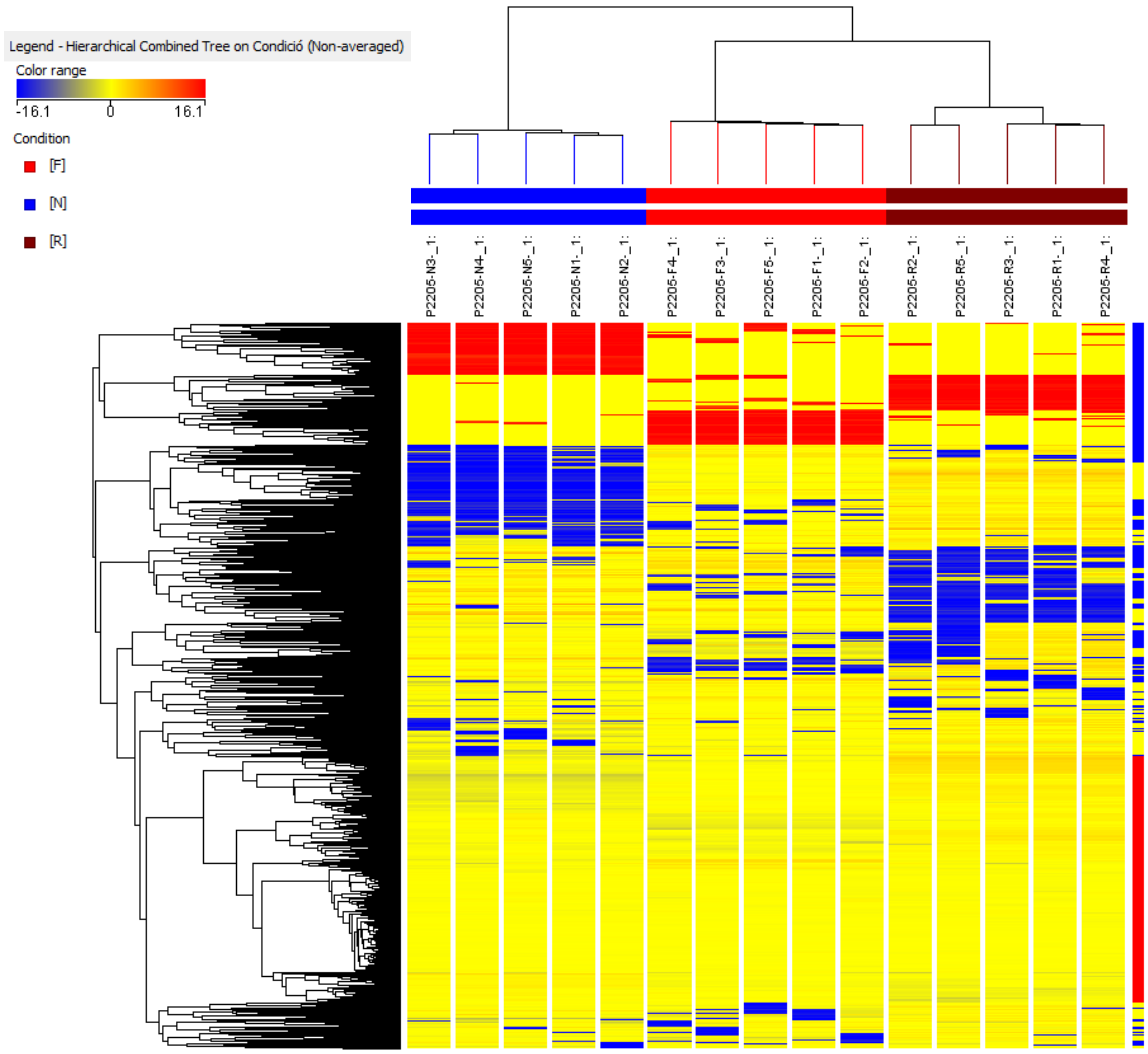

Figure 45. Clustering by normalised intensity values of intracellular metabolite content (n = 691) of yeast acclimated cells after Friedman statistical test ($P < 0.05$). Negative ionisation mode (ESI-). R, LSA-R, Active Dry Yeast Rehydrated in water during 30 min; F; P1, activation phase (24h); N, NOXI, Semi-anaerobiosis + sugar addition.

The energy metabolism of cells was summarised by the total accumulation of AMP, ADP and ATP (Figure 46). As seen in Figure, the total ATP content is higher in the case of P1 and in NOXI conditions because the metabolism was more active in comparison to LSA-R (conditions with cells just rehydrated for 30 min in water). In the case of NOXI conditions, cells were observed to be fermenting because they were under the *Crabtree* effect (Borrull *et al.* 2016). In the case of P1, although there is a constant aeration of the culture, cells did not respire because they were exposed to a high concentration of sugar (150 g/L). Our mitochondrial morphology analysis showed that cells were disorganised, which may mean that mitochondria were not close enough to the cell wall membrane to capture oxygen.

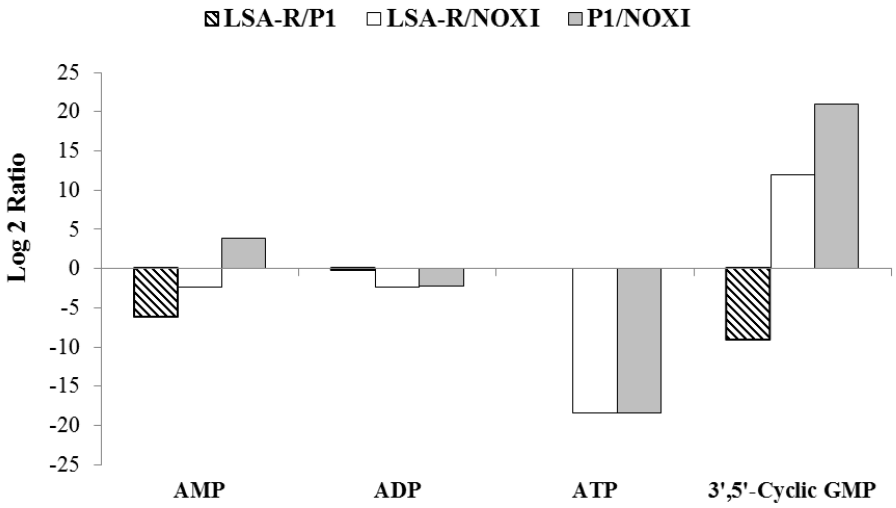

Figure 46. Metabolites from the energy metabolism of *S. cerevisiae* (AMP, ADP, ATP and 3',5'-Cyclic GMP) detected by UPLC relative's concentrations. The ratios between conditions were represented with coloured bars following legend in log 2. LSA-R, cells rehydrated for 30 min in water; P1, cells from phase 1 acclimation process and NOXI, at the end of acclimation, cells with sugar but with limiting oxygen.

3',5'-Cyclic GMP has regulatory functions in eukaryotic cells (Davis *et al.* 2006) and it was also identified in the present study under all of the conditions. This molecule is quite difficult to quantify and the intracellular level of cGMP depends on the metabolic conditions, such as glucose and oxygen supply to the media (Eckstein *et al.* 1988). According to the same author, cGMP increased significantly in yeast, growing exponentially compared to pressed baker's yeast and also fermenting yeasts.

Our results showed that the accumulation of this molecule was higher in cells under LSA-R and P1 conditions than in cells under NOXI conditions. In any case, cells were not observed in exponential growth under any of our conditions. Glucose is described as a positive effector on the cGMP level, and consistently, the highest level of sugar was found in conditions P1 (150 g/L sugar). The 2', 3'-cyclic GMP had the same accumulation pattern in cells placed in these conditions.

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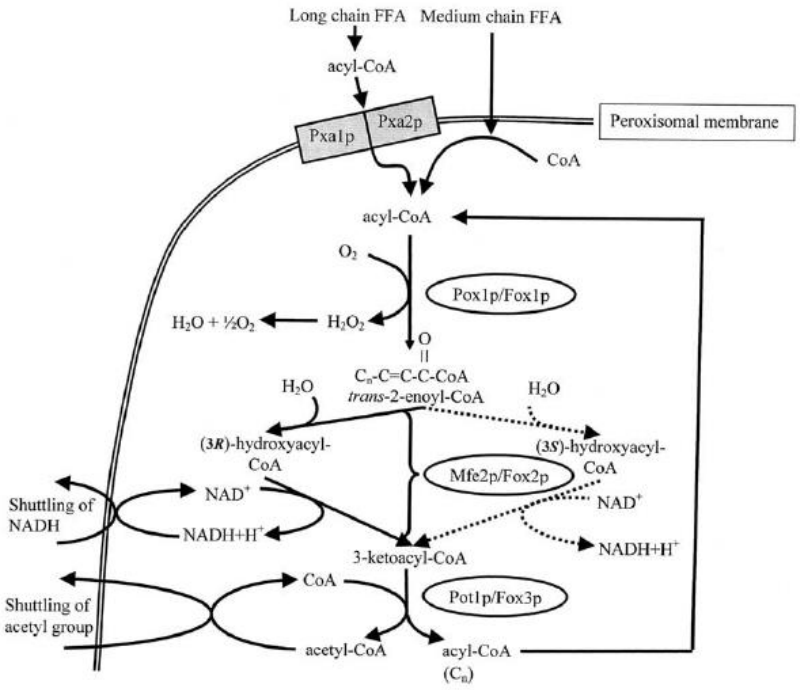

Figure 47. Diagram of β-oxidation in *S. cerevisiae* peroxisomes (Hiltunen *et al*. 2003).

β-oxidation is the catabolic process by which fatty acid molecules are broken down in peroxisomes to generate acetyl-CoA that enters the citric acid cycle (Figure 47). Normally, the β-carbon to the carbonyl group of fatty acid is oxidised. The first two steps to convert the fatty acid in hydroxyl fatty acid are as follows: first, the action of enoyl CoA isomerase catalyses the conversion of the *cis*- or *trans*- double bond of the fatty acid at γ-carbon (position 3) to a *trans* double bond at the β-carbon (position 2), the second important enzyme is enoyl-CoA-hydratase which catalyses the hydration of the double bond between the second and third carbon on acyl-CoA. The result is the addition of a hydroxyl group in carbon in position 3 (β) on acyl-CoA.

As seen in Figure 49, hydroxyl- or keto- medium-chain fatty acids were mainly accumulated in cells rehydrated in water (LSA-R) or activated for one day in the presence of a high level of glucose and aerobiosis (P1). This phenomenon started to reverse from long-chain fatty acid, C14, to give a higher 2-hydroxy-C16 accumulation in cells acclimated under NOXI conditions.

Curiously, with our untargeted metabolic profiling by UPLC approach, several α-hydroxy fatty acids were significantly found in yeast cells and differently expressed in the different condtions. In contrast, β-hydroxy fatty acids do not present significant changes

between the different working conditions. Only statistically significant differences were taken into account in the final results, and thus, it was possible that β-hydroxy fatty acids were present in the cells but without significant differences between the conditions. Although they had the same retention time and exact mass, and matched the corresponding compounds on the database correctly, further work should be conducted to confirm whether cerain α- hydroxyl fatty acids could be produced and accumulated in *S. cerevisiae* by doing a target analysis. In Figure 48, with respect to 2-hydroxy-C6, 2-hydroxy-C14 and 2-keto-C8, the lowest concentration was achieved at the end of the acclimation for NOXI conditions. As seen in the Figure, the concentration of the different fatty acids in each condition was compound dependant. In contrast to other kinetic points, 2-hydorxy-C16 was significantly higher at the end of the process under NOXI conditions.

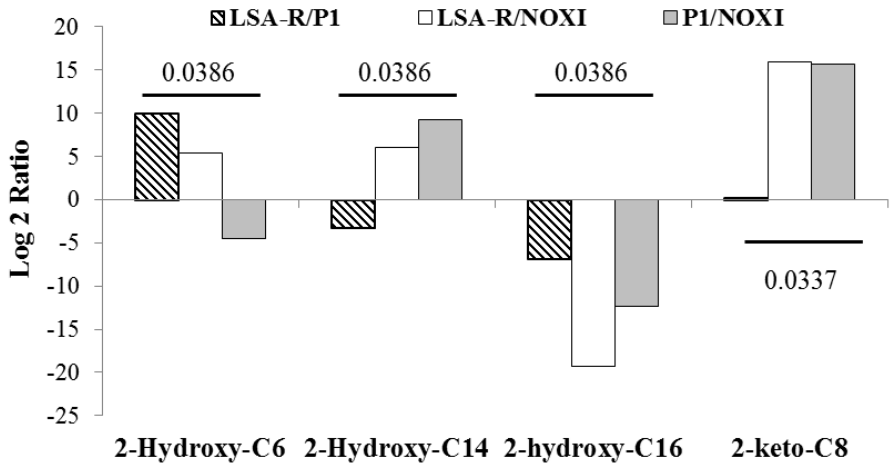

Figure 48. Metabolites from β-oxidation fatty acids process from *S. cerevisiae* metabolism (2-hydroxy-C6, 2-hydroxy-C14, 2-hydroxy-C16 and 2-keto-C8) detected by UPLC relative's concentrations. The ratios between conditions were represented with coloured bars following legend in log 2. LSA-R, cells rehydrated for 30 min in water; P1, cells from phase 1 acclimation process and NOXI, at the end of acclimation, cells with sugar but with limiting oxygen.

As shown in Figure 49, the accumulation of palmitic acid (C16) and its corresponding hydroxy- and keto-fatty acids varied depending on the experimental conditions. C16 was detected at a higher concentration under LSA-R and P1 condition than in NOXI conditions. However, in the case of the latter, cells accumulated more 2-hydroxy-C16. Other oxidised molecules are increased for LSA-R and P1. It is worth noting that 3-keto fatty acids were also present in the cells, which may show that (i) there was a β-

oxidation process in the cell and/or (ii) the identification of these metabolites, 2-hydroxy fatty acids, is correct.

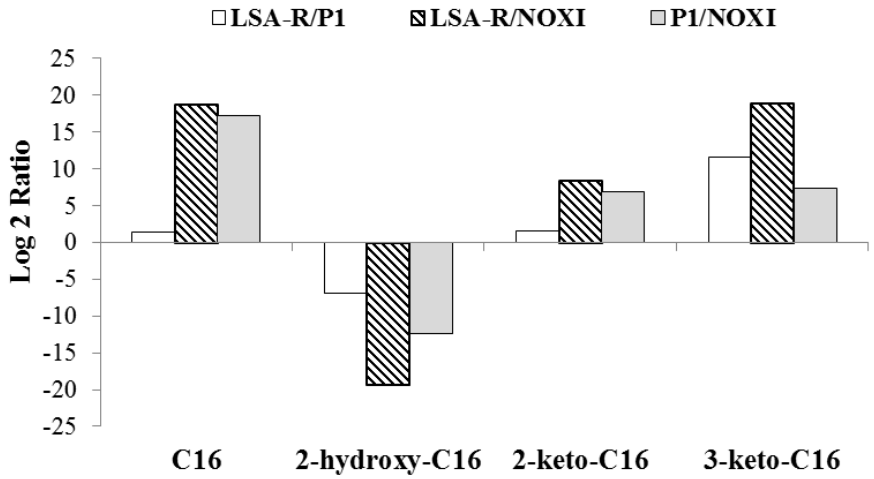

Figure 49. Metabolites from β-oxydation fatty acids process from *S. cerevisiae* metabolism (C16, 2-hydroxy-C16, 2-keto-C16, 3-keto-C16) detected by UPLC relative's concentrations. The ratios between conditions were represented with coloured bars following legend in log 2. LSA-R, cells rehydrated for 30 min in water; P1, cells from phase 1 acclimation process and NOXI, at the end of acclimation, cells with sugar but with limiting oxygen.

1.2 Intracellular metabolites accumulated at the final of the acclimation process (NEOV)

With the aim of elucidating the metabolic differences between the studied conditions at the end point of the acclimation process, we prepared new studyof UPLC analysis of samples in N, E, O and V sets of conditions. The entities detected and identified in this experiment can be seen in Table 27.

Table 27. Number of entities found with the analysis of N, E, O and V by UPLC. The analysis was carried out with positive (ESI+) and negative (ESI-) ionisation.

Study	Nº of initial entities	Nº of filtered entities	Nº of entities after statistical filtration	Database Hits
NEOV ESI+	7335	2015	312	102
NEOV ESI-	1762	1119	365	126

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For this experiment a total of 7,335 entities were detected by positive ionisation (ESI+), whilst only 1,762 were detected by negative ionisation (ESI-) (Table 27). Analogously to the experiment commented above, the entities found were filtered by to keep only those with similar results between replicates (Nº of filtered entities). Next, search was refined to metabolites displaying significant differences between the sets of conditions studied, and these metabolites were represented in Figure 50 and 51. Finally, only 102 (for ESI+) and 126 (for ESI-), could be identified from the database used. In contrast to RFN studio, the negative ionisation is the one which detected many metabolites which could be well separated by doing the PCA statistic analysis. (Figure 58b in Supplementary information).

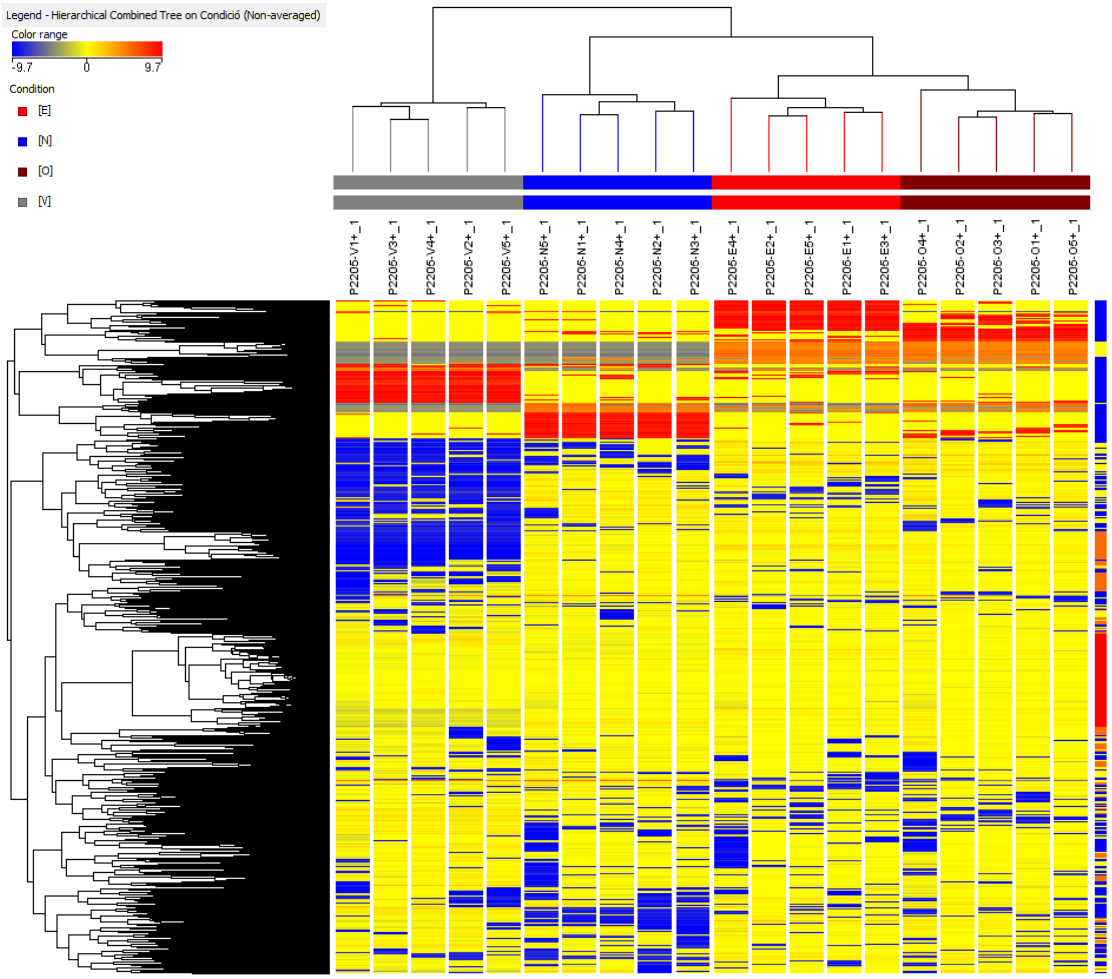

Figure 50. Clustering by normalised intensity values of intracellular metabolite content (n = 312) of acclimatedyeasts cells using the Kruskal-Wallis statistical test (P < 0.05). Positive ionisation mode (ESI+). V, LSA-W, Active Dry Yeast directly added to base wine for 30 min; O, OXI, Aerobiosis + sugar addition; N, NOXI, Semi-anaerobiosis + sugar addition; E, ETOXI, Aerobiosis without sugar addition.

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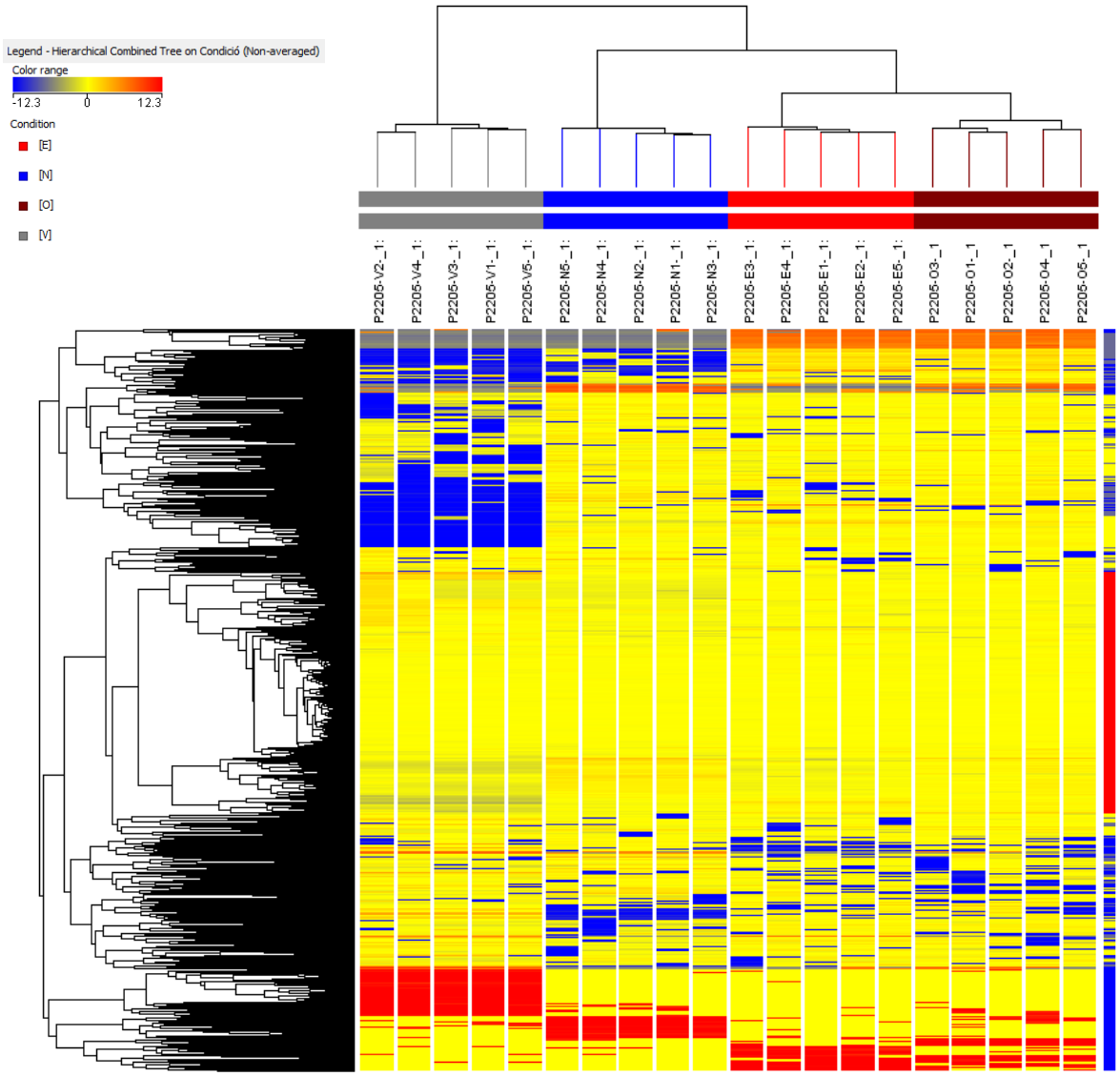

Figure 51. Clustering by normalised intensity values of intracellular metabolite content (n = 365) of acclimated yeast cells using the Kruskal-Wallis statistical test ($P < 0.05$). Negative ionisation mode (ESI-). V, LSA-W, Active Dry Yeast directly added to base wine for 30 min; O, OXI, Aerobiosis + sugar addition; N, NOXI, Semi-anaerobiosis + sugar addition; E, ETOXI, Aerobiosis without sugar addition.

The concentration of AMP decreased significantly in NOXI in comparison to other conditions. NOXI conditions have been shown to give certain advantages to yeast cells when they are inoculated in base wine to perform secondary fermentation (Borrull *et al.* 2016) in comparison to the other acclimated cells. In the case of ADP content, although some differences were detected, ADP levels did not show different profiles. Finally, ATP was higher under NOXI, ETOXI and OXI conditions than in the case of LSA-W and P1.

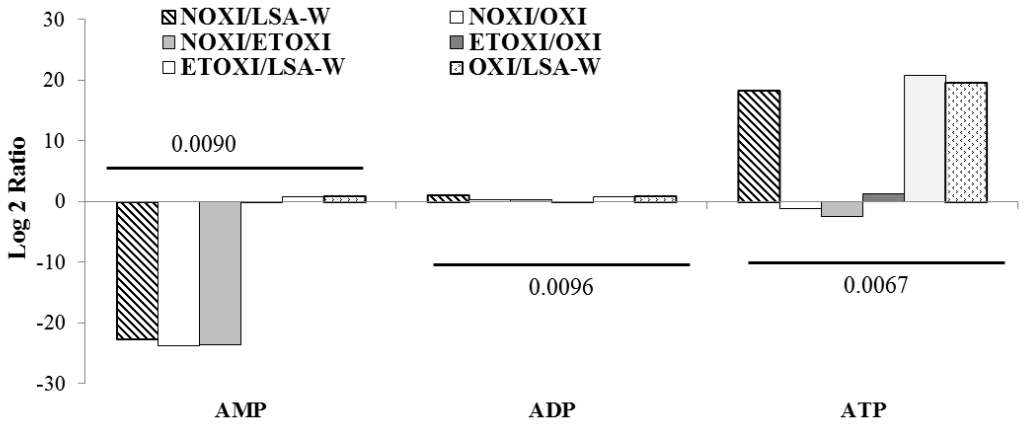

Figure 52. Metabolites from energy metabolism of *S. cerevisiae* (AMP, ADP and ATP) detected by UPLC relative's concentrations. The ratios of log2 between conditions were represented with patterned bars following legend in log 2. LSA-W, cells rehydrated directly to wine media; NOXI, cells with sugar but limiting oxygen; OXI cells with sugar and constant aeration; ETOXI cells with constant aeration but limiting sugar.

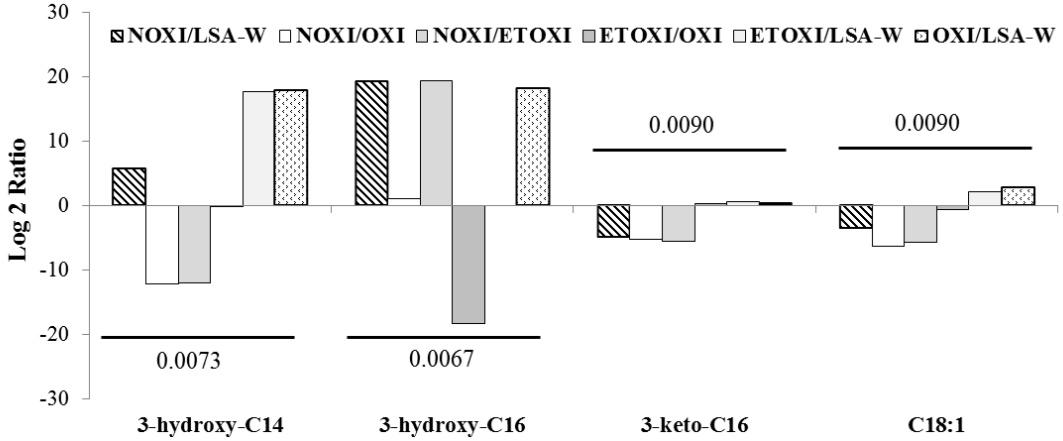

Figure 53. Metabolites from β-oxydation fatty acids process from *S. cerevisiae* metabolism (3-hydroxy-C14, 3-hydroxy-C16, 3-keto-C16 and C18:1) detected by UPLC relative's concentrations. The ratios between conditions were represented with coloured bars following legend in log 2. LSA-W, cells rehydrated directly to wine media; NOXI, cells with sugar but limiting oxygen; OXI cells with sugar and constant aeration; ETOXI cells with constant aeration but limiting sugar.

In contrast with the commented above, comparing the four sets of conditions at the end of acclimation, cells accumulated significant levels of 3-hydroxy and 3-keto fatty acids (Figure 53). Interestingly enough, 3-hydroxy-C14 relative concentration was higher in the active cells (NOXI, OXI and ETOXI conditions) than in the case of rehydrated cells in base wine (LSA-W). For 3-hydroxy-C16, the accumulation was superior in OXI and NOXI conditions than in ETOXI one, e.g. in fermenting cells than in respiring cells. However, the

3-keto-C16 was accumulated more in cells of LSA-W, OXI and ETOXI than in NOXI acclimated cells.

NOXI conditions also displayed the lowest content of C18:1 which could be related to the fact that the culture was not subject to constant aeration. Oxygen is required to synthetise UFAs, which would have seen to confer better cell wall stability for cells exposed to ethanol stress. However, C18:1 has been shown to confer protection against ethanol. A started by Ding *et al.* (2009), some UFAs protect cells against the stereo-chemical effect caused by the reasonably lipophilic ethanol on the head-groups of the phospholipid bilayer. The mechanism by whic fatty acids can stabilise cell membranes is still unclear.

2. GC-MS analysis of intracellular metabolites during the acclimation process

Torija *et al*. (2003a) demostrated that the addition of tartaric acid ensured complete fermentation, regardless of the initial pH (3.5 -5.5). Tartaric acid (5 g/L in the base wine) was accumulated inside the cells throughout the fermentation and reached maximal concentration at the end of the process (Figure 54).

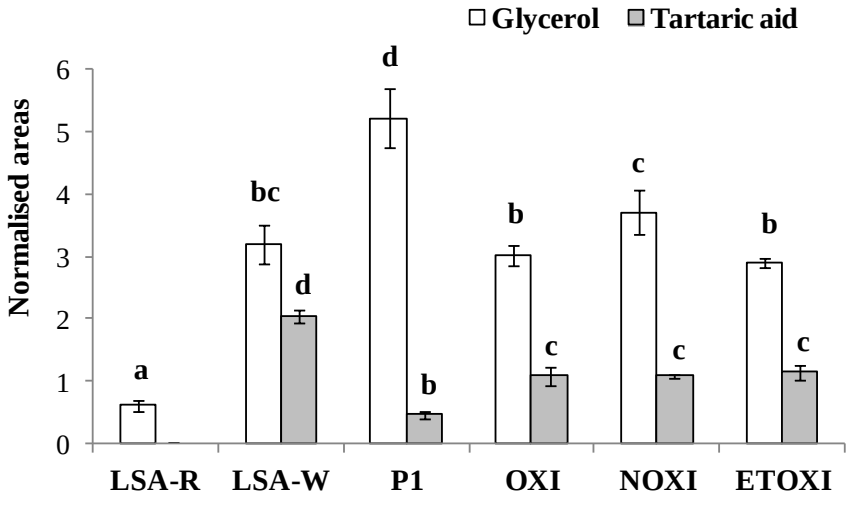

Figure 54. Representation of glycerol (white bars) and tartaric acid (grey bars) content in cells with its normalised area by the internal standard. The relative concentration is represented for all of the studied conditions of the acclimation process (LSA-R, Active Dry Yeast rehydrated; LSA-W, yeast inoculated directly in wine; P1, phase 1 acclimation (24 h); and then at the end of the acclimation changing their conditions: OXI, shaked culture with sugar added; NOXI, static culture with sugar added and ETOXI, aerated culture with no sugar added at phase 2.

No tartaric acid accumulation was found in the case of LSA-R (cells after rehydration in water) which may indicate that the commercial ADYs were not cultivated in tartaric-supplemented medium. It displayed progressive accumulation, as observed by Torija *et al.* (2003a) from this condition to P1 and finally at the acclimation end points (OXI, NOXI and ETOXI). No significant differences were found between the sets of conditions. In the case of LSA-W, the highest intracellular tartaric acid content was found, surely due to the loss of membrane stability.

LSA-R cells had low glycerol content, but a high increase was observed in cells under P1 conditions followed by a small decrease for cells under OXI, NOXI and ETOXI conditions. NOXI was the set of conditions that display the highest intracellular content, which could really give an advantage to cells in terms of adapting to the changing environmental conditions (Blomberg and Adler 1989).

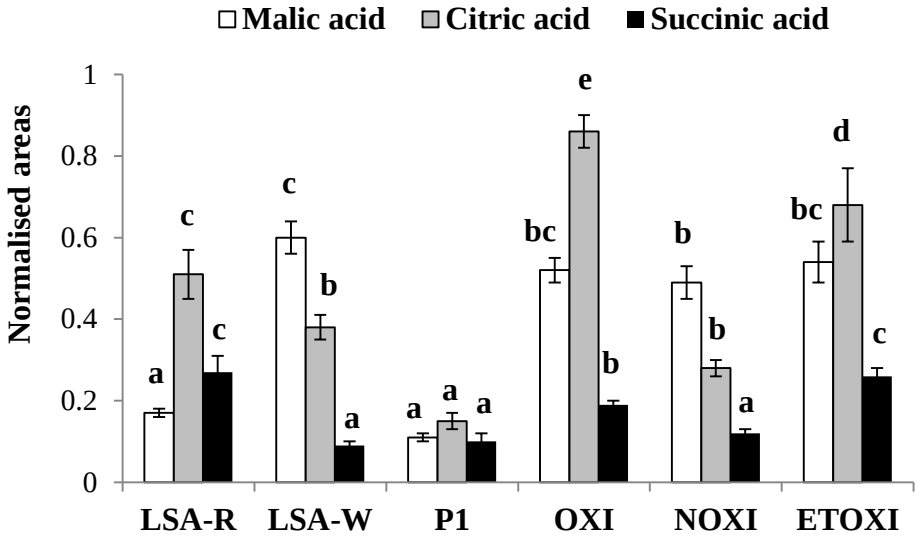

Figure 55. Representation of malic acid (white bars), citric acid (grey bars) and succinic acid (dark bars) content in cells with its normalised area by the internal standard. The relative concentration is represented for all of the studied conditions of the acclimation process (LSA-R, Active Dry Yeast rehydrated; LSA-W, yeast inoculated directly in wine; P1, phase 1 acclimation (24 h); and then at the end of the acclimation changing their conditions: OXI, shaked culture with sugar added; NOXI, static culture with sugar added and ETOXI, aerated culture with no sugar added at phase 2.

Organic acids such as malic, citric and succinic acids were also present in the acclimation medium and can be accumulated in yeast cells. The undissociated form of organic acids can be found in a free form in acidic media (e. g. wine), and can pass across

the plasma membrane and dissociate in the neutral cytoplasm by means of passive diffusion, causing a decrease in intracellular pH (Viegas and Sá-Correia 1997). In contrast, in the physiological medium, these acids will require a transporter to enter yeast cells. Focusing on the acids represented in Figure 55; citric acid and malic acids have the lowest pKa, 3.09 and 3.46, respectively, while succinic acid has a pKa of 4.18 (Ribéreau-Gayon *et al.* 2006). Permease which is responsible for the dissociated form entering is not very efficient (Ribéreau-Gayon *et al.* 2006) and therefore, only non-dissociated molecules would enter the cell easily. Malic and citric acids with a pKa near the base wine pH would have approximately 50% of the acid in dissociated form and the remaining 50% in non-dissociated form. In contrast, succinic acid, with its higher pKa would have around 90% non-dissociated form, which means that this acid would enter the cell easily and it can cause cytoplasm acidification. However, it must be taken into account that the concentration of malic acid is higher in media (2 g/L) in comparison to malic and citric acids (0.5 g/L). Based on these features, malic acid would be most accumulated in the cell due to the high concentration in culture medium. Succinic would be in second place because practically all the content was in the undissociated form, followed by citric acid in last place. This is not happening under our conditions; other aspects should be taken into accound to further explain this unexpected behaviour.

Comparing conditions, NOXI conditions had a lower concentration of citric acid than ETOXI and OXI. This may be due to the respiratory metabolism; NOXI conditions were the only case with clearly fermentation while ETOXI had a respiratory metabolism and OXI conditions also initiated respiration (Figure 55).

It is interesting to note that the only point at which an inorganic nitrogen source was added during the acclimation process, was at the beginning of Phase 1. Intracellular accumulation of glutamate family compounds and amino acids are shown in Figure 56 and were related to nitrogen metabolism. It has been reported the amino acids increase in concentration when exposed to ethanol (Li *et al.* 2012; Ding *et al*. 2009). In our metabolic study, amino acid pathways were also affected by ethanol stress. However, looking in depth, it can be seen in Figure 56 that there was a high amino acid accumulation at P1, but there was a lower concentration at the end of the acclimation. There were no differences in amino acid accumulation between OXI, NOXI and ETOXI conditions. This may be due to the addition of ammonia to the P1 media, whereas no addition was made in the case of LSA-R or P2 points under any of the conditions.

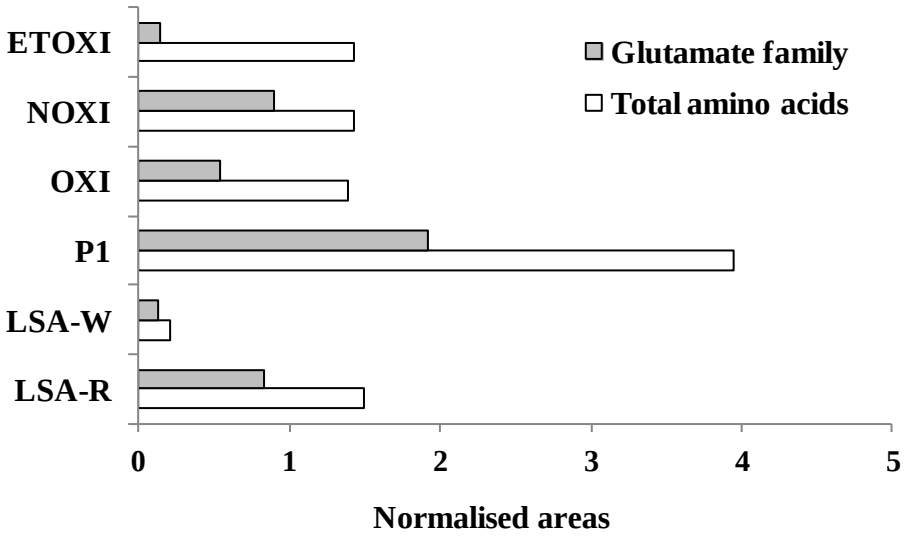

Figure 56. Glutamate family intracellular compounds (glutamic acid, glutamine, pyroglutamic acid and GABA, γ-aminobutyric acid) from cells acclimated under different conditions were represented in grey and total amino acids content in white, both in normalised areas. Conditions were ETOXI (+oxygen, -sugar), NOXI (-oxygen, +sugar), OXI (+oxygen, +sugar), P1 (phase 1 acclimation), LSA-W (Active Dry yeast Rehydrated directly in wine) and LSA (Active Dry Yeast rehydrated in water).

From the sole inorganic nitrogen source added in Phase 1, amino acids and L-glutamate was synthesised. LSA-W displayed a decrease in both amino acids and glutamate compounds; which may be due to the dissolution of some of these compounds due to the strong shock to its membrane caused by ethanol (López-Martínez *et al*. 2013).

The accumulation of amino acids was not significantly different between OXI, NOXI and ETOXI conditions, but there were some differences in the case of glutamate family compounds (Figure 57). The higher content of amino acids under NOXI conditions may give them and advantage by stabilising membrane structures (Ding *et al*. 2009, Takagi *et al.* 2005). These differences can also be seen in Figure 57 in comparison to the other conditions.

The asparagine degradation pathway results in L-glutamate production. There were no significant differences between intracellular glutamate family compounds for NOXI, OXI and ETOXI conditions, remaining at a concentration similar to those found in LSA-R conditions, but there was a high accumulation in cells under P1 conditions (Figure 57).

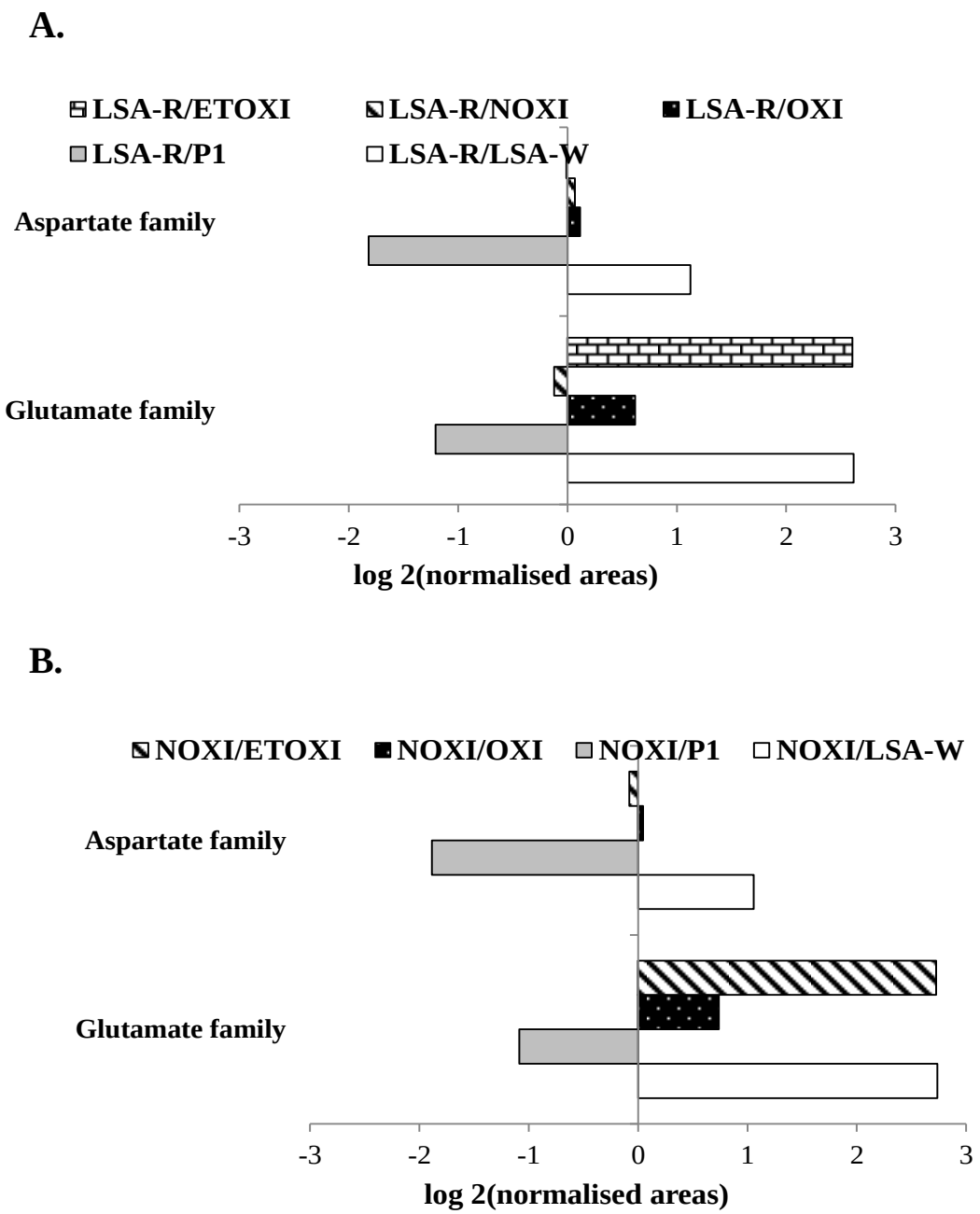

Figure 57. Glutamate and aspartate (aspartic acid + asparagine) families were represented in log2 of normalised areas. Aspartate family compounds were composed by aspartic acid and asparagine, whereas: glutamate family was composed by glutamic acid, glutamine, pyroglutamic acid and GABA (γ-aminobutyric acid). Areas were represented normalised (log 2). Conditions were: ETOXI (+oxygen, -sugar), NOXI (-oxygen, +sugar), OXI (+oxygen, +sugar), P1 (phase 1 acclimation), LSA-W (active dry yeast rehydrated directly in wine) and LSA (active dry yeast rehydrated in water). In (A) there is LSA-R condition compared to other and in (B) NOXI is compared with the rest of conditions.

3. Combination of UPLC and GC results

To complete our metabolomic analysis, results found using both UPLC and GC methodologies were combined. In this way, it was possible to complete some metabolic

pathways according to the effect of time (RFN comparison in Table 30) and the final step of cell acclimation (NEOV comparison in Table 31).

Table 28. *S. cerevisiae* pathways with metabolites detected in RFN analysis by UPLC and/or GC-MS.

Pathway	Matched Entities	Nº Entities by Pathway	Frequency (%)
Sc_Leucine_Degradation_WP354_70265	2	2	100.00
Sc_Asparagine_degradation_WP432_71892	2	2	100.00
Sc_Isoleucine_Degradation_WP178_69780	1	1	100.00
Sc_Serine_Biosynthesis_WP459_69877	3	4	75.00
Sc_Aspartate_Biosynthesis_WP1518_76276	6	9	66.67
Sc_Serine_and_Glycine_biosynthesis_WP218_69824	4	6	66.67
Sc_Glutamate_degradation_I_WP556_69958	2	3	66.67
Sc_Isoleucine_Biosynthesis_WP250_69831	2	3	66.67
Sc_Trehalose_Biosynthesis_WP239_70272	2	3	66.67
Sc_Glycogen_Catabolism_WP478_70263	3	5	60.00
Sc_Asparagine_Biosynthesis_WP67_71349	3	5	60.00
Sc_Trehalose_Degradation,_Low_Osmolarity_WP70_70048	3	5	60.00
Sc_Phospholipid_Biosynthesis_WP9_69581	3	5	60.00
Sc_Tryptophan_Degradation_WP301_67723	3	5	60.00
Sc_Glutamate_biosynthesis_WP77_71350	4	7	57.14
Sc_Glutathione_Biosynthesis_WP196_76277	4	7	57.14
Sc_Superpathway_of_Glutamate_Biosynthesis_WP191_69812	4	7	57.14
Sc_Riboflavin,_FMN,_and_FAD_Biosynthesis_WP381_71896	5	9	55.56
Sc_Isoleucine,_Leucine,_and_Valine_biosynthesis_WP198_69820	4	8	50.00
Sc_Glutamate_degradation_VIII_WP99_70055	4	8	50.00
Sc_Tryptophan_Biosynthesis_WP165_71899	4	8	50.00
Sc_Isoleucine_and_Valine_biosynthesis_WP359_69861	4	8	50.00
Sc_Glycine_biosynthesis_WP261_77412	3	6	50.00
Sc_Threonine_Biosynthesis_WP331_70271	3	6	50.00
Sc_Trehalose_Anabolism_WP398_69664	2	4	50.00
Sc_NAD_Biosynthesis_WP84_71345	2	4	50.00
Sc_Cell_Cycle_and_Cell_Division_WP414_76251	1	2	50.00
Sc_Phenylalanine,_Tyrosine,_Tryptophan_biosynthesis_WP27_70250	8	17	47.06
Sc_Folic_acid_biosynthesis_WP555_69957	6	13	46.15
Sc_Glutamate_degradation_VII_WP559_70041	5	11	45.45
Sc_Serine-isocitrate_lyase_pathway_WP390_69869	5	11	45.45
Sc_TCA_Cycle_WP490_78137	5	11	45.45
Sc_Arginine_degradation_WP54_70247	4	9	44.44
Sc_Glutamate_degradation_III_WP503_69932	3	7	42.86
Sc_Leucine_Biosynthesis_WP180_69807	2	5	40.00
Sc_Tyrosine_Biosynthesis_WP538_69941	2	5	40.00
Sc_Pathways_of_Chorismate_WP213_71175	9	24	37.50
Sc_Aerobic_Glycerol_Catabolism_WP224_71353	3	8	37.50
Sc_Arginine_Biosynthesis_WP275_69844	4	11	36.36
Sc_De_Novo_NAD_Biosynthesis_WP541_69942	4	11	36.36
Sc_Histidine_Biosynthesis_WP514_71894	3	9	33.33
Sc_TCA_Cycle_-_Detailed_WP296_76239	3	9	33.33
Sc_Heme_Biosynthesis_WP102_72196	2	6	33.33
Sc_Polyamine_Biosynthesis_WP290_69849	2	6	33.33
Sc_Phenylalanine_Biosynthesis_WP194_69817	1	3	33.33
Sc_UDP-Glucose_Conversion_WP109_69750	1	3	33.33
Sc_Galactose_Metabolism_WP549_69945	1	3	33.33
Sc_Tricarboxylic_acid_cycle_WP674_74129	5	16	31.25
Sc_De_Novo_Biosynthesis_of_Purine_Nucleotides_WP203_71893	5	16	31.25
Sc_Purine_Fermentation_WP463_73550	3	10	30.00
Sc_Phenylalanine_and_Tyrosine_Biosynthesis_WP120_77891	3	10	30.00
Sc_Phosphatidic_Acid_and_Phospholipid_Biosynthesis_WP472_71340	3	10	30.00

Chapter IV

As seen in Table 28 and Table 29; most of the affected pathways were related to amino acid metabolism, both in terms of degradation and synthesis. It is interesting to note that no amino acids were added to the media during the acclimation process and so cells performed the *novo* synthesis from the inorganic ammonia. In additon, reserve carbohydrates (glycogen and trehalose) seemed to be influenced by the acclimation conditions. This finding has been described previously by Borrull *et al.* (2015a).

Table 29. *S. cerevisiae* pathways with metabolites detected in NEOV analysis by UPLC and/or GC-MS.

Pathway	Matched Entities	Nº Entities by Pathway	Frequency (%)
Sc_Leucine_Degradation_WP354_70265	2	2	100.00
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Sc_Glutamate_degradation_I_WP556_69958	2	3	66.67
Sc_Isoleucine_Biosynthesis_WP250_69831	2	3	66.67
Sc_Tryptophan_Degradation_WP301_67723	3	5	60.00
Sc_Asparagine_Biosynthesis_WP67_71349	3	5	60.00
Sc_Glutamate_biosynthesis_WP77_71350	4	7	57.14
Sc_Superpathway_of_Glutamate_Biosynthesis_WP191_69812	4	7	57.14
Sc_Glutathione_Biosynthesis_WP196_76277	4	7	57.14
Sc_Glutamate_degradation_VIII_WP99_70055	4	8	50.00
Sc_Tryptophan_Biosynthesis_WP165_71899	4	8	50.00
Sc_Isoleucine_and_Valine_biosynthesis_WP359_69861	4	8	50.00
Sc_Isoleucine,_Leucine,_and_Valine_biosynthesis_WP198_69820	4	8	50.00
Sc_Glycine_biosynthesis_WP261_77412	3	6	50.00
Sc_Threonine_Biosynthesis_WP331_70271	3	6	50.00
Sc_NAD_Biosynthesis_WP84_71345	2	4	50.00
Sc_Cell_Cycle_and_Cell_Division_WP414_76251	1	2	50.00
Sc_Folic_acid_biosynthesis_WP555_69957	6	13	46.15
Sc_Glutamate_degradation_VII_WP559_70041	5	11	45.45
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Sc_Arginine_degradation_WP54_70247	4	9	44.44
Sc_Aspartate_Biosynthesis_WP1518_76276	4	9	44.44
Sc_Riboflavin,_FMN,_and_FAD_Biosynthesis_WP381_71896	4	9	44.44
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Sc_Phenylalanine,_Tyrosine,_Tryptophan_biosynthesis_WP27_70250	7	17	41.18
Sc_Leucine_Biosynthesis_WP180_69807	2	5	40.00
Sc_Glycogen_Catabolism_WP478_70263	2	5	40.00
Sc_Phospholipid_Biosynthesis_WP9_69581	2	5	40.00
Sc_Trehalose_Degradation,_Low_Osmolarity_WP70_70048	2	5	40.00
Sc_Arginine_Biosynthesis_WP275_69844	4	11	36.36
Sc_Pathways_of_Chorismate_WP213_71175	8	24	33.33
Sc_TCA_Cycle_-_Detailed_WP296_76239	3	9	33.33
Sc_Histidine_Biosynthesis_WP514_71894	3	9	33.33
Sc_Polyamine_Biosynthesis_WP290_69849	2	6	33.33
Sc_Phenylalanine_Biosynthesis_WP194_69817	1	3	33.33
Sc_UDP-Glucose_Conversion_WP109_69750	1	3	33.33
Sc_Galactose_Metabolism_WP549_69945	1	3	33.33
Sc_Trehalose_Biosynthesis_WP239_70272	1	3	33.33
Sc_De_Novo_Biosynthesis_of_Purine_Nucleotides_WP203_71893	5	16	31.25
Sc_Purine_Fermentation_WP463_73550	3	10	30.00

With respect to the intracellular amino acid content cited specifically by authors to promote ethanol tolerance, our findings revealed some discrepancy. Our observations that were in contrast to Hu *et al.* (2005) were as follows: Many metabolites from the (i) isoleucine pathways related to both, biosynthesis and degradation were encountered, and many synthesis routes of (ii) phenylalanine for both RFN kinetic and NEOV comparative analysis of final acclimation conditions. Controversy, these authors also found differences in the (iii) methionine metabolism that has not been confirmed in our study. We have also confirmed for both analyses the changes in the synthesis precursors of (i) glycine reported by Li *et al*. (2012). (ii) Glutamine and (iii) lysine, which decreased in accumulation in the study, was not detected in our case; (iv) tyrosine was detected only in the RFN study and not at the end of the process; it was pointed out as important to counteract ethanol stress. Finally, Li *et al.* (2012) found (v) alanine and (vi) proline, which were identified in our study. Interestingly, metabolites from L-proline synthesis were not observed, which has been cited by many authors (Takagi *et al.* 2005; Ma and Liu 2010; Li *et al.* 2012) as a cell protectant against ethanol stress.

With respect to the carbon source metabolism, some metabolites of galactose degradation were found and, in the case of trehalose, even synthesis and degradation pathways were observed, as well as glycogen catabolism.

Conclusion

To summarise, the characterisation of the metabolomic approach in cells during and at the end of the acclimation process revealed that the main changes were similar to those attributed to ethanol stress exposure. The carbohydrate, lipid and amino acid pathways were modified.

Many amino acids increased in concentration. However, the ones most affected differed depending on the culture media and conditions. NOXI conditions displayed an increase in glutamate family compounds in comparison to the other conditions. In terms of carbon reserves, such as glycogen or trehalose, were accumulated inside cells under ethanol stress and help cells to overcome it. β-oxidation of FA occurred throughout the acclimation process and energetic metabolism (ATP pathways) was activated differently

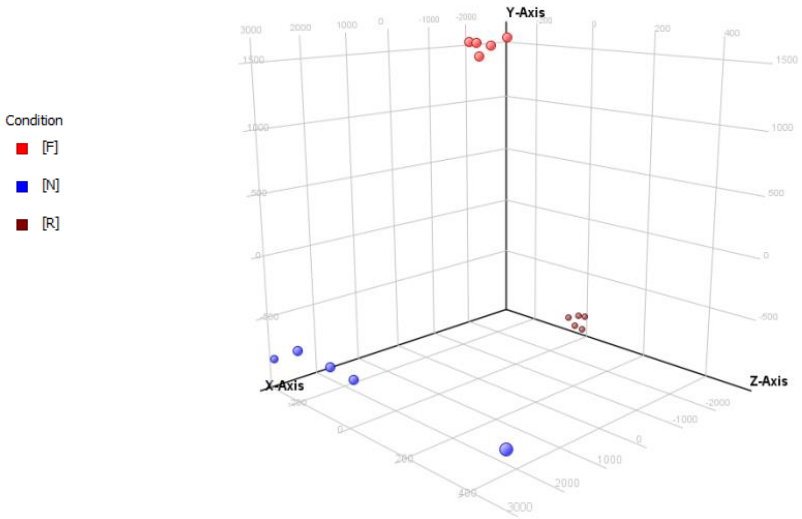
depending on the conditions. The concentration of organic acids from the media decreased inside the cells, except from tartaric acid, which increased in concentration.

However, a metabolic target analysis with deuterated or even radioactive compounds would be required to identify some metabolites accurately. This could be an approach for the future to confirm certain features of cells acclimated to ethanol.

Chapter IV

Supplementary information

A.



B.

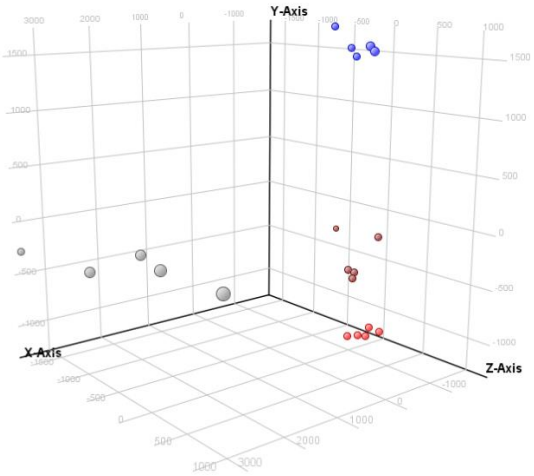


Figure 58. PCA statistical analysis from compounds detected by UPLC (A) ESI+, RFN samples (B) PCA ESI-, NOEV samples.

CHAPTER V

DISCUSSION

Stress factors from the primary and secondary fermentations in sparkling wine production on the yeast *Saccharomyces cerevisiae* were evaluated in the present thesis. The primary fermentation converts grape juice into the base wine. The second fermentation, which involves transforming sugar into ethanol and CO_2 in bottles, is a hard challenge for *S. cerevisiae*, due to an accumulation of stressful factors which act in synergy and, consequently, it requires an extra effort for cells trying to survive and ferment. Some stress factors are the same as those found in first alcoholic fermentation, although with a different intensity: high ethanol level, low pH, low nitrogen content, accumulation of toxic fermentation by-products such as MCFA and organic acids. However, others are especially found in the second fermentation, such as the low fermentation temperature, CO_2 overpressure, high total acidity and, sometimes, high SO_2 concentration (Juroszek *et al.* 1987).

Although winemakers apply often empirical procedures which are efficient for acclimating the yeast to ethanol and other stress factors, there is always a lag phase after inoculation, followed by a short cell proliferation, indicating that cells are still not completely adapted to the second fermentation media. However, without this acclimation process, cells are highly susceptible to death. A progressive acclimation of *S. cerevisiae* is required before being inoculated into the base wine (Tai *et al.* 2007; Laurent and Valade 1994, 1996, 2007). Commercial wine yeasts display different behaviour when subjected to a range of abiotic stress factors. However, the level of ethanol remains the truly differential factor between them, affecting yeast membrane fluidity and interacting with proteins adhered to it. The effect of ethanol on yeasts is well documented. In contrast, the synergic effect with other stress factors during alcoholic fermentation or, even more, during secondary fermentation is not fully understood, probably due to the complexity of the scenario.

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During primary fermentation in our base growth medium (BGM), some abiotic factors may influence the yeast's capacity to grow and ferment because of the acidic pH due to the presence of weak organic acids and MCFA produced and excreted by the yeasts themselves. In the presence of MCFA, C8 and C10, the viability decreases drastically in yeast due to the higher accumulation of reactive oxygen species (ROS). Being this intracellular accumulation higher for C10 (Borrull et al. 2015b). Both MCFAs display a synergic effect because they are dissociated in culture media due to the acid pH and can enter the cell in a passive way, easily acidifying the intracellular pH with drastic effects.

With regard to ROS compound accumulation, cysteine was tested due to its intracellular antioxidant properties inherent to the thiol group (Kumar *et al.* 2006). It seems basing on results that this amino acid can repair damaged produced by MCFA or other oxidants in the cells affected. To ascertain the real effect of cysteine, further research should be conducted to test its concentration dependence and analogue molecules, such as glutathione. The determination of intracellular ROS shows that, throughout the first days of yeast growth, values of ROS significantly increased for all strains, regardless of the conditions, except in the case of cysteine addition, when cells maintained the lowest ROS percentage. Furthermore, the ROS level of cells fell after two days. There seemed to be a relation, as longer as the lag phase, the higher the ROS.

Meanwhile, to the best of our knowledge, this is the first time that a negative synergistic effect between ethanol and glycerol has been described. High extracellular glycerol content in the presence of ethanol seems to mimic the effect of the absence of the aquaporin channel on the growth parameter. Further work is necessary to confirm the intracellular glycerol accumulation, which could modify the redox balance.

Oxygen availability in the growth medium enables yeast cells to synthetise UFAs from phospholipids and sterols (Alexandre *et al.* 1994; Ding *et al.* 2009). These changes may be responsible for an important physical characterisation of membranes, regulating membrane permeability and fluidity (Daum *et al.* 1998). However; oxygen it is directly related to ROS accumulation. There were significant differences in the MCFA, SFA and UFA compositions between yeast strains under our growth conditions, which mimic the industrial acclimation for sparkling wine production. However, no significant differences were found for the estimations of NIC and MIC. It also seems that a small increase in MCFA combined with the decrease in the UFA level, due to the hypoxic conditions,

allowed yeast cells to maintain proper membrane fluidity by limiting the membrane-fluidising effect of ethanol. For some of the studied strains, the estimated MIC was even higher without constant aeration.

The yeast acclimation process in the line with the traditional *Champenoise* method to produce sparkling wines was adapted to laboratory scale. The main objective was to study the importance of a physiologic al diagnostic for yeast cells after acclimation to improve the onset and accomplishment of secondary fermentation in bottle.

During the acclimation process, yeast was progressively adapted to ethanol and other stresses. Active Dry Yeast (ADY) was rehydrated in water (R) and then inoculated in Phase 1 (P1) with 2% (v/v) of ethanol for 24 h and, finally in Phase 2 (P2) with 10% (v/v) of ethanol for 3 days. The acclimation changes its parameters during P2 depending on the conditions, so different sugar availabily and oxygen exposures were studied: (i) OXI (+sugar/+oxygen), (ii) NOXI (+sugar/-oxygen) and (iii) ETOXI (-sugar/+oxygen).

Growth curves were carried out and specific fluorochromes for dying mitochondria made it possible to ascertain which kind of respiratory metabolism corresponded to each condition. Mitochondria are known to be the organelles related to energy metabolism, cell signalling and pathways of programmed cell death.

In the case of OXI conditions, even though there is constant aeration of the culture, there is no respiration due to the high amount of sugar, responsible for the *Crabtree* effect. At the end of the acclimation, sugars are practically consumed under OXI conditions. It was uncertain before testing whether cells would be respiring or fermenting. Based on growth curves, cells seem to begin a respiro-fermentative metabolism. There is little organisation in some of the cells evidencing that the cells start respiring. Yeast under NOXI conditions may be subject to the *Crabtree* effect, with sugar remaining at the end of P2. This is confirmed by mitochondrial analysis. Its disorganisation confirms the fermentative model. Finally, in the case of ETOXI conditions, sugars were completely consumed within 60 h of P2 and then developed a respiratory metabolism, using a non-fermentable carbon source, ethanol, resulting in a decrease in ethanol content. The mitochondrial analysis shows cells with mitochondria remaining organised as near to the cell membrane as possible in order to capture oxygen, confirming respiratory metabolism.

Chapter V

To observe a reliable phenotypic response due to the acclimation conditions, the secondary fermentations in bottles were conducted in a base wine with OXI, NOXI and ETOXI acclimated cells. To complete this study, direct inoculation with the active dry yeast was used without rehydration in water (LSA-W condition). Secondary fermentation was considered to be concluded when the sparkling wine reached 6 bars in bottle. It was only completed in the case of NOXI acclimated cells, in 35 days, while, in the other cases, the fermentation stuck (experiment stopped after 40 days). NOXI conditions showed the highest degree of alcohol, lowest sugar level and it was the only case with live cells at a pressure of 4 bars. Furthermore, under our conditions, the secondary fermentation of the defined base wine (DBW) was conducted without the addition of nitrogen, which could be the key factor, in association with the yeast strain and fermentation temperature, for correctly ensuring the secondary fermentation. Our results showed that not only these factors have to be taken into account, but also the type of yeast metabolism during the acclimation process.

With regard to conditions differing in terms of oxygen availability (OXI and NOXI), it can be concluded at this point that the supply of oxygen to the culture should be avoided during acclimation because cells acclimated under OXI conditions (i) have a growth curve profile very similar to NOXI and, even start respiring by the end of acclimation (confirmed by mitochondrial organisation), they are not respiring during the process, so, oxygen is not giving them advantages; (ii) make cells accumulate more ROS; (iii) have a worse organelle organisation which makes them less suitable to carry on with secondary fermentation; and (iv) have a worse phenotype.

Having established the best acclimated cells based on the phenotype, many other parameters were analysed in order to make a cell diagnostic before being inoculated to carry out the secondary fermentation.

By staining the vacuole, its morphology and activity were determined. Vacuoles are involved in many processes, including the homeostasis of cell pH (Cebollero *et al.* 2005). The best state of cells could be observed in those acclimated under NOXI set of conditions and are very similar to LSA-R: (i) they displayed the best microvacuolation (fragments into multiple small vesicles) so, when diving, they could transfer a proportion of vacuoles to a new cell, and this phenomenon also indicates that cells are ready to overcome certain general stress and not being into apoptosis state; (ii) they have the highest vacuolar

activity, which means better capacity for maintaining vacuolar pH; and (iii) the lowest ROS accumulation.

Glutathione (GSH) plays a role in protection against oxidative stress and is a non-enzymatic scavenger of ROS. GSH (90% in the reduced form) is oxidised to GSSG, removing the reactive oxidants from cells (Penninckx *et al.* 2002). Cells that contained the highest level of GSH were acclimated under LSA-R and NOXI conditions. Interestingly, GSH content decreased when yeast was directly rehydrated in wine (LSA-W), compared to water (LSA-R). A relationship could be established between this decrease in GSH and the ROS accumulation. Unfortunately, the ratio between GSH and GSSG for LSA-W and LSA-R are similar and, consequently, cells placed under LSA-W conditions were unable to use GSH to counteract this harmful stress. The non-utilisation of GSH by LSA-W cells could be explained by the fact that these cells presented low vacuolar activity. The GSH in cells is equally distributed between the cytosol and vacuoles.

Glycogen and trehalose are the two glucose carbohydrate reserves of yeast. Their presence confers survival and reproductive advantages (François and Parrou 2001). High glycogen content was observed in the case of LSA-R, NOXI and ETOXI conditions. Trehalose content in cells depends on the acclimation process, with the highest being LSA-R and NOXI. In the end, by comparing the average rehydration for the ADY (water vs ethanol), a rapid depletion of the two storage carbohydrates was observed in the presence of ethanol.

With respect to lipid composition, the relation between UFAs and SFAs was significantly different depending on the acclimation process. In the presence of oxygen, cells had a UFA/SFA ratio much higher than in the case of NOXI. Differences observed were mainly due to the relation between oleic and stearic acids. Not only does oxygen have a real effect on the lipid biosynthesis, but so does the type of carbon source and its level in the growth medium. The membrane structure becomes slack under ethanol stress and, to retain its structural integrity, the membrane should counteract the effect as much as possible by producing more SFAs, or less UFAs with a concomitant increase in MCFA, depending on oxygen availability and increasing ergosterol (Ding *et al.* 2010). SFA concentration is higher in NOXI condition in comparison to others; however, C18:1 is also increased. Concretely C18:1 was reported to confer ethanol stress tolerance (Ding *et al.* 2010). The role of UFAs is complex and their function is not straightforward. With respect

to the sterol composition, it was interesting to note that no changes occurred in the ergosterol percentage regardless of the conditions. In the case of NOXI conditions, the ergosterol/squalene ratio was significantly lower than P1, demonstrating that either an absence of oxygen or the presence of a high sugar content in the growth medium lead to an increase in squalene. Steryl esters increase when yeast cells grow in nutrient depletion (ETOXI). This fact was observed by the ergosterol/zymosterol ratio. Cells directly inoculated in wine (LSA-W) had a significantly lower ergosterol/squalene ratio than in the case of the water-rehydrated cells.

Regarding the transcriptomic, metabolomic and lipidomic results, several features can be identified. Undoubtedly, the lipid metabolism is affected by the acclimation conditions of the cells to ethanol, mainly depending on the presence of oxygen and a fermentable source, such as glucose.

With respect to the metabolic approach, the increased levels of some amino acids can also confer ethanol tolerance to *S. cerevisiae* by stabilising membrane structures (Li *et al.* 2012; Ding *et al.* 2009). In our study, despite to the fact that most amino acids increased its concentration, the amino acid level was depending on culture media and conditions. NOXI conditions presented high concentration of glutamate family compounds. Pathways as β-oxydation of FA and energetic metabolism (ATP pathways) were differently activated depending on conditions. The concentration of organic acids decreased into cells except from tartaric acid, which increased its intracellular concentration, whereas the condition. Tartaric acid cannot be metabolised by *S. cerevisiae*. Some results, not shown in this manuscript, enable us to assume that this accumulation is concentration-dependent but also dependent on other conditions of the culture, such as temperature, fermentable sugar, lipid composition, etc.

In the transcriptome analysis, there was a slight overexpression of genes related with lipid metabolism (*ERG5, ERG10, ERG13, OLE1, YEH1, EEB1*) in cells acclimated under fermentative conditions (NOXI). In contrast, some genes, such as *GUT1* or *MDH1*, are preferentially activated under respiratory (ETOXI) and fermentative-respiratory (OXI) conditions, respectively. In addition, it seems that the level of sterol esterification, the type of sterols and the relation between them in the cells during the acclimation may enable cells to achieve the secondary fermentation correctly in bottles at 25ºC. As perspectives for further studies, effect of low temperatures should be tested to ensure NOXI acclimation

conditions will be the bests for acclimation process. Finally, the phospholipid content of cells decreased throughout the acclimation process due to the increasing presence of ethanol in the medium. Little modification of the proportion of phospholipid classes is found.

Taking into account the physiological parameters analysed and the secondary fermentation performance of the cells, optimisation indexes (OI) were calculated, grading each parameter from 0 to 5 (from worst to best). Seven parameters (chosen based on the correlation results between parameters) were used to determine the OI of the acclimation conditions. They were ranked as follows: NOXI>ETOXI>OXI>LSA-W. By determining both optimisation indexes, for the secondary fermentation and physiological parameters, a relationship was found with a good coefficient of determination. This correlation was improved by changing the preference order in relation to the two lipid ratios, ergosterol/squalene and C18:1/C18. It was assumed that a slight rigidity in terms of the cell membrane would be an advantage for cells in an ethanol-growing medium. The figures and our hypothesis support this assumption.

In terms of the nitrogen level during the acclimation process, yeast cells from inorganic nitrogen added at the beginning are able to synthesise completely all of the amino acids needed for its metabolism. As recently shown by Valade *et al.* (2007), the inorganic nitrogen source can be added at two times, the first during the activation phase (P1) and the second during the proper acclimation phase with the same total level of 400 mg/L of inorganic nitrogen.

In conclusion, the acclimation of *S. cerevisiae* cells to ethanol for producing sparkling wines should always be carried out in the presence of a fermentable source of carbon, with or without oxygen. In other words, yeast cells have to maintain fermentative activity throughout the process with a low availability of oxygen, as shown in the preparation of *Champagne* in terms of the *prise de mousse* from ADY (Valade *et al.* 2007), as well as our results. Oxygen availability is important for maintaining a minimal biosynthesis of fatty acids and ergosterol in yeast cells during the acclimation process. However, excessive aeration, especially without sugar in the acclimation medium, can induce increasing oxidative stress, mainly by increasing the ROS species, which drastically affects the lifespan of the yeast.

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ANNEX

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