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STUDY OF THE PRODUCTION AND RELEASE OF AROMAS DURING WINEMAKING CARRIED OUT BY DIFFERENT *Saccharomyces* SPECIES AND HYBRIDS

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Instituto de Agroquímica y Tecnología de Alimentos (IATA-CSIC)

Grupo de Microbiología Molecular de Levaduras Industriales

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Tesis doctoral presentada por:

Amparo Gamero Lluna

para optar al grado de doctor en Ciencia, Tecnología y Gestión Alimentaria

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La Dra. Amparo Querol Simón, Profesora de Investigación, y la Dra. Carmela Belloch Trinidad, Científico Titular, del Consejo Superior de Investigaciones Científicas (CSIC) en el Instituto de Agroquímica y Tecnología de los Alimentos (IATA)

CERTIFICAN

Que la presente memoria "Study of the production and release of aromas during winemaking carried out by different *Saccharomyces* species and hybrids" constituye la tesis doctoral de Dña. Amparo Gamero Lluna para optar al grado de doctor en Ciencia, Tecnología y Gestión Alimentaria por la Universidad Politécnica de Valencia. Asimismo, certifican haber dirigido y supervisado tanto los distintos aspectos del trabajo como su redacción.

Y para que conste a los efectos oportunos, firmamos el presente certificado en Valencia a 15 de marzo de 2011

Fdo. Amparo Querol Simón Fdo. Carmela Belloch Trinidad

STUDY OF THE PRODUCTION AND RELEASE OF AROMAS DURING WINEMAKING CARRIED OUT BY DIFFERENT *Saccharomyces* SPECIES AND HYBRIDS

ABSTRACT

Aroma is one of the most important attributes involved in wine quality. Current trend in winemaking consists of producing wines with different aroma nuances to offer variety of wines to a developing market. Several studies have demonstrated that low temperature fermentations favours aroma synthesis and retention. In this background, new wine yeasts able to perform fermentation at low temperatures improving wine aroma while maintaining good fermentation rates are necessary. This doctoral thesis explores the oenological traits of different *Saccharomyces* species and hybrids relevant for present-day wine industry, especially regarding aroma production, as well as the molecular bases underneath. This exploration has been possible using different biochemical, analytical chemistry and molecular techniques to perform enzymatic activity detection, aroma profile determination and transcriptome analysis in wine fermentations. Through this doctoral thesis the abilities of different *Saccharomyces* species and hybrids regarding primary aroma release and secondary aroma production, especially at low temperatures, has been elucidated in order to know the different possibilities that these yeasts offer to create new wines with different aromatic nuances. One of the general conclusions of this doctoral thesis is that production and release of aromas in winemaking depends on the strain carrying out the fermentation process. Nevertheless, sometimes there was a species tendency. On the other hand, the fact that fermentation temperature affects aroma synthesis but not always in the direction to aroma increase has been demonstrated.

ESTUDIO DE LA PRODUCCIÓN Y LIBERACIÓN DE AROMAS DURANTE LA VINIFICACIÓN POR PARTE DE DISTINTAS ESPECIES E HÍBRIDOS DEL GÉNERO *Saccharomyces*

RESUMEN

El aroma es uno de los más importantes atributos implicados en la calidad del vino. La actual tendencia en vinificación consiste en producir vinos con diferentes matices aromáticos para ofrecer variedad de vinos a un mercado en desarrollo. Varios estudios han demostrado que las fermentaciones a baja temperatura favorecen la síntesis y la retención de los aromas. En este contexto, son necesarias nuevas levaduras vínicas capaces de llevar a cabo fermentaciones a bajas temperaturas y que mejoren el aroma del vino, manteniendo buenas tasas fermentativas. Esta tesis doctoral explora las características enológicas de diferentes especies e híbridos del género *Saccharomyces* relevantes para la industria vínica actual, especialmente en lo referente a la producción del aroma, así como las bases moleculares subyacentes. Esta exploración ha sido posible usando diferentes técnicas bioquímicas, de química analítica y moleculares para llevar a cabo detección de actividad enzimática, determinación del perfil aromático y análisis transcriptómico en fermentaciones vínicas. A través de esta tesis doctoral, las habilidades de las diferentes especies e híbridos del género *Saccharomyces* en lo que se refiere a la liberación del aroma primario y a la producción del aroma secundario, especialmente a bajas temperaturas, han sido dilucidadas para conocer las diferentes posibilidades que estas levaduras ofrecen para crear nuevos vinos con diferentes matices aromáticos. Una de las conclusiones generales de esta tesis doctoral es que la producción y liberación de aromas en vinificación depende de la cepa que lleva a cabo el proceso fermentativo. Sin embargo, en ocasiones hay una tendencia de especie. Por otra parte, se ha demostrado el hecho de que la temperatura de fermentación afecta a la síntesis de aromas pero no siempre en la dirección del aumento.

ESTUDI DE LA PRODUCCIÓ I ALLIBERAMENT D´AROMES DURANT LA VINIFICACIÓ PER PART DE DIFERENTS ESPÈCIES I HÍBRIDS DEL GÈNERE *Saccharomyces*

RESUM

L´aroma és un dels més importants atributs implicats en la qualitat del vi. L´actual tendència en vinificació consisteix en produïr vins amb diferents matisos aromàtics per a oferir varietat de vins a un mercat en desenvolupament. Diversos estudis han demostrat que les fermentacions a baixa temperatura afavoreixen la síntesi i la retenció dels aromes. En aquest context, són necessaris nous llevats vínics capaços de dur a terme fermentacions a baixes temperaturas i que milloren l´aroma del vi, mantenint bones taxes fermentatives. Aquesta tesi doctoral explora les característiques enològiques de diferents espècies i híbrids del gènere *Saccharomyces* rellevants per a l´indústria vínica actual, especialmente en allò referent a la producció de l´aroma, així com les bases moleculars subjacents. Aquesta exploració ha sigut posible usant diferents tècniques bioquímiques, de química analítica i moleculars per a dur a terme detecció d´activitat enzimática, determinació del perfil aromàtic i anàlisi transcriptòmic en fermentacions víniques. A través d´aquesta tesi doctoral, les habilitats de les diferents espècies i híbrids del gènere *Saccharomyces* en allò referent a l´alliberament de l´aroma primari i a la producció de l´aroma secundari, especialment a baixes temperatures, han sigut dilucidades per a conèixer les diferents possibilitats que aquestos llevats ofereixen per a crear nous vins amb diferents matisos aromàtics. Una de les conclusions generals d´aquesta tesi doctoral és que la producció i alliberament d´aromes en vinificació depèndeix del cep que du a terme el procés fermentatiu. Tanmateix, en ocasions hi ha una tendència d´espècie. Per altra banda, s´ha demostrat el fet que la temperatura de fermentació afecta a la síntesi d´aromes però no sempre en la direcció de l´augment.

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INTRODUCTION

1. Chemical composition of grape must

Freshly expressed grape must consists of 70 to 80% water and many dissolved solids. These soluble solids include numerous organic and inorganic compounds. The important groups of compounds, from the winemaking point of view, are the following:

Sugars

In grapes, a large portion of the soluble solids are sugars, mainly glucose and fructose in equal amounts. The sugar content of the must of ripe grapes varies between 150 to 250 g/L. During the course of fermentation, yeasts convert fermentable sugars (glucose and fructose) into alcohol and carbon dioxide. The amount of alcohol produced is related to the amount of sugar initially present in the must; thus, by controlling the amount of sugar in the juice, it is possible to control the amount of alcohol in the resulting wine.

Organic acids

Organic acids are the most abundant solids present in grape juice. They are responsible for the tart taste and have a marked influence on wine stability, color, and pH. The principal organic acids found in grapes are tartaric, malic, and to a small extent, citric. Many other organic acids are also found in must and wines, but tartaric and malic acid account for over 90% of the total acids present. During the early period of berry growth, concentration of both acids increases in the fruit. With the onset of ripening, sugars accumulate in the fruit and acids concentration decreases. Generally the reduction in malic acid is greater, and consequently, at maturity, the fruit

contains more tartaric acid than malic. Tartaric acid is present as free acid and as a salt, such as potassium bitartrate, which is an important constituent affecting pH and cold stability of wine.

Acid composition of grapes is influenced by many factors such as variety, climatic region, and cultural practices. Generally in ripe grapes, the acid levels are lower in a warm climatic region than in a cold region. The acidity is expressed as titratable acidity (TA), which is an important parameter used in quality evaluation of must and wine. Acid content of the juice has importance on must and wine pH. However, the relationship is neither direct nor predictable due to the presence of various kinds of acids and their salts. Understanding the role of pH in winemaking is crucial to make good wines.

Phenolic compounds

Phenolic compounds are important constituents of grapes and wines. They are the most abundant constituents present in grapes after sugars and acids. Phenolic compounds are a group of substances structurally diverse and present in different amounts. They play a vital role in determining the wine's color and flavor. They are involved in browning reactions in grapes and wines and also play a key role in the aging and maturation of wines. Phenolic substances are primarily located in seeds and skins of the berry. As white wines are usually produced from must with low skin and seed contact, their phenolic content is low (100-250 mg/L gallic acid equivalent, GAE). On the contrary, red wines are commonly produced with skin and

seed contact. Depending on the contact time, the phenolic content of a red wine generally varies between 1000 to 3500 mg/L GAE.

The two main substances included in this group of compounds are anthocyanins and tannins. Anthocyanins are pigments responsible for the red and purple color of the grapes and wines. They exist in both colored and colorless forms. In young red wines, most of the colored anthocyanins are present in free (uncombined) forms. As the red wine ages, the anthocyanins combine with other phenolic compounds. In a combined state, the pigment contributes to color stability.

Tannins are very large and complex compounds with a molecular weight over 500. They are yellow, brown, and red colored and give astringency and bitterness. During processing and aging, the tannins polymerize, increasing their molecular size, being astringency more perceived than bitterness. However, increase in molecular size also makes these compounds insoluble, precipitating and causing decrease of wine´s astringency.

Nitrogenous compounds

Grapes contain ammonium cations and organic nitrogenous compounds, such as amino acids, peptides, and proteins. Nitrogen content of the grapes depends on grape variety, climate, soil, fertilization, and other cultural practices. Total nitrogen concentration of the fruit increases during maturation period. Nitrogen containing compounds are important because they serve as the nutrient for yeast and lactic acid bacteria, influencing

biomass formation, fermentation rate, and byproducts synthesis, thus affecting sensory wine attributes.

Proteins are involved in wine stability. In addition, insufficient nitrogen in must can cause sluggish or stuck fermentations and H_2S formation ("rotten egg" odour). To avoid this problem, must is often supplemented with diammonium phosphate (DAP).

Aroma Compounds

Aromatic compounds in grapes are largely present in the skin and the layers of cells immediately beneath it. Their concentration tends to increase during ripening. It is important that the grapes be harvested when the flavor is at its peak. Many factors affect the concentration of aroma compounds in grapes. Manipulation and control of these factors is necessary for attaining the desired flavor level at harvest.

Minerals

Minerals are taken up by the vine from the soil. They usually constitute approximately from 0.2 to 0.6% of the fresh weight of the fruit. The main mineral compounds include: potassium, sodium, iron, phosphates, sulfate, and chloride, being potassium the most important (50-70% of must cations). During ripening, potassium grape content increases. The formation of potassium bitartrate reduces acidity and increases pH of the must, but this salt is involved in wine instability problems.

Pectic Substances

Pectin substances are cementing agents present in the cell wall. Chemically, these compounds are complex polysaccharides made of galacturonic acid molecules linked together. During ripening, pectin is hydrolyzed by naturally occurring pectolytic enzymes, softening the berry. In must, pectin causes turbidity by holding the particles of fruit pulp in suspension. To allow the suspended solids to settle and clarify the juice, commercial preparations of pectolytic enzymes are often used.

2. Winemaking process

Winemaking process (Fig.1) which starts in vineyard is an ancient art influenced by several factors such as viticulture practices, soil quality, and the cultivar of *Vitis vinifera*. All of these factors are of crucial importance for wine quality (Vivier & Pretorius, 2002). The first step in wine production consists of grapes harvesting. After that, grapes are subjected to destemming, crushing, macerating and pressing.

In the case of red wines, alcoholic fermentation occurs at the same time of maceration, whereas Rosé wine is made equally to red one but reducing macerating time. However, white wines are usually fermented without maceration directly after crushing.

Alcoholic fermentation is the anaerobic transformation of sugars of the must (glucose and fructose) into ethanol and carbon

dioxide (Zamora, 2009). It can be summarized by this overall reaction:

$C_6H_{12}O_6 \rightarrow 2\ CH_3CH_2OH + 2CO_2$

In addition to ethanol and carbon dioxide, other important compounds are produced throughout alcoholic fermentation, such as glycerol, organic acids, acetaldehyde, alcohols and esters.

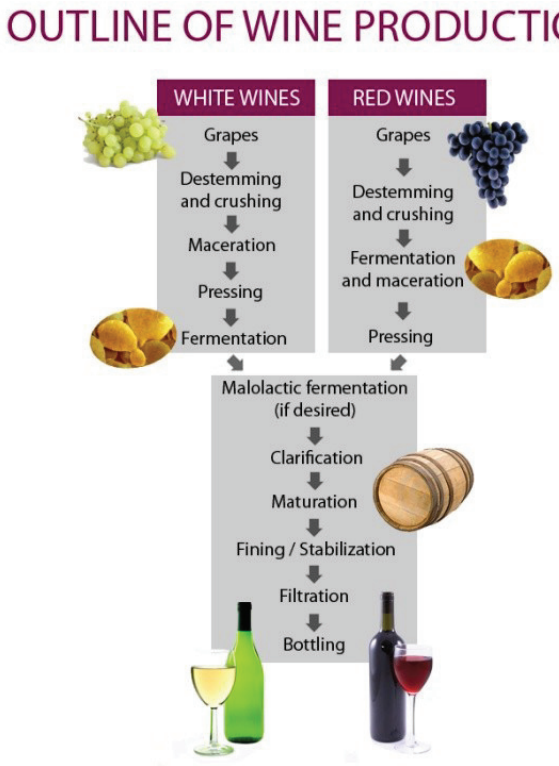

Fig 1. Winemaking process (adapted from Pretorius, 2000).

Alcoholic fermentation is carried out by yeasts. At the early stages of spontaneous alcoholic fermentation, genera *Kloeckera*, *Hanseniaspora* and *Candida* predominate and *Pichia* and *Metschnikowia* prevail in the middle stages. Finally, *Saccharomyces*

cerevisiae is the predominant yeast species because of its greater resistance to high ethanol concentration (Fleet, 1993; Fleet & Heard, 1993). Some other yeast genera, such as *Torulaspora*, *Kluyveromyces*, *Schizosaccharomyces*, *Zygosaccharomyces* and *Brettanomyces* may also be present during alcoholic fermentation and even in the wine (Peynaud & Domercq, 1959; Ribéreau-Gayon et al., 2000). In order to prevent developing of undesirable yeast, wineries add sulphur dioxide to the grape juice and inoculate selected dry yeasts, mainly *Saccharomyces cerevisiae*, which is more resistant to this chemical product (Lafon-Lafourcade & Peynaud, 1974; Romano & Suzzi, 1993).

In red wines subjected to a maturation process, after alcoholic fermentation another fermentation conducted by acid lactic bacteria occurs. This process is called malolactic fermentation and it is carried out by bacteria of the genus *Lactobacillus*, *Leuconostoc* and *Pediococcus* and the species *Oenococcus oeni*, which is the main bacteria conducting this fermentation according to Riberéau-Gayon et al. (2006). Through this fermentation, malic acid is decarboxylated to lactic acid. This constitutes a beneficial process, since it leads to acidity reduction and microbial stability of wines. Both fermentations can ocurr spontaneously or by the addition of commercial selected yeasts and bacteria.

The wine is now subjected to clarification, maturation in oak barrels, fining and stabilization, filtration and bottling.

3. Main metabolites in wine

Wine is constituted by chemical compounds whose principal origin is grapes and yeast alcoholic fermentation (Fig. 2).The main component of this alcoholic beverage is water, which constitutes about 85%. In addition, several compounds also can be found in wines:

Ethanol

Ethanol is one of the main compounds synthesized in wine fermentation. This compound decreases wine aroma and flavor perception by increasing aromatic compounds solubility in the wine and lowering the volatile fraction (Ferreira, 2007). Furthermore, in the last years, there is an increasing demand to produce wines with lower ethanol contents, due to its negative effects on health (Pereira et al., in press; Zhang et al., 2011).

Glycerol

Glycerol is involved in osmoregulation (Ansell et al., 1997; Nevoigt and Stahl, 1997) and in low-temperature tolerance in yeasts (Izawa et al., 2004). The amount of glycerol produced by *S. cerevisiae* in wines is typically between 4 and 9 g/l, with average values approximately of 7 g/l (Ough et al., 1972; Scanes et al., 1998). The oenological importance of glycerol lies in its contribution to wine quality by providing slight sweetness, smoothness and fullness, reducing wine astringency (Ishikawa and Noble, 1995; Llaudy, 2006; Remize et al., 2000).

Organic acids

Organic acids constitute about 1% of wine. Tartaric acid is the major acid that is derived from grapes. Volatile acids like acetic, succinic, lactic, etc. are produced during fermentation. Acetic acid is the main volatile acid of wine, and its presence at high concentrations gives a vinegar odour and a disagreeable sensation in mouth. Acetic acid can be produced by yeast, lactic acid bacteria and acetic acid bacteria. *Saccharomyces cerevisiae* only produce small quantities of this compound in fermentation (0.1-0.3 g/l). Conversely, stuck and sluggish fermentations can generate large amounts of this acid due to high production by yeasts or lactic disease development (Zamora, 2009). Besides, there are other organic acids such as tartaric, malic, lactic, succinic and citric acids.

Acetaldehyde

Acetaldehyde, also called ethanal, is an intermediary of alcoholic fermentation obtained by the decarboxylation of pyruvate. Later on, acetaldehyde is reduced to ethanol although a little quantity always remains in the wine. Excessive acetaldehyde amount contributes to perception of oxidation in wine, although in some wines such as Fino and Manzanilla from Jerez high quantity of this compound is desirable (Zamora, 2009).

Sugars

Sweet wines have about 10% sugar content whereas dry wines have only about 0.1%. Sugars are the responsible for the wine sweetness.

Minerals

Minerals constitute from 0.2 to 0.4% of wine. Common mineral salts that are found in wines are sodium, potassium, magnesium, calcium, and iron.

Sulfites

Sulfites are added in winemaking process to achieve sterilization and wine preservation.

Phenolic compounds

Phenols form about 0.01 to 0.5% of wine. The main phenolic compounds are anthocyanins and tannins, which give red color and astringency to wines, respectively.

Aromatic compounds

Alcohols and esters, in addition to volatile organic acids and to lesser extent aldehydes, constitute the main compounds of secondary wine aroma (Rapp & Versini, 1991).

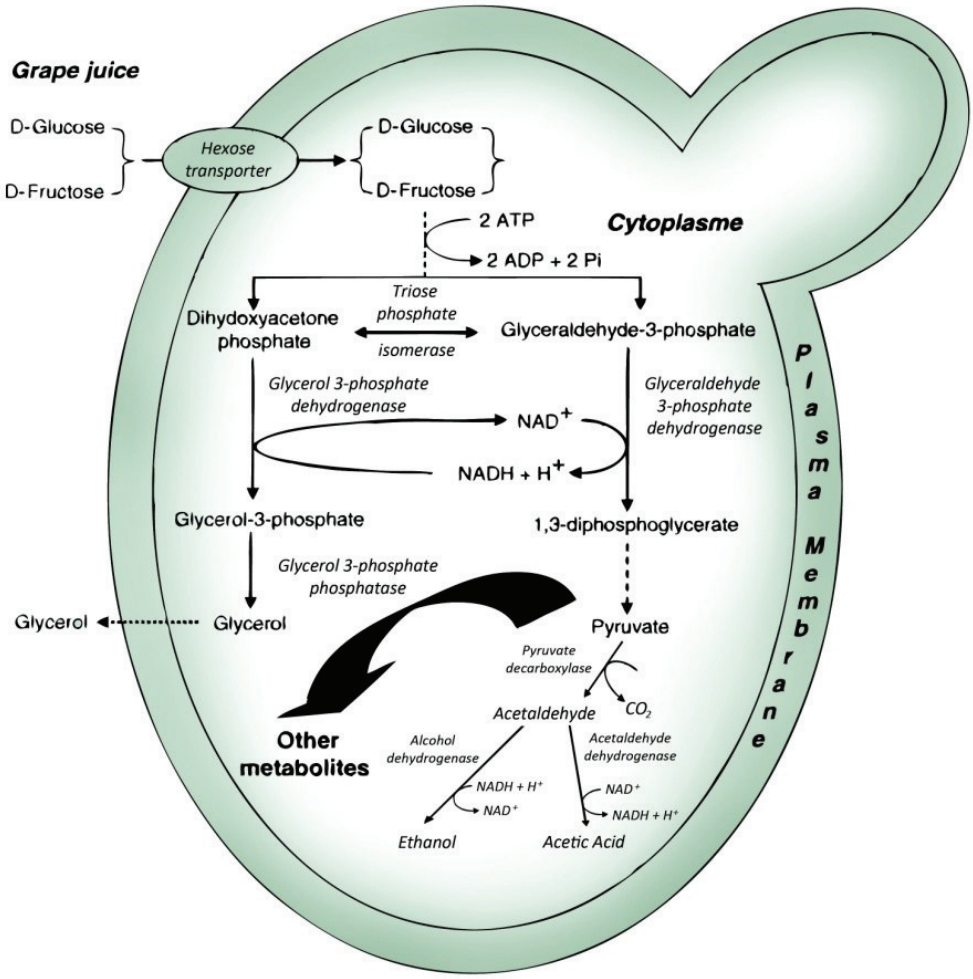

Fig 2. Alcoholic fermentation (adapted from Zamora, 2009).

4. Genus *Saccharomyces* in winemaking

Winemaking is a complex chemical and biological process in which different genera of yeast and bacteria are involved. The principal species of alcoholic fermentation in grape wine is *S. cerevisiae*, but the closely related *Saccharomyces uvarum*

(*Saccharomyces bayanus var. uvarum*) can also participate (Demuyter et al., 2004; Massoutier et al., 1998; Naumov et al., 2000, 2001; Sipiczki, 2002, 2008). Both *S. cerevisiae* and *S. uvarum* are able to grow on substrates characterized by high sugar and ethanol content, low pH, high sulphur dioxide concentrations and remains of fungicides, demonstrating that their genomes are well adapted to the oenological conditions (Sipiczki, 2008). However, *S. cerevisiae* has higher resistance to high temperature stress (up to 37°C) and higher ethanol resistance (up to 15%) than *S. uvarum* (Belloch et al., 2008). From the oenological point of view, these *Saccharomyces* species differ in several properties. Comparison between *S. uvarum* and *S. cerevisiae* reveals that the former is more cryotolerant, produces smaller acetic acid quantities, lower amounts of amyl alcohols, but higher amounts of glycerol, succinic acid, malic acid, isobutyl alcohol, isoamyl alcohol and numerous secondary compounds (Sipiczki, 2002). Wines produced by *S. uvarum* strains have a higher aromatic intensity than those produced by *S. cerevisiae* (Coloretti et al., 2006; Henschke et al., 2000). Specifically, *S. uvarum* produces higher amount of 2-phenylethanol, 2-phenylethyl acetate and ethyl lactate (Antonelli et al., 1999; Di Stefano et al., 1981; Gangl et al., 2009). On the other hand, *S. uvarum* is less common and appears mainly in fermentations at low temperatures (Antunovics et al., 2003; Demuytier et al., 2004; Masneuf-Pomarède et al., 2010; Sipiczki et al., 2001).

Other members of the genus *Saccharomyces* (*S. cariocanus, S. kudriavzevii, S. mikatae, S. paradoxus, S. arboricolus, S. pastorianus*) are not likely to play important roles in wine fermentation (Sipiczki,

2008). Nevertheless, *S. paradoxus* has been found in grapes in the north-western region of Croatia and it is currently used to ferment wines (Redzepovic et al., 2002). Likewise, *S. kudriavzevii* species has only been isolated in natural environments, like decayed leaves (Naumov et al., 2000) or oak barks (Sampaio & Gonçalves, 2008; Lopes et al., 2010). However, *S. kudriavzevii* participates in hybrid formation with *S. cerevisiae* and *S. bayanus* species, which are present in industrial fermentations. Physiological characterization of *S. kudriavzevii* strains has showed that they are able to grow at low temperatures (10ºC) and at higher ones (up to 30ºC) however they are not able to tolerate more than 5% of ethanol (Belloch et al., 2008).

Natural hybrids of *S. cerevisiae, S. bayanus* and *S. kudriavzevii* conducting wine fermentations have been recently discovered and characterized by genetic approaches (Belloch et al., 2009; Dunn et al., 2008; González et al., 2006, 2008; Horinouchi et al., 2010; Masneuf et al., 1998; Nguyen et al., 2000; Sipiczki, 2008). Hybridization process between *Saccharomyces* species has been proposed as an adaptation mechanism of yeasts to ferment at low temperatures (de Barros Lopes et al., 2002; Barrio et al., 2008; Sipiczki, 2008). Physiological data suggest that *Saccharomyces* hybrids might have inherited the ability to grow at high temperatures (30-37ºC) and ethanol tolerance from their *S. cerevisiae* parental and ability to grow at low temperatures (10-16ºC) from their *S. bayanus* and *S. kudriavzevii* parental. These physiological characteristics point to *Saccharomyces* hybrids as better adapted to meet the new winemakers' tendencies, such as conducting wine fermentation at

low temperatures, which causes wine aroma improvement (Lambrecht & Pretorius, 2000; Torija et al., 2003; Llauradó et al., 2002, 2005; Novo et al., 2003).

Oenological characterization of hybrids between *S. cerevisiae* and *S. kudriavzevii* has demonstrated that the hybrids are well adapted to ferment at low and intermediate conditions of temperature, giving intermediate or higher amounts of glycerol, less acetic acid and higher amounts of higher alcohols with regard to reference strains of *S. cerevisiae* and *S. kudriavzevii* (Gangl et al., 2009; González et al., 2007). Concerning oenological characterization of natural hybrids between *S. bayanus* and *S. cerevisiae*, there is scarce information in spite of having been described by some authors in wine and cider some years ago (Masneuf et al., 1998; Nguyen et al., 2000). However, artificial *S. cerevisiae* x *S. bayanus* hybrids have been constructed and characterized. These hybrids seem to have inherited the cryotolerance from *S. bayanus* (Kishimoto et al., 1994) and they produce intermediate glycerol concentrations with respect to their parental species (Zambonelli et al., 1997). Regardless of the limited studies on the fermentative potential of *Saccharomyces* hybrids, several strains (Lalvin W27, Lalvin W46, Lalvin S6U) are being commercialized to perform fermentations at low temperature enhancing varietal aromas (Lallemand Inc. WEB page).

5. Wine aroma

Aroma profile is constituted by the interaction of different groups of compounds. Ethanol content in wine and major volatile compounds form an "aromatic buffer", which is not easy to break. Only certain molecules of group of molecules acting in a synergic way can break it (Escudero et al., 2004; Ferreira et al., 2002). This buffer is constituted by 22 chemical substances and it is present in all wines in concentrations above their sensory threshold. The most relevant aromatic nuances of the most important wines are the consequence of complex odorants associations acting as contributors, suppressors or enhancers (Ferreira, 2007).

Wine aromatic buffers can be broken through one unique molecule in enough concentration (impact compound), through one group of homologue molecules (groups with impact), through one big group of molecules with similar aromatic note (subtle groups or compounds) or with the help of an aroma enhancer (Ferreira, 2007; Fretz et al., 2005; Guth et al., 1997a, 1997b; López et al., 1999, 2003). The more complex aromatic profile the wine has, the more important are odorant interactions.

Furthermore, wine aroma is not static, since it is changing constantly due to new odorant molecules derived from aromatic precursors. Grapes play a crucial role in wine aroma profile as source of volatile compounds and aromatic precursors (Hernández-Orte et al., 2008; Loscos et al., 2007). In addition, aromas produced by yeasts as a result of carbohydrate and aminoacid metabolism, have a very

important contribution to the final wine aroma (Lambrechts & Pretorius, 2000; Swiegers et al., 2005; Swiegers & Pretorius, 2005).

In order to distinguish the origin of molecule odorants, wine aroma can be divided in primary or varietal aroma, secondary or fermentative aroma and tertiary aroma, also called "bouquet".

5.1. Primary or varietal aroma

Primary or varietal aroma is constituted by different chemical compounds whose origin is grapes. These chemical compounds can be in two forms:

- Free aromatic molecules: methoxypyrazines, varietal thiols and monoterpenes (Loscos, 2009) (Table 1).

- Precursors: unsaturated fatty acids, phenolic acids, S-cysteine conjugates, dimethylsulfide precursors, carotenoids and glycoconjugates (Baumes, 2009; Loscos, 2009) (Table 2).

Table 1. Free aromatic molecules found in musts.

Free aromatic molecules	Definition	Main compounds	Aroma	Odour threshold (ng/L)
Methoxypyrazines				
Cabernet Sauvignon, Sauvignon Blanc, Cabernet Franc, Merlot	Nitrogenated heterocyclic compounds from leucine, isoleucine, valine and glioxal metabolism	3-Isobutyl-2-methoxypyrazine	herbaceous	10[a*]
		3-Sec-butyl-2-methoxypyrazine	herbaceous	1-2[b*]
		3-Isopropyl-2-methoxypyrazine	herbaceous	2[c*]
Varietal tiols				
Sauvignon Blanc, Verdejo, Merlot	Sulphur compounds with thiol function	4-Mercapto-4-methyl-2-pentanone	box tree	0.8[d*]
		3-Mercaptohexanol	grapefruit, passion fruit	60[e*]
		3-Mercaptohexyl acetate	exotic fruit	4[f*]
Monoterpenes				
Moscatel, Riesling, Gewürztraminer	Simple hydrocarbures, aldehydes, alcohols, acids, oxides and esters with 10 carbon atoms	Linalool	floral, moscatel	25[g**]
		α-Terpineol	floral, lily	250[g**]
		Nerol	floral, rose	400[h**]
		Geraniol	rose, geranium	30[i**]
		Hotrienol	lime	110[j**]

a Kotseridis et al., 1998; b Sala et al., 2004; c Maga, 1990; d Darriet et al., 1995; e Tominaga et al., 1998; f Tominaga et al., 1996; g Ferreira et al, 2000; h Ribéreau-Gayon et al., 1975; i Guth, 1997a,b; j Simpson, 1978b; * results expressed in ng/l; ** results expressed in µg/l.

The presence of these chemical compounds in grapes is related to grape variety, characteristics of the soil, climatic conditions and viticulture practices. In some cases, several of these aromatic compounds have been related to certain grape varieties, although most of them appear in most wines in varying proportion according to the different grape varieties (Ribéreau-Gayon et al., 1975; Simpson, 1978a; Lacey et al., 1991; Tominaga et al., 2004).

Grape varieties, such as Moscatel, Riesling or Gewürztraminer, present a typical aroma similar to the one found in the resulting wines, whereas non aromatic or neutral grape varieties produce musts without a typical aroma, although the resulting wines develop specific aromatic notes depending on each grape variety (Günata et al., 1985).

5.1.1. Glycosidic precursors

Synthesis of glycosidic precursors during grape ripening is affected by environmental temperature changes (Park et al., 1991) and solar exposition, having less synthesis with less solar exposition (Bureau et al., 2000a, 2000b). In general, aroma compounds and aromatic precursors are more concentrated in grape skins; therefore an increase in the time of contact between grape skins and must means an increase in the transference of these compounds to the must (Sánchez-Palomo et al., 2006; Tamborra et al., 2004; Castro-Vázquez et al., 2002). Moreover, an increase in maceration time would conduct to colour enhancement, bitterness and astringency due to extraction of phenolic compounds.

Glycosidic precursors are a potential source of aromatic compounds, which can be released during winemaking or wine aging by biological or chemical action (Sefton et al., 1993, 1994; Sefton, 1998). Sometimes the released molecule is aromatic (some monoterpenols, volatile phenols, vanillins and aliphatic alcohols), but sometimes additional reactions are necessary to form an aromatic molecule (β-damascenone, β-ionone, TDN, TPB, Riesling acetal and vitispirane). Enzymatic hydrolysis is quantitatively more important (Loscos et al., 2009), whereas acid hydrolysis has more qualitative importance (Francis et al., 1992; Sefton et al., 1993; Williams et al., 1989).

In wines, several diglycosides can appear (Voirin et al., 1990; Williams et al., 1982):

- β-D-glucopyranose
- α-L-arabinofuranosyl-β-D-glucopyranose
- α-L-rhamnopyranosil-β-D-glucopyranose
- β-D-apiofuranosyl-β-D-glucopyranose

Enzymatic hydrolysis of diglycosides is carried out in two steps (Figure 3). First, the enzymatic activity of α-arabinosidase, α-rhamnosidase or β-apiosidase breaks the bond between the two sugars, and second, β-glucosidases release the aromatic molecule (Günata et al., 1988). However, enzymes able to release the aromatic molecule in only one step have been described (Günata et al, 1998).

Table 2. Aromatic precursors found in musts.

Aromatic precursors	Definition	Compounds formed	Aroma	Odour threshold (µg/l)
Unsaturated fatty acids				
(linoleic acid, linolenic acid)	Carboxylic acid with a long unbranched aliphatic unsaturated chain	C-6 Alcohols: hexanol	herbaceous, bitterness	500[a]
		C-6 Aldehydes: hexanal, 2-hexenal	herbaceous, bitterness	5-17[b,c]
		γ- and δ-Lactones	coconut, vanilla, berry, coffee, dark chocolate	1-30[d,e]
Phenolic acids				
(caleic acid, p-coumaric acid, ferulic acid)	Hydroxylated derivatives of benzoic and cinnamic acids	*Volatile phenols:*		
		4-vinylphenol + 4-vinylguaiacol	medicine, smoked, leather	725[i]
		4-ethylphenol	medicine, smoked, leather	350-1000[g]
		4-ethylguaiacol	medicine, smoked, leather	47[h]
S-cysteine conjugates				
(S-4-(4-methylpentan-2-one)-l-cysteine, S-3-(hexan-1-ol)-l-cysteine)	S-cysteine conjugates	*Varietal thiols:*		
		4-mercapto-4-methyl-2-pentanone	box tree	0.0042[r]
		3-mercaptohexanol	grapefruit, passion fruit	0.0008[r]
		3-mercaptohexyl acetate	exotic fruit	0.060[r]
Dimethyl sulphide precursors				
(sulphur aminoacids, cystine, cysteine, glutation, dimethylsulfoxide)	Sulphur compounds	Dimethyl sulphide	fruity, truffle	25[l], 27[k]
Carotenoids				
(lutein, β-carotene, neoxantine, flavoxantine, violaxantine, 5,6-epoxilutein, luteoxantine)	Tetraterpenoid organic pigments	*C-13 Nor-isoprenoids:*		
		β-damascenone	floral, compote	0.05[j]
		β-ionone	violet	0.09[m]
		TDN	kerosene	200[n]
		TPB	kerosene	0.04[o]
		3-oxo-α-ionol	tobacco	nd[j]
		actinidiols	camphor, eucalyptus	nd[j]
		vitispirane	camphor, eucalyptus	800[p]
		Riesling acetal	fruity	nd[j]
Glycoconjugates				
(β-D-glucopyranoside, α-L-rhamnopyranosil-β-D-glucopyranoside, α-L-arabinofuranosil-β-D-glucopyranoside, α-L-apiofuranosil-β-D-glucopyranoside)	Aromatic compounds linked to sugar compounds	Monoterpenes	floral	25-400[**]
		C-13 Nor-isoprenoids	various	0.04-800[***]
		Volatile phenols	medicine, smoked, leather	47-1000[***]
		Shikimic derivatives:		
		Ethyl dihydrocinnamate	flowery	1.6[m]

*nd: not determined; ** see Table 1; *** see above; a Ferreira et al., 1995; b Colagrande, 1989; c Flath et al., 1967; d Abott et al., 1995; e Nakamura et al., 1988; f Chatonnet et al., 1993; g Tchovanov et al., 2008; h Chatonnet et al., 1990; i Tominaga et al., 2000; j Spedding & Raut et al., 1982; k Anocibar Beloqui, 1998; l Guth, 1997a,b; m Ferreira et al., 2000; n Simpson, 1978c; o Janusz et al., 2003; p Simpson et al, 1977; q Culleré et al., 2004; r Maga, 1973.

In winemaking there are several natural sources for glycosidases: grapes, yeasts and bacteria, however exogenous addition of commercial enzymes is also common. Glycosidase activity in yeasts, *Saccharomyces* and non-*Saccharomyces* has been described by several authors (Delcroix et al., 1994; Esteve-Zarzoso et al., 1998; Fleet, 2008; Günata et al., 1993; Hernández et al., 2003; Mateo et al., 1997; McMahon et al., 1999; Ugliano et al., 2006; Zoecklein et al., 1997). Recent studies have demonstrated yeasts are capable of releasing aromatic molecules from glycosidic precursors in winemaking conditions (Hernández-Orte et al., 2008; Loscos et al., 2007; Ugliano et al., 2006). Moreover, an additional result of glycosidase activity has been described associated to colour decrease in wines due to anthocyanidin release as a consequence of anthocyanin hydrolysis (Blom, 1983; Huang, 1955; Manzanares et al., 2000; Wightman & Wrolstad, 1996; Wrolstad et al., 1994).

In addition to hydrolysis of glycosides of monoterpenes and other aromas, *Saccharomyces* yeasts are able to carry out terpenes biotransformation into other terpenes (Gramatica et al., 1982; King and Dickinson, 2000; Zea et al., 1995; Zoecklein et al., 1997) (Figure 4).

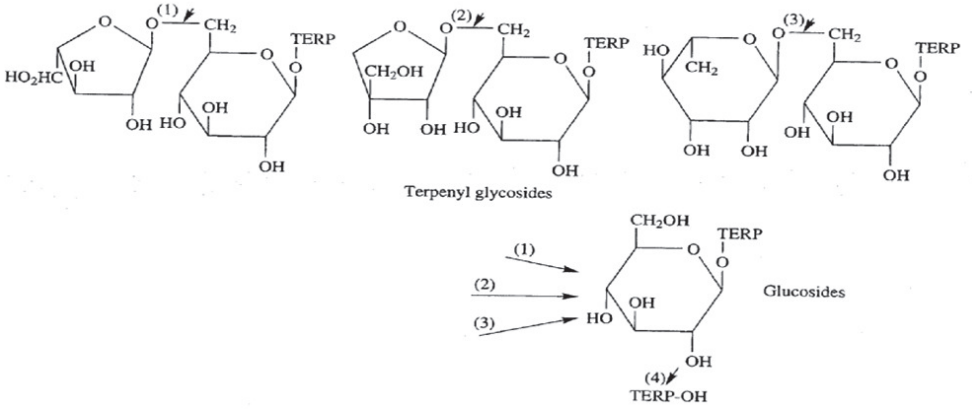

Figure 3. Hydrolysis of terpenyl glycosides (King&Dickinson, 2000).

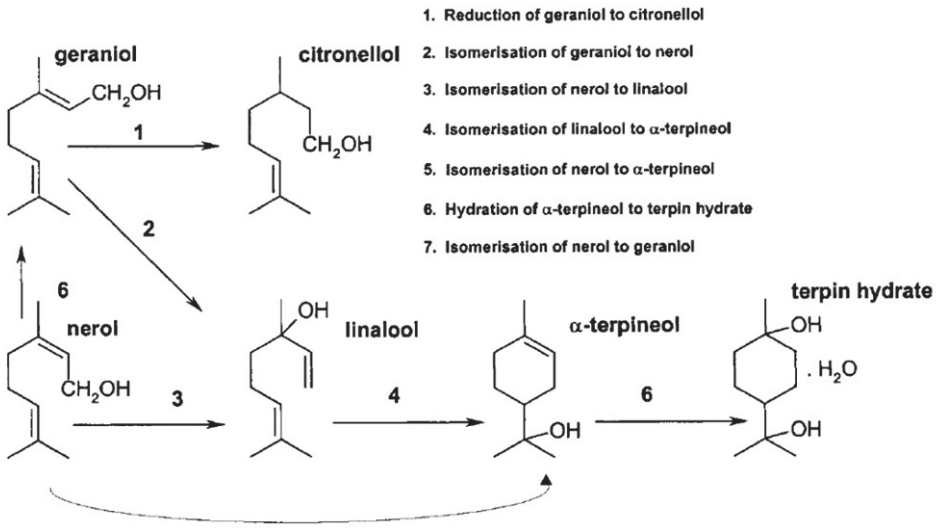

Figure 4. Terpenes biotransformation carried out by yeasts (King&Dickinson, 2000).

5.2. Secondary or fermentative aroma

Aromatic compounds synthesized by yeasts and lactic acid bacteria during winemaking process constituted the secondary aroma (Table 3). The most important compounds within fermentative aroma are higher alcohols, acetate esters and ethyl esters (Mountounet, 1969; Rapp & Mandery, 1986). However, other low sensory impact compounds such as aldehydes (acetaldehyde), ketones and organic acids (acetone, acetoine, 2,3-pentadione and acetic acid), volatile phenols (4-vinylphenol, 4-vinilguaiacol) and sulfurous compounds (hydrogen sulfide) are also important (Lambrechts & Pretorius, 2000).

5.2.1. Higher alcohols

Higher or fusel alcohols are alcohols with two or more carbon atoms with molecular weight and boiling point higher than those of ethanol (Lambrechts & Pretorius, 2000). From a quantitative point of view, higher alcohols are the most important group of volatile compounds produced by yeast during wine fermentation. Higher alcohols have an intense aroma in wine and in other alcoholic beverages. According to Rapp & Versini (1991), concentrations of higher alcohols below 300 mg/l add desirable complexity to wine aroma, whereas higher concentrations (400 mg/l) can be detrimental to wine quality by disguising fruity aromas given by esters.

Higher alcohols are classified in aliphatic like isobutanol, hexanol and isoamyl alcohol and aromatics like 2-phenylethanol and benzyl alcohol. The concentration of each higher alcohol acting positively or negatively on wine aroma depends on aroma intensity and wine style. In spite of having aroma themselves, the main oenological importance of higher alcohols lies in the fact that they are precursors of acetate esters (Soles et al., 1982).

Higher alcohols are synthesized by the Ehrlich pathway from branched-chain amino acids, leucine, valine and isoleucine, aromatic amino acids, phenylalanine, tyrosine and trytophan, and the sulfur-containing amino acid methionine (Fig.3). In this metabolic pathway the aminoacids are transaminated to the corresponding α-ketoacid, followed by decarboxylation to aldehydes. Finally, these aldehydes are reduced to higher alcohols, whereas NADH becomes NAD^+. These chemical reactions are carried out by aminoacid permeases, transaminases, decarboxylases and dehydrogenases. Aminoacid permeases are codified by the genes *GAP1*, *BAP2*, BAP3, MMP1 and MUP3 (Didion et al., 1998; Grauslund et al., 1995; Isnard et al., 1996; Jauniaux & Grenson, 1990; Mai & Lipp, 1994; Rouillon et al., 1999), branched-chain aminoacids transaminases by *BAT1* and *BAT2* and aromatic aminoacids transaminases by *ARO8* and *ARO9* (Dickinson & Norte, 1993; Eden et al., 2001; Hazelwood et al., 2008; Kispal et al., 1996; Lilly et al., 2006; Ugliano & Henschke, 2009), decarboxylases by *PDC1*, *PDC5*, *PDC6*, *THI3* and *ARO10* (Dickinson et al., 2003; Hazelwood et al., 2008; Vuralhan et al., 2003), and dehydrogenases by *ADH1*, *ADH2*, *ADH3*, *ADH4*, *ADH5*, *ADH6*, *ADH7* and *SFA1* (Delneri et al., 1999; Hazelwood et al., 2008). Furthermore,

aryl alcohol dehydrogenases, *AAD10* and *AAD14*, are believed to be responsible for the degradation of aromatic aldehydes into their corresponding higher alcohols (Delneri et al., 1999). Higher alcohols can be also produced *de novo* through carbohydrate metabolism (Äyräpää, 1968, 1971).

Many factors affect the levels of higher alcohols in wine, such as yeast strain and species, initial sugar content, pH and composition of the grape juice, fermentation temperature, assimilable nitrogen, aeration, grape variety and maceration time (Fleet & Heard, 1993; Houtman et al., 1980; Houtman & du Plessis, 1981). Regarding yeast species carrying out the fermentation, higher alcohol production is strain dependent (Giudici et al., 1990; Longo et al., 1992; Mateo et al., 1991).

Several studies have demonstrated that *Saccharomyces bayanus* produces higher amounts of several higher alcohols (2-phenylethanol, isobutyl alcohol and isoamyl alcohol) than *S. cerevisiae* (Antonelli et al., 1999; Massoutier et al., 1998). Other authors observed greatest higher alcohol production by *Saccharomyces* species with respect to non-*Saccharomyces* species (Gil et al., 1996; Herraiz et al., 1990).

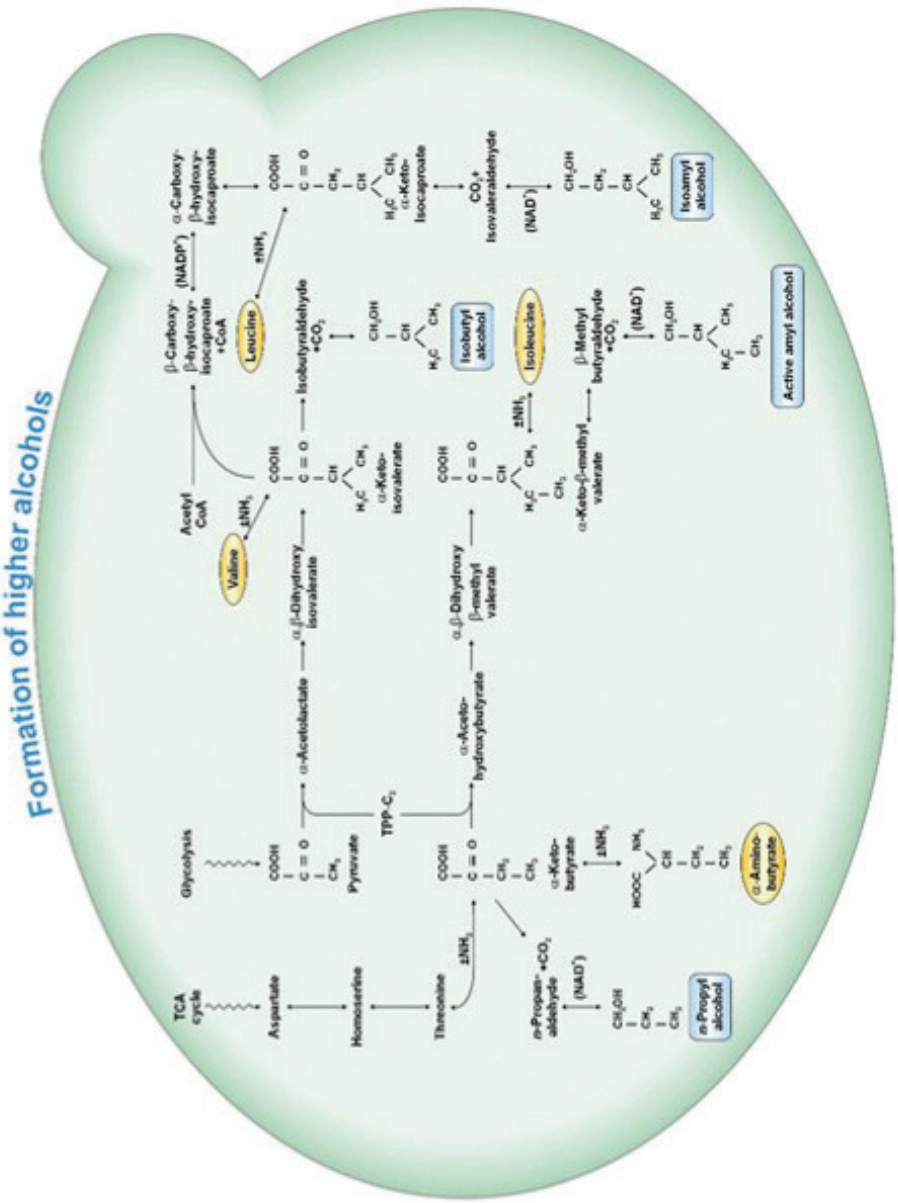

Fig.4. Schematic representation of the biosynthesis of higher alcohols in wine yeast (Boulton et al., 1998).

5.2.2. Acetate esters

Acetate esters such as ethyl acetate, isoamyl acetate, isobutyl acetate, 2-phenylethyl acetate, hexyl acetate and benzyl acetate give desirable fruity and floral aromas to the wines (Lambrechts & Pretorius, 2000; Swiegers et al., 2005).

Acetate esters are synthetised by a condensation between higher alcohols and acetyl-CoA (Fig.4). This reaction is mediated by acetyltransferases codified by genes *ATF1* and *ATF2* (Fujii et al., 1994, 1996; Fujiwara et al., 1999; Lilly et al., 2006; Saerens et al., 2008, 2010; Verstrepen et al., 2003). Besides, the effect of esterases codified by *IAH1* and *TIP1* is also important for the final concentration of acetate esters in the wine (Horsted et al., 1998; Lilly et al., 2006; Saerens et al., 2008, 2010).

Concentration of acetate esters in wine is affected by different factors such as maturity and sugar content of the grapes (Houtman et al., 1980a, 1980b), yeast strain and species, fermentation temperature (Piendl and Geiger, 1980), alcoholic and malolactic fermentation, winemaking method (Herraiz and Ough, 1993; Gómez et al., 1994) or the presence of non- soluble material in the must (Edwards et al., 1985). Besides, different factors after the fermentative process, such as time and temperature of aging and storage, affects ester content in wine (Marais and Pool, 1980; Ramey and Ough, 1980). Regarding yeast species carrying out the fermentation, acetate ester production depends on each strain (Antonelli et al., 1999; Mateo et al., 1992). Some studies have demonstrated that *S. cerevisiae* produces high amounts of several

acetate esters such as isopenthyl acetate, phenylethyl acetate, isoamyl acetate, hexyl acetate (Nykänen & Nykänen, 1977; Soles et al., 1982; Suomalainen & Lehtonen, 1979), whereas *S. bayanus* has demonstrated to be a good 2-phenylethyl acetate producer (Soles et al., 1982). Comparison between *Saccharomyces* and non-*Saccharomyces* regarding acetate esters production showed species dependence in the production of these aromatic compounds (Gil et al., 1996; Lema et al., 1996; Rojas et al., 2001).

Ethyl acetate is the most common ester in wine, being the product of condensation of acetic acid and ethanol generated during fermentation. The aroma of ethyl acetate is most vivid in younger wines and contributes towards the general perception of "fruitiness", although excessive amounts of this acetate ester, from 150 to 200 mg/l, are considered a wine fault (Amerine & Cruess, 1960; Corison et al., 1979).

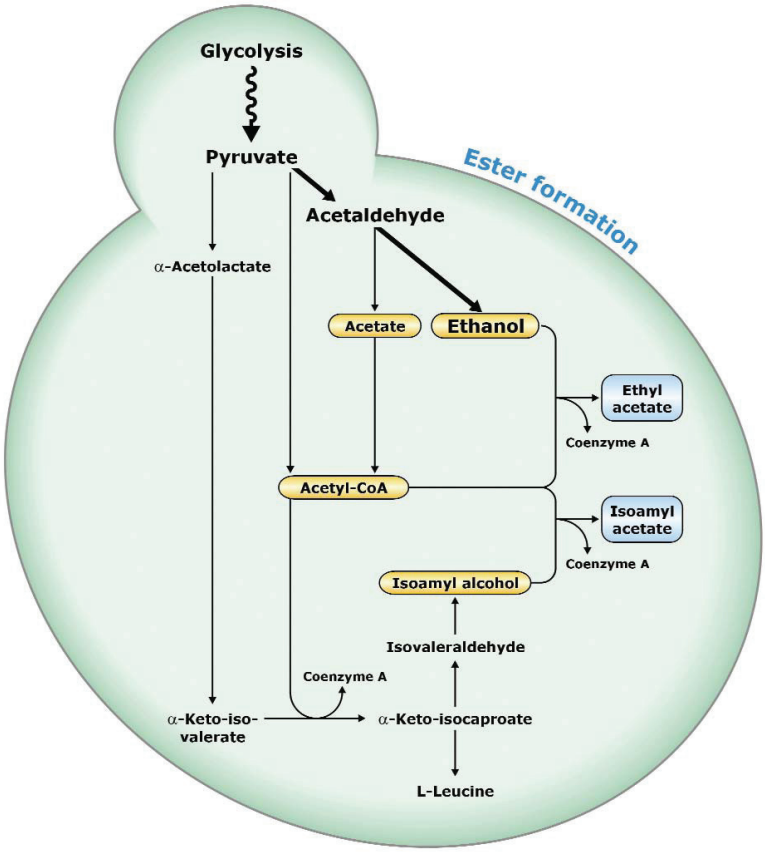

Fig. 5. Schematic representation of the formation of ethyl acetate and isoamyl acetate in wine yeast (Swiegers & Pretorius, 2005).

5.2.3. Ethyl esters

Ethyl esters such as ethyl propanoate, ethyl butanoate, ethyl hexanoate (ethyl caprylate), ethyl octanoate (ethyl caproate), ethyl decanoate (ethyl caprate) and ethyl lactate give desirable fruity and flowery aroma to the wine. They are produced by condensation between ethanol and acyl-CoA, reaction mediated by acyltransferases. These acyltransferases are codified by the genes

EHT1, *EEB1* and *YMR210W* (Rossouw et al., 2008; Saerens et al., 2006, 2008, 2010). Besides, in the final concentration of ethyl esters in the wine it must be taken into account the effect of esterases codified by *IAH1* and *TIP1* (Horsted et al., 1998; Lilly et al., 2006; Saerens et al., 2008, 2010).

Ethyl esters content in wines are affected by the same factors commented before in the case of acetate esters. Regarding yeast species carrying out the fermentation, ester production depends on each strain (Mateo et al., 1992). Several studies have demonstrated that *Saccharomyces cerevisiae* produced high amounts of several ethyl esters such as ethyl caproate, ethyl caprylate and ethyl caprate (Antonelli et al., 1999; Nykänen & Nykänen, 1977; Soles et al., 1982; Suomalainen & Lehtonen, 1979), whereas *S. bayanus* has demonstrated to be a good ethyl caprate and ethyl lactate producer (Antonelli et al., 1999; Soles et al., 1982). Comparison between *Saccharomyces* and non-*Saccharomyces* ethyl ester production showed *Saccharomyces* species produced equal or higher ethyl esters amounts (Gil et al., 1996; Lema et al., 1996).

Table 3. Genes involved in flavor-active compound formation (Bisson & Karpel, 2010).

Compound	Enzymatic activity	Genes identified
Higher alcohols	Branched-chain amino acid transferases	*BAT1, BAT2*
	Aromatic amino acid transferases	*ARO8, ARO9*
	Decarboxylases	*ARO10, PDC1, PDC5, PDC6, THI3*
	Alcohol dehydrogenases	*ADH1, ADH2, ADH3, ADH4, ADH5, ADH6, ADH7, SFA1*
Esters	Alcohol acetyl transferases	*ATF1, ATF2*
	Acyl transferases	*EEB1, EHT1, YMR210W*
	Esterases	*IAH2, EEB1, EHT1*
Volatile aglycones	β-glucosidases	*BGL2, EXG1, EXG2*

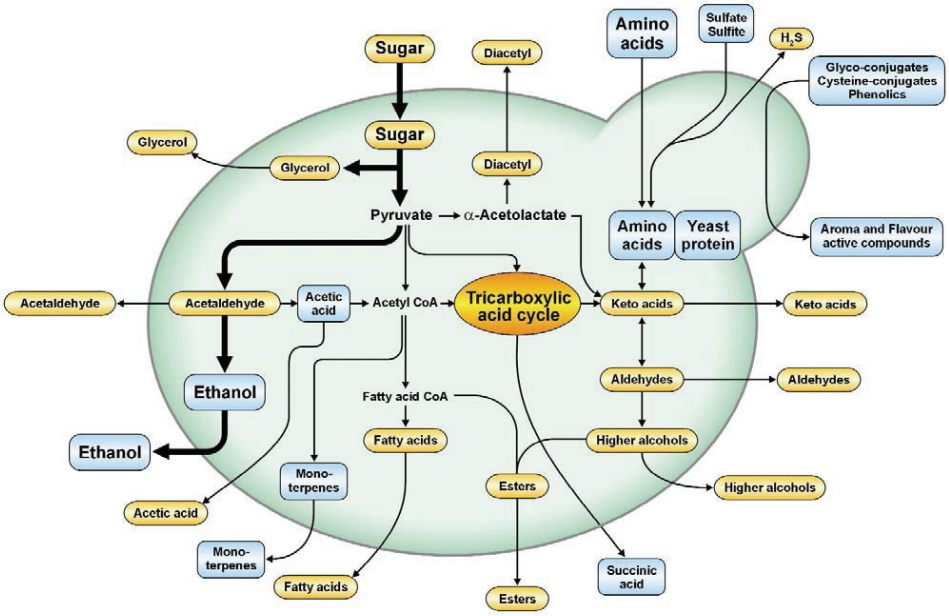

Table 3. Genes involved in flavor-active compound formation (Bisson & Karpel, 2010).

Table 4. Most important secondary aroma compounds in wines.

Aromatic compounds	Aroma	Odour threshold (mg/l)	Concentr. in wine (mg/l)[f]
Higher alcohols			
Isobutanol	wine, solvent, bitter	40[a]	9.0-174
Isoamyl alcohol	whiskey, malt, burnt	30[a]	6.0-490
1-Hexanol	resin, flower, green	8[a]	0.3-12.0
2-Phenylethanol	honey, spice, rose, lilac	10[a] , 14[b]	4.0-197
Acetate esters			
Ethyl acetate	pineapple	12.26[c]	22.5-63.5
Isobutyl acetate	floral	1.600[d]	0.01-1.6
Isoamyl acetate	banana	0.030[a]	0.1-3.4
Hexyl acetate	pear, fruity	0.115[e]	0-4.8
Phenylethyl acetate	rose, honey, tobacco	0.250[a]	0-18.5
Ethyl esters			
Ethyl caproate (ethyl hexanoate)	apple peel, fruit	0.005[a] , 0.014[b]	0.03-3.4
Ethyl caprylate (ethyl octanoate)	fruit, fat	0.002[a] , 0.005[b]	0.05-3.8
Ethyl caprate (ethyl decanoate)	grape	0.200[b]	0-2.1

[a] Guth, 1997a,b; [b] Ferreira et al., 2000; [c] Etiévant, 1991; [d] González, 2005; [e] Takeoka et al., 1996; [f] Swiegers et al., 2005.

5.3. Tertiary aroma or "bouquet"

Tertiary aroma is the consequence of several enzymatic and chemical reactions which take place during wine aging. Depending on the kind of aging it is possible to distinguish between oxidative "bouquet" (in wood barrels) and reductive "bouquet" (in bottles). During these processes some aromatic compounds are synthesized and some are modified, increasing or decreasing their levels (Oliveira et al., 2008; Rapp et al., 1985; Simpson, 1978a).

Oxidative "bouquet" consists of acetaldehydes and acetals synthesis and extraction of several wood compounds, such as phenolic compounds from lignin degradation or lactones like 3-methyl-ϒ-octalactone (Masuda & Nishimura, 1971).

Reductive "bouquet" is based on the interaction among wine compounds produced in fermentation. In this wine ageing, reduced sulphurous compounds, like dimethyl sulfide are produced (Marais et al., 1979). Furthermore, most primary aroma compounds show a significant increase and later a steady decrease. Even the levels of some volatile compounds related to wine ageing such as vitispiranes, Riesling acetal, 1,1,6-trimethyl-1,2-dihydronaphthalene (TDN), and trans-1-(2,3,6-trimethylphenyl)buta-1,3-diene (TPB) were found to decrease after the initial increase. However, vanillin derivatives, furan linalool oxides, 3-oxo-β-ionone, actinidiols, 4-ethylphenol and guaiacol showed a continuous increase along the ageing process. Levels of lactones, benzenes, guaiacol, terpenes and volatile phenols after ageing depend on grape variety used to carry out the fermentation process (Loscos et al., 2010).

Monoterpenes, such as linalool, geraniol and citronellol decrease, whereas there is an increase in the levels of nerol oxide, hotrienol, hydroxylinalool, and hydroxycitronellol (Rapp & Mandery, 1986).

In addition, higher alcohols remain constant or diminish (Simpson, 1979; Marais & Pool, 1980), whereas acetate esters diminish, except for ethyl acetate, dimethyl succinate, ethyl caproate and ethyl caprylate. Consequently, at the end of the ageing process there is a loss of fresh character and fruitiness (Uber, 2006).

6. Expression analysis of genes involved in aroma production

After the genome sequence of *S. cerevisiae* (Goffeau et al., 1996), research has focused on its comprehensive analyses including transcriptomic, proteomic, metabolomic, and phenotypic analyses. DNA array (also commonly known as gene or genome chip, DNA chip, or gene array) is one of the most powerful tools to monitor the expression of genes from a whole genome in one single experiment (Lashkari et al., 1997). A DNA array is a collection of DNA spots, commonly representing genes arrayed on a solid surface. Complementary DNA (cDNA) fragments that correspond to mRNAs, are spotted onto a glass surface. The array is hybridized with cDNA from two samples to be compared, being labelled with two different fluorophores. Relative intensities of each fluorophore are used in ratio-based analysis to identify up-regulated and down-regulated genes. Absolute levels of gene expression cannot be determined,

but relative differences in expression among different spots (genes) can be estimated (Pérez-Ortín et al., 2009).

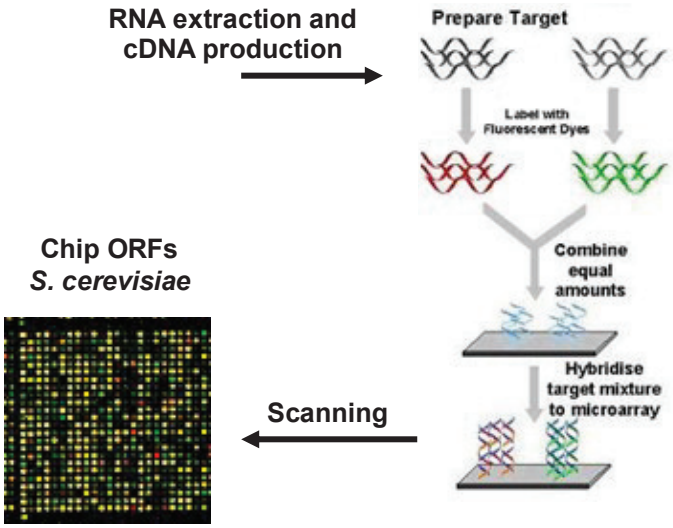

Fig. 7. Microarray analysis.

In the case of *S. cerevisiae*, many studies have been reported on genome-wide expression analysis using DNA microarrays to better understanding winemaking processes (Rossignol et al., 2003; Varela et al., 2005), temperature influence in growth or in aroma production (Pizarro et al., 2008; Beltrán et al., 2006), genes involved in aroma production (Rossouw et al., 2008), general or sugar stress response (Marks et al., 2008; Erasmus et al., 2003) or the response to nitrogen depletion (Backhus et al., 2001).

BACKGROUND & JUSTIFICATION

Aroma is one of the most important quality attributes of wines. Current trend in winemaking consists of producing wines with different aroma nuances to offer variety of wines to a developing market. In order to achieve this goal, new wine yeasts able to perform fermentation at low temperatures increasing wine aroma production and retention while maintaining good fermentation rates are necessary (Feuillat et al., 1997; Lallemand Inc. WEB page). The improved quality of wines produced at low temperatures can be attributed to greater retention of terpens and increase in the proportion of ethyl and acetate esters (Novo et al., 2003; Torija et al., 2003).

Recently, natural *Saccharomyces* hybrids have been isolated from low temperature fermentations (González et al., 2006 and 2008; Masneuf et al., 1998; Nguyen et al., 2000; Sipiczki, 2008). *Saccharomyces* hybrids seem to be better suited to ferment at low and intermediate conditions of temperature yielding intermediate or higher amounts of glycerol, less acetic acid and greater amounts of higher alcohols comparing to strains of parental species *S. cerevisiae* and *S. kudriavzevii* or *S. bayanus* (Gangl et al., 2009; González et al., 2007). Therefore, *Saccharomyces* hybrids would be better adapted to meet the new winemakers' tendencies (Lambrecht & Pretorius, 2000; Llauradó et al., 2002, 2005; Novo et al., 2003; Torija et al., 2003). Regardless of the limited studies on the potential of *Saccharomyces* hybrids to be used in winemaking, several strains (Lalvin W27, Lalvin W46, Lalvin S6U) are being commercialized to perform fermentations at low temperature and enhance varietal aromas (Lallemand Inc. WEB page).

This doctoral thesis explores the oenological traits of *Saccharomyces* species and hybrids relevant for present-day wine industry, especially regarding aroma production, as well as the molecular bases underneath. This exploration has been possible using different biochemical, analytical chemistry and molecular techniques to perform enzymatic activity detection, aroma profile determination and transcriptome analysis in wine fermentations. Through this doctoral thesis the abilities of the different *Saccharomyces* species and hybrids regarding primary aroma release and secondary aroma production, especially at low temperatures, has been elucidated in order to know the different possibilities that these yeasts offer to create new wines with different aromatic nuances.

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OBJECTIVES

1. Analysis of varietal aroma release from glycosilated precursors by the different *Saccharomyces* species and hybrids.

2. Analysis of monoterpene alcohols profile in wine by the different *Saccharomyces* species and hybrids: β-D-glucosidase activity and terpene bioconversion.

3. Study of secondary aroma production in wine by different cryotolerant *Saccharomyces* species and hybrid strains.

4. Expression analysis of genes related with secondary aroma production by DNA microarrays: parental species versus hybrid strains.

<u>OBJECTIVE 1</u>. Analysis of varietal aroma release from glycosilated precursors by the different *Saccharomyces* species and hybrids.

Chapter 1

Effect of aromatic precursor addition to wine fermentations carried out with different *Saccharomyces* species and their hybrids

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Abstract

This work explores the ability of different yeast strains from different species of the genus *Saccharomyces* (*S. cerevisiae, S. uvarum* and *S. kudriavzevii*) and hybrids between these species to release or form varietal aroma compounds from fractions of grape odourless precursors. The *de novo* synthesis by the yeasts of some of the varietal aroma compounds was also evaluated. The study has shown that *de novo* synthesis affects some lipid derivatives, shikimic derivatives and terpenes in all species and hybrids, with some remarkable differences amongst them. The release or formation of aroma compounds from precursors was found to be strongly linked to the yeast or hybrid used, and the triple hybrid *S. cerevisiae* x *S. bayanus* x *S. kudriavzevii* in particular and secondarily the hybrid *S. cerevisiae* x *S. bayanus* were highly efficient in the production of most varietal aroma compounds, including γ-lactones, benzenoids, volatile phenols, vanillin derivatives and terpenols. The presence of precursors in the fermenting media caused a surprising levelling effect on the fermentative aroma composition. Altogether, these results suggest that it is possible to modulate wine aroma by employing different yeast species in order to create new wines with different aromatic notes.

Keywords: *Saccharomyces*, hybrids, wine yeasts, aromatic precursors.

1. Introduction

From a chemical point of view, the volatile fraction of wines is mainly composed of higher alcohols and esters formed by yeast secondary metabolism, but there are many other wine aroma compounds in which formation yeast play a relevant role. The so called wine primary or grape-varietal aroma consists of lactones, benzenes, volatile phenols, vanillins, norisoprenoids, terpenes and some polyfunctional mercaptans present at low concentrations in the ng L^{-1}-µg L^{-1} range (Loscos et al. 2007; Mateo-Vivaracho et al. 2010; Tominaga et al. 1998). Most of these aroma compounds are accumulated in the grape under the form of odourless precursors (glycosides, polyhydroxylated molecules or cysteinil-derivatives), which implies that the aroma will be effectively released only after the precursor molecule is transformed. As certain yeast strains are able to release those aroma compounds by cleavage of the precursor molecules or are even able to synthesize new aroma molecules similar to the ones present in the grape, it can be affirmed that yeast can enhance wine varietal aroma.

It should be noted that a limited number of varietal aroma compounds in an also limited number of wines, really play a predominant aroma role. This is the case of some polyfunctional mercaptans on some white wines (Mateo-Vivaracho et al. 2010; Tominaga et al. 1998), of linalool and other terpenols on Muscat wines (Ribéreau-Gayon et al. 1989) or of cis-rose oxide on wines from Gerwurtztraminer (Guth, 1997). In most wines, varietal aroma is formed by combinations of many grape and yeast-derived

compounds, none of which play a predominant aroma role, and it is the aroma profile, i.e., the particular relative levels of aroma compounds, which is really aromatically significant and responsible for varietal and origin related difference (Escudero et al. 2007; Loscos et al. 2007, 2010). The aroma compounds derived from odourless glycosides are one of the most important constitutional parts of wine aroma and will be the subject of the present research.

The contribution of yeast to the formation of the wine varietal aroma by action on grape glycosidic precursors is well documented in the scientific literature (Darriet et al. 1988; Delcroix et al. 1994; Delfini et al. 2001; Fernández-González et al. 2003; Fernández-González & Di Stefano, 2004; Hernández et al. 2003; Hernández-Orte et al. 2008; Loscos et al. 2007; Mateo and Di Stefano, 1997; Spagna et al. 2002; Ugliano et al. 2006; Ugliano & Moio, 2008), but the role played by hybrids from different species is not well known. To the best of our knowledge, only in one work from Hernández-Orte et al. (2008) a *S. cerevisiae* x *S. bayanus* hybrid was tested.

Notwithstanding the fact that *S. cerevisiae* is the predominant species responsible for alcoholic fermentation, other species of the genus *Saccharomyces* seem to have an important role during fermentation processes (for a revision see Blondin et al. 2009). *S. bayanus var. uvarum* (or simply *S. uvarum*) has been described as adapted to low temperature fermentations during winemaking whereas the other four species included in the genus, *S. arboriculus*, *S. cariocanus*, *S. mikatae,* and *S. kudriavzevii*, have only been isolated from natural environments. In addition, some hybrids between the species of the genus *Saccharomyces* can participate

in fermentation processes. The first and best known examples are *S. pastorianus* strains involved in ale beer production. In the case of wine, other hybrids potentially involved in the alcoholic fermentation are the commercial wine strain *S. cerevisiae* x *S. uvarum* S6U (Masneuf et al. 1998) and several hybrids *S. cerevisiae* x *S. kudriavzevii* (González et al. 2006). These strains are better adapted to the present winemakers' tendency to decrease the wine fermentation temperature which strongly affects yeasts metabolism, but has been empirically shown to improve the aroma, taste and flavour density of the wine. Some basic research about the oenological characterization of the different species from *Saccharomyces* and hybrids between them have recently been published (Gangl et al. 2009; González et al. 2007; Masneuf-Pomarède et al. 2010), but none of them addressed the question of varietal aroma formation.

Despite the important role of the *Saccharomyces* genus in wine fermentation, this genus is not known as a good enzyme producer for releasing varietal aroma compounds from glycosidic precursors (glycosidases), although there are some scientific publications about certain *Saccharomyces* strains which are capable of doing this action in wine. Mateo and Di Stefano, (1997) analysed β-glucosidase activity in three *S. cerevisiae* and three *S. bayanus* strains; Fia et al. (2005) analysed the production of this enzyme by *S. cerevisiae* and non-*Saccharomyces* strains and Gamero et al. (2011) (Objective 2, Chapter 1) studied the activity of this enzyme in different *Saccharomyces* species and hybrids.

In the present work we have studied the ability of different yeast strains from the different species from the genus *Saccharomyces* (*S. cerevisiae, S. uvarum and S. kudriavzevii*) and hybrids between these species to release or form varietal aroma compounds from glycosidic precursors or from other sources during fermentation (*de novo* synthesis).

2. Materials and methods

2.1. Reagents and standards

Dichloromethane and methanol (LiChrosolv quality) were purchased from Merck (Darmstadt, Germany), pentane from Fluka (Buchs, Switzerland), ethyl acetate, absolute ethanol, sodium hydroxide, sodium fluoride, L(+)-ascorbic acid, ammonium sulfate, sodium dihydrogenphosphate1-hydrate, and disodium hydrogenphosphate 12-hydrate were supplied by Panreac (Barcelona, Spain). Pure water was obtained from a Milli-Q purification system (Millipore, U.S.). LiChrolut EN resins were purchased from Merck. The chemical standards were supplied by Aldrich (Gillingham, UK), Sigma (St. Louis, MO), ChemService (West Chester, PA), PolyScience (Niles, IL), Firmenich (Geneva, Switzerland), Panreac, Merck, Fluka, and Lancaster (Strasbourg, France) as shown in Table 1.

Table 1. Retention Indexes and Chemical Standards used for identification and quantification of volatile compounds.

	RI[b]	Source, purity	Compounds
			Terpenes
1	1447	Tentatively identified	(Z)-linalool oxide
2	1476	Tentatively identified	(E)-linalool oxide
3	1556	Fluka, 98.5%	Linalool
4	1565	Tentatively identified	Linalyl acetate
5	1608	Tentatively identified	Terpinen-4-ol
6	1613	Tentatively identified	2,6-dimethyl-1,7-octadiene-3,6-diol
7	1664	Tentatively identified	δ-terpineol
8	1705	Fluka, 97%	α-terpineol
9	1775	Fluka, 90-95%	β-citronellol
10	1858	Fluka, 99.5%	Geraniol
11	2366	Tentatively identified	Neric acid
			Norisoprenoids
12	1526	Tentatively identified	Vitispirane A[a]
13	1529	Tentatively identified	Vitispirane B[a]
14	1637	Tentatively identified	Riesling acetal[a]
15	1748	Tentatively identified	1,1,6-trimethyl-1,2-dihydronaphthalene (TDN)
16	1832	Tentatively identified	t-1-(2,3,6-trimethylphenyl)but-1,3-diene (TPB)
17	1829	Firmenich, 90%	β-damascenone
18	1939	Tentatively identified	3-oxo-β-ionone
19	1950	Sigma, 98%	β-ionone
20	1952	Tentatively identified	Actinidols[a]
21	2657	Tentatively identified	3-oxo-α-ionol
			Volatile phenols
22	1876	Aldrich, 98%	Guaiacol
23	2068	Lancaster, 98%	4-ethylguaiacol
24	2237	Aldrich, 99%	Eugenol
25	2244	Aldrich, 99%	4-ethylphenol
26	2262	Aldrich, 98%	4-vinylguaiacol
27	2317	Aldrich, 99%	2,6-dimethoxyphenol
28	2279	Lancaster, 97%	(E)-isoeugenol
29	2404	Lancaster, 10% soln.	4-vinylphenol
30	2563	Aldrich 90%	4-allyl-2,6-dimethoxyphenol
			Vanillin derivatives
31	2592	Panreac, 99%	Vanillin
32	2629	Aldrich, 99%	Methyl vanillate
33	2654	Lancaster, 97%	Ethyl vanillate
34	2664	Aldrich, 98%	Acetovanillone
35	2829	Aldrich, 96%	Zingerone
36	2892	Aldrich, 99%	Homovanillyl alcohol
37	3040	Aldrich, 98%	Syringaldehyde
38	3099	Tentatively identified	Homovanillic acid
39	3123	Aldrich, 97%	Acetosyringone

Table 1. (continuation).

	RI[b]	Source, purity	Compounds
			Benzenes
40	1520	Fluka, 99%	Benzaldehyde
41	1659	Aldrich, 90%	Phenylacetaldehyde
42	1891	Aldrich, 99%	Benzyl alcohol
43	1908	Aldrich, 99%	Ethyl dihydrocinnamate
44	1926	Fluka, 99%	β-phenylethanol
45	2081	Aldrich, 99%	Ethyl cinnamate
46	2219	Fluka, 98%	2-phenoxyethanol
47	2725	Tentatively identified	1,2-dimethoxy-4-propylbenzene
			Lactones
48	1988	Lancaster, 98%	δ-octalactone
49	2068	Aldrich, 97%	γ-nonalactone
50	2141	Aldrich, 98%	γ-decalactone
51	2260	Lancaster, 98%	δ-decalactone
			Miscellaneous
52	1390	Aldrich, 98%	(Z)-3-hexen-1-ol
53	1672	Lancaster, 98%	3-methylbutyric acid
54	1677	Aldrich, 98%	2-methylbutyric acid

[a]Actinidols: 2,2,6-trimethyl-8-(1-hydroxy)ethyl-7-oxabicyclo[4.3.0]nona-4,9-dienes;
Riesling Acetal: 2,2,6,8-tetramethyl-7,11-dioxatricyclo[6.2.1.0(1,6)]undec-4-ene;
Vitispirane: 2,10,10-trimethyl-6-methylen-1-oxaspiro-[4,5]dec-7-ene.
[b] Retention index calculated in a DBWAXetr column.

2.2. *Samples*

Grapes from *Vitis Vinifera* vars. Macabeo, Muscat, Verdejo, Tempranillo, Parellada and Parraleta cultivated in different regions of Spain in 2007, were harvested by hand and they were stored frozen at -30°C.

2.3. *Precursor extract preparation*

Precursors were extracted from six different floral and non-floral grape varieties (Macabeo, Muscat, Verdejo, Tempranillo, Parellada and Parraleta) to obtain a complex "multivarietal" pool of

precursors. The procedure is based on that described in Ibarz et al. (2006). Grapes were treated in batches of 500 g of a single variety, and they were destemmed by hand and homogenized with a mixer Ultra Turrax T25 Basic (Ika, Labortechnik) in the presence of 0.13 M NaF and 50 mg/l ascorbic acid. The triturate was centrifuged at 4500 rpm for 15 min at 10°C to separate the must from the skin, followed by a filtration through filter paper under vacuum. The mashes of skin obtained (around 80 g per batch) were suspended in 380 mL of a buffer solution (0.1 M Na_2HPO_4/NaH_2PO_4) at pH 7 and 13% ethanol and allowed to macerate in the dark (24 h, 20°C, nitrogen atmosphere) to extract the precursors. This solution was centrifuged at 4500 rpm for 15 min at 20°C, and the supernatant was filtered through filter paper under vacuum. Ethanol was then removed at room temperature by vacuum distillation in a rotary evaporator. This solution (260 mL per batch) is the "macerate". The must (300 mL per batch) and the macerate were percolated through two LiChrolut EN (650 mg) resin beds (previously conditioned with 16 mL of dichloromethane, 16 mL of methanol, and 32.5 mL of Mili-Q water). In both cases the column was washed with 20 mL of Mili-Q water, dried and washed again with 20 mL of dichloromethane. The retained precursors were finally eluted with 25 mL of an ethyl acetate: methanol (9:1 v/v) The ethyl acetate extracts were mixed and evaporated under vacuum to dryness. These dry extracts were reconstituted in 7 mL of a 50% ethanol solution. Finally, the macerate and must extracts coming from each variety were mixed and put together to form the multivarietal mix used to spike the musts.

2.4. Yeasts and fermentation conditions

Yeasts from different *Saccharomyces* species and hybrids were used in this study (Table 2). One representative belonging to each species and hybrids was selected to carry out fermentation without precursor addition in order to see the effect of that addition and to see if there was *de novo* synthesis of aromatic compounds by the yeasts (blanks of fermentation). These representatives were Lalvin T.73 (*S. cerevisiae*), BM 58 (*S. bayanus var. uvarum*), IFO 1802 (*S. kudriavzevii*), Lalvin W27 (*S. cerevisiae* x *S. kudriavzevii* hybrid), Lalvin S6U (*S. cerevisiae* x *S. bayanus* hybrid) and CBS 2834 (triple hybrid). One non-fermented synthetic media with precursors was also included, in which the presence of aroma compounds should be attributed exclusively to the natural acid hydrolysis of glycosides.

For the fermentations, yeasts were inoculated at OD 600 nm equal at 0.2. Laboratory fermentations were carried out in duplicate using 200 ml-bottles filled with 100 mL of sterile synthetic grape must (*major constituents*: $CaCl_2$ 0.1g/l, NaCl 0.1g/l, KH_2PO_4 1g/l, $MgSO_4 \cdot 7H_2O$ 0.5g/l, $(NH_4)_2SO_4$ 0.944g/l, tartaric acid 3g/l, fructose 100g/l, glucose 100g/l; *minor compounds:* $MnCl_2 \cdot 4H_2O$ 0.198 mg/l, $ZnCl_2$ 0.135 mg/l, $Co(NO_3)_2$ $6H_2O$ 0.029 mg/l, $NaMoO_4$ $2H_2O$ 0.0242 mg/l, $CuCl_2$ 0.0136 mg/l, KIO_3 0.0108 mg/l, H_3BO_3 0.0057 mg/l; *vitamins*: ClH piridoxin 400 µg/l, ClH tiamin 400 µg/l, mioinositol 200 µg/l, biotin 20 µg/l, calcium pantoteate 400 µg/l, p-aminobenzoic acid 200 µg/l; *fatty acids*: tween 80 1 ml/l, ergosterol 15 mg/l). pH was adjusted to 3.5 with KOH.

Synthetic grape juice was sterilized by filtration (0.45 μm Schleicher & Schull, Postfch, Germany) and 30 mL/L of the precursor extract (see Precursor extract preparation section) was added. The samples were incubated at 20°C. The fermentation process was monitored by weight loss. Immediately following the alcoholic fermentation, yeast lees were removed by centrifugation and samples for quantitative analysis were then taken and analyzed.

Table 2. Strains' denomination and origin.

	Strain	Isolation source
S. cerevisiae	Lalvin T.73	wine (Alicante, Spain)
	Fermol cryophile	wine (AEB, France)
	Fermol Reims Champagne	sparkling wine (AEB, France)
S. bayanus	BM 58	wine (Utiel-Requena, Spain)
	CECT 1969	redcurrant (Holland)
	CECT 12600	sweet wine (Alicante, Spain)
	CECT 12629	must (Zaragoza, Spain)
	CECT 12638	must (Cádiz, Spain)
	CECT 12669	grapes (La Rioja, Spain)
	CECT 12930	wine (Spain)
S. kudriavzevii	IFO 1802	decayed leaves (Japan)
Sc x Sb	Lalvin S6U	wine (Italy)
Sc x Sk	Lalvin W27	wine (Switzerland)
	HA1841	wine (Perchtoldsorf, Austria)
	Assmanhausen	wine (Geisenheim, Germany)
	VIN7	wine (Cape Town, South Africa)
	Uvaferm CEG	wine (France)
	SPG 16-91	wine (Switzerland)
Sc x Sb x Sk	CBS 2834	wine (Switzerland)

Scxsb: hybrid between *S.cerevisiae* and *S.bayanus*; ScxSk: hybrids between *S.cerevisiae* and *S.kudriavzevii*; ScxSbxSk: triple hybrid.

2.5. Extraction and analysis of minor volatile compounds (SPE and GC-Ion Trap-MS analysis)

This analysis was carried out using the method proposed and validated by López et al. (2002). The method was modified to use a smaller quantity of sample and also incorporated a new washing step to improve the chromatographic resolution. In accordance with this method, 15 mL of wine, containing 10 μL of a surrogate standards solution (isopropyl propanoate, 3-octanone, heptanoic acid, and β-damascone, 2000 μg/g in ethanol), was passed through a 50 mg LiChrolut EN cartridge (previously conditioned with 6 mL of dichloromethane, 2 mL of methanol, and 2 mL of 12% hydroalcoholic ethanol solution in Mili-Q) at about 2 mL min^{-1}. The sorbent was washed with 5 mL of 40% methanol solution and dried by letting air pass through (-0.6 bar, 10 min). Analytes were recovered by elution with 600 μL of dichloromethane. An internal standard solution (4-methyl-4-pentanol, 4-hydroxy-4- methyl-2-pentanone, and 2-octanol, at a concentration of 350, 450, and 500 μg/g, respectively, in dichloromethane) was added to the eluted sample. The extract was then analyzed by GC with Ion Trap-MS detection under the conditions described below.

2.6. Gas Chromatography-Mass Spectrometry conditions

Gas chromatographic analysis was performed with a CP-3800 chromatograph coupled to a Saturn 2200 ion trap mass spectrometric detection system from Varian (Sunnyvale, CA). A DB-

WAXetr capillary column (J&W Scientific, Folsom, CA) (60 m x 0.25 mm I.D., film thickness 0.5 μm) preceded by a 3 m x 0.25 mm uncoated (deactivated, intermediate polarity) precolumn from Supelco (Bellefonte, PA) was used. Helium was the carrier gas at a flow rate of 1 mL min^{-1}. The oven temperature program was 3 min at 40°C, 4°C/min up to 80°C, 2°C/min up to 230°C, and finally held at this temperature for 37 min. Initially the injector was kept at 35°C during 0.3 min and a pressure pulse of 25 psi during 2.60 min was applied. The injector was then heated to 250°C at rate of 200°C min^{-1}. The splitless time was 2.60 min. Silanized glass wood was used as a packing material in the insert. The injection volume was 4 μL. The global run time was recorded in full scan mode (40-220 *m/z* mass range). The chromatographic data was analyzed by Varian Saturn GC-MS version 6.3 software.

2.7. Extraction and analysis of major volatile compounds (microextraction and GC-FID analysis)

Quantitative analysis of major compounds was carried out using the method proposed and validated by Ortega et al. (2001) with some modifications: 2.7 mL of wine and 6.3 mL of Mili-Q water were mixed with 4.05 g of ammonium sulphate and extracted with 250 µL of dichloromethane. The extract was then analyzed by GC with FID detection using the conditions described in Ortega et al. (2001). Quantitative data was obtained by interpolation of relative peak areas in the calibration graphs built by the analysis of synthetic wines containing known amounts of the analytes. 2-butanol, 4-

methyl-2-pentanol, 4-hydroxy-4-methyl-2-pentanone and 2-octanol, at a concentration of 200 µg/g in dichloromethane, which were used as internal standards.

2.8. Wine descriptive analysis

The sensory panel was composed of 7 females and 3 males, 25–40 years of age, all of them belonging to the laboratory staff and with a long experience in sensory analysis. Nine aroma terms (sulphurous, sweet fruit, citric and herbaceous, fusel, toasty, oxidized, flowery, sweet, sweet wood) were selected by the panel for the descriptive analysis of the wines following standardized practices (ISO 6564:1985 and 4121:1987). Due to the small amount of sample available, panellists exclusively evaluated the odour of the samples, scoring the intensity of each of the nine attributes using a seven-point structured scale. In all cases, wines (20 mL at 20°C) were presented in coded, black tulip-shaped wine glasses covered by glass Petri dishes. Samples were randomly divided into five subsets of 5 or 6 samples. Each subset was in a different tasting booth, and the ten members of the sensory panel had to evaluate the five subsets following a random order. All the evaluations were carried out in less than 90 minutes.

2.9. Data treatment and statistical analysis

Data is presented as the average of the amount of aroma compounds yielded by the strains belonging to the same species. Due to there sometimes being a high variation between the strains from the same species, in some cases deviations are equal or higher than the average. The quantitative data of aromatic compounds and sensorial descriptors were analyzed by analysis of variance (ANOVA). Yeast strain was the factor. The analyses were carried out using SPSS (SPSS Inc., Chicago, IL) for Windows, version 17.0. Component principal analysis (PCA) was also done with the Unscrambler 9.7 (Camo, Norway).

3. Results

3.1. Role of yeast species on formation of aroma compounds from grape odourless precursors

Most grape-derived aroma compounds are accumulated in the form of odourless polar precursors which only after some chemical transformations (most usually the cleavage of a glycosidic bond), become truly aromatic. In the so called neutral grapes, most of these compounds are present at very low quantities, even in reference to their odour thresholds. This means that in isolation these compounds will not produce strong aromas, but as there are several tens of them, altogether may exert a clear contribution to the sweet, floral and even fruity notes of wines (Loscos et al. 2007). Experimental evidences showing that mixtures of aroma compounds at sub-

threshold levels exert a cooperative action were first published nearly fifty years ago (Guadagni et al. 1963).

The relevance of yeasts in the production of the aromatic molecules from the odourless precursors is clearly demonstrated in the Principal Component plot shown in Figure 1. As can be seen, samples fermented in the absence of precursors (B Sc, B Sb, B Sk, B ScxSb, B ScxSk, B ScxSbxSk) lie on the left part of the plane; the control containing precursors but not yeast (B) is found centred-north; and only samples fermented in the presence of precursors are in the right part of the plane, where most aroma compounds are.

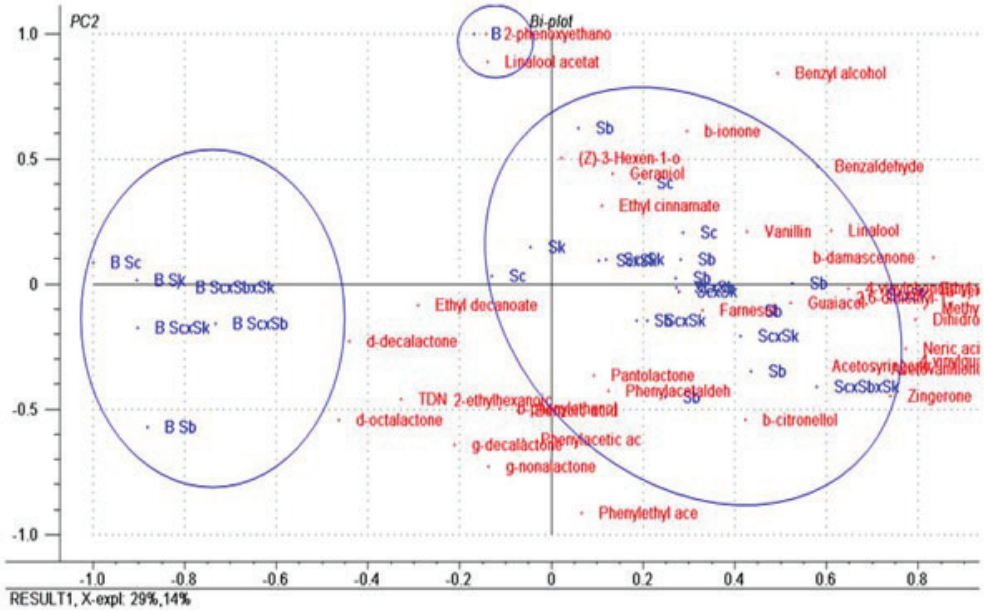

Fig. 1. Principal component plot with the sample loadings and variable weights. Samples denoted with B yeast are samples fermented without precursors. Sample denoted B are the unfermented control. Samples without B are samples with precursor addition and inoculated. B: blank; Sc: *S.cerevisiae*, Sb: *S.bayanus*, Sk: *S.kudriavzevii*, Scxsb: hybrid between *S.cerevisiae* and *S.bayanus*; ScxSk: hybrids between *S.cerevisiae* and *S.kudriavzevii*; ScxSbxSk: triple hybrid.

3.1.1. De novo synthesis by yeasts

The first interesting finding is the powerful *de novo* synthesis of some aromatic compounds by yeast species (Table 3). Nearly all yeasts were able to form ϒ and δ-lactones and 2-ethylhexanoic acid, benzoic and phenylacetic acids, phenylacetaldehyde, β-ionone and some terpenes, such as linalool and its acetate, α-terpineol and β-citronellol, while some aroma compounds were formed just by one or two yeasts. On the contrary, with the exception of the small amount of guaiacol formed by *S. cerevisiae* x *S kudriavzevii*, it can be clearly seen that yeasts were not able to produce volatile phenols or vanillin derivatives.

In most cases, there were significant differences in the levels of *de novo* synthesis of each yeast, as ANOVA study revealed. Of particular quantitative relevance are the huge amounts of farnesol produced by *S. cerevisiae*, the high levels of γ-nonalactone, 2-ethylhexanoic acid, benzaldehyde, phenylacetaldehyde, phenylacetic acid and β-ionone produced by *S. bayanus* and of linalool and β-citronellol in the case of *S. cerevisiae* x *S. bayanus* hybrid.

3.1.2. Aroma compounds released by yeasts from precursors

The aroma composition of the media in which fermentation took place in the presence of grape aroma precursors is shown in Table 4. As shown in the table, and according to ANOVA study, significant differences were found in all of the chemical groups

tested. It is particularly noticeable that in most cases the highest amounts of these compounds were found in the case of the triple hybrid strain. The second most powerful strain at releasing compounds from precursors was the *S. cerevisiae* x *S. bayanus* hybrid.

A closer look at Table 4, shows that in some cases the levels of aroma compounds found in the unfermented control (B, first column), are as important as those found in the fermented media. This presence must be attributed to the natural acid hydrolysis of the precursor molecules and it is particularly important in the cases of benzaldehyde, 2-phenoxyethanol, 4-vinylphenol, ethyl vanillate, β-ionone, β-damascenone and linalyl acetate.

In order to focus our attention on the role strictly played by the yeast on the precursor molecules, data in Table 5 shows the neat amount of aroma molecules formed by yeast from precursors. Data has been obtained simply by subtracting the amount found in the unfermented control (first column in Table 4) and the amount of compound formed in the absence of precursor (Table 3) to data in Table 4. The higher the role played by a given yeast on the formation of a given compound, the higher the value in Table 5. On the contrary, if the compound has been formed mainly by simple acid hydrolysis or has been formed *the novo* by yeast it will bear a negative sign in that Table. As can be seen, lactones were mainly formed *de novo* by the yeast and only the triple hybrid and the *S. kudriavzevii* hybrid were able to form a significant amount of γ-decalactone (both of them) and of γ-nonalactone (only the former).

Most benzoic derivatives were also mainly formed *de novo* by yeasts and only *S. kudriavzevii* and its hybrids seemed to have ability to form tiny amounts of phenylacetaldehyde from precursors. In the case of nor-isoprenoids, in contrast to other previous experiences (Madhour et al. 2005) no yeast was able to produce significant amounts of nor-isoprenoids above those found in the acid hydrolysis or in *de novo*-formation.

On the contrary, the ability of forming or releasing volatile phenols, vanillins and terpenols from precursors is more general. Leaving out vanillin, geraniol and linalyl acetate which were not formed at all, most yeasts were able to form or release the other compounds with some exceptions. *S. cerevisiae* was not able to form guaiacol, methyl and ethyl vanillates and farnesol from the precursors, *S. bayanus* did not form farnesol, *S. kudriavzevii* did not form guaiacol, 4-vinylphenol and neric acid, the ScxSb hybrid failed in the production of guaiacol, ScxSk hybrids failed in the formation of farnesol, and only the triple hybrid was able to form all of these compounds. From a quantitative point of view, while it can be said that in general, *S. kudriavzevii* hybrids produced higher significant increases compared to the rest of yeasts, the ability of the triple hybrid to form or release aroma compounds from precursors really outperforms that of any other yeast strain. The levels formed of γ-nona and decalactones, 4-vinylphenol and 4-vinylguaiacol, methyl vanillate, acetovanillone, acetosyringone, linalool, 2,6-dimethyl-1,7-octadien-3,6-diol and neric acid are clearly the highest.

3.2. Role of yeast species on fermentation-volatiles formation

Major aroma compounds formed during fermentation are higher alcohols, acetate esters, ethyl esters and acids. As shown in Table 3, the levels of these compounds in the absence of precursor fractions were in all cases found to be significantly dependent on the yeast. Only the most relevant cases will be commented. Three major compositional patterns are clearly seen. The most markedly different is *S. bayanus*, which produced the greatest amounts of isoamyl alcohol, β-phenylethanol and of their acetates and also of acetoine and acetaldehyde, while produced small amounts of fatty acids and of their ethyl esters. *S. cerevisiae* and *S. kudriavzevii* follow the opposite behaviour, since they have formed relatively small amounts of those alcohols and acetates, and maxima amounts of fatty acids and their ethyl esters. The hybrids show a pattern that is intermediate between those previously described extreme patterns.

The most surprising fact is that these patterns seem to be blurred out when the fermentation is carried out in the presence of grape odourless precursors, which seem to exert a certain "levelling" effect, as can be seen in Table 4. Even if some significant differences remain, their magnitude is greatly reduced. Now, it is the triple hybrid the one producing maxima amounts of isobutanol and isoamyl alcohol, *S. cerevisiae* and *S. kudriavzevii* retain their ability to form more fatty acids (but not for forming more ethyl esters) and *S. bayanus* forms again the highest levels of acetoin.

Table 3. Aroma composition of the wines obtained by fermenting a synthetic must with different yeasts without precursor addition (de novo synthesis).

	B *S.cerevisiae*	B *S.bayanus*	B *S.kudriavzevii*	B *ScxSb*	B *ScxSk*	B *ScxSbxSk*
Lipids derivatives						
δ-Octalactone	7.61 ± 0.44^{b}	3.51 ± 0.86^{a}	3.97 ± 0.18^{a}	3.59 ± 0.23^{a}	4.41 ± 0.67^{ab}	3.25 ± 0.74^{a}
γ-Nonalactone	13.0 ± 0.73^{ab}	23.8 ± 6.63^{b}	nd^{a}	15.1 ± 0.32^{ab}	15.9 ± 0.34^{ab}	12.4 ± 2.29^{ab}
γ-Decalactone	6.90 ± 0.42	6.72 ± 1.95	5.81 ± 0.51	7.22 ± 0.21	nd	nd
δ-Decalactone	9.48 ± 0.74	3.68 ± 1.44	5.49 ± 1.00	4.56 ± 0.00	5.65 ± 0.47	4.71 ± 2.07
2-Ethylhexanoic acid	9.15 ± 1.17^{a}	49.1 ± 9.78^{b}	3.82 ± 3.53^{a}	9.13 ± 0.69^{a}	7.90 ± 0.71^{a}	7.83 ± 2.01^{a}
Shikimic derivatives						
Benzoic acid	20.9 ± 1.69^{a}	31.6 ± 4.99^{a}	22.5 ± 0.06^{a}	63.6 ± 0.91^{b}	20.4 ± 0.12^{a}	22.1 ± 0.53^{a}
Benzaldehyde	nd^{a}	4.31 ± 1.43^{b}	nd^{a}	nd^{a}	nd^{a}	nd^{a}
Phenylacetaldehyde	0.28 ± 0.15	3.38 ± 1.80	nd	0.53 ± 0.11	0.25 ± 0.06	0.39 ± 0.08
Ethyl cinnamate	nd	nd	nd	nd	nd	nd
2-Phenoxyethanol	5.75 ± 5.75	nd	nd	nd	nd	nd
Phenylacetic acid	8.49 ± 0.02^{a}	45.4 ± 4.11^{b}	12.2 ± 0.43^{a}	14.7 ± 1.16^{a}	9.55 ± 0.28^{a}	13.9 ± 1.64^{a}
Volatile phenols						
Guaiacol	nd^{a}	nd^{a}	nd^{a}	nd^{a}	0.88 ± 0.02^{b}	nd^{a}
4-Vinylguaiacol	nd	nd	nd	nd	nd	nd
4-Vinylphenol	nd	nd	nd	nd	nd	nd
Dihydromethyleugenol*	nd	nd	nd	nd	nd	nd
Vanillins						
Vanillin	nd	nd	nd	nd	nd	nd
Methyl vanillate	nd	nd	nd	nd	nd	nd
Ethyl vanillate	nd	nd	nd	nd	nd	nd
Acetovanillone	nd	nd	nd	nd	nd	nd
Zingerone	nd	nd	nd	nd	nd	nd
Acetosyringone	nd	nd	nd	nd	nd	nd
Nor-isoprenoids						
ß-Damascenone	nd	nd	nd	nd	nd	nd
ß-Ionone	nd^{a}	1.88 ± 0.04^{c}	1.70 ± 0.00^{bc}	1.65 ± 0.04^{b}	nd^{a}	1.56 ± 0.08^{b}
TDN*	nd	0.31 ± 0.24	nd	nd	nd	nd

Table 3. (Continuation).

	B *S.cerevisiae*	B *S.bayanus*	B *S.kudriavzevii*	B *ScxSb*	B *ScxSk*	B *ScxSbxSk*
Terpenes						
Linalool	5.41 ± 1.40^{b}	6.82 ± 0.58^{b}	0.93 ± 0.93^{a}	14.9 ± 0.54^{c}	4.55 ± 0.02^{ab}	5.31 ± 0.07^{b}
α-Terpineol	2.30 ± 0.53	1.95 ± 0.34	1.17 ± 0.23	2.45 ± 0.10	1.66 ± 0.28	1.57 ± 0.31
Geraniol	nd	nd	nd	nd	nd	nd
β-Citronellol	nd^{a}	4.27 ± 1.23^{b}	2.26 ± 0.47^{ab}	7.93 ± 0.36^{c}	3.50 ± 0.17^{b}	4.36 ± 0.41^{b}
Farnesol	355 ± 28.2^{b}	nd^{a}	nd^{a}	nd^{a}	nd^{a}	nd^{a}
Linalyl acetate*	1.93 ± 1.26	0.83 ± 0.05	0.39 ± 0.39	0.78 ± 0.04	0.50 ± 0.01	0.73 ± 0.12
2,6-Dimethyl-1,7-octadien-3,6-diol*	nd^{a}	0.12 ± 0.04^{b}	nd^{a}	nd^{a}	nd^{a}	nd^{a}
Neric acid*	nd	nd	nd	nd	nd	nd
Miscellaneous						
Pantolactone	nd	nd	nd	nd	nd	nd
Fermentative compounds						
Higher alcohols						
Isobutanol**	10.1 ± 0.19^{a}	12.1 ± 0.44^{a}	8.37 ± 0.47^{a}	23.1 ± 1.03^{b}	9.50 ± 1.37^{a}	12.2 ± 1.35^{a}
1-Butanol**	0.32 ± 0.03^{bc}	0.26 ± 0.01^{abc}	0.50 ± 0.03^{d}	0.36 ± 0.02^{c}	0.17 ± 0.01^{a}	0.22 ± 0.00^{ab}
Isoamyl alcohol**	54.6 ± 2.75	73.9 ± 1.24	47.6 ± 2.90	61.2 ± 5.99	44.7 ± 11.1	49.5 ± 5.05
1-Hexanol**	0.25 ± 0.00	0.23 ± 0.02	0.25 ± 0.00	0.23 ± 0.02	0.20 ± 0.00	0.25 ± 0.00
ß-Phenylethanol**	9.72 ± 0.00^{a}	19.3 ± 2.47^{b}	9.72 ± 0.00^{a}	8.40 ± 2.20^{a}	4.43 ± 0.66^{a}	9.72 ± 0.00^{a}
Benzyl alcohol	2.72 ± 2.72	nd	nd	2.92 ± 0.23	nd	2.75 ± 0.17
Acetate esters						
Isoamyl acetate**	0.13 ± 0.00	0.13 ± 0.01	0.08 ± 0.01	0.09 ± 0.00	0.10 ± 0.03	0.08 ± 0.02
Hexyl acetate**	0.08 ± 0.04	nd	nd	nd	nd	nd
Phenylethyl acetate**	0.04 ± 0.00^{a}	0.24 ± 0.01^{b}	0.03 ± 0.00^{a}	0.08 ± 0.00^{a}	0.06 ± 0.02^{a}	0.07 ± 0.01^{a}

Table 3. (Continuation).

	B *S.cerevisiae*	B *S.bayanus*	B *S.kudriavzevii*	B *ScxSb*	B *ScxSk*	B *ScxSbxSk*
Ethyl esters						
Ethyl butyrate**	0.11 ± 0.01	0.06 ± 0.00	0.07 ± 0.00	0.08 ± 0.00	0.06 ± 0.01	0.07 ± 0.02
Ethyl hexanoate**	0.17 ± 0.02^{c}	0.07 ± 0.00^{a}	0.14 ± 0.01^{bc}	0.06 ± 0.01^{a}	0.08 ± 0.01^{ab}	0.07 ± 0.01^{a}
Ethyl lactate**	4.33 ± 0.10	4.06 ± 0.40	3.98 ± 0.13	3.52 ± 0.03	0.30 ± 0.30	4.29 ± 0.03
Ethyl octanoate**	0.44 ± 0.02^{c}	0.10 ± 0.03^{a}	0.37 ± 0.02^{bc}	0.14 ± 0.06^{a}	0.24 ± 0.01^{ab}	0.16 ± 0.04^{a}
Ethyl decanoate**	5.25 ± 0.65	3.02 ± 1.73	4.59 ± 1.01	3.20 ± 0.32	3.47 ± 0.54	3.40 ± 0.30
Ethyl 3-hydroxybutyrate**	0.05 ± 0.00	0.04 ± 0.01	0.05 ± 0.00	0.05 ± 0.01	0.05 ± 0.02	0.05 ± 0.00
Diethyl succinate**	0.31 ± 0.01	0.38 ± 0.06	0.31 ± 0.01	0.29 ± 0.01	0.31 ± 0.00	0.28 ± 0.00
Acids						
Isobutyric acid**	0.45 ± 0.01^{ab}	0.58 ± 0.02^{b}	0.45 ± 0.01^{ab}	0.38 ± 0.03^{a}	0.43 ± 0.05^{ab}	0.58 ± 0.05^{b}
Butiric acid**	0.54 ± 0.02^{b}	0.32 ± 0.02^{a}	0.50 ± 0.03^{ab}	0.45 ± 0.04^{ab}	0.33 ± 0.04^{a}	0.37 ± 0.04^{ab}
Hexanoic acid**	1.58 ± 0.19^{b}	0.65 ± 0.01^{a}	1.83 ± 0.03^{b}	0.47 ± 0.06^{a}	0.60 ± 0.04^{a}	0.80 ± 0.15^{a}
Octanoic acid**	1.21 ± 0.09	0.71 ± 0.37	1.35 ± 0.07	0.49 ± 0.04	0.57 ± 0.07	0.69 ± 0.17
Decanoic acid**	1.43 ± 0.11	0.73 ± 0.03	1.68 ± 0.18	0.88 ± 0.30	0.89 ± 0.08	0.83 ± 0.25
Miscellaneous						
Acetaldehyde**	10.9 ± 8.58	34.2 ± 2.19	27.2 ± 7.10	13.0 ± 11.6	21.4 ± 2.85	6.61 ± 0.74
Diacethyl**	0.56 ± 0.00	0.72 ± 0.16	0.56 ± 0.00	nd	0.50 ± 0.01	nd
Acetoine**	3.43 ± 0.17	127 ± 105	10.8 ± 0.05	1.47 ± 0.64	3.94 ± 1.76	1.56 ± 0.27
Y-Butyrolactone**	0.74 ± 0.03^{ab}	0.54 ± 0.12^{a}	0.29 ± 0.04^{a}	1.24 ± 0.19^{b}	0.35 ± 0.09^{a}	1.19 ± 0.06^{b}

*Relative areas (to 4-hydroxy-4-methyl-2-pentanone *1000) of the volatile compounds for which pure references were not available.

Except where indicated, concentration data is in µg/L. The compound identification is based on the work of Ibarz et al. (2006).

Data is average of three replicates ± standard deviation. ** Data in mg/L

A,b,c Different letters indicate significant differences (significant level 95%); nd: not detected.

B: blank; B *S.cerevisiae*: *S.cerevisiae* blank (Lalvin T.73), B *S.bayanus*: *S.bayanus* blank (BM 58), B *S.kudriavzevii*: *S.kudriavzevii* blank (IFO 1802), B ScxSb: hybrid between *S.cerevisiae* and *S.bayanus* blank (Lalvin S6U); B ScxSk: hybrid between *S.cerevisiae* and *S.kudriavzevii* blank (Lalvin W27); ScxSbxSk: triple hybrid blank (CBS 2834); nd: not detected

Table 4. Aroma composition of the wines obtained by fermenting a synthetic must containing a precursor extract with different yeasts.

	B	*S.cerevisiae*	*S.bayanus*	*S.kudriavzevii*	*ScxSb*	*ScxSk*	*ScxSbxSk*
Lipid derivatives							
δ-Octalactone	nd^{a}	2.96 ± 0.83^{b}	2.39 ± 0.47^{b}	2.57 ± 0.11^{b}	2.42 ± 0.09^{b}	3.16 ± 0.77^{b}	3.46 ± 0.10^{b}
γ-Nonalactone	1.53 ± 0.20	8.13 ± 6.67	5.83 ± 8.07	nd	nd	9.45 ± 8.12	16.61 ± 0.37
γ-Decalactone	nd^{a}	5.41 ± 0.64^{ab}	4.42 ± 2.55^{ab}	nd^{a}	nd^{a}	5.07 ± 3.10^{ab}	7.13 ± 0.02^{b}
δ-Decalactone	2.46 ± 2.13^{ab}	3.61 ± 0.80^{ab}	1.14 ± 0.66^{a}	2.62 ± 0.03^{ab}	1.97 ± 0.13^{ab}	4.52 ± 1.70^{b}	5.13 ± 0.48^{b}
2-Ethylhexanoic acid	9.64 ± 0.32^{ab}	8.39 ± 2.91^{ab}	5.87 ± 3.07^{a}	4.88 ± 0.33^{a}	8.08 ± 1.64^{ab}	12.4 ± 6.05^{ab}	17.26 ± 0.38^{b}
Shikimic derivatives							
Benzoic acid	19.0 ± 1.05^{a}	20.6 ± 0.30^{ab}	21.6 ± 1.32^{ab}	21.0 ± 0.36^{ab}	23.2 ± 0.06^{b}	22.2 ± 1.77^{ab}	23.80 ± 0.34^{b}
Benzaldehyde	6.96 ± 0.07^{b}	3.64 ± 3.18^{ab}	3.27 ± 0.35^{a}	3.67 ± 0.00^{ab}	2.63 ± 0.01^{a}	3.59 ± 0.68^{ab}	4.14 ± 0.00^{ab}
Phenylacetaldehyde	nd	0.68 ± 0.67	1.81 ± 0.88	1.73 ± 0.56	nd	0.24 ± 0.58	0.95 ± 0.02
Ethyl cinnamate	nd	0.33 ± 0.47	nd	nd	nd	nd	nd
2-Phenoxyethanol	11.7 ± 0.59^{b}	2.48 ± 5.56^{a}	nd^{a}	nd^{a}	nd^{a}	nd^{a}	nd^{a}
Phenylacetic acid	6.99 ± 0.01^{a}	13.9 ± 3.79^{ab}	24.0 ± 9.17^{b}	16.6 ± 1.50^{ab}	17.9 ± 0.93^{ab}	10.7 ± 1.60^{ab}	18.50 ± 0.34^{ab}
Volatile phenols							
Guaiacol	nd	0.82 ± 0.58	0.55 ± 0.48	nd	nd	1.07 ± 0.56	1.04 ± 0.06
4-Vinylguaiacol	3.75 ± 1.27^{a}	17.8 ± 5.23^{ab}	22.1 ± 10.9^{ab}	7.37 ± 0.12^{a}	19.0 ± 0.32^{ab}	16.6 ± 8.45^{ab}	37.99 ± 3.42^{b}
4-Vinylphenol	88.2 ± 64.8^{ab}	144 ± 31.0^{ab}	175 ± 69.4^{ab}	60.6 ± 1.59^{a}	150 ± 1.82^{ab}	130 ± 59.5^{ab}	243.62 ± 27.38^{b}
Dihydromethyleugenol	1.31 ± 0.36^{a}	6.27 ± 0.60^{abc}	6.82 ± 1.75^{bc}	4.36 ± 0.08^{ab}	8.38 ± 1.36^{bc}	8.18 ± 2.83^{bc}	10.85 ± 0.07^{c}
Vanillins							
Vanillin	0.36 ± 0.02	0.42 ± 0.34	0.32 ± 0.40	nd	nd	0.18 ± 0.41	nd
Methyl vanillate	1.53 ± 0.45^{a}	5.52 ± 3.92^{ab}	7.74 ± 1.84^{bc}	6.77 ± 1.00^{abc}	9.83 ± 0.37^{bc}	7.92 ± 1.89^{bc}	12.68 ± 0.25^{c}
Ethyl vanillate	0.74 ± 0.05^{ab}	0.59 ± 0.43^{a}	1.04 ± 0.26^{ab}	0.80 ± 0.11^{ab}	1.44 ± 0.07^{b}	1.22 ± 0.22^{ab}	1.33 ± 0.09^{b}
Acetovanillone	2.34 ± 2.21^{a}	32.9 ± 3.95^{bc}	36.8 ± 12.9^{bc}	19.7 ± 0.95^{ab}	38.7 ± 4.81^{bc}	38.8 ± 10.5^{bc}	53.27 ± 0.59^{c}
Zingerone	0.80 ± 0.22^{a}	8.70 ± 1.26^{abc}	9.16 ± 3.33^{bc}	3.53 ± 0.73^{ab}	14.1 ± 1.20^{c}	7.19 ± 3.57^{abc}	11.49 ± 1.09^{bc}
Acetosyringone	nd^{a}	4.17 ± 0.22^{b}	4.31 ± 0.81^{b}	1.78 ± 0.17^{a}	5.19 ± 0.62^{bc}	4.24 ± 0.93^{b}	6.36 ± 0.42^{c}

Table 4. (Continuation).

	B	*S.cerevisiae*	*S.bayanus*	*S.kudriavzevii*	*ScxSb*	*ScxSk*	*ScxSbxSk*
Nor-isoprenoids							
ß-Damascenone	1.63 ± 0.08^{bc}	0.81 ± 0.11^{a}	1.05 ± 0.34^{ab}	1.27 ± 0.10^{abc}	1.41 ± 0.10^{abc}	1.45 ± 0.30^{abc}	1.88 ± 0.42^{c}
ß-Ionone	2.80 ± 0.08^{b}	2.21 ± 0.53^{ab}	1.49 ± 0.63^{a}	1.85 ± 0.04^{ab}	1.57 ± 0.02^{a}	1.74 ± 0.09^{ab}	1.74 ± 0.02^{ab}
TDN*	nd	nd	nd	nd	nd	nd	nd
Terpenes							
Linalool	11.3 ± 0.66^{a}	18.5 ± 2.09^{ab}	23.1 ± 4.51^{bc}	18.4 ± 0.13^{ab}	31.7 ± 1.84^{c}	27.0 ± 4.54^{bc}	30.87 ± 0.00^{c}
α-Terpineol	4.45 ± 0.03^{a}	6.95 ± 0.75^{ab}	7.85 ± 1.16^{bc}	7.42 ± 0.06^{bc}	10.1 ± 0.72^{c}	8.29 ± 1.12^{bc}	9.30 ± 0.65^{bc}
Geraniol	12.6 ± 1.84	nd	2.85 ± 8.61	nd	nd	nd	nd
β-Citronellol	nd^{a}	4.99 ± 0.62^{bc}	7.09 ± 0.78^{d}	3.93 ± 0.21^{b}	10.1 ± 0.59^{e}	4.71 ± 0.82^{b}	6.92 ± 0.34^{cd}
Farnesol	nd	nd	139 ± 186	100 ± 10.6	582 ± 5.02	378 ± 337	317.69 ± 49.24
Linalyl acetate*	3.17 ± 0.04^{c}	1.72 ± 1.22^{bc}	0.64 ± 0.46^{ab}	0.49 ± 0.02^{ab}	nd^{a}	0.54 ± 0.40^{ab}	nd^{a}
2,6-Dimethyl-1,7-octadien-3	1.67 ± 0.45^{a}	3.58 ± 0.57^{b}	4.58 ± 0.71^{bc}	4.39 ± 0.10^{bc}	5.82 ± 0.14^{c}	4.93 ± 0.81^{bc}	6.16 ± 0.18^{c}
Neric acid*	14.2 ± 4.40^{a}	21.6 ± 2.72^{ab}	20.4 ± 8.93^{ab}	13.6 ± 1.22^{a}	20.2 ± 0.89^{ab}	26.6 ± 9.90^{ab}	37.95 ± 0.39^{b}
Miscellaneous							
Pantolactone	nd	nd	nd	nd	nd	nd	nd
Fermentative compounds							
Higher alcohols							
Isobutanol**		6.78 ± 1.85^{a}	7.49 ± 2.42^{a}	5.50 ± 0.03^{a}	20.2 ± 1.35^{b}	9.56 ± 3.40^{a}	15.71 ± 1.99^{b}
1-Butanol**		0.27 ± 0.08	0.38 ± 0.21	0.48 ± 0.06	0.35 ± 0.02	0.22 ± 0.06	0.23 ± 0.01
Isoamyl alcohol**		47.4 ± 2.97^{a}	49.1 ± 4.15^{a}	44.4 ± 0.00^{a}	48.7 ± 3.60^{a}	48.8 ± 14.7^{a}	61.83 ± 1.97^{b}
1-Hexanol**		0.25 ± 0.00^{b}	0.24 ± 0.11^{b}	nd^{a}	0.25 ± 0.00^{b}	0.25 ± 0.07^{b}	0.23 ± 0.02^{b}
ß-Phenylethanol**		9.72 ± 0.00	11.4 ± 3.79	9.72 ± 0.00	9.72 ± 0.00	9.72 ± 2.69	9.19 ± 0.53
Benzyl alcohol		3.98 ± 1.67	2.88 ± 0.23	2.95 ± 0.02	2.53 ± 0.08	2.94 ± 0.23	3.25 ± 0.09
Acetate esters							
Isoamyl acetate**		0.09 ± 0.02	0.11 ± 0.04	0.08 ± 0.01	0.07 ± 0.01	0.09 ± 0.04	0.12 ± 0.03
Hexyl acetate**		nd	nd	nd	nd	nd	nd
Phenylethyl acetate**		0.05 ± 0.01	0.16 ± 0.07	0.09 ± 0.00	0.06 ± 0.00	0.05 ± 0.01	0.13 ± 0.01

Table 4. (Continuation).

	B	*S.cerevisiae*	*S.bayanus*	*S.kudriavzevii*	*ScxSb*	*ScxSk*	*ScxSbxSk*
Ethyl esters							
Ethyl butyrate**		0.08 ± 0.02	0.06 ± 0.02	0.07 ± 0.01	0.07 ± 0.01	0.07 ± 0.03	0.09 ± 0.01
Ethyl hexanoate**		0.11 ± 0.02^{bc}	0.06 ± 0.02^{a}	0.13 ± 0.01^{c}	0.06 ± 0.00^{a}	0.07 ± 0.02^{ab}	0.08 ± 0.01^{ab}
Ethyl lactate**		3.79 ± 0.20^{ab}	3.80 ± 0.93^{ab}	3.63 ± 0.01^{a}	3.58 ± 0.04^{a}	3.93 ± 1.13^{ab}	4.24 ± 0.03^{b}
Ethyl octanoate**		0.28 ± 0.04^{a}	0.18 ± 0.14^{a}	0.64 ± 0.06^{b}	0.16 ± 0.00^{a}	0.17 ± 0.07^{a}	0.13 ± 0.04^{a}
Ethyl decanoate**		3.67 ± 0.91	0.76 ± 0.39	2.94 ± 0.63	3.30 ± 0.31	3.04 ± 1.93	.54 ± 0.11
Ethyl 3-hydroxybutyrate**		0.06 ± 0.01	0.05 ± 0.02	0.05 ± 0.00	0.05 ± 0.00	0.06 ± 0.02	0.06 ± 0.01
Diethyl succinate**		0.27 ± 0.01	0.34 ± 0.05	0.30 ± 0.00	0.28 ± 0.01	0.29 ± 0.08	0.28 ± 0.01
Acids							
Isobutyric acid**		0.29 ± 0.01^{a}	0.33 ± 0.05^{ab}	0.28 ± 0.00^{a}	0.32 ± 0.01^{ab}	0.29 ± 0.09^{ab}	0.38 ± 0.02^{b}
Butyric acid**		0.39 ± 0.06	0.29 ± 0.02	0.28 ± 0.00	0.33 ± 0.01	0.30 ± 0.10	0.33 ± 0.02
Hexanoic acid**		0.82 ± 0.15^{b}	0.28 ± 0.07^{a}	0.88 ± 0.01^{b}	0.27 ± 0.00^{a}	0.45 ± 0.20^{ab}	0.46 ± 0.04^{ab}
Octanoic acid**		0.68 ± 0.09^{bc}	0.25 ± 0.10^{a}	0.72 ± 0.02^{c}	0.27 ± 0.01^{a}	0.41 ± 0.19^{abc}	0.39 ± 0.03^{ab}
Decanoic acid**		1.06 ± 0.11^{ab}	0.69 ± 0.33^{a}	1.44 ± 0.15^{b}	1.00 ± 0.01^{ab}	0.94 ± 0.38^{ab}	0.72 ± 0.11^{a}
Miscellaneous							
Acetaldehyde**		39.9 ± 33.8	25.6 ± 21.0	18.3 ± 5.75	1.39 ± 0.00	15.3 ± 12.0	9.45 ± 3.25
Diacethyl**		0.56 ± 0.00	0.63 ± 0.31	nd	0.56 ± 0.28	0.56 ± 0.28	0.56 ± 0.28
Acetoine**		14.3 ± 15.4	62.0 ± 74.0	6.33 ± 1.06	0.83 ± 0.00	10.2 ± 11.9	12.06 ± 9.34
Y-Butyrolactone**		0.43 ± 0.21^{a}	0.46 ± 0.16^{a}	0.25 ± 0.00^{a}	1.31 ± 0.09^{b}	0.44 ± 0.16^{a}	0.96 ± 0.06^{b}

*Relative areas (to 4-hydroxy-4-methyl-2-pentanone *1000) of the volatile compounds for which pure references were not available. Except where indicated,
^{a,b,c} Different letters indicate significant differences (significant level 95%), nd: not detected.

B: blank; B Sc: *S.cerevisiae* blank (Lalvin T.73), B Sb: *S.bayanus* blank (BM 58), B Sk: *S.kudriavzevii* blank (IFO 1802),

B ScxSb: hybrid between *S.cerevisiae* and *S.bayanus* blank (LalvinS6U); B ScxSk: hybrid between *S.cerevisiae* and *S.kudriavzevii* blank (LalvinW27);

ScxSbxSk: triple hybrid blank (CBS 2834); nd: not detected

Table 5. Effect of the presence of precursors in the fermentation media on the volatile composition of the wines obtained from different yeasts.

	Sc (Lalvin T.73)	Sb (BM 58)	Sk (IFO 1802)	ScxSb (Lalvin S6U)	ScxSk (Lalvin W27)	ScxSbxSk (CBS 2834)
Lipid derivatives						
δ-Octalactone	*-4.03 ± 0.16^{a}*	*-1.03 ± 0.12^{b}*	*-1.40 ± 0.16^{b}*	*-1.17 ± 0.13^{b}*	*-0.64 ± 1.37^{b}*	0.21 ± 0.14^{b}
γ-Nonalactone	*-4.46 ± 0.97^{bc}*	*-7.14 ± 5.80^{ab}*	*-1.53 ± 0.00^{bc}*	*-16.7 ± 0.00^{a}*	*-2.29 ± 0.08^{bc}*	2.70 ± 0.53^{c}
γ-Decalactone	*-1.82 ± 0.71^{b}*	*-0.37 ± 1.12^{b}*	*-5.81 ± 0.00^{a}*	*-7.22 ± 0.00^{a}*	6.66 ± 0.12^{c}	7.13 ± 0.03^{c}
δ-Decalactone	*-8.31 ± 0.25^{a}*	*-4.67 ± 0.24^{c}*	*-5.33 ± 0.05^{b}*	*-5.05 ± 0.19^{b}*	*-3.01 ± 1.42^{bc}*	*-2.04 ± 0.67^{c}*
2-Ethylhexanoic acid	*-11.7 ± 3.78^{b}*	*-52.1 ± 2.70^{a}*	*-8.59 ± 0.47^{bc}*	*-10.7 ± 2.32^{b}*	0.77 ± 0.64^{d}	*-0.21 ± 0.54^{cd}*
Shikimic derivatives						
Benzoic acid	*-19.0 ± 0.01^{cd}*	*-28.5 ± 1.58^{b}*	*-20.5 ± 0.50^{c}*	*-59.4 ± 0.09^{a}*	*-15.4 ± 0.38^{e}*	*-17.3 ± 0.48^{de}*
Benzaldehyde	*-6.96 ± 0.00^{a}*	*-7.71 ± 0.52^{a}*	*-3.29 ± 0.01^{c}*	*-4.33 ± 0.02^{b}*	*-2.59 ± 0.14^{c}*	*-2.82 ± 0.01^{c}*
Phenylacetaldehyde	*-0.12 ± 0.10^{ab}*	*-0.87 ± 0.83^{a}*	1.73 ± 0.80^{b}	*-0.53 ± 0.00^{ab}*	1.16 ± 0.86^{ab}	0.56 ± 0.03^{ab}
2-Phenoxyethanol	*-17.5 ± 0.00^{b}*	*-11.7 ± 0.00^{a}*	*-11.7 ± 0.00^{a}*	*-11.7 ± 0.00^{a}*	*-11.7 ± 0.00^{a}*	*-11.7 ± 0.00^{a}*
Phenylacetic acid	*-3.92 ± 0.22*	*-15.3 ± 16.4*	*-2.62 ± 2.12*	*-3.78 ± 1.32*	*-3.02 ± 0.04*	*-2.42 ± 0.48*
Volatile phenols						
Guaiacol	*nd^{a}*	0.87 ± 0.15^{b}	*nd^{a}*	*nd^{a}*	0.72 ± 0.32^{b}	1.04 ± 0.08^{b}
4-Vinylguaiacol	9.44 ± 3.37^{a}	15.4 ± 3.49^{a}	3.62 ± 0.17^{a}	15.2 ± 0.46^{a}	15.3 ± 3.71^{a}	34.2 ± 4.83^{b}
4-Vinylphenol	34.9 ± 37.5^{a}	80.8 ± 31.8^{ab}	*-31.4 ± 2.25^{a}*	61.9 ± 2.57^{ab}	52.4 ± 39.7^{ab}	155 ± 38.7^{b}
Dihydromethyleugenol	4.97 ± 0.33^{ab}	6.08 ± 2.33^{ab}	3.05 ± 0.12^{a}	7.07 ± 1.92^{ab}	7.76 ± 0.09^{ab}	9.54 ± 0.10^{b}
Vanillins						
Vanillin	*-0.36 ± 0.00^{a}*	0.35 ± 0.10^{b}	*-0.36 ± 0.00^{a}*	*-0.36 ± 0.00^{a}*	*-0.36 ± 0.00^{a}*	*-0.36 ± 0.00^{a}*
Methyl vanillate	*-1.53 ± 0.00^{a}*	8.39 ± 2.44^{bc}	5.24 ± 1.41^{b}	8.30 ± 0.53^{bc}	7.59 ± 1.46^{bc}	11.2 ± 0.35^{c}
Ethyl vanillate	*-0.74 ± 0.00^{a}*	0.40 ± 0.31^{bc}	0.06 ± 0.15^{b}	0.70 ± 0.11^{c}	0.68 ± 0.03^{bc}	0.59 ± 0.13^{bc}
Acetovanillone	27.1 ± 1.02^{ab}	39.9 ± 10.9^{bc}	17.4 ± 1.34^{a}	36.3 ± 6.80^{abc}	46.9 ± 2.87^{bc}	51.0 ± 0.83^{c}
Zingerone	7.69 ± 0.11^{ab}	13.4 ± 3.19^{b}	2.73 ± 1.03^{a}	13.3 ± 1.70^{b}	8.84 ± 1.35^{ab}	10.7 ± 1.54^{b}
Acetosyringone	3.99 ± 0.06^{ab}	4.48 ± 0.80^{b}	1.78 ± 0.24^{a}	5.19 ± 0.88^{b}	5.27 ± 0.77^{b}	6.36 ± 0.59^{b}

Table 5. (Continuation).

	Sc (Lalvin T.73)	Sb (BM 58)	Sk (IFO 1802)	ScxSb (Lalvin S6U)	ScxSk (Lalvin W27)	ScxSbxSk (CBS 2834)
Nor-isoprenoids						
ß-Damascenone	*-0.92 ± 0.04*^{a}	*-0.53 ± 0.30*^{ab}	*-0.36 ± 0.15*^{ab}	*-0.22 ± 0.14*^{ab}	*-0.27 ± 0.18*^{ab}	0.25 ± 0.59^{b}
ß-Ionone	*-0.87 ± 0.47*^{c}	*-4.68 ± 0.00*^{a}	*-2.65 ± 0.06*^{b}	*-2.88 ± 0.03*^{b}	*-1.04 ± 0.03*^{c}	*-2.63 ± 0.03*^{b}
TDN	*nd*^{a}	*-0.31 ± 0.00*^{b}	*nd*^{a}	*nd*^{a}	*nd*^{a}	*nd*^{a}
Terpenes						
Linalool	1.46 ± 1.27^{a}	1.67 ± 5.54^{a}	6.18 ± 0.19^{ab}	5.47 ± 2.61^{ab}	11.3 ± 3.99^{ab}	14.3 ± 0.01^{b}
α-Terpineol	0.73 ± 0.54	1.10 ± 2.12	1.80 ± 0.09	3.20 ± 1.02	3.85 ± 1.90	3.27 ± 0.92
Geraniol	*-12.6 ± 0.00*	*-12.6 ± 0.00*	*-12.6 ± 0.00*	*-12.6 ± 0.00*	*-12.6 ± 0.00*	*-12.6 ± 0.00*
β-Citronellol	4.52 ± 0.46^{b}	1.97 ± 0.22^{a}	1.67 ± 0.30^{a}	2.14 ± 0.84^{a}	0.71 ± 0.35^{a}	2.56 ± 0.49^{a}
Farnesol	*-355 ± 0.00*^{a}	*nd*^{b}	100 ± 14.9^{b}	582 ± 7.10^{d}	*nd*^{b}	318 ± 69.6^{c}
Linalyl acetate	*-4.22 ± 0.87*	*-3.26 ± 0.00*	*-3.07 ± 0.03*	*-3.95 ± 0.00*	*-2.92 ± 0.07*	*-3.90 ± 0.00*
2.6-Dimethyl-1.7-octadie	1.71 ± 0.69^{a}	2.23 ± 1.03^{ab}	2.72 ± 0.14^{ab}	4.15 ± 0.20^{ab}	4.02 ± 1.11^{ab}	4.49 ± 0.26^{b}
Neric acid	5.20 ± 1.13^{a}	6.87 ± 4.67^{a}	-0.58 ± 1.72^{a}	6.02 ± 1.26^{a}	19.10 ± 1.14^{b}	23.8 ± 0.55^{b}

*Relative areas (to 4-hydroxy-4-methyl-2-pentanone *1000) of the volatile compounds for which pure references were not available. Rest of the data in B: blank; Sc: *S.cerevisiae*. Sb: *S.bayanus* blank. Sk: *S.kudriavzevii*, Scxsb: hybrid between *S.cerevisiae* and *S.bayanus*; ScxSk: hybrids between *S.cerevisiae* and *S.kudriavzevii*; ScxSbxSk: triple hybrid; nd: not detected.

3.3. Sensory differences

Nine aroma terms were evaluated in the sensory tests (sulphurous, sweet fruit, citric and herbaceous, fusel, toasty, oxidized, flowery, sweet and sweet wood). Results are shown in Figure 2.

The study showed an apparently quite limited sensory effect of the presence of precursors. In fact, only in the cases of *S. bayanus* and the triple hybrid a significant effect linked to the presence of precursors was found. Moreover, the effect was in each case opposite to the other: while the fusel note increased (30.5%) in the case of the triple hybrid, it decreased by 37.4% in *S. bayanus.* This fact was perfectly correlated to chemical analysis, since comparing Tables 3 and 4 the total amount of higher alcohols increased in the case of triple hybrid from 71.79 mg/l to 87.19 mg/l (increase of 21.5%) and in the case of *S. bayanus,* they decreased from 105.74 mg/l to 68.63 mg/l (decrease of 35.1%).

With regards to the impact of the yeast species on the sensory attributes, significant differences were found in the sulphurous and sweet fruit aroma nuances (data not shown). While *S. bayanus* showed the lowest levels of sulphurous aroma, *S. cerevisiae* had the highest scores in fruity notes and the triple hybrid appeared as the most sulphurous and the least fruity.

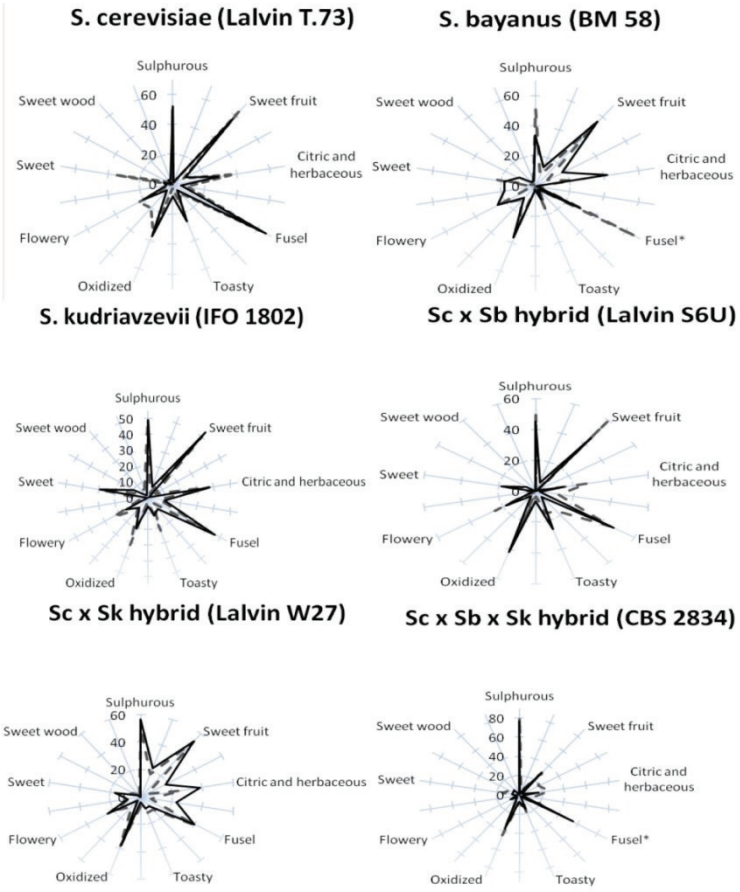

Fig. 2. Spider webs showing the differences in the measured aromatic descriptors between fermentation with and without precursor addition. Scxsb: hybrid between *S.cerevisiae* and *S.bayanus*; ScxSk: hybrids between *S.cerevisiae* and *S.kudriavzevii*; ScxSbxSk: triple hybrid; Black line: fermentations with precursor addition, grey line: blank fermentations without precursor addition. *Aroma term in which appeared significant differences.

4. Discussion

The ability to release or form varietal aroma compounds from a pool of odourless precursors extracted from different grape varieties by different species of the genus *Saccharomyces* (*S. cerevisiae, S. uvarum* and *S. kudriavzevii*) and hybrids between them have been tested in the present study. Table 6 summarises the most relevant results. One of the most noticeable findings of this study is

the relatively intense *de novo* formation of some minor aroma compounds by all or some of the species and hybrids considered in the study. *De novo* synthesis of aliphatic lactones by yeasts was documented by Endrizzi et al. (1996), who tried to elucidate the mechanisms of *de novo* formation of these compounds by biotransformation from fatty acids. Wanikawa et al. (2000) established that *Saccharomyces* yeasts are able to form lactones from hydroxyacids formed by previous oxidation of fatty acids by lactic bacteria in whisky. In addition, the presence of specific precursor molecules of lactones, such as glycerol esters of the corresponding hydroxy acids, in the grape odourless precursor fraction has also been suggested (Loscos et al. 2007). In the case of monoterpenes, Carrau et al. (2005) demonstrated the existence of *de novo* synthesis of these compounds by *Saccharomyces cerevisiae* wine yeasts, while different researchers have shown that terpenes can be biotransformed into other ones by different yeast genera (King and Dickinson, 2000) and by different species of *Saccharomyces* genus and hybrids between these species (Gamero et al. 2011, Objective 2, Chapter 1). De Carvalho and da Fonseca (2006) demonstrated the possibility of terpene biotransformation by different microorganisms at industrial scale. Finally, Bode and Birnbaum (1981) worked in a better understanding of shikimate pathway enzymes in *S. cerevisiae* and in other genera. The ability to form some nor-isoprenoids was already observed in *S. cerevisiae* by Hernández-Orte et al. (2008) and Loscos et al. (2007). Besides, some yeasts are able to synthesize carotenoids under oxidative stress (Madhour et al. 2005), which can be degraded by enzymatic

(dioxygenases, glycosidases) or non-enzymatic reactions yielding norisoprenoids (Mendes-Pinto, 2009). To the best of our knowledge, the *de novo* synthesis of volatile phenols has not been previously documented, but the amount of guaiacol detected in *S. cerevisiae* x *S. kudriavzevii* blank was so low that we cannot rule out the possibility that their presence may arise from some impurities present in the reagents used to prepare the synthetic media or even in the inoculum.

Furthermore, our results have shown that *de novo* synthesis depends on the yeast species and hybrids which carried out the fermentation process. The species and hybrids whose *de novo* production was significantly higher were *S. bayanus*, in the case of certain lipid derivatives, shikimic derivatives, nor-isoprenoids and terpenes; *S. cerevisiae*, in the case of certain lipid derivatives and terpenes; *S. cerevisiae* x *S. bayanus* hybrid, in the case of certain shikimic derivatives and terpenes; and *S. cerevisiae* x *S. kudriavzevii*, in the case of one volatile phenol. *S. kudriavzevii* and triple hybrid did not point out in *de novo* synthesis of any of the minor aromatic compounds tested.

Going in depth through the different compounds formed *de novo* by yeasts what was remarkable was the high farnesol levels found in *S. cerevisiae* wines without precursor addition (355 ± 28.2 µg/l, Table 3), whereas the other species was not able to synthesize this compound. What is particularly noteworthy is the fact that the single species which failed in the release of farnesol was the single one presenting *de novo* synthesis of this compound. This fact was

also seen by Loscos et al. 2007 in the case of β-ionone, linalool, δ-decalactone and 4-vinyl-2-methoxyphenol. The farnesol levels reached by *S. cerevisiae* were much higher than the usual concentration of this compound in wines (from 6 to 113 µg/l according to Coelho et al. 2008, 2009). In our study, acid hydrolysis of farnesol could not be detected, so the high amount in *S. cerevisiae* wines was completely consequence of *de novo* synthesis. The explanation for the high farnesol levels could be a deficiency in squalene synthase activity or an overexpression in mevalonate pathway enzymes (Ohto et al. 2010). The production of a high amount of farnesol is a very interesting fact, since this compound and its derivatives have been described to have relevant biological properties (Downward, 1998; Hohl et al. 1998; Inoue et al. 2004; Liang et al. 2002; Shchepin et al. 2003; Xie et al. 2000).

Phenylacetaldehyde was another compound presenting *de novo* synthesis by all of the species and hybrids except for *S. kudriavzevii*. *De novo* synthesis of this aromatic compound is particularly noteworthy, since phenylacetaldehyde is commonly related to ageing (Oliveira et al. 2008). Degradation of the amino acids formed by sugar retroaldolization in the presence of dicarbonylic compounds during ageing leads to the formation of volatile aldehydes, such as methional and phenylacetaldehyde (Oliveira et al. 2008).

On the other hand, more than 40 compounds belonging to different classes, such as lactones, cinnamates, volatile phenols, vanillin-derivatives, nor-isoprenoids and terpenols are formed from

precursors (Hernández-Orte et al. 2008; Loscos et al. 2007), which was confirmed in our study. Taking into account primary aroma compounds released by the different species and hybrids, there were significant differences according to the ANOVA analysis in many aroma compounds belonging to all of the chemical groups tested (Tables 4 and 5). It is noticeable that the highest amounts of these compounds were yielded by the triple hybrid strain in most of the cases. This hybrid was not particularly efficient in *de novo* synthesis, but it seems to have the highest levels of glycosidic enzymatic activity. It is also remarkable that species belonging to genus *Saccharomyces*, in spite of being considered to have a low enzymatic activity in the wine (Fia et al. 2005; Mateo and Di Stefano, 1997), showed in general a major ability to release aroma compounds from precursors. In any case, data confirms that the species or hybrids carrying out the fermentation have an outstanding role on varietal aroma formation, affecting to nearly all aroma families, except perhaps nor-isoprenoids.

Some of the most studied varietal aroma compounds are terpenes. Regarding terpene profile in wines, it is worth mentioning that all the species and hybrids of this study were able to release linalool, whereas only *S. bayanus* was able to release geraniol, in accordance with Gamero et al. (2011) (Objective 2, Chapter 1). In spite of being produced by acid hydrolysis, geraniol was not found in any of the wines, except for *S. bayanus* wines. Lack of geraniol could be explained by terpene biotransformation, since geraniol can be transformed into linalool and α-terpineol by yeasts (Gamero et al. 2011, Objective 2, Chapter 1; King and Dickinson, 2000).

In spite of the weak activity of yeast β-glucosidases at wine pH (Delcroix et al. 1994; Günata et al. 1986), enzymes of *S. cerevisiae*, *S. bayanus*, *S. kudriavzevii* and hybrids between these species have been shown to be able to hydrolyze Muscat glycosilated terpenes even at high glucose concentrations (Darriet et al. 1988; Gamero et al. 2011, Objective 2, Chapter 1; Mateo and Di Stefano, 1997). Likewise, analysis of the wines resulting from fermentations with precursor addition carried out by *S. cerevisiae*, *S. bayanus* and *S. cerevisiae* x *S. bayanus* hybrid showed breakage of glycosilated compounds (Hernández-Orte et al. 2008; Loscos et al. 2007).

In this study, fermentations with precursor addition were carried out, but with different *Saccharomyces* species and hybrids, being particularly remarkable, first, the triple hybrid (*S. cerevisiae* x *S. bayanus* x *S. kudriavzevii*), and second, the remaining hybrids. From a genetic point of view, none of the ORFs present in the *S. cerevisiae* genome sequence database can be included in the β-D-glucosidase families GH1 and GH3 (Coutinho and Henrissat, 1999). However, there are three exo-1,3-β-glucanase activities encoded by the *EXG1* (Vázquez de Aldana et al. 1991) and *EXG2* (Nebreda et al. 1986) which show β-D-glucosidase activity since they act on synthetic glucosides such as *p*-nitrophenyl-β-D-glucoside (Nebreda et al. 1986). Exoglucanase activities could be responsible of aroma compounds release from glycosylated precursors. In this regard some authors consider these two enzymes codified by *EXG1* and *EXG2* to be β-glucosidases that are able to act on the 1,3-β and 1,6-β linkages of glucan (Ridruejo et al. 1989). Several research works over-expressing or deleting *EXG1* gene have been carried out to confirm

this hypothesis (Daenen et al. 2008; Gil et al. 2005; Schmidt et al. in press). In addition, *SPR1* gene and the ORF *YIR007W* have also been related to β-D-glucosidase activity. Deletion of SPR1 led to the loss of the ability of hydrolyzing flavonoid glucosides being annotated as exo-1,3-β-glucanase, whereas the function of *YIR007W* has not been classified yet (Schmidt et al. in press).

On the other hand, notwithstanding of the fact that most *S. cerevisiae* strains do not have genes coding for β-glucosidases (EC 3.2.1.21), this seems not to be true for certain *S. cerevisiae* yeasts. The β-glucosidase gene of a *S. cerevisiae* strain AL41 isolated on arbutin by Spagna et al. (2002) was recently partially sequenced by Quatrini et al. (2008). The translated amino acid sequence contained one of the conserved patterns, namely FGYGLSY, which is typical for most yeast β-glucosidases (Rojas and Romeu, 1996).

In some cases, natural acid hydrolysis seems to be more efficient than enzymatic activity of yeasts in the release/formation of some aroma compounds (Table 4). This has been previously observed by Loscos et al. (2007). The fact that in some cases the levels of some compounds (benzaldehyde, 2-phenoxiethanol, β-damascenone, β-ionone and linalyl acetate) were higher in blank with precursor addition but without inoculum than in the fermentations with precursor addition (Table 4) reinforced the idea of bioconversion carried out by yeasts, as already seen by de Carvalho and da Fonseca (2006), Endrizzi et al. (1996), King and Dickinson (2000) and Wanikawa et al. (2000).

In the case of pure fermentative aroma, significant differences were found in all of the chemical groups studied, except for acetate esters in the case of fermentations carried out with precursor addition. A surprising result was the major effect exerted by the presence of precursors on the compositional profiles of fermentative compounds. Although an effect of this kind has been previously observed (Hernández-Orte et al. 2008; Loscos et al. 2007), the strong levelling effect exerted by the presence of precursors on the fermentative aroma profile, has not been previously reported and clearly suggests that the yeast metabolism is being altered, inhibiting or promoting certain enzymatic activities related to secondary aroma production.

Regarding sensory analysis, part of the lack of clear effects linked to the addition of precursors, must be attributed to the complexity of the overall aroma interactions and to limitations in the experimental approach. The sensory study has to be done very soon after the fermentation has taken place, since the small volumes handled cannot be safely stored for long. However, this implies that the aroma profile is very often dominated and distorted by the sulphurous aroma nuances (see Fig. 2) most likely elicited by volatile sulphur compounds, whose disappearance takes several weeks or even months. This is surely the cause of why the triple hybrid, which reached highest scores on sulphurous aroma nuances, was found the less fruity in the present experiment,. The highest amount of relevant wine aroma compounds that it contains, however, make us think that once the sulphur aromas disappear, that wine would

develop very interesting flavour properties, but that hypothesis will have to be checked in larger volume fermentations.

As a general conclusion, *S. bayanus* was the most remarkable species in *de novo* synthesis, while in primary aroma release the triple hybrid was the most effective. The rest of the hybrids and *S. cerevisiae* were also good primary aroma releasers. In secondary aroma production a strong levelling effect was noted by the presence of precursors, although *S. bayanus* retained a high production ability of acetoine, which could have nasty sensory consequences. The limited sensory analysis carried out in the present work, does not make it possible to get a reliable assessment of the potential sensory effect linked to the ability of the triple hybrid to form and release varietal aroma compounds.

Moreover, this research has confirmed that yeast can increase varietal aroma by releasing aromatic compounds from grape odourless precursors and that yeast have the ability for synthetising *de novo* aroma compounds. These observations allow us to conclude that yeasts not only have a crucial role in secondary (fermentative) aroma production but also in primary (varietal) aroma increase. The differential ability showed by the yeast species or hybrids to release varietal aroma compounds during fermentation, together with their known ability to modulate secondary aroma, strongly suggests that it is possible to modulate wine aroma by employing different yeasts in order to create new wines with different aromatic notes, according to consumer preferences.

Table 6. Summary of the most relevant results of this study.

	de novo synthesis	minor aroma compounds release	major aroma compounds	sensory evaluation*
Sc	lipid derivatives terpenes	terpenes	*w/o precursors*: ethyl esters, fatty acids *with precursors*: fatty acids	high: sweet fruit
Sb	lipid derivatives shikimic derivatives nor-isoprenoids terpenes	volatile phenols vanillins	*w/o precursors*: higher alcohols, acetate esters, acetoin, acetaldehyde *with precursors*: acetoin	low: fusel note, sulphurous
Sk	-	shikimic derivatives	*w/o precursors*: ethyl esters, fatty acids *with precursors*: fatty acids	-
ScxSb	shikimic derivatives terpenes	vanillins terpenes	-	-
ScxSk	volatile phenols	lipid derivatives volatile phenols vanillins terpenes	-	-
ScxSbxSk	-	lipid derivatives volatile phenols vanillins nor-isoprenoids terpenes	*with precursors*: higher alcohols	high: fusel note, sulphurous low: sweet fruit

Sc: *Saccaromyces cerevisiae*; Sb: *Saccharomyces bayanus*: Sk: *Saccharomyces kudriavzevii*;
ScxSb: hybrid between *S. cerevisiae* and *S. bayanus*; ScxSk: hybrids between *S. cerevisiae* x *S. kudriavzevii;*
ScxSbxSk: hybrid between *S. cerevisiae*, *S. bayanus* and *S. kudriavzevii*; * Sensory evaluation in wines with precursor addition.

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<u>OBJECTIVE 2</u>. Analysis of monoterpene alcohols profile in wine by the different *Saccharomyces* species and hybrids: β-D-glucosidase activity and terpene bioconversion.

Chapter 1

Monoterpene alcohols release and bioconversion by *Saccharomyces* species and hybrids

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Abstract

Terpene profile of Muscat wines fermented by *Saccharomyces* species and hybrid yeasts was investigated. The amount of geraniol decreased in most wines with respect to the initial must except for *S. bayanus* wines. On the other hand, alpha-terpineol amount was higher in wines fermented by *S. cerevisiae* and hybrid yeasts. The amount of linalool was similar in all wines and comparable to the amount in the initial must. Lower levels of beta-D-glucosidase activity were found in the hybrid yeasts with respect to *S. cerevisiae*. Moreover, no relationship between beta-D-glucosidase activity and terpenes profile in Muscat wines fermented with *Saccharomyces* species and hybrids was found. Growth of yeasts on minimum medium supplemented with geraniol showed bioconversion of geraniol into linalool and alpha-terpineol. Percentages of geraniol uptake and bioconversion were different between *Saccharomyces* species and hybrids. Strains within *S. bayanus*, *S. kudriavzevii* and hybrids showed higher geraniol uptake than *S. cerevisiae*, whereas the percentage of produced linalool and alpha-terpineol was higher in *S. cerevisae* and hybrid yeasts than in *S. bayanus* and *S. kudriavzevii*. The relationship between geraniol uptake and adaptation of *Saccharomyces* species to grow at low temperature is discussed.

Keywords: *Saccharomyces*, hybrids, beta-D-glucosidase, terpenes, geraniol bioconversion

1. Introduction

The yeasts responsible for alcoholic fermentation had been traditionally identified as strains pertaining to the species *S. cerevisiae* and *S. bayanus* var. *uvarum* (Demuyter et al. 2004; Naumov et al. 2000; Nguyen and Gaillardin, 2005; Pretorius, 2000). Recent studies based on genetic characterization of wine yeast strains have revealed the presence of hybrids *S. cerevisiae* x *S. bayanus* and *S. cerevisiae* x *S. kudriavzevii* conducting wine fermentations in Central Europe (González et al. 2006; Masneuf et al. 1998; Nguyen et al. 2000). Moreover, several *Saccharomyces* hybrids are presently being commercialized for performing fermentations at low temperature and enhancing of varietal aroma in wines (Lallemand, Montreal, Canada).

Varietal aromas in wines are attributed to grape-derived flavour-active precursor compounds. These compounds are in free form in the "aromatic" grapes, although most of them are present as non-volatile and odourless glycoconjugates (Baumes, 2009; Loscos et al. 2007; Rensburg and Pretorius, 2000; Ugliano and Henschke, 2009). Among the most important key odorants in the so-called "aromatic" grape varieties (e.g., Muscat) are monoterpenes such as linalool, geraniol, nerol, citronellol and α-terpineol. (Gunata et al. 1985; Loscos et al. 2007; Maicas and Mateo, 2005; Strauss et al. 1986; Ugliano and Henschke, 2009).

During must fermentation the grape glycosidic precursors are hydrolysed by action of glycosidases and the aromatic volatile compounds released into the wine. Among the most important

glycosidases are β-glucosidases, α-L-arabinofuranosidases, α-L-rhamnosidases and β-D-xylosidases (Maicas and Mateo, 2005; Rensburg and Pretorius, 2000; Sarry and Gunata, 2004). Characterization of wine yeasts with regard to glycosidase activities and release of varietal aromas has been done by different research groups on *S. cerevisiae* and non-*Saccharomyces* (Charoenchai et al. 1997; Esteve-Zarzoso et al. 1998; Fernández et al. 2000; Fleet, 2008; McMahon et al. 1999; Straus et al. 2001, Ugliano et al. 2006; Zoecklein et al. 1997). Unfortunately, there are very few studies concerning the production of glycosidic enzymes and increase of terpene metabolites by different species within the genus *Saccharomyces*. Mateo and Di Stefano (1997) analysed the β-D-glucosidase activity of three *S. cerevisiae*, one *S. bayanus* and one hybrid *S. bayanus* x *S. cerevisiae* strain, and Ugliano et al. (2006) studied the β-D-glucosidase, α-L-rhamnosidase and α-L-arabinosidase activity and the volatile compounds released by two *S. cerevisiae* and one *S. bayanus* strains.

Wines produced under laboratory conditions with natural *Saccharomyces* hybrids have been found more aromatic than those produced by *S. cerevisiae* (Gangl et al. 2009; González et al. 2007). Moreover, a slight colour loss in *S. kudriavzevii* wines attributable to anthocyanin-β-D-glucosidase (Manzanares et al. 2000; Rensburg and Pretorius, 2000) was indicative of potential enzymatic activity related to terpene release. Consequently, as the new *Saccharomyces* hybrids start playing a role in wine fermentation it is interesting to analyze the glycosidases spectrum and terpenes profile displayed by these hybrid yeasts in comparison with their parental species.

Grape aroma compounds are potential subjects to biochemical transformation by yeast enzymatic activity (Ugliano and Henschke, 2009). According to Darriet (1992) change in terpene content along alcoholic fermentation is the result of the joint action of several phenomena including mutual conversions, formation of terpene oxides, sweeping by CO_2 released during alcoholic fermentation, enzymatic and chemical hydrolysis of glycosidic bounds and adsorption on yeast cell walls.

Nevertheless, the differences in terpenes profile during fermentations performed by different yeasts cannot be fully accounted for, unless a direct effect of the physiological activity of the yeasts is considered (Zea et al. 1995). Limited studies concerning biotransformation of terpenes by *S. cerevisiae* have shown reduction of geraniol to citronellol, translocation of geraniol to linalool, isomerization of nerol to geraniol and cyclicizations of linalool to α-terpineol (Gramatica et al. 1982; King and Dickinson, 2000; Zea et al 1995; Zoecklein et al. 1997).

The aim of the present study was to characterize *Saccharomyces* species and hybrids attending to their glycosidase activities, terpene release profiles and changes in terpene composition derived from yeast metabolism.

2. Materials and methods

2.1. Yeast strains

22 yeast strains belonging to the species *Saccharomyces cerevisiae, Saccharomyces kudriavzevii, Saccharomyces bayanus* and hybrids between them were tested. Strains denomination and origin are listed in Table 1.

Yeast cultures used in all assays were grown overnight on GPY liquid medium (0.5% yeast extract, 0.5% peptone and 4% glucose; pH 6.5).

2.2. Plate assays of glycosidase activities

Plate assays of glycosidase (β-D-glucosidase, β-D-xylosidase, α-L-arabinofuranosidase and α-L-rhamnosidase) activities were done following the methodology of Manzanares et al. (1999) and Strauss et al. (2001).

2.3. β-D-Glucosidase activity, cellular location and enzymatic assays

Cells of *Saccharomyces* hybrids and parental species growing on 5 mL Muscat must were used to test the production of glycosidic enzymes. Must was sterilized by adding 1 mg/l of dimethyl-dicarbonate (Fluka, Switzerland) and allowed to settle overnight. Sterile must was inoculated with 10^6 cells and incubated at 16ºC for 72 hours. Experiments were carried out in duplicate.

Enzyme activities were investigated in the culture supernatants (extracellular activity), cell extracts (intracellular activity) and in the whole cells (cell wall bound activity) as described by Manzanares et al. (1999). Activity was measured using p-nitrophenyl-β-D-glucopiranoside (pNPG) as substrate following the protocol of Manzanares et al. (1999). Release of

p-nitrophenol was measured at 405 nm using a spectrophotometer (Ultrospec 2100pro, G.E. Healthcare). Enzymatic measurements were done in duplicate and the results were expressed as milliunits of enzyme activity per milligram of cells (mU/mg cells) (Table 1). One unit of enzyme activity (U) was defined as the amount of enzyme that released 1 μmol of p-nitrophenol per hour at 40ºC in McIlvaine buffer at pH 5.

2.4. Microfermentations

Microfermentations were carried out in Muscat must (reducing sugars 250 g/L and pH 3.2). Must was sterilized by adding 1 mg/L of dimethyl-dicarbonate (Fluka, Switzerland) and allowed to settle overnight. The sterilized must was distributed in 50mL flasks, inoculated with 10^6 cells and incubated at 16ºC. Fermentations were carried out in duplicate. Must fermentation was completed when reducing sugars were below 2 g/L. Fermentation days are indicated in Table 1.

2.5. Quantification of released terpenes

Four main monoterpene alcohols, nerol, citronellol, geraniol, linalool and α-terpineol were determined. Extraction of volatile terpenes was performed using headspace solid phase-microextraction sampling (SPME) with poly-dimethylsiloxane (PDMS) fibres (Supelco, Sigma-Aldrich, Barcelona, Spain) following the protocol of Rojas et al. (2001). Terpenes separation was carried out by gas chromatography using a Hewlett-Packard (HP) 5890 Series II gas chromatograph with a flame ionization detector (FID) using a HP-INNOWAX 30m x 0.25mm capillary column coated with 0.25 µm layer of cross-linked polyethylene glycol (Agilent Technologies Inc.). Carrier gas was helium (flow 1 mL/min), and the oven temperature program was: 5 min at 60°C, 5°C/min to 190°C, 20°C/min to 250°C and 2 min at 250 °C. Detector temperature was 280°C and injector temperature 220°C in splitless conditions. Chromatographic signal was registered by a HP Vectra QS/16S detector and HP3365 Chemstation program. Internal standard was 2-heptanone (0.05% w/v).

Retention times of eluted peaks were compared to those of commercial monoterpene alcohol standards. Terpenes concentrations in µg/ml were quantified using calibration graphs (R^2 value > 0.99) of standards.

The amount of released terpenes was quantified at the end of fermentation. Free terpenes in Muscat must were determined before fermentation assays.

2.6. Terpenes bioconversion

Screw cap glass tubes containing 5 ml of minimum medium (2% glucose, 0.5% ammonium sulphate and 0.17% of yeast nitrogen base) supplemented with geraniol at 25 µg/mL were inoculated with 10^6 overnight yeast cultures and incubated at 16ºC. After 72 hours of incubation, yeast cells were removed by centrifugation, supernatants were extracted using a syringe and stored in new glass tubes. The tubes were stored at -20ºC for further analysis. Extraction of volatiles, terpene separation and quantification was performed as specified above.

Terpenes quantification is given as the average of two independent growth experiments (Table 2). Control tubes of medium without geraniol were included in the study to test "*de novo*" synthesis of terpenes by the yeasts. A control tube of medium supplemented with geraniol but not inoculated was included to test the chemical transformation of geraniol into linalool and/or α-terpineol.

2.7. Statistical treatment of data

Statistic analysis and graphics were generated using SPSS Statistics v.17.0 (Statistical Package for the Social Sciences, IBM, USA).

3. Results

3.1. Enzymatic activities

Preliminary plate assays of glycosidase activities indicated very low or non activity for most of the enzymes (data not shown) except for β-D-glucosidase. Assays to determine the location of the maximum enzymatic activity showed the highest values in the whole cell fraction indicating that this enzyme was mainly associated to cell wall. Table 1 shows the amount of cell-wall associated β-D-glucosidase activity in mU/mg of cells.

All tested strains showed β-D-glucosidase activity lower than the one of the control strain, a genetically modified *S. cerevisiae* ($YCB3_5$) for β-D-glucosidase production (Sanchez-Torres et al. 1998). Comparison of β-D-glucosidase activity among the different *Saccharomyces* species and hybrids revealed the highest values for *S. cerevisiae* strains and the lowest values for *S. kudriavzevii* and *S. cerevisiae* x *S. kudriavzevii* hybrid strains (Table 1). ANOVA test for dependence between β-D-glucosidase and *Saccharomyces* species or hybrids groups showed significant differences between *S. cerevisiae* and the rest of species and hybrids (significance < 0.05).

Table 1. List of strains of *Saccharomyces* species and hybrids used in this study, reference numbers in culture collections, commercial names and isolation sources. Terpene amounts (µg/ml) were detected by GC at the end of Muscat must fermentation. Enzymatic activities (mU/mg cells) were detected in cell wall of whole cells.

Species	Strain	Isolation source	Terpenes in wine			Enzymatic activity
			geraniol	linalool	α-Terpineol	β-D-Glucosid.
S. cerevisiae	control[a]		0.15 ± 0.06	0.60 ± 0.02	2.87 ± 0.08	1.90 ± 0.35
	Lalvin T73	wine, Spain	0.18 ± 0.00	0.58 ± 0.10	2.22 ± 0.43	0.99 ± 0.15
	Fermiblanc Arom	wine, France	0.18 ± 0.06	0.48 ± 0.04	1.55 ± 0.19	1.02 ± 0.23
	Fermicru Primeur	wine, France	0.16 ± 0.01	0.47 ± 0.02	1.09 ± 0.01	0.88 ± 0.32
	UCLM S-377	wine, Spain	0.14 ± 0.03	0.64 ± 0.04	0.92 ± 0.09	1.24 ± 0.12
S. bayanus	CECT 12627	wine, Spain	0.55 ± 0.03	0.39 ± 0.11	0.23 ± 0.03	0.72 ± 0.04
	CECT 12629	grape must, Spain	0.55 ± 0.00	0.39 ± 0.00	0.21 ± 0.01	0.61 ± 0.03
	CECT 12638	grape must, Spain	0.91 ± 0.05	0.53 ± 0.06	0.29 ± 0.07	0.51 ± 0.08
	CECT 12669	grape, Spain	0.52 ± 0.07	0.38 ± 0.07	0.25 ± 0.03	0.60 ± 0.12
	CECT 12930	wine, Spain	1.08 ± 0.02	0.48 ± 0.06	0.38 ± 0.06	0.20 ± 0.07
S. kudriavzevii	IFO 1802	decayed leaf, Japan	0.05 ± 0.01	0.50 ± 0.09	0.13 ± 0.04	0.29 ± 0.08
S.c. x S.b.	Lalvin S6U	wine, Switzerland	0.05 ± 0.02	0.42 ± 0.05	0.80 ± 0.01	1.08 ± 0.20
	CECT 11000	beer, Belgium	nd	0.40 ± 0.03	1.77 ± 0.36	0.66 ± 0.16
	CECT 11037	beer, UK	0.07 ± 0.01	0.41 ± 0.04	1.73 ± 0.25	0.30 ± 0.06
	CECT 1885	wine, Spain	nd	0.49 ± 0.05	1.18 ± 0.80	0.26 ± 0.09
S.c. x S.k.	Lalvin W27	wine, Switzerland	0.10 ± 0.02	0.52 ± 0.02	1.69 ± 0.17	0.26 ± 0.09
	Lalvin W46	wine, Switzerland	0.11 ± 0.02	0.50 ± 0.11	2.07 ± 0.34	0.26 ± 0.08
	SPG 16-91	wine, Switzerland	nd	0.38 ± 0.08	2.66 ± 0.24	0.22 ± 0.06
	SPG 441	wine, Switzerland	0.09 ± 0.02	0.44 ± 0.01	2.06 ± 0.08	0.24 ± 0.06
	CECT 1388	beer, UK	nd	0.46 ± 0.04	1.62 ± 0.26	0.32 ± 0.06
	CECT 1990	beer, UK	0.05 ± 0.02	0.48 ± 0.15	2.00 ± 0.34	0.29 ± 0.08
S.c. x S.b. x S.k.	CBS 2834	wine, Switzerland	0.03 ± 0.01	0.41 ± 0.03	1.89 ± 0.47	0.30 ± 0.09
	CID 1	cider, France	0.32 ± 0.09	0.42 ± 0.03	0.75 ± 0.17	0.33 ± 0.11

[a] *S. cerevisiae* recombinant strain $YCB3_5$ (Sánchez-Torres et al. 1998) was used as a control for β-D-glucosidase production; nd: not detected

3.2. Monoterpene alcohols release

Free geraniol and linalool concentrations in Muscat must were 0.166 µg/ml and 0.433 µg/ml respectively, whereas free nerol, citronellol and α-terpineol were not detected. Average of terpene amounts found in the final wines determined from two independent fermentations at 16ºC is shown in Table 1. In all fermentations linalool level was above its odour threshold (0.100 µg/ml), whereas only geraniol levels yielded by *S. cerevisiae* and *S. bayanus* species and the triple hybrid CID 1 were above sensorial threshold (0.132 µg/ml). Finally, all species produced α-terpineol above odour threshold (0.460 µg/ml) except *S. bayanus* and *S. kudriavzevii*.

Total amount of terpenes in the final wines was appreciably higher than in the original must (Figure 1). This is mainly due to the increment in α-terpineol, which was absent as free terpene in the original must. Wines showing the highest increase in α-terpineol were produced by strains of the species *S. cerevisiae* and the double and triple hybrids (*S. cerevisiae* x *S. bayanus*, *S. cerevisiae* x *S. kudriavzevii* and *S. cerevisiae* x *S. bayanus* x *S. kudriavzevii*). In contrast, *S. bayanus* and *S. kudriavzevii* wines showed very moderate increments in the amount of α-terpineol. Wines fermented with *S. bayanus* strains and the triple hybrid CID1 showed a remarkable increment in their geraniol content, whereas, wines fermented with *S.cerevisiae* showed geraniol content similar to the original must. *S. kudriavzevii* and all of the hybrids, except triple hybrid CID 1, produced wines displaying a remarkable decrease in geraniol content with respect to the initial must. Linalool was quite stable through the fermentation

process and only minor increases or decreases with respect to the amount in the original must were observed in all of the wines. Finally, nerol and citronellol were not detected in any of the wines.

ANOVA test for dependence between geraniol, linalool and α-terpineol amount and the *Saccharomyces* species or hybrids showed differences between the groups for geraniol and α-terpineol (significance < 0.05). *S. bayanus* strains were different with respect to the remaining species and hybrids in geraniol content, whereas *S. cerevisiae* and the hybrids were different in their α-terpineol content.

3.3. Relationship between terpenes in wines and β-D-glucosidase activity

Pearson correlation coefficient between β-D-glucosidase activity and ∑terpenes in wines provided a very low value (+0.190). This result points to a very low relationship between both variables, therefore suggesting that the amount of terpenes found in the final wines would not depend on the β-D-glucosidase activity of the strains. Similarly, Pearson correlation coefficient was used to evaluate the relationship between β-D-glucosidase activity and geraniol or α-terpineol. Pearson correlation coefficient values, -0.043 and +0.135 respectively, could not confirm any relationship between these terpenes and β-D-glucosidase enzymatic activity. These results would suggest either different β-D-glucosidases specificity in *S. cerevisiae* and the hybrids or that part of the terpenes released by β-D-glucosidase activity are being transformed into α-terpineol during the fermentative process.

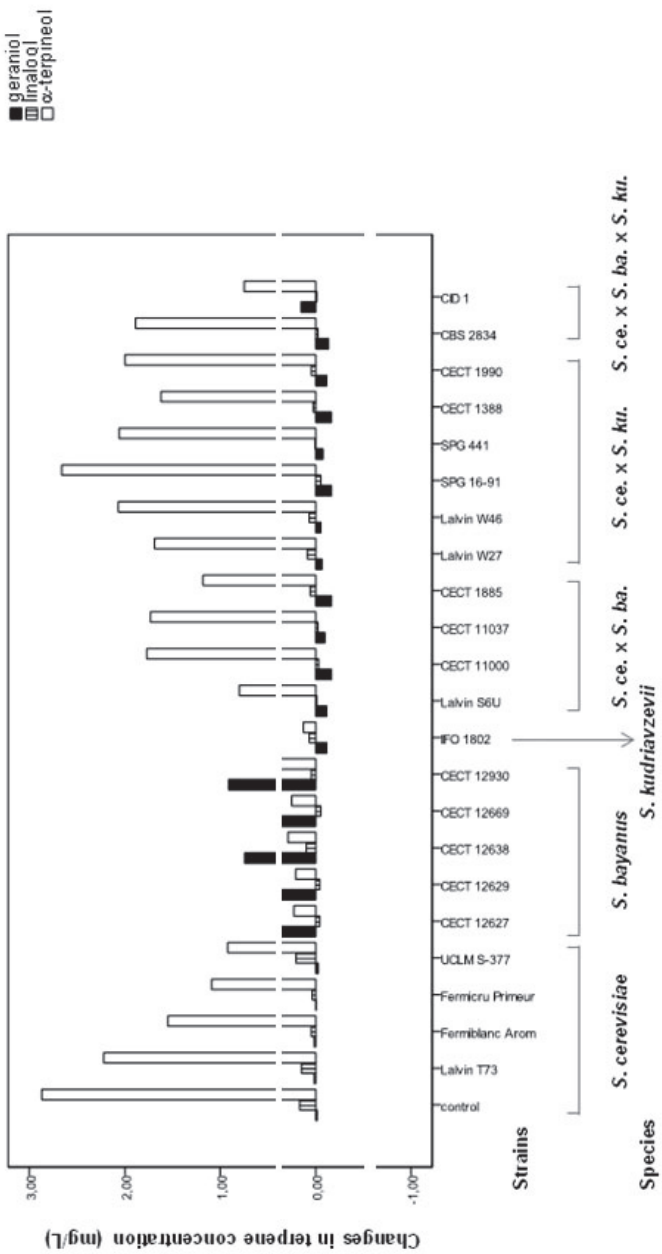

Figure 1.- Changes of terpenes concentration in Muscat wines with respect to the amounts detected in the must.

Further analysis of *S. cerevisiae* and its hybrids terpene profile was performed on synthetic minimum medium supplemented with geraniol.

3.4. Terpenes biotransformation

Table 2 summarizes the amount of geraniol, linalool and α-terpineol in minimum medium supplemented with 25 µg/ml of geraniol after 72 hours of incubation at 16ºC.

Terpenes measurement from minimum medium without geraniol revealed that "*de novo*" synthesis of terpenes was not carried out by any tested yeast after 72 hours of incubation (data not shown). Quantification of terpenes in minimum medium supplemented with geraniol but not inoculated with yeasts showed linalool (0.48±0.01 µg/ml) and α-terpineol (0.37±0.01 µg/ml). These results indicate slow chemical transformation of geraniol into linalool and α-terpineol. Moreover, decrease of geraniol in the minimum medium, from 25 µg/ml to 20.56 ± 0.02 µg/ml, after 72 hours would indicate evaporation during tubes incubation and handling.

Table 2. Quantification of terpenes in µg/ml after 72 hours growth on minimum medium supplemented with geraniol 25 µg/ml.

Species	strain	geraniol	linalool[a]	α-terpineol[a]	% geraniol uptake[b]	% geraniol bioconv.[e]	% into linalool[c]	% into α-terpineol[d]	% geraniol not bioconv.
S. cerevisiae	Lalvin T.73	10.30 ± 3.82	2.45 ± 0.35	0.77 ± 0.28	49.90	15.64	11.89	3.75	34.27
	Fermiblanc Arom	9.85 ± 0.77	3.31 ± 0.47	0.82 ± 0.42	52.09	20.04	16.07	3.96	32.05
	Fermicru Primeur	9.90 ± 1.14	2.92 ± 0.29	0.96 ± 0.55	45.16	24.32	16.10	8.22	20.84
	UCLM S-377	9.16 ± 1.10	2.71 ± 0.89	0.45 ± 0.08	55.45	15.37	13.18	2.19	40.08
S. bayanus	CECT 12627	6.59 ± 0.94	1.50 ± 0.16	0.27 ± 0.04	67.97	8.58	7.27	1.31	59.39
	CECT 12629	5.38 ± 0.22	1.65 ± 0.06	0.40 ± 0.06	73.86	9.97	8.03	1.95	63.89
	CECT 12638	5.01 ± 0.59	2.25 ± 0.01	0.65 ± 0.10	75.66	14.11	10.94	3.16	61.55
	CECT 12669	5.08 ± 0.53	1.10 ± 0.25	0.22 ± 0.11	75.32	6.40	5.33	1.07	68.92
	CECT 12930	6.33 ± 0.66	1.60 ± 0.01	0.43 ± 0.01	69.24	9.87	7.78	2.09	59.36
S. kudriavzevii	IFO 1802	2.92 ± 0.75	1.27 ± 0.52	0.95 ± 0.62	85.80	10.80	6.18	4.62	75.00
S.c. x S.b.	Lalvin S6U	5.72 ± 0.26	2.14 ± 0.52	1.28 ± 0.26	72.70	16.63	10.41	6.23	55.57
	CECT 11000	6.51 ± 0.11	2.52 ± 0.59	0.92 ± 0.51	68.34	16.68	12.23	4.45	51.65
	CECT 11037	6.11 ± 0.27	0.98 ± 0.49	0.38 ± 0.18	70.31	6.59	4.77	1.82	63.72
	CECT 1885	7.13 ± 0.52	2.08 ± 0.53	0.59 ± 0.38	65.35	12.96	10.09	2.87	52.38
S.c. x S.k.	Lalvin W27	6.93 ± 0.76	1.59 ± 0.38	0.24 ± 0.52	66.29	8.88	7.73	1.14	57.42
	Lalvin W46	8.29 ± 0.58	2.48 ± 0.48	0.87 ± 0.18	59.68	16.27	12.06	4.21	43.41
	SPG 16-91	6.06 ± 0.10	2.18 ± 0.44	0.91 ± 0.47	70.53	15.00	10.58	4.43	55.52
	SPG 441	6.38 ± 0.76	3.11 ± 0.64	1.69 ± 0.39	68.99	23.37	15.15	8.22	45.62
	CECT 1388	6.45 ± 0.09	4.07 ± 0.25	0.88 ± 0.95	68.65	22.35	19.41	2.94	46.30
	CECT 1990	6.46 ± 0.52	2.07 ± 0.20	0.47 ± 0.02	68.58	12.35	10.07	2.29	56.23
S.c. x S.b. x S.k.	CBS 2834	5.62 ± 0.41	3.20 ± 0.86	2.03 ± 0.85	72.67	25.41	15.56	9.85	47.25
	CID 1	7.42 ± 0.34	2.73 ± 0.02	0.89 ± 0.01	63.91	17.61	13.25	4.35	46.30

[a]. Linalool and α-terpineol amounts given are the result of detected amounts in minimum medium minus average terpene amount due to chemical transformation measured in control tube. b. percentage of geraniol entering the cell with respect to the total amount measured in control tube.
[c]. percentage of geraniol biotransformed into linalool, [d]. percentage of geraniol biotransformed into α-terpineol. e. ∑c+d, f. geraniol entering the cell but not biotransformed into linalool and α-terpineol: = b-e.

S. cerevisiae cells take around 50% of geraniol from the minimum medium and transform nearly 20% into linalool and α-terpineol. *S. bayanus* cells take more than 70% of geraniol and transform less than 10% into linalool and α-terpineol. The hybrids display an intermediate behaviour between *S. cerevisiae* and *S. bayanus* or *S. kudriavzevii* for geraniol transformation into linalool and α-terpineol, although geraniol uptake is similar to *S. bayanus* and *S. kudriavzevii*.

ANOVA test for dependence between geraniol, linalool and α-terpineol and *Saccharomyces* species or hybrid groups showed differences in geraniol content (significance < 0.05). Firstly, *S. cerevisiae* strains take less geraniol than the hybrids, although the amount of resulting linalool is very similar in both cases. Moreover, *S. bayanus* strains take more geraniol than *S. cerevisiae* but the resulting linalool amount is lower in the former than in the latter. These results point to different uptake and transformation percentages of geraniol by the different species (Table 2).

4. Discussion

The most abundant terpenes found in Muscat grapes are geraniol, linalool, nerol, citronellol and α-terpineol. The levels of glycosidically bounded terpenols in Muscat grapes are usually higher than the free forms. Consequently, glycosidases release the aglycones increasing the levels of free aromatic terpenes during fermentation. Enzymatic release of bounded terpenes in Muscat

wines can increment geraniol content from few hundreds to more than thousand micrograms per liter, whereas the release of less abundant bounded α-terpineol can increase to barely one hundred micrograms per liter (Gunata et al. 1985).

Screening of glycosidase activity in several yeast species have shown that *S. cerevisiae* is not a good enzyme producer. Moreover, glycosidase activities different than β-D-glucosidase are usually low or not detectable (Charoenchai et al. 1997; McMahon et al. 1999; Ugliano et al. 2006). Our results regarding glycosidase activity of *S. cerevisiae* strains are in agreement with the studies above. Furthermore, most of the non S. *cerevisiae* species and hybrids used in our study display β-D-glucosidase activities significantly lower than *S. cerevisiae* as indicated with ANOVA analysis. Consequently, the increase in the aroma found in wines fermented by *Saccharomyces* hybrids (González el al. 2007) would not be explained by an increment in the β-D-glucosidase activity.

Despite differences in β-D-glucosidase activity demonstrated by ANOVA analysis, wines produced by *S. cerevisiae* and its hybrids were very similar in their terpene profile, displaying low geraniol and high α-terpineol amounts. In contrast, *S. bayanus* wines contained high geraniol and low α-terpineol amounts. These results indicate no relationship between β-D-glucosidase activity and terpene profile which is confirmed by a low Pearson correlation coefficient (+0.190).

Similar experiments from Mateo and Di Stefano (1997) demonstrated that *S. bayanus* strain S1U (S. *bayanus* var. *uvarum*) displayed lower β-D-glucosidase activity than *S. cerevisiae* strains or

S. cerevisiae x *S. bayanus* double hybrid strain S6U (formerly *S. bayanus* var. *uvarum*), although higher geraniol amount was released from glycosides extracts in case of this strain S1U. The authors also observed that the high β-D-glucosidase activity in the S6U hybrid cell extracts corresponded with a lower amount of released geraniol, what is also in agreement with our data.

The low Pearson correlation coefficient between β-D-glucosidases and terpenes would suggest different specificity in β-D-glucosidases of *S. cerevisiae* and *S. bayanus* which would account for some of the differences in the amount of released geraniol. However, differences in β-D-glucosidases activity levels could not explain geraniol disappareance from the original must, nor the large differences in the amount of α-terpineol between different *Saccharomyces* species and hybrids wines, as confirmed by ANOVA analysis. A probable explanation for our results might be that most of released geraniol by β-D-glucosidase activity of *S. cerevisiae* and the hybrids is being transformed into other terpenes, mainly linalool and α-terpineol, during the fermentative process. In this case, differences in geraniol concentration in Muscat wines would not depend exclusively on β-D-glucosidase activity but on different rates of terpenes biotransformation for the different *Saccharomyces* species and hybrids.

Biotransformation of terpenes by yeasts during fermentation has been previously studied (Bishop et al. 1998; Croteau, 1984; Darriet, 1992; Di Stefano et al. 1992). Zea et al. (1995) and Zoeckline et al. (1997) analysed the changes in free terpenes in yeast cells and

musts during vinification using *S. cerevisiae* and *S. bayanus* strains. These authors found that at the end of *S. cerevisiae* fermentation the levels of α-terpineol increased much more than linalool or geraniol levels. The only exception was *S. bayanus* wines (Zea et al. 1995), whose α-terpineol levels were as low as at the beginning of the fermentation whereas geraniol level increased. Our results indicate the same trend in the variation of α-terpineol and geraniol amounts in *S. cerevisiae* and hybrids Muscat wines, which are rich in α-terpineol and poor in geraniol. In the case of S. bayanus wines, there are low amounts of α-terpineol and high amounts of geraniol. These results would support the supposition that part of the geraniol released by β-D-glucosidase activity is being transformed into linalool and α-terpineol by *Saccharomyces* yeast metabolism. Moreover, our results indicate that terpene bioconversion rates seem to be different for the different yeast species and hybrids.

Dynamics of terpene biotransformation has been studied by King and Dickinson (2000) in three yeasts, *S. cerevisiae*, *Torulaspora delbrueckii* and *Kluyveromyces lactis* growing on terpene supplemented medium. The authors observed that the geraniol entering the cell was converted into linalool and α-terpineol. Our experiment using geraniol supplemented medium demonstrated the same conversion into linalool and α-terpineol by *Saccharomyces* species and hybrids. ANOVA test for dependence between geraniol uptake and the different *Saccharomyces* species and hybrids showed differences between the groups, indicating that geraniol enters the cell with different rate and outcome depending on the

Saccharomyces group. *S. cerevisiae* strains show a moderate uptake of geraniol which is mainly converted into linalool and α-terpineol. *S. bayanus* and *S. kudriavzevii* strains show the largest geraniol uptake and the lowest conversion rate into linalool and α-terpineol indicating that geraniol is stored within the cell. The hybrid strains show geraniol bioconversion and accumulation percentages between the parental species *S. cerevisiae*, *S. bayanus* and *S. kudriavzevii*. In light of these results, the low geraniol amounts detected in the minimum medium after growth of *S. bayanus*, *S. kudriavzevii* and hybrids can not be solely the result of geraniol bioconversion; therefore another physiological activity must be taken into account. Interestingly, *S. bayanus* and *S. kudriavzevii*, as well as the *Saccharomyces* hybrids have demonstrated their ability to grow at low temperatures when compared to *S. cerevisiae* (Arroyo-Lopez et al. 2010; Belloch et al. 2008; Kishimoto, 1994; Lopes et al. 2009; Sampaio and Gonçalves, 2008). Studies in yeast and bacteria have shown that organisms are adapted to grow at low temperature in part through altering the lipid and sterol content of their membranes (Beltrán et al. 2008; Thieringer et al. 1998). A single desaturase that introduces one double bond into the fatty acid chain has been found in *S. cerevisiae* (Stukey et al. 1989). Consequently, modification of sterols might play a much more important role in the cold adaptation responses of yeast (Loertscher et al. 2006).

Zea et al. (1995) proposed that during the growth phase, when oxygen is still present in the medium, yeast use terpenes as biosynthetic intermediate for sterol synthesis (Coolbear and Threlfall,

1989). Additional studies on the effect of fermentation temperature on sterol composition in *S. cerevisiae* revealed that at 13ºC the proportions of sterols in the membrane were higher than at 25ºC (Beltrán et al. 2008). Subsequently, yeasts with the ability to produce sterols rapidly would be better equipped to grow faster at low temperatures. In our study, the strains best adapted to grow at low temperatures are the ones accumulating more geraniol within the cell, thus providing advantages for wine fermentation at low temperatures.

In conclusion, differences in terpene profile of wines would depend on β-D-glucosidase activity, terpenes bioconversion rate and percentage of terpenes accumulation by different *Saccharomyces* species and hybrids yeasts. Yeast strains with high β-D-glucosidase activity would release bound terpenes improving the terpene profile of a wine. Afterwards, free terpenes entering the cell could be transformed into other terpenes showing different aromatic characteristics or odour thresholds, thus influencing the organoleptic properties of wines.

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<u>OBJECTIVE 3</u>. Study of secondary aroma production in wine by different cryotolerant *Saccharomyces* species and hybrid strains.

Chapter 1

Oenological characterization of cryotolerant *Saccharomyces* species and hybrids at low and moderate fermentation temperatures

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Abstract

Among the most important factors influencing wine quality are yeast strains and fermentation temperature. Fermentation at low temperature is presently used in winemaking to improve both aroma formation and retention, as well as to produce wines with more glycerol and less ethanol content. In this study, we have analysed the oenological characteristics of Tempranillo wines produced at 12ºC and 28ºC by different *Saccharomyces* species and hybrids. Low temperature had a strong influence on fermentation kinetics. The increase in glycerol production seemed to be directly influenced by fermentation at low temperature. Analysis of aroma composition of wines produced by diferent *Saccharomyces* species and hybrids revealed large differences depending on fermentation temperature. Non-cryophilic *S. cerevisiae* was one of the best aroma producers at 28ºC. The non-*S. cerevisiae* cryophilic strains excelled producing aroma compounds at 12ºC. Production of acetate esters was favoured in all strains at 28ºC whereas production of ethyl esters was apparently stimulated by low fermentation temperatures in the case of cryophilic yeast strains. Our results suggest that fermentation temperature and yeast species are of crucial importance for production of metabolites influencing wine quality.

Keywords: *Saccharomyces*, hybrids, cryotolerant, glycerol, ethanol, aroma

1. Introduction

Wine fermentation is a complex ecological and biochemical process involving the sequential development of different yeasts, fungi and bacteria strains. Although different yeast species and genera are present in musts, only the species of *Saccharomyces* genus are responsible for the alcoholic fermentation (Lambrechts and Pretorius, 2000). The physiological characterization of industrial *Saccharomyces* strains has showed that, in addition to their high fermentative capabilities, these yeasts also produce valuable secondary metabolites, which have an essential influence on wine quality. Among those metabolites of high importance are glycerol, ethanol, higher alcohols, acetates and esters (Fleet and Heard, 1993; Lambrechts and Pretorius, 2000).

S. cerevisiae is the main yeast species responsible for the alcoholic fermentation, but closely related species, such as *Saccharomyces bayanus var. uvarum* (or *S. uvarum*) and natural hybrids between species of the *Saccharomyces* complex have been also found conducting wine fermentation at low temperatures (González et al., 2006; Sipiczki, 2002, 2008). Recently described *Saccharomyces* hybrids seem to be better adapted than *S. cerevisiae* to carry out fermentations at low temperatures (Gonzalez et al., 2007; Gangl et al., 2009). However, wine fermentation processes at low temperatures increases the probabilities of sluggish or stuck fermentations, a problem well reported in the case of fermentations conducted by *S. cerevisiae* (Novo et al., 2003). These risks can be reduced by selection of new wine yeasts able to ferment

at low temperatures, producing at the same time wines of good quality.

Natural hybrids between *S. cerevisiae* and *S. kudriavzevii* have proven to be well adapted to ferment at low and intermediate conditions of temperature, giving higher amounts of glycerol and higher alcohols with respect to reference strains of *S. cerevisiae* and *S. kudriavzevii* (Gangl et al., 2009; González et al., 2007). Other cryotolerant yeasts such as *S. uvarum* produces low quantity of acetic acid, but high amount of glycerol, as well as numerous secondary aroma compounds (Antonelli et al., 1999; Giudici et al., 1995; Sipiczki, 2002; Tosi et al., 2009). There is scarce information about the oenological characteristics of natural hybrids between *S. bayanus* and *S. cerevisiae*. However, artificial *S. cerevisiae* x *S. bayanus* hybrids seem to have inherited the cryotolerance from *S. bayanus* (Kishimoto, 1994) whereas they produce intermediate glycerol concentrations with respect to their parental species (Zambonelli et al., 1997). Regardless of the limited studies on the fermentative potential of *Saccharomyces* hybrids, several strains (Lalvin W27, Lalvin W46, Lalvin S6U) are being commercialized to perform fermentations at low temperature enhancing varietal aromas

(http://www.lallemandwine.com/catalog/products/view/48;http://www.lallemandwine.us/cellar/gewurztraminer.php;http://www.lallemandwine.us/products/yeast_chart.php).

Secondary wine aroma is a complex mix of different families of compounds. The principal ones in young wines are higher alcohols

(fusel, marzipan and floral aromas) and acetate and ethyl esters (fruity and floral aromas). These compounds are produced by yeast secondary metabolism (Lambrechts and Pretorius, 2000; Swiegers and Pretorius, 2005). Higher alcohols can be synthesised either from intermediates of sugar metabolism, through anabolic reactions, or from branched-chain amino acids, through a multi-step catabolic reaction, the Ehrlich pathway (Boulton et al., 1996; Dickinson et al., 1997, 2003; Eden et al., 2001). Ethyl ester compounds are produced by condensation of an alcohol and a coenzyme-A-activated acid (acyl-CoA) (Swiegers and Pretorius, 2005). Acetate esters result from the combination of acetyl-CoA with an alcohol, by the action of the alcohol acetyl transferases (Lambrechts and Pretorius, 2000). The nature and amount of these aroma compounds depend on multiple factors, such as the nitrogen content of the must, the temperature of fermentation and the yeast strain used (Lambrechts and Pretorius, 2000; Swiegers et. al, 2006).

This work focuses on the influence of temperature fermentation on the final aromatic quality of wine produced by different cryophilic *Saccharomyces* species and hybrids compared with mesophilic *S. cerevisiae* Lalvin T73 strain. Fermentations of natural Tempranillo must were performed at low 12ºC and moderated 28ºC temperatures. Concentration of wine metabolites ethanol, glycerol, acetic acid and acetaldehyde as well as higher alcohols, acetate esters and ethyl esters were measured.

2. Materials and methods

2.1. Yeast strains

The yeasts used in the present study belong to different species of the genus *Saccharomyces* as well as diverse natural interspecific hybrids among these species. Table 1 shows references and origin of these yeasts.

2.2. Microvinifications

Fermentations were carried out in duplicate using 450 ml of 'Tempranillo' must. The must was previously clarified by sedimentation for 24 h at 4ºC to separate the clear juice from the sediment in presence of 60 mg/l of sulphur dioxide. After filtration glucose and fructose were added to reach a concentration of 250 g/l. The must (pH of 3.5 ± 0.1) was then supplemented with 0.25 g/l of yeast nutrients (Lallemand). Yeast assimilable nitrogen (YAN) was determined by the formol index method (Aerny, 1997) and diammonium sulphate was added to reach a final concentration of 250 mg/l. Finally, must was sterilized by adding 1 ml/l of dimethyldicarbonate (Fluka, Switzerland).

Sterile tempranillo must in 500 ml flasks were fermented at two different temperatures, 12ºC and 28ºC. Fermentation evolution was monitored by sugar consumption. Glucose and fructose concentration were determined enzymatically in an Echo-Enosys analyzer (Tecnova S.A., Madrid). Measurements were done in

duplicate. Fermentations were finished when reducing sugars concentration was lower than 2 g/l. Samples taken at the last day of wine fermentation were used to determine concentrations of different metabolites.

2.3. Glycerol, ethanol, acetic acid and acetaldehyde determination

Glycerol was quantified by liquid chromatography consisting of a GP40 gradient pump, an ED40 pulsed electrochemical detector and an AS3500 autosampler system (Dionex Corporation, Sunnyvale, CA, USA). The mobile phase consisted of water and sodium hydroxide 1M (52:48, V/V) at a flow rate of 0.4 ml/min. The anion-exchange CarboPac MA1 column (Dionex, 4 x 250nm) with guard (4 x 50nm) was used for chromatographic separation. Ethanol concentration in wine was determined enzymatically (Boehringer Mannheim, Germany) using a spectrophotometer (Ultrospec 2100 pro, Amersham Biosciences). Acetic acid and acetaldehyde concentrations in wine were determined enzymatically in a refrigerated Echo-Enosys analyzer (Tecnova S.A., Madrid, Spain). Measurements were done in duplicate.

2.4. Higher alcohols and esters determination

Analysis of higher alcohols and esters from wine samples was carried out by headspace solid phase-microextraction sampling (SPME) using poly-(dimethylsiloxane) (PDMS) fibres (Supelco, Sigma-

Aldrich, Barcelona, Spain) and gas chromatography (Rojas et al., 2001). Gas chromatography was performed using a Hewlett-Packard (HP) 5890 Series II gas chromatograph with a flame ionization detector (FID) and an HP-INNOWAX 30 m x 0.25 mm capillary column coated with 0.25 μm layer of cross-linked polyethylene glycol (Agilent Technologies Inc.). Carrier gas was helium (1 mL/min) and oven temperature program utilized was: 5 min at 35°C, 2°C/min to 150°C, 20°C/min to 250°C and 2 min at 250°C. Detector temperature was 300°C and injector temperature 220°C (splitless). Chromatographic signal was registered by a HP Vectra QS/16S detector and HP3365 Chemstation program.

Volatile compound concentrations were determined using calibration graphs of the corresponding standard volatile compounds. Concentrations are given as the average of two independent fermentations. 2-heptanone (0.05% w/v) was used as internal standard. The analysed compounds in elution order were: ethyl acetate, isobutyl acetate, isobutanol, isoamyl acetate, isoamyl alcohol, ethyl caproate (ethyl hexanoate), hexyl acetate, ethyl lactate, 1-hexanol, ethyl caprylate (ethyl octanoate), ethyl caprate (ethyl decanoate), benzyl acetate, phenylethyl acetate, benzyl alcohol and 2-phenylethanol.

2.5. Statistics

ANOVA analysis of glycerol and ethanol production at significant level ($p>0.05$) (Tukey test) was done using SPSS Statistics

v.17.0 (Statistical Package for the Social Sciences, IBM, USA). ANOVA analysis was performed excluding strains CECT 1969 and BMV58 (stuck fermentations).

3. Results

3.1. Fermentation kinetics

In this work, we have studied the ability of cryophilic *Saccharomyces* strains and hybrids to ferment 'Tempranillo' must at two different temperatures 12ºC and 28ºC. Fermentation days are indicated in Table 1. As expected, fermentations carried out at 12ºC were slower than at 28ºC. *S. kudriavzevii* IFO 1802 was the fastest fermenting at 12ºC (11 days), whereas at 28ºC was *S. cerevisiae* FCry (3 days). Strain *S. uvarum* CECT 1969 did not finish fermentation at any temperature and strain *S. uvarum* BMV58 did not finish fermentation at 28ºC. The slowest fermenters at any temperature were double hybrid AMH and triple hybrid CBS 2834.

Table 1.- List of strains used in this study. Duration of microvinifications with 'Tempranillo' must at 12ºC and 28ºC.

Species	Yeast strains	Origin	Days 12ºC	Days 28ºC
S. cerevisae	Lalvin T73	wine, Spain	21	6
	FCry[d]	wine, France	17	3
	FRCh[e]	sparkling wine, France	15	4
S. bayanus	BMV58	wine, Spain	21	6[a]
	CECT 12600	wine, Spain	17	4
	CECT 1969	red currant, Holland	24[a]	4[a]
S. kudriavzevii	IFO 1802	decayed leaves, Japan	11	11
S.c. x S.b.	Lalvin S6U	wine, Switzerland	14	6
S.c. x S.k.	Lalvin W27	wine, Switzerland	14	5
	AMH[f]	wine, Germany	20	11
	HA 1841	wine, Austria	21	7
	VIN7	wine, South Africa	23	6
S.c. x S.b. x S.k.	CBS 2834	wine, Switzerland	25	8

[a] stuck fermentations; [d] Fermol Cryophile; [e] Fermol Reims Champagne; [f] Assmanhausen

3.2. Main metabolites in wine

Glycerol, ethanol, acetic acid and acetaldehyde were determined to assess the effect of temperature and different *Saccharomyces* species and hybrid strains on the production of these compounds. Figure 1 shows glycerol and ethanol concentration at both temperatures 12ºC and 28ºC.

Comparisons between strains at both temperatures pointed *S. uvarum*, *S. kudriavzevii* and the hybrid *S. cerevisiae* x *S. uvarum* as the best glycerol producers at 12ºC. Likewise, the same species, with the exception of *S. uvarum* CECT 1969 and *S. kudriavzevii*, produced the highest amount of glycerol at 28ºC. Furthermore, ANOVA analysis of glycerol showed significant differences between temperatures ($p>0.05$) for the group *S. uvarum*, *S. kudriavzevii* and the hybrid *S. cerevisiae* x *S. uvarum*. This group of strains produces significantly higher concentration of glycerol at 12ºC than at 28ºC.

Comparison between strains shows that strain *S. cerevisiae* Fermol Cryophile is the lowest ethanol producer at 28ºC (10%), whereas strains *S. uvarum* CECT 12600 and *S. kudriavzevii* IFO 1802 are the lowest ethanol producers at 12ºC (10%). ANOVA analysis of ethanol showed no significant differences between temperatures. However, in the case of *S. cerevisiae* Fermol Cryophile and double hybrid *S. cerevisiae* x *S. kudriavzevii* HA 1841 differences of two ethanol degrees can be seen at 12ºC with respect to 28ºC (Figure 1). On the contrary, double hybrid *S. cerevisiae* x *S. kudriavzevii* W27 yield higher ethanol amount at 28ºC.

In all wines, the levels of acetic acid and acetaldehyde were below the sensorial thresholds, 0.7 g/L and 100 mg/L respectively (Berg et al., 1955; Corison et al., 1979; Dubois, 1994; Schreier, 1979; Swiegers et al., 2005).

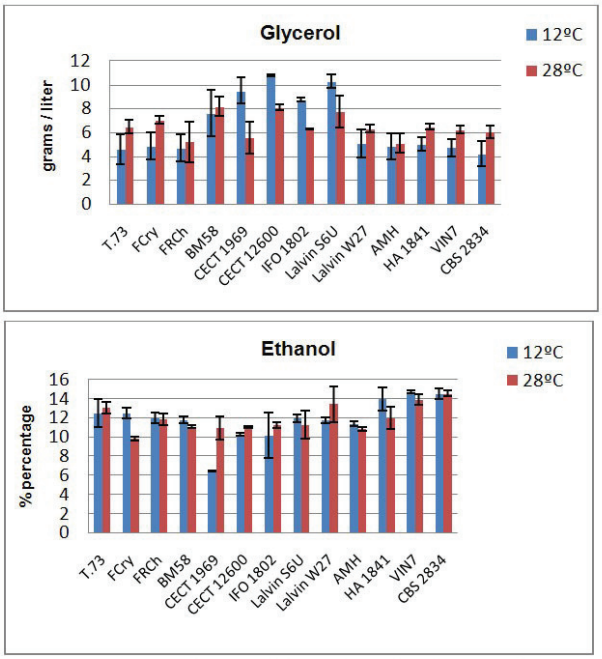

Figure 1. Glycerol and ethanol production by the different strains in the fermentations at 12°C and 28°C.

3.3. Aroma compounds

Higher alcohols, acetate esters and ethyl esters were analysed by headspace solid phase-microextraction sampling and gas chromatography (SPME-GC). Table 2 shows the total concentration of higher alcohols, acetate esters and ethyl esters produced by *Saccharomyces* strains and hybrids at 12ºC and 28ºC. Ethyl acetate was excluded from the total sum of esters due to its distinctive

contribution to the aroma of wine (Cabrera et al., 1998; Lema et al., 1996).

Table 2. Total concentration of aroma compounds produced by the strains at 28ºC and 12ºC fermentation temperatures.

	28ºC			12ºC		
	Higher alcohols	Acetate esters*	Ethyl esters	Higher alcohols	Acetate esters*	Ethyl esters
Lalvin T73	312.7	5.4	1.79	308.3	2.0	0.35
FCry	365.8	9.4	0.59	164.0	3.6	1.38
FRCh	243.5	4.0	2.39	159.1	1.2	0.63
BMV58	176.3	3.4	0.57	553.3	4.1	0.35
CECT 1969	109.7	1.4	0.57	291.1	1.8	0.14
CECT 12600	274.6	4.2	0.75	491.9	3.7	1.09
IFO 1802	181.7	3.5	0.25	342.7	3.0	0.61
Lalvin S6U	192.9	5.2	0.20	366.4	0.4	0.16
Lalvin W27	381.8	4.7	0.78	415.9	0.9	0.56
AMH	243.5	0.4	0.00	188.5	1.5	0.15
HA 1841	415.5	2.5	0.72	263.2	0.5	0.81
VIN7	365.3	3.7	0.35	252.9	5.4	1.57
CBS 2834	321.1	1.3	0.48	290.2	0.4	1.06

* Total acetate esters without ethyl acetate.

Most of the higher alcohols analysed were found in concentrations above the odour threshold at both temperatures (Figure 2), whereas only few acetate esters and ethyl esters (Figure 3) were produced above sensorial thresholds. Nevertheless, large differences were found between strains regarding concentration of different aroma compounds.

Non-cryophilic *S. cerevisiae* Lalvin T73 produced similar amount of total higher alcohols at both temperatures (Table 2), however differences in production of isoamyl alcohol and 2-phenylethanol were found between temperatures (Figure 2). Production of isoamyl alcohol was higher at 12ºC than at 28ºC, whereas concentration of 2-phenylethanol increased four times at

28ºC comparing with 12ºC. Regarding total acetate esters Lalvin T73 produced around three more times at 28ºC than at 12ºC, whereas ethyl esters concentration increased five times at 28ºC with respect to 12ºC (Table 2). The highest differences in the concentration of acetate esters were found in isoamyl acetate and acetate of 2-phenylethanol (Figure 3) which increased 2 times and 6 times respectively at 28ºC with respect to 12ºC. Finally, the concentration of ethyl caprylate increased five times at 28ºC with respect to 12ºC (Figure 3).

S. cerevisiae Fermol Cryophile (FCry) and Fermol Remis Champagne (FRCh) strains showed substantial differences in the total amount of higher alcohols at both temperatures. These strains produced similar amounts of total higher alcohols at 28ºC whereas at 12ºC they produced around twice less amount (Table 2). These differences were mainly due to the amount of isoamyl alcohol, which increased at 28ºC and decreased at 12ºC (Figure 2). *S. cerevisiae* FCry was the only *Saccharomyces* producing isobutanol above sensorial threshold at 28ºC (Figure 2). Regarding acetate esters, both strains behaved similar to the non-cryophilic *S. cerevisiae* Lalvin T73, although the increment in the concentration of all acetate esters at 28ºC was superior in the case of strain FCry (Table 2). Differences between both cryophilic *S. cerevisiae* strains were also observed in the production of ethyl esters. Strain FRCh produced more ethyl esters at 28ºC than at 12ºC likewise *S. cerevisiae* Lalvin T73 (Table 2). However, FCry produced more ethyl esters at 12ºC than at 28ºC, being the largest increment in the amount of ethyl caprylate (Figure 3).

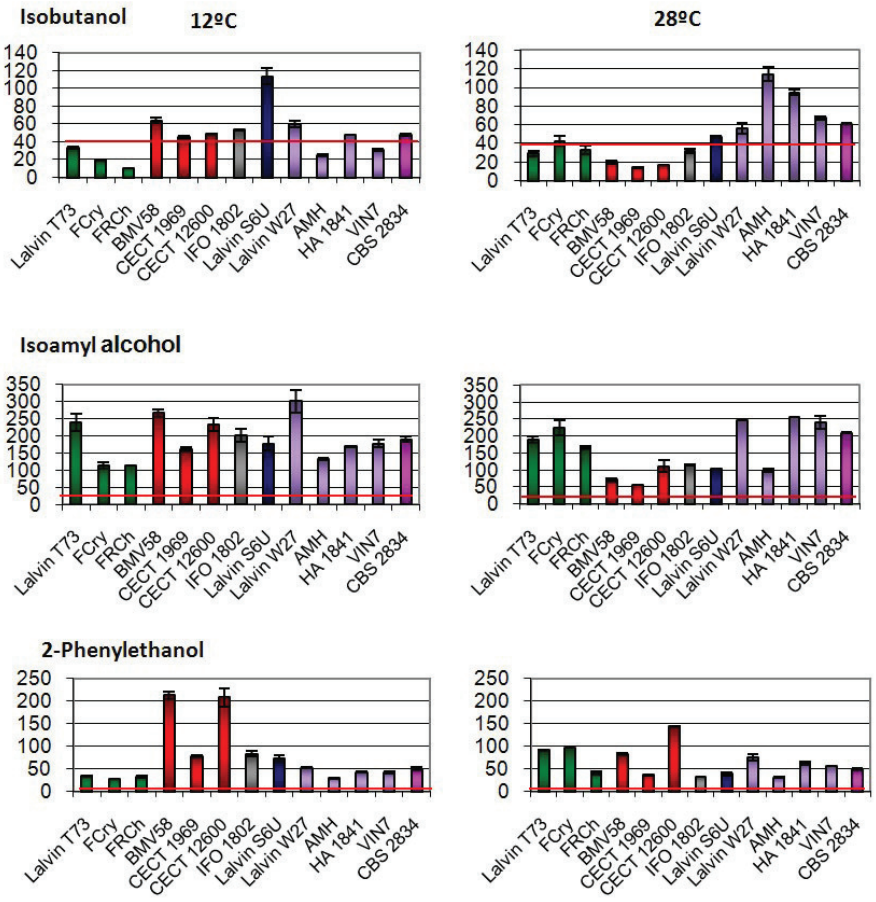

Figure 2. Concentration of higher alcohols (mg/l) produced above the sensorial threshold at 12°C and 28°C. Blue lines indicate sensory threshold. Green columns: *S. cerevisiae*, red columns: *S. uvarum*, grey column: *S. kudriavzevii*, blue column: *S. cerevisiae* x *S. uvarum*, purple column: *S. cerevisiae* x *S. kudriavzevii*, and pink column: triple hybrid *S. cerevisiae* x *S. uvarum* x *S. kudriavzevii*.

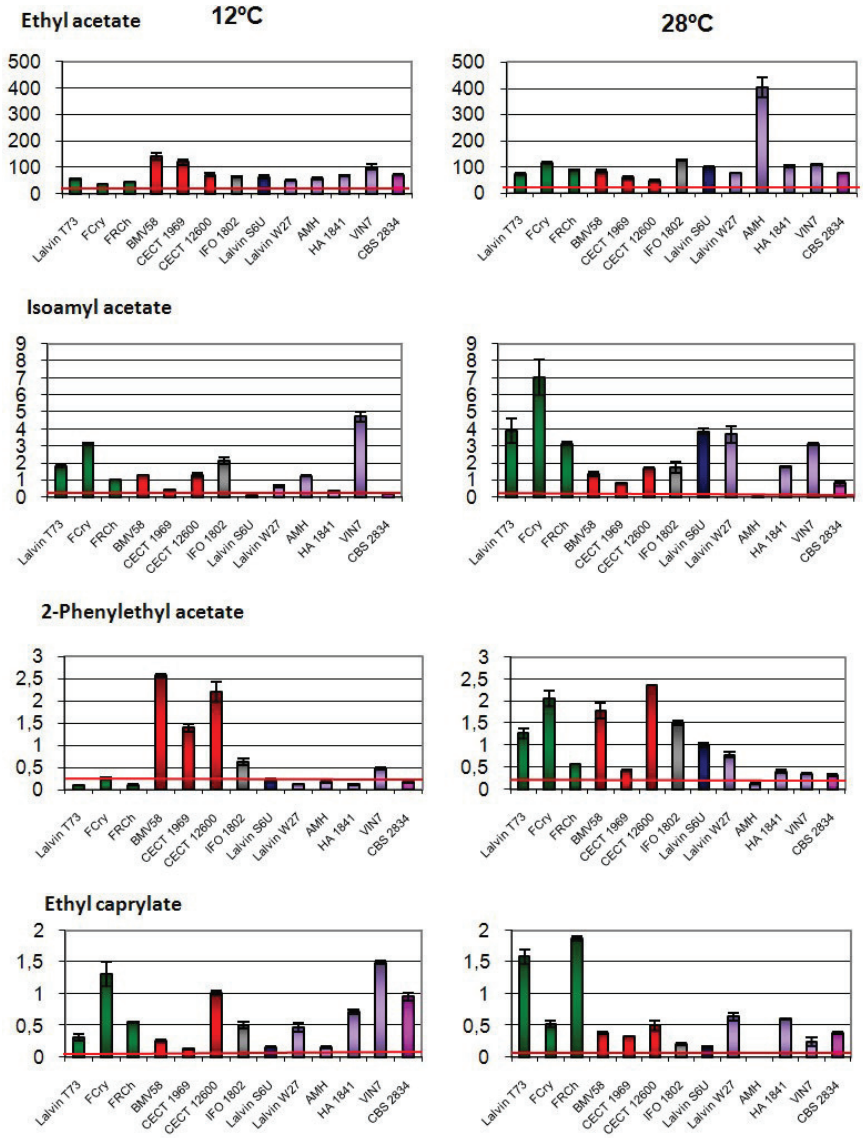

Figure 3. Concentration of acetate and ethyl esters (mg/l) produced above the sensorial threshold at 12°C and 28°C. Blue lines indicate sensory threshold. Green columns: *S.cerevisiae*, red columns: *S. uvarum*, grey column: *S. kudriavzevii*, blue column: *S. cerevisiae* x *S. uvarum*, purple column: *S. cerevisiae* x *S. kudriavzevii*, and pink column: triple hybrid *S. cerevisiae* x *S. uvarum* x *S. kudriavzevii*.

Aroma production by *S. uvarum* strains was difficult to analyse as fermentation with strain CECT BMV58 was stuck at 28ºC and CECT 1969 was stuck at both temperatures. Nevertheless, *S. uvarum* strains produced substantially more higher-alcohols at 12ºC than at 28ºC (Table 2). Isobutanol concentration was produced by all *S. uvarum* above sensorial threshold only at 12ºC (Figure 2). The highest increments were observed in the concentration of isoamyl alcohol and 2-phenylethanol, which were more than two times superior at 12ºC than at 28ºC (Figure 2). Differences between *S. uvarum* strains regarding the production of acetate esters were observed also. *S. uvarum* BMV58 produced more acetate esters at 12ºC than at 28ºC (stuck fermentation), whereas CECT 12600 produced similar amounts of acetate esters at both temperatures. Differences between *S. uvarum* BMV58 and CECT 12600 were also observed in the production of ethyl esters (Table 2). *S. uvarum* CECT 12600 produced twice more ethyl caprylate at 12ºC than at 28ºC, whereas BMV58 produced more of this compound at 28ºC (stuck fermentation) (Figure 3).

S. kudriavzevii IFO 1802 displayed similar behaviour to *S. uvarum* in the case of higher alcohols production, which was favoured at 12ºC with respect to 28ºC (Table 2). However, *S. kudriavzevii* wines contained higher concentration of acetate esters at 28ºC with respect to 12ºC except in the case of isoamyl acetate (Figure 3). On the contrary, *S. kudriavzevii* increased the production of ethyl esters at 12ºC with respect to 28ºC as occurs in the case of *S. uvarum* CECT 12600 (Table 2).

Double hybrid *S. cerevisiae* x *S. uvarum* S6U showed similar aroma production as *S. uvarum* and *S. kudriavzevii* in the case of higher alcohols, which was favoured at 12ºC with respect to 28ºC. However, double hybrid S6U showed higher production of acetate esters at 28ºC than at 12ºC. Finally, production of ethyl esters by double hybrid S6U was similar at both fermentation temperatures (Table 2).

Aroma production by the double hybrids between *S. cerevisiae* x *S. kudriavzevii* revealed very important differences between them (Table 2). All the hybrids *S. cerevisiae* x *S. kudriavzevii* produced more higher alcohols at 28ºC than at 12ºC, except strain Lalvin W27 which produced slightly more higer alcohols at 12ºC than at 28ºC due to the increment in isoamyl alcohol production at 12ºC (Figure 2). Regarding acetate esters, double hybrids Lalvin W27 and HA 1841 showed five times increase in the concentration of these compounds at 28ºC, whereas AMH and VIN7 produced higher concentrations at 12ºC. Production of ethyl esters by double hybrids *S. cerevisiae* x *S. kudriavzevii* was higher at 12ºC except in case of Lalvin W27 (Table 2).

Triple hybrid CBS 2834 showed similar aroma prodution as *S. cerevisiae* in case of higher alcohols and acetate esters, which was favoured at 28ºC with respect to 12ºC, whereas production of ethyl esters was higher at 12ºC with respect to 28ºC (Table 2).

4. Discussion

Several authors have demonstrated that wines produced at low temperatures (10-15ºC) develop improved characteristics of taste and aroma due to greater retention of terpenes, a reduction in higher alcohols and an increase in the proportion of ethyl and acetate esters (Feuillat, 1997; Llauradó et al., 2002, 2005; Torija et al., 2003). Alcoholic fermentations carried out at low temperatures are becoming more frequent due to the current winemakers tendency to produce wines with more pronounced aromatic profiles.

S. cerevisiae yeasts are good fermenters at moderate and high temperatures (Bertolini et al., 1996; Gonzalez et al., 2007; Kishimoto, 1994) however, some strains of this species have been commercialized to ferment at low temperatures (http://www.vignevin.com). Other *Saccharomyces* species such as *S. bayanus* and *S. uvarum* as well as *S. kudriavzevii* are considered cryotolerant yeasts (Giudici et al., 1998; Naumov et al., 2000; Pulvirenti et al., 2000; Belloch et al., 2008). Recent isolation of *S. kudriavzevii* from oak bark in Portugal and Spain at 10ºC supports the cryophilic character or this species (Lopes et al., 2010; Sampaio and Gonçalves, 2008). Regarding the hybrids between *S. cerevisiae* and *S. uvarum* or *S. kudriavzevii*, several studies have demonstrated the ability of these strains to grow and ferment at low temperatures (Arroyo-Lopez et al., 2010; Belloch et al., 2008; Gangl et al., 2009; Gonzalez et al. 2007; Kishimoto, 1994; Zambonelli et al., 1997).

In this study we have investigated the fermentation dynamics as well as metabolite and aroma production by several cryophilic *S.*

cerevisiae, *S. uvarum*, *S. kudriavzevii* and natural hybrids between these species at 12ºC and 28ºC temperatures. Differences in the fermentative behavior and aroma components production between cryophilic *Saccharomyces* strains and non-cryophilic *S. cerevisiae* Lalvin T73 have been investigated.

Most of the cryophilic strains used in our study have been isolated from wine fermentations at low temperatures. However, comparison of fermentating days between fermentations at both temperatures revealed that all strains fermented faster at 28ºC than at 12ºC except *S. kudriavzevii* IFO 1802. This strain was the slowest at 28ºC and the fastest at 12ºC, therefore could be considered an authentic cryophilic strain and, consequently, the remaining strains could be considered cryotolerant. Nevertheless, most of the *Saccharomyces* strains and hybrids were able to consume all reducing sugars in must at both fementing temperatures except *S. uvarum* CECT 1969 and *S. uvarum* BMV58. Our results suggest that strain CECT 1969 isolated from red currants might be stressed by other fermentation variables than temperature, whereas BMV58 seems to be stressed at moderate temperatures. Previous biometric studies based on physiological and technological properties of *S. bayanus* var. *uvarum* (or *S. uvarum*) strains clearly indicated that this species had lower capacity to ferment at unlimited sugar concentrations at 24ºC than *S. cerevisiae* (Masneuf-Pomarede et al., 2010). Moreover, earlier published data suggested that cryotolerant wine strains had low ethanol resistance at 25ºC (Kishimoto, 1994). Our results would be in agreement with this conclusion solely in the case of *S. bayanus* var. *uvarum* strains, whereas other cryotolerant

Saccharomyces species and hybrids would not be inhibited by ethanol at moderate or intermediate fermentation temperatures. Production of different ethanol and glycerol levels by cryotolerant *S. bayanus* var. *uvarum* at low and moderate fermentation temperatures has been reported previously (Masneuf-Pomerade et al., 2010; Kishimoto, 1994). Glycerol is one of the main metabolites produced in wine fermentation contributing to wine quality by providing slight sweetness, smoothness and fullness, reducing wine astringency (Ishikawa and Noble, 1995; Remize et al., 2000). Furthermore, glycerol is involved in osmoregulation (Ansell et al., 1997; Nevoight and Stahl, 1997) and low-temperature tolerance in yeasts (Izawa et al., 2004). Early studies suggested that high levels of glycerol production were a trait common to thermotolerant (non-cryotolerant) *S. cerevisiae* strains (Castellari et al., 1994; Rainieri et al., 1998). Our results suggest that cryotolerant *Saccharomyces* species well adapted to growth at low temperatures such as *S. uvarum*, *S. kudriavzevii* and double hybrid *S. cerevisiae* x *S. uvarum* Lalvin S6U were significantly the highest glycerol producers at 12ºC as demonstrated by ANOVA analysis. Besides, previous reports comparing glycerol production by non-cryotolerant *S. cerevisiae*, cryophilic *S. kudriavzevii* and cryotolerant *S. uvarum* and *Saccharomyces* hybrids support our findings (Arroyo-López et al., 2010; Bertolini et al., 1996; Gonzalez et al., 2007; Kishimoto, 1994; Zambonelli et al., 1997).

Previously published data suggested that cryotolerant wine strains produced low ethanol levels at moderate or intermediate temperatures (Kishimoto, 1994; Masneuf-Pomerade et al., 2010).

Nowadays, there is a clear trend in the wine industry to reduce the ethanol content due to the negative effect of this metabolite on human health, therefore, yeasts capable of quick sugar degradation but yielding low ethanol amounts are very desirable for the wine industry (Pereira et al., 2010; Zhang et al., 2011). Our results demonstrated that the majority of cryotolerant *S. bayanus* var. *uvarum* strains produced less ethanol than non-cryotolerant *S. cerevisiae* at any temperature as already had been seen by other authors (Castellari et al., 1994). Nevertheless, different strain behaviours regarding ethanol production and fermentation temperature could be observed in case of other cryotolerant *Saccharomyces* species and hybrids. Moreover, ANOVA analysis of our data demonstrated no significative differences regarding ethanol production between strains at any temperature. Therefore, our results would suggest that low or high ethanol production might happen at intermediate or moderate fermentation temperatures in case of cryotolerant *Saccharomyces* species or hybrids.

Low fermentation temperatures affect yeast metabolism and therefore final composition and quality of wines (Beltrán et al., 2008; Llauradó et al., 2005). Comparison of aroma compounds produced by cryotolerant yeasts in natural Tempranillo must fermentation at low and moderated temperatures revealed large differences. Non-cryotolerant *S. cerevisiae* Lalvin T73 was one of the best aroma producers at 28ºC. Cryotolerant *S. cerevisiae* strains were the best acetate and ethyl ester producers at 28ºC. On the contrary, non-*S. cerevisiae* cryotolerant strains were better aroma producers at 12ºC. The best higher alcohol producers were *S. uvarum* strains, whereas

double hybrid *S. cerevisiae* x *S. kudriavzevii* VIN7 was the best producer of acetate and ethyl esters.

Aromatic patterns distinguishing between different species or hybrids were not found at any fermentation temperature; on the contrary, aroma production was revealed as strain dependent. Nevertheless, differences between groups of strains in the concentration of higher alcohols, acetate esters and ethyl esters were noticeable at both temperatures. *S. uvarum*, *S. kudriavzevii* and double hybrid *S. cerevisiae* x *S. uvarum* produced fewer higher alcohols at 28ºC than the rest of strains. On the contrary, cryotolerant *S. cerevisiae* strains produced fewer higher alcohols than the rest of the strains at 12ºC.

A trend in the variation of the aroma compounds concentration due to the different fermentation temperatures was also not found. Remarkable differences in the aroma production performance were found between the four hybrids *S. cerevisiae* x *S. kudriavzevii*. Aroma production by double hybrid Lalvin W27 at several temperatures was already investigated by Gonzalez et al. (2007). These authors found that double hybrid Lalvin W27 produced intermediate concentrations of higher alcohols with respect to the parental species at 14ºC and fewer amounts than both parentals at 32ºC. With respect to the total esters, the double hybrid Lalvin W27 produced higher amounts than both parentals at 14ºC, whereas at 32ºC displayed an intermediate production between both parentals. In our study, double hybrid Lalvin W27 produced larger amounts of higher alcohols and acetate esters and less ethyl esters than the

parental species at 12ºC. On the other hand, production of acetate and ethyl esters by Lalvin W27 was intermediate between the parental species at 28ºC, whereas production of higher alcohols was higher than in case of both parentals at the same temperature.

Aroma production by double hybrid HA 1841 at several temperatures was recently investigated by Gangl et al. (2009). These authors found that aroma composition of wines fermented by double hybrid HA 1841 and *S. cerevisiae* overlaps at high temperatures, whereas at low temperatures HA 1841 wines show similarities with *S. kudriavzevii* wines. Our study reveals similar results, double hybrid HA 1841 as well as non-cryotolerant *S. cerevisiae* produced larger amounts of higher alcohols and acetate esters at 28ºC, whereas more similarities were found with *S. kudriavzevii* in the production of ethyl esters at 12ºC.

Among the most important aromatic compounds in wine providing desirable fruity and floral aromas are 2-phenylethanol and 2-phenylethyl acetate. Important differences in production of both compounds were found between the studied strains at both temperatures. Production of these compounds by *S. cerevisiae* was favoured at 28ºC. In case of *S. kudriavzevii* and double hybrid Lalvin S6U, production of 2-phenylethanol was increased at 12ºC whereas production of the corresponding acetate was favoured at 28ºC. Nevertheless, the best producers of both compounds at any temperature were the *S. uvarum* strains. Increased production of 2-phenylethanol and 2-phenylethyl acetate by *S. uvarum* has proven to be a characteristic trait of this species (Antonelli et al., 1999;

Bertolini et al., 1996; Gangl et al., 2009; Masneuf et al., 1998; Tosi et al., 2009).

In conclusion, the largest differences in the production of aroma compounds between the yeast strains at both temperatures were observed in the amounts of acetate and ethyl esters. Production of acetate esters was favoured in all strains at 28ºC and production of ethyl esters was apparently stimulated by lower fermentation temperatures in case of the cryotolerant yeast strains. Several authors have attributed the improvement in the quality of wine aroma at low temperatures to a reduction in higher alcohols production and an increase in acetate and ethyl esters (Lambrecht and Pretorius, 2000; Torija et al., 2003; Llauradó et al., 2002, 2005; Novo et al., 2003). Our study demonstrates that aromatic profile of wines is not only significantly affected by fermentation temperature but also by the different yeast strains.

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<u>OBJECTIVE 4</u>. Expression analysis of genes related with secondary aroma production by DNA microarrays: parental species versus hybrid strains.

Chapter 1

Correlation between wine aroma profile and gene expression in different *Saccharomyces* species

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Abstract

Members of the *Saccharomyces* genus are the main yeasts involved in wine fermentations. These yeasts also participate in the production and release of aromatic compounds. In addition, lowering fermentation temperature to achieve wines with improved aromatic profiles is desirable. In this background, it is interesting to find new yeast strains able to ferment at low temperatures efficiently, yielding wines with unique aromatic profiles. In this research work, we have analysed the expression of genes involved in flavour compounds production in three different cryotolerant *Saccharomyces* strains from the species *S. cerevisiae*, *S. uvarum* and *S. kudriavzevii* under low and moderate fermentation temperatures. Agreement between flavour-related compounds concentration and transcriptome was investigated by DNA microarrays. Transcriptome analysis of the genes related to aroma production can give us an idea of the compounds that are going to be synthesised during the fermentation process but several factors affecting aroma synthesis must be taken also into consideration. The conclusions of this study are that acetate esters synthesis seems to be influenced by higher alcohol availability in a significant way, being this factor sometimes more important than acetyltransferase levels of expression. Furthermore, the higher importance of *ADH1* with respect to *ADH4*, and *EEB1* with respect to *EHT1* was confirmed.

Keywords: *Saccharomyces* species, gene expression, microarrays, aroma profile.

1. Introduction

Wine is an alcoholic beverage profusely studied through the years. One of its more important quality traits is aroma profile. Yeasts play a crucial role in the development of the so called wine secondary aroma. The main aromatic compounds which constitute wine secondary aroma are higher alcohols, acetate esters and ethyl esters, all of them produced by yeasts during alcoholic fermentation process (Lambrechts & Pretorius, 2000; Swiegers et al., 2005; Swiegers & Pretorius, 2005). On the other hand, *Saccharomyces* yeasts can also participate in primary aroma release through glycosidases or carbon sulfur lyases. This enzymatic activity yields to the release of several aromatic compounds, such as monoterpenes or volatile thiols (Delcroix et al., 1994; Dubourdieu et al., 2006; Hernández et al., 2003; Mateo et al., 1997; Tominaga et al., 1995, 1998a,b).

During the winemaking process, ethanol, glycerol, acetic acid, acetaldehyde and hydrogen sulfide can be synthesised by yeasts. Ethanol is a majority compound which decreases aroma perception by increasing the solubility of aroma compounds in wine (Ferreira, 2007). The tendency in cellars is lowering ethanol quantity due to its negative effects on health (Pereira et al., in press; Zhang et al., 2011). Glycerol is involved in cryotolerance (Izawa et al., 2004) and osmoregulation (Ansell et al., 1997; Nevoigt & Stahl, 1997) and contributes to wine quality giving sweetness, smoothness and fullness, reducing wine astringency (Ishikawa & Noble, 1995; Llaudy, 2006; Remize et al., 2000). Acetic acid (volatile acidity) in high concentration, as occurs in case of stuck and sluggish fermentations,

gives undesirable odour to wine (Zamora, 2009). Acetaldehyde is obtained by pyruvate decarboxylation, and although can be reduced to ethanol, a small quantity can remains producing wine oxidation (Zamora, 2009).

The main yeasts responsible for wine production belong to the *Saccharomyces* genus; however, not all of the species belonging to this genus are involved in industrial processes. For instance, *S. kudriavzevii* species was isolated from decayed leaves in Japan (Naumov et al., 2000) and recently from oak barks in Portugal (Sampaio & Gonçalves, 2008) and Spain (Lopes et al., 2010). In spite of not being involved in winemaking, *S. kudriavzevii* participate in the hybridization processes with other *Saccharomyces* species like *S. cerevisiae* or *Saccharomyces bayanus var. uvarum* (*Saccharomyces uvarum*) (Belloch et al., 2009; González et al., 2006; Sipiczki, 2008). Besides, this species produce higher glycerol and lower ethanol amounts than *S. cerevisiae* (Arroyo-López et al., 2010; Gangl et al., 2009; González et al., 2007) and is able to grow at low temperatures (10ºC) and at higher ones (up to 30ºC), however they are not able to tolerate more than 5% of ethanol (Belloch et al., 2008). Contrarily, *S. cerevisiae* is the most important species involved in winemaking and the closely related species *Saccharomyces uvarum* can also participate (Demuyter et al., 2004; Massoutier et al., 1998; Naumov et al., 2000, 2001; Sipiczki, 2002, 2008). From the oenological point of view, these *Saccharomyces* species differ in several properties. Comparison between *S. uvarum* and *S. cerevisiae* reveals that the former is more cryotolerant and produces smaller acetic acid quantities (Sipiczki, 2002). Wines produced by *S. uvarum* strains have

a higher aromatic intensity than those produced by *S. cerevisiae* (Coloretti et al., 2006; Henschke et al., 2000). Specifically, *S. uvarum* produces higher amounts of 2-phenylethanol, 2-phenylethyl acetate and ethyl lactate (Antonelli et al., 1999; Di Stefano et al., 1981; Masneuf et al., 1998; Tosi et al., 2009). On the other hand, *S. uvarum* is less common and appears mainly in fermentations at low temperatures (Antunovics et al., 2003; Demuytier et al., 2004; Masneuf-Pomarède et al., 2010; Sipiczki et al., 2001).

Nowadays, there is a trend in winemaking which consists of decreasing fermentation temperatures in order to improve the aromatic profile of the wines. Previous studies have demonstrated lower fermentation temperatures results in a higher aroma retention, higher alcohols and a volatile acidity decrease and a volatile ester and fatty acid esters increase (Lambrecht & Pretorius, 2000; Torija et al., 2003; Llauradó et al., 2002, 2005; Novo et al., 2003). However, lowering fermentation temperatures has some disadvantages, including an increase in the duration of the process and a greater risk of halted or sluggish fermentation (Bisson, 1999). As commented before, both *S. kudriavzevii* and *S. uvarum* are characterized as cryotolerant and they constitute a potential tool to carry out low temperature fermentations efficiently. It is remarkable that low temperatures induce cold-responsive genes, whose role in the winemaking process has not yet been addressed (Kondo & Inouye, 1991; Kondo et al., 1992; Kowalski et al., 1995; Rodríguez-Vargas; et al., 2002; Sahara et al., 2002; Schade et al., 2004; Beltrán et al., 2006; Pizarro et al., 2008).

After the genome DNA sequence of *S. cerevisiae* was reported (Goffeau et al., 1996), a lot of research has been done related to its comprehensive analyses, including transcriptomic, proteomic, metabolomic, and phenotypic analyses. DNA array (also commonly known as gene or genome chip, DNA chip, or gene array) is one of the most powerful tools to monitor the expression of genes from a whole genome in one single experiment (Lashkari et al., 1997). In the case of *S. cerevisiae*, many studies have been reported on genome-wide expression analysis using DNA microarray to better understand the winemaking processes (Rossignol et al., 2003; Varela et al., 2005), temperature influence in growth or in aroma production (Pizarro et al., 2008 and Beltrán et al., 2006, respectively), genes involved in aroma production (Rossouw et al., 2008), general or sugar stress response (Marks et al., 2008 and Erasmus et al., 2003, respectively) or the response to nitrogen depletion (Backhus et al., 2001). Only in few of these studies, genomic expression data was correlated with the phenotypical one. Although there were several studies of genome-wide expression analysis using DNA microarray technology in *S. cerevisiae*, there was no equivalent information available regarding other species of the genus.

In this study, we have analysed the expression of genes involved in flavour compounds production in fermentations carried out by three different cryotolerant *Saccharomyces* strains from the species *S. cerevisiae*, *S. uvarum* and *S. kudriavzevii* under low and moderate fermentation temperatures. Correlation between gene expression and phenotypical data was investigated.

2. Material and methods

2.1. Yeast strains

The yeast strains used in this study belong to different species from genus *Saccharomyces,* Lalvin T.73 and Fermol cryophile *(S. cerevisiae*), IFO 1802 (*S. kudriavzevii*) and CECT 12600 (*S. bayanus var. uvarum* or *S. uvarum*) (Supplementary material Table S1).

2.2. Total RNA extraction and cDNA labeling with Cy3 and Cy5

Cells were collected by centrifugation (4000 rpm/min, 5 min) from two independent fermentations at 12ºC and 28ºC at the beginning of stationary phase, determined when 50% of reducing sugars were consumed. RNA extraction method was based on consecutive treatments with phenol-tris, phenol-chloroform (5:1) and chloroform-isoamyl alcohol (24:1), and a final precipitation with ethanol and sodium acetate (García-Martínez et al., 2004). RNA concentrations and purity were determined using a Nanodrop spectrophotometer ND-1000 (Nanodrop Technologies™, Wilmington, DE). RNA integrity was determined by electrophoresis in 1% agarose gel. 2-4 µg of total RNA from each sample was linearly amplified using the Low RNA Input Fluorescent Linear Amplification kit (Agilent Technologies™, Ca, USA). 2-3 ug of amplified cRNA was used as template for cDNA synthesis. cDNA was marked indirectly with "SuperScript™ Indirect cDNA Labeling System" (Invitrogen™, San Diego, CA). The fluorophores used were Cy3 and Cy5 mono-reactive

Dye (Amersham GE Healthcare™, Amersham UK) and dye incorporation was monitored by a Nanodrop spectrophotometer.

2.3. Microarrays hybridization, washing and scanning

A mixture of 200 to 300 pmol of the two samples labelled was concentrated in a Concentrator Plus (Eppendorf™, Hamburg, Germany). Competitive hybridization was performed on a Yeast 6.4K Array, PCR-amplified ORFs of yeast S288c strain, (Microarray Centre, UHN, Toronto, Ontario, Canada) in hybridization chambers AHC (ArrayIt Corporation, CA, USA) at 42°C overnight. Prehybridization solution contained 3X SSC, 0.1% SDS and 0.1 mg/ml BSA; hybridization solution contained 5X SSC, 0.1% SDS and 0.1 mg/ml of salmon DNA. Microarrays were washed manually with different solutions containing different SSC 20X and SDS 10% concentrations (Sol.1: 2X SSC-0.1% SDS; Sol.2: 0.1X SSC-0.1% SDS; Sol.3: 0.1 SSC; Sol4: 0.01X SSC). Signal intensities of Cy3 and Cy5 were acquired with an Axon GenePix 4100A scanner (Molecular Devices, CA, USA) using GenePix Pro v.6.1 software, at a resolution of 10 µm.

2.4. Microarray data analysis

Microarray data were derived from three independent experiments for cDNA hybridization. Raw data with a global background subtraction were generated from GenePix pro 6.0. Analyses were done using Acuity 4.0 software (Molecular Devices,

CA, USA).The individual data sets were normalized to a log_2 ratio value of 1. After normalization, data were filtered to remove spots flagged as not found. Only spots with at least two replicates were considered. Finally, replicates were combined and their medians were calculated.

Genes with a two-fold log_2 ratio values were considered to be significantly expressed, in cDNA hybridization. For these genes "GO terms" enrichment analysis was done using the GO Term Finder, in *Saccharomyces* Genome Database

(http://www.yeastgenome.org/cgi-bin/GO/goTermFinder.pl).

3. Results

Microarray analysis was carried out with RNA extracted from cells harvested at the beginning of stationary phase from wine microfermentations at 12ºC and 28ºC in Tempranillo must. Oenological parameters appear in Table S1 (data extracted from Objective 3, Chapter 1). In the microvinifications at 12ºC, *S. uvarum* CECT 12600 stood out in acetaldehyde production, whereas reference strain Lalvin T73, in acetic acid synthesis. Furthermore, *S. uvarum* excelled in higher alcohols, acetate esters and ethyl esters production, *S. cerevisiae* Fermol cryophile, in ethyl esters synthesis and *S. kudriavzevii* IFO 1802, in acetate esters. In microvinifications carried out at 28ºC, *S. uvarum* again stood out in acetaldehyde production, *S. kudriavzevii*, in acetic acid synthesis and the reference strain yielded the highest ethanol amount. The production of higher

alcohols and acetate esters by *S. cerevisiae* was remarkable. *S. kudriavzevii* also excelled in acetate esters formation, and the reference strain synthesized the highest amount of ethyl esters.

3.1. Global analysis of genes presenting changes in expression

The two more divergent species used in this study are *S. cerevisiae* and *S. uvarum* which display approximately 80% identity of coding and 74% identity of noncoding sequences (Cliften et al., 2003; Kurtzman, 2003). Hybridization of cDNA from the three *Saccharomyces* species was achieved using heterologous hybridization conditions on *S. cerevisiae* microarrays. Gene expression was determined at the beginning of stationary phase in fermentations carried out at 12ºC and 28ºC. Gene expression of the three cryophilic strains used in this study (*S. cerevisiae* Fermol cryophile, *S. uvarum* CECT 12600 and *S. kudriavzevii* IFO 1802) was compared with gene expression of reference mesophilic strain, Lalvin T73. Only genes with a fold change in expression greater than 2 (positive or negative) regarding *S. cerevisiae* Lalvin T73 were taken into consideration for further analysis. Figure 1 shows the amount of up and down regulated genes found in each species with respect to Lalvin T73. Aproximately 30% of the genes of the three cryophilic strains were differently expressed at 12ºC or 28ºC with respect to the mesophilic *S. cerevisiae* Lalvin T73. The first point standing out is the high number of up and down regulated genes shared by the three cryophilic species at 12ºC, 306 and 236 respectively. However, comparison of up and down regulated genes at 28ºC reveals only 77

up regulated and 41 down regulated genes shared by the three cryophilic strains.

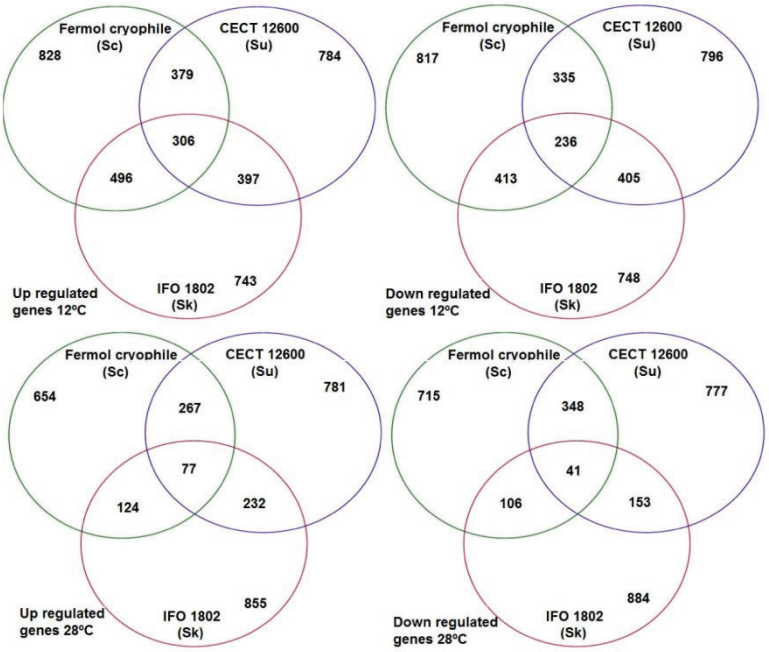

Figure 1. Global genetic expression analysis at 12ºC and 28ºC. Sc: *S. cerevisiae*; Su: *S. bayanus var. uvarum*; Sk: *S. kudriavzevii*.

Go terms show the metabolic functions in which a significant number of genes up or down regulated are involved. Go terms were done with the up and down regulated genes for each species at both temperatures (Supplementary material Table S3). No significant Go terms were found in any cryophilic strain among the up regulated genes at 12ºC. Conversely, common down regulated functions at 12ºC among the cryophilic strains were observed. These include several basic metabolic pathways such as catalitic activity and oxydoreductase activity. It is worth noting that *S. cerevisiae* Fermol Cryophile shows down regulated metabolic functions related to transmembrane transport activity. Furthermore, *S. cerevisiae*

Fermol Cryophile and *S. kudriavzevii* present down regulated most genes involved in aryl-alcohol dehydrogenase activity.

On the other hand, at 28ºC, no significant Go terms were found in any cryophilic strain among the down regulated genes (Supplementary material Table S3). On the contrary, common up regulated functions at 28ºC were observed between *S. cerevisiae* Fermol Cryophile and *S. uvarum strains*, amongst them structural functions of the ribosome, nucleic acid binding, translation factor activity and oxidoreductase activity. Finally, no significant GO terms were found in the case of *S. kudriavzevii*.

3.2. Analysis of the expression of genes related to aroma production

Expression level of genes involved in aminoacids, higher alcohols, acetate esters, ethyl esters, ethanol, acetaldehyde, acetate metabolism and enzymes involved in wine primary aroma release appear in Figure 2.

3.2.1. Genes related to aminoacid and higher alcohol metabolism

Higher alcohols are secondary aroma compounds produced by yeasts during the fermentation process. They contribute to the flowery wine aromas, being desirable in amounts below 300 mg/l. Production of these compounds occur throughout transamination of branched-chain aminoacids (leucine, isoleucine, valine), aromatic

amino acids (phenylalanine, tyrosine, tryptophan) and the sulfur-containing amino acid methionine to the corresponding α-ketoacid, followed by decarboxylation to aldehydes. Finally, those aldehydes are reduced to higher alcohols and NADH becomes NAD^+. These reactions are carried out by transaminases, decarboxylases and dehydrogenases.

The main higher alcohol producer at 12ºC was *S. uvarum* (491.9 mg/l, Supplementary material Table S2). These high values can be explained by up regulation of alcohol dehydrogenase gene *ADH6*, decarboxylase gene *ARO10* and aminoacid permease gene *BAP3*. However, *S. uvarum* also presented specific down regulation in *ARO9* gene, related to aminoacids biosynthesis, in comparison with the other three cryophilic strains. Conversely, *S. cerevisiae* cryophilic strain presented the lowest levels of higher alcohols (164.1 mg/l, Supplementary material Table S2). This low amount can be explained by absence of any up regulated gene related to higher alcohol production, except for *ARO1* gene involved in aromatic aminoacids metabolism. *S. kudriavzevii* strain presented an intermediate higher alcohol production (342.7 mg/l, Supplementary material Table S2). This strain presented up regulation of several genes related to production of higher alcohols (alcohol dehydrogenase gene *ADH7* and decarboxylase gene *ARO10*) and *ARO1* gene involved in aromatic aminoacid metabolism. On the other hand, branched-chain aminoacids transaminase gene *BAT1*, dehydrogenase gene *SFA1* and gene *ARO7* related to aminoacids biosynthesis were down regulated in comparison with the other cryophilic strains. It is worth noting that *GAP1* gene codifying the general aminoacid permease,

ILV2 gene involved in branched chain aminoacid synthesis and most of ADH genes were down regulated in the three strains.

The highest alcohol production (365.8 mg/l) at 28ºC was showed by *S. cerevisiae* strain, whereas *S. kudriavzevii* strain produced the lowest amount (181.7 mg/l). *S. uvarum* strain produced an intermediate amount of higher alcohol (274.6 mg/l) (Supplementary material Table S2). *S. cerevisiae* strain presented up regulation in most genes related to higher alcohol production, such as *ADH4*, *ADH6* genes codifying alcohol dehydrogenases, branched-chain aminoacids transaminases (*BAT1*, *BAT2*), enzymes involved in branched-chain aminoacids biosynthesis (*ILV2*, *ILV3*, *ILV5*, *LEU1*, *LEU4*) and aromatic aminoacids metabolism (*ARO7*, *ARO8*). Furthermore, piruvate decarboxylase gene *PDC5* was also up regulated. The remaining genes involved in higher alcohol synthesis presented no changes in their expression compared to reference strain. *S. uvarum* and *S. kudriavzevii* showed fewer up regulated genes involved in higher alcohol synthesis than *S. cerevisiae* cryophilic strain. In the case of *S. uvarum* the up regulated genes were alcohol dehydrogenases genes (*ADH4*, *ADH6*, *ADH7*), transaminase gene *BAT1* (higher alcohol production) and *LEU1* (branched-chain aminoacids biosynthesis). Besides, *S. uvarum* strain, the main 2-phenylethanol producer (Supplementary material Table S2), showed up regulation of the genes *ARO7* and *ARO8* related to synthesis of this higher alcohol. *S. kudriavzevii*, the species with the lowest higher alcohol production, showed few up regulated genes, *ADH7* (alcohol dehydrogenase) and *PDC6* (decarboxylase gene). On the other hand, *S. kudriavzevii* showed numerous down regulated

genes involved in high alcohol synthesis *ADH1-3* and *ADH5* genes (alcohol dehydrogenases), *THI3* and *PDC1* genes (decarboxylases), ARO9 gene (aromatic aminoacids transaminase) and genes related to branched-chain aminoacids biosynthesis *ILV1*, *LEU1* and *LEU4,* when compared to the other two species. Furthermore, *S. kudriavzevii* presented the general aminoacid permease gene *GAP1* down regulated.

3.2.2. Genes related to acetate ester production

Acetate esters are another group of aromatic compounds which constitute wine secondary aroma. Acetate esters contribute to desirable fruity and floral wine aromas. They are synthesized by a condensation between higher alcohols and acetyl-CoA mediated by acetyltransferases.

The highest acetate ester producer at 12ºC was, as in the case of higher alcohols, *S. uvarum* (76.60 mg/l), followed by *S. kudriavzevii* (66.79 mg/l) and *S. cerevisiae* (41.20 mg/l) (Supplementary material Table S2). Analysis of the up and down regulated genes revealed no correspondence between transcriptome and aroma production data. *S. uvarum* showed up regulation in *ATF2* gene, but also in esterase gene *TIP1*. However, this esterase has only been related to ethyl esters (Horsted et al., 1998). *S. cerevisiae* strain did not present any gene up regulated or down regulated involved in the synthesis of acetate esters compared to the reference strain, although *S. cerevisiae* yielded lower acetate ester amounts than the reference strain. Finally, *S. kudriavzevii*

presented, like *S. uvarum*, up regulated the gene codifying esterase *TIP1*.

The main acetate ester producers at 28ºC were *S. kudriavzevii* strain (128.8 mg/l) and *S. cerevisiae* strain (124.8 mg/l) (Supplementary material Table S2). In this case, concordance between transcriptome and chemical data was observed for *S. kudriavzevii* which presented up regulation in acetyltransferase gene *ATF2*. *S. cerevisiae* strain showed down regulation of acetyltransferase gene *AYT1*, indicating that this enzyme plays a less important role in acetate esters synthesis than the others. Finally, despite *S. uvarum* yielding the lowest amount of acetate esters, differences in gene expression were not found.

3.2.3. Genes related to ethyl ester production

Ethyl esters are other of the main chemical compounds which constituted secondary aroma in wines. Likewise acetate esters, ethyl esters contribute to desirable fruity and flowery wine aroma. Ethyl esters are synthesized by condensation between ethanol and acyl-CoA, reaction mediated by acyltransferases.

All the species tested showed higher ethyl ester production at 12ºC than the reference strain (0.35 mg/l, Supplementary material Table S2). The highest ethyl ester producer was *S. cerevisiae* (1.38 mg/l), followed by *S. uvarum* (1.10 mg/l) and *S. kudriavzevii* (0.61 mg/l) (Supplementary material Table S2). These differences could not be explained by a change in the expression of the genes involved in

ethyl esters production. *S. cerevisiae* showed down regulation in acyltransferase (ORF *YMR210W)* involved in the biosynthesis of ethyl esters. *S. uvarum* showed up regulation in esterase activity gene *TIP1*. Whereas *S. kudriavzevii* showed down regulation in two acyltranferases codified by *EEB1* and *YMR210W* genes and up regulation in esterase gene *TIP1*.

All cryophilic strains showed lower ethyl ester production at 28ºC than the reference strain (1.8 mg/l, Supplementary material Table S2). *S. uvarum* and *S. kudriavzevii* presented the same gene expression differences compared to the reference strain, up regulation in *EHT1* acyltransferase gene and down regulation in *EEB1* acyltransferase gene.

3.2.4. Genes related to ethanol, acetaldehyde and acetate metabolism

Ethanol, acetate and acetaldehyde levels are oenological parameters defining wine quality. Ethanol, a product of alcoholic fermentation made during wine production, can be oxidized to acetaldehyde by alcohol dehydrogenases. Acetaldehyde can be oxidized to acetate by aldehyde dehydrogenases. Acetate can react with CoA to form acetyl-CoA. Excessive acetaldehyde and acetate levels (>100 mg/l and >0.7-1.1 g/l, respectively) (Swiegers et al., 2005) are considered negative in wine. Besides, the current trend in oenology is to achieve wines with less ethanol content due its negative health effects.

All strains showed down regulation in most alcohol dehydrogenases at 12ºC. Down regulation of ADH genes would lead to a higher acetaldehyde and lower ethanol production than in case of the reference strain. According to chemical data (Supplementary material Table S1), all species produced more acetaldehyde than the reference strain, except for *S. kudriavzevii*. Regarding ethanol, all strains produced fewer amounts than the reference strain with the exception of *S. cerevisiae* cryophilic strain. On the other hand, down regulation of *ALD3*, *ALD4* and *ALD6* genes in cryophilic *S. cerevisiae* would lead to lower acetic acid and higher acetaldehyde production than the reference strain, which is in accordance with chemical data (Supplementary material Table S1). Besides, up regulation of piruvate decarboxylase gene *PDC2* supports the higher acetaldehyde production in *S. cerevisiae* and *S. uvarum* when compared with the control strain. Conversely, up regulation of *ALD4*, *ALD5* and *ALD6* in *S. uvarum* was not in accordance with chemical data (Supplementary material Table S1). A possible explanation for the low acetate levels detected in wines produced by *S. uvarum* strain could be that this compound is used to ethyl acetate production, since this species produce the highest ethyl acetate amount at this temperature (see Supplementary material Table S2). In case of *S. kudriavzevii* down regulation of *ALD3* gene would lead to lower acetic acid and higher acetaldehyde production than reference strain, confirmed by volatile acidity values. On the contrary, up regulation of piruvate decarboxylase gene *PDC2* would not be in accordance with the acetaldehyde level detected.

S. cerevisiae and *S. uvarum* showed up regulation of alcohol dehydrogenase gene *ADH4* at 28ºC, which might lead to a higher ethanol and lower acetaldehyde production than the reference strain; however, this is not confirmed by chemical data (Supplementary material Table S1). In *S. kudriavzevii*, down regulation of ADH genes would lead to higher acetaldehyde and lower ethanol production than the reference strain which is in accordance with chemical data (Supplementary material Table S1). Regarding aldehyde dehydrogenases, *S. cerevisiae* strain showed down regulation of *ALD3* and *ALD6*, which would lead to higher acetic acid and lower acetaldehyde production than the reference strain which was confirmed by chemical data. On the contrary, *S. uvarum* strain showed up regulation of aldehyde dehydrogenases *ALD4* and *ALD6* genes, which would lead to higher acetic acid and lower acetaldehyde production than the reference strain, although this is not in accordance with chemical data (Supplementary material Table S1). In the case of *S. uvarum* and *S. kudriavzevii* strains, up regulation of acetyl CoA synthetase genes, *ACS1* and also *ACS2* in *S. uvarum* strain, might lead to an increase in acetyl-CoA and acetate ester synthesis, as seen in *S. kudriavzevii* (Supplementary material Table S2). Finally, in the case of *S. cerevisiae* up regulation of piruvate decarboxylase *PDC5* gene was in accordance with the higher acetaldehyde production by this species when compared to the reference strain (Supplementary material Table S1).

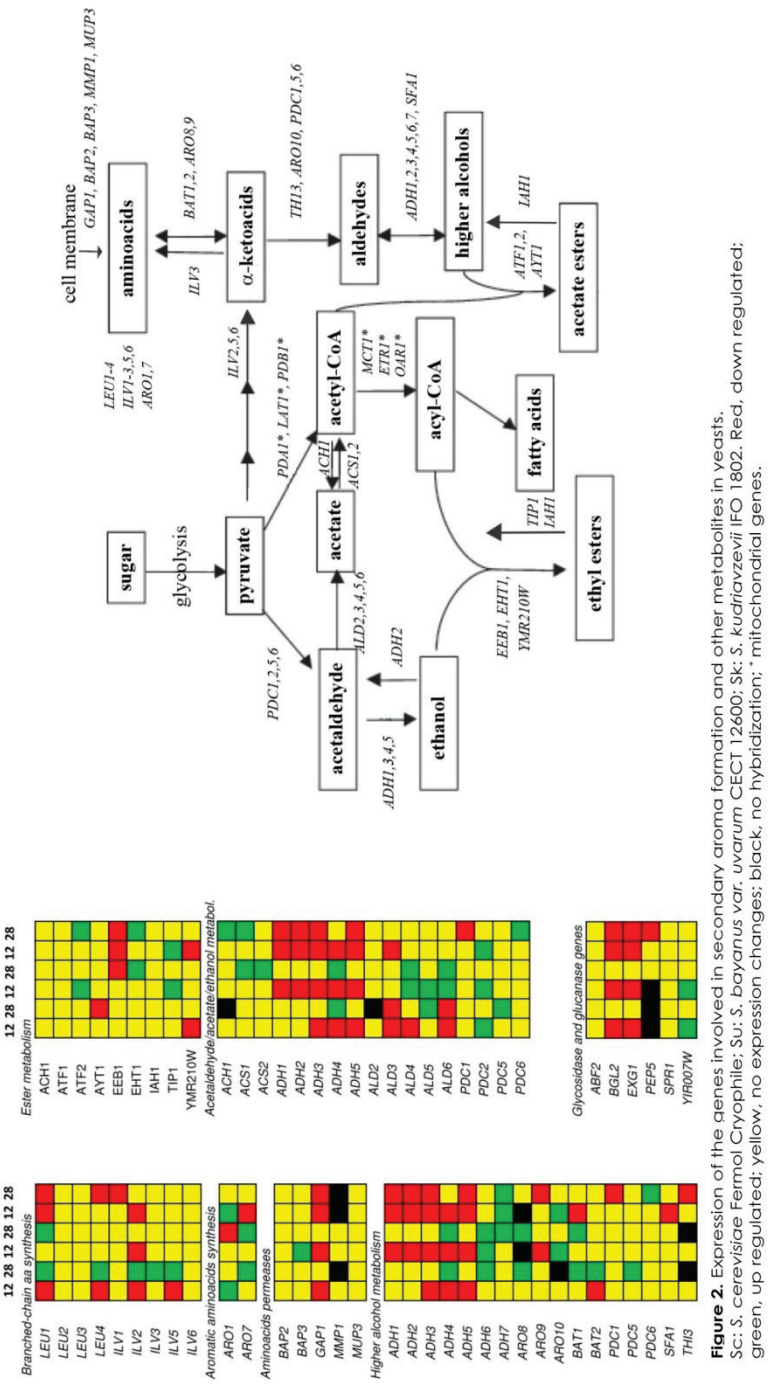

Figure 2. Expression of the genes involved in secondary aroma formation and other metabolites in yeasts. Sc: *S. cerevisiae* Fermol Cryophile; Su: *S. bayanus var. uvarum* CECT 12600; Sk: *S. kudriavzevii* IFO 1802. Red, down regulated; green, up regulated; yellow, no expression changes; black, no hybridization; * mitochondrial genes.

3.2.5. Genes related to glycosidase and glucanase activities

Yeasts glycosidases, such β-D-glucosidases and α-L-arabinofuranosidades, are important to wine aroma since they are involved in breaking glycosidic linkage between aromatic compounds and sugars. The aromatic compounds released become volatile increasing wine primary aroma. Glucanases accelerate yeast autolysis and mannoproteins release at the end of fermentation (van Rensburg & Pretorius, 2000). The protein component of the mannoproteins is important for overall aroma stabilization, improving aroma intensity (Lubbers et al., 1994).

Differences in expression levels of *BGL2* (β-glucosidase and endoglucanase activities) and *EXG1* (exoglucanase) could be observed at both temperatures compared to the reference strain. All species presented down regulation of both genes at 12ºC. *S. cerevisiae* and *S. uvarum* presented up regulation of ORF YIR007W (glycosidase) at 12ºC. Additionally, *S. kudriavzevii* presented down regulation of *BGL2*, *EXG1* and *PEP5* gene (glucanase) at 28ºC.

4. Discussion

Functional genomic approaches, such as microarray technology, are powerful tools for the analysis of gene expression at the whole genome level, providing a comprehensive view of yeast physiology (De Risi et al., 1997; Holstege et al., 1998; Spellman et al., 1998). However, yeast secondary metabolism is a complex network of biochemical pathways, which, although well mapped from a

biochemical point of view, is not well understood with regards to its physiological roles and genetic and biochemical regulation (Rossouw et al., 2008). Besides, most of the genes encoding the enzyme activities of the aroma network are also co-regulated by transcription factors that are related to total nitrogen and amino acid availability (Vollbrecht et al., 1973). Further difficulties arise from the lack of knowledge about the kinetics of individual enzymes involved in these pathways, as several of these enzymes are capable of catalyzing both the forward and reverse reactions, depending on the ratios of substrates to end products, as well as the prevailing redox balance of the cell (Ribéreau-Gayon et al., 2000; van Dijken et al., 1986; Zoecklein et al., 1995).

The genetic profile of the yeast carrying out the alcoholic fermentation, mainly from *Saccharomyces* genus, is of obvious importance in the formation of metabolites that confer specific flavors to wine (Bisson & Karpel, 2010). Besides, other factors, such as composition of the grapes, nitrogen metabolism, fermentation rate and temperature, can influence the aromatic quality of wine.

Several authors have observed that low temperature fermentations drive to higher aroma retention, a decrease in higher alcohols and volatile acidity and an increase in volatile esters (Lambrecht & Pretorius, 2000; Torija et al., 2003; Llauradó et al., 2002, 2005; Novo et al., 2003). However, our data suggests that the way in which fermentation temperature affects wine aroma profile is dependent on the strain carrying out the process. Comparing fermentations at 28ºC with 12ºC, higher alcohol decrease only

appeared in the case of *S. cerevisiae* cryophilic strain whereas increase of acetate esters increase only occurred in the case of *S. uvarum* strain. However, ethyl esters increase appeared in all of the strains and acetic acid levels (volatile acidity) underwent a slight decrease, except for *S. uvarum* strain.

Expression of genes related to aroma production was determined at the beginning of the stationary phase, since the most active period of aroma compound accumulation appears to be in the earlier stages of fermentation (Bisson & Karpel, 2010; Rossouw et al., 2008).

The species studied in the present investigation were selected due to their remarkable aroma production during wine microfermentations in Tempranillo must at 12ºC and 28ºC, and due to their adaptation to ferment well at low temperature. Mesophilic strain Lalvin T.73 was used as a reference for gene expression comparison.

Comparison of chemical data with transcriptome demonstrated limited correlation for some aroma families. Higher alcohol levels produced by the different species can be explained by gene expression at both temperatures, whereas ester amounts were not possible to correlate to gene expression data in all cases. In the case of ethanol, acetic acid and acetaldehyde the same difficulties to correlate phenotypic and transcriptomic data appeared. Nevertheless, transcriptome might not reflect the proteome, as mRNA levels of non-growing cells might or not reflect differences in protein levels (Backhus et al., 2001) or maybe proteins

need a postranslational modification to become active. In addition, correlation of gene regulation with exo-metabolome is difficult without taking into consideration that metabolic pathways are sometimes interconnected. Metabolic pathways related to aroma production are interconnected among them and with other pathways such as fatty acid metabolism, glycolysis, stress tolerance and detoxification to name a few (Rossouw et al., 2008).

Taking into account these premises, several explanations can be found to achieve better correlation between transcriptome and phenotypic data. For instance, alcohol dehydrogenases *ALD4*, *ALD5* and *ALD6* (involved in acetaldehyde conversion into acetate) appeared up regulated in *S. uvarum* strain at 12ºC. A possible explanation for the low acetate levels detected in wines produced by this strain is possible if part of this compound is being used to ethyl acetate production, since this species produce the highest ethyl acetate amount at this temperature. On the other hand, the fact that some genes with the same enzymatic function might have more importance in aroma formation than others must be also taken into account to analyse the correlation between chemical data and transcriptome. For example, *S. uvarum* and *S. kudriavzevii* strains at 28ºC presented up regulation in acyltranferase *EHT1* and down regulation in acyltranferase *EEB1* (genes involved in ethyl esters formation). The low ethyl ester production in *S. uvarum* and *S. kudriavzevii* strains compared to the reference strain suggests that acyltransferase *EEB1* is more important in the production of these aromatic compounds than *EHT1* (Rossouw et al., 2008). Besides, both acyltransferases have been related to esterase activity (Saerens et

al., 2006). Likewise, alcohol dehydrogenase *ADH4* gene up regulation in *S. cerevisiae* and *S. uvarum* strains would lead to more ethanol and less acetaldehyde than the reference strain, which was not in agreement with the chemical data. However, in the conversion of acetaldehyde to ethanol, the role of *ADH4* is not well demonstrated as *ADH1* is the main responsible gene for this transformation (De Smidt et al., 2008). Finally, up regulation of piruvate decarboxylase *PDC5* gene at 28ºC in cryophilic *S. cerevisiae* was in accordance with its higher acetaldehyde production than the reference strain. Conversely, down regulation of piruvate decarboxylase *PDC1* gene in case of *S. kudriavzevii* strain was not in accordance with chemical data, which could indicate that *PDC5* has more importance in acetaldehyde synthesis than *PDC1*.

In higher alcohol synthesis different families of aminoacids are involved, branched-chain aminoacids valine and leucine are necessary for isobutanol and isoamyl alcohol production, respectively; whereas aromatic aminoacid phenylalanine is necessary for 2-phenylethanol synthesis. Up regulation of the genes codifying permeases, transaminases and other enzymatic activities involved in branched-chain aminoacids metabolism was observed in *S. uvarum* strain at both temperatures and *S. cerevisiae* strain at 28ºC, and according to chemical data, higher levels of isobutanol and/or isoamyl alcohol than in the reference strain were found in *S. uvarum* at 12ºC and *S. cerevisiae* at 28ºC. However, no increase of any of these compounds was observed in *S. uvarum* strain at 28ºC, although this strain presented alcohol dehydrogenases up regulated.

Discrepancy between chemical data and transcriptome in *S. uvarum* at 28ºC could be due to utilization of 2-phenylethanol to produce the corresponding acetate. Higher amount of this acetate detected in *S. uvarum* at 28ºC with regards to the reference strain supported this hypothesis. Relevant production of 2-phenylethanol and the corresponding acetate, phenylethyl acetate, is a typical trait of *S. uvarum* species (Antonelli et al., 1999; Di Stefano et al., 1981; Masneuf et al., 1998; Tosi et al., 2009). On the contrary, down regulation of genes codifying enzymes involved in branched-chain aminoacids metabolism in *S. cerevisiae* strain at 12ºC and *S. kudriavzevii* strain at both temperatures was in agreement with the production of isobutanol and/or isoamyl alcohol. Furthermore, up regulation of genes codifying transaminases and other enzymes related to aromatic aminoacids metabolism were found in *S. cerevisiae* at both temperatures, in *S. kudriavzevii* strain at 12ºC and in *S. uvarum* at 28ºC. Up regulation of transaminases genes was correlated with higher 2-phenylethanol production by all of the species than by the reference strain except for *S. cerevisiae* at 12ºC. This exception could be justified because of the down regulation of the general aminoacid permease codified by *GAP1*.

In our study, the best acetate ester producers at 12ºC, *S. uvarum* and *S. kudriavzevii*, did not presented up regulation in any acetyltransferase gene or even showed up regulation in esterases. Similar results were obtained by Beltrán et al. (2006) analysing the correspondence between *ATF1* and *ATF2* with acetate ester levels in fermentations conducted by *S. cerevisiae* at 13ºC and 25ºC. As those species were the best higher alcohol producers at this

temperature, acetate ester synthesis might be more dependent on substrate availability, higher alcohols, than on acetyltransferase or esterase enzymatic activities. Accordingly, several authors observed that mutants and transformants overproducing certain higher alcohols also showed a clear increase in the synthesis of the respective acetate ester (Lee et al., 1995; Yoshimoto et al., 2001). Nevertheless, ester synthesis cannot be explained through higher alcohol availability solely. For instance, high oxygen and unsaturated fatty acid levels are known to increase fusel alcohol production, but to decrease ester levels (Hammond, 1993; Quain et al., 1985; Taylor et al., 1979).

In conclusion, transcriptome analysis of the genes related to aroma production can give us an idea of the compounds that are going to be synthetised during fermentation process. This is in accordance with Rossouw et al. (2008). However other factors that can affect aroma synthesis must be taken into account such as interconnections between the different metabolic pathways and co-regulation by transcription factors, postraductional modifications of the enzymes, importance of the enzymes with the same activity or the presence of non-growing cells in the culture, parameters that make interpretation of individual gene and enzyme contributions problematic in the context of aroma compound production. However, important conclusions of this study are that acetate esters synthesis seems to be influenced by higher alcohol availability in a significant way, being this factor sometimes more important than acetyltransferase levels of expression. Furthermore, the higher

importance of *ADH1* with respect to *ADH4*, and *EEB1* with respect to *EHT1* was confirmed.

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Supplementary material

Table S1. ANOVA analysis of oenological parameters of the fermentations at 12ºC and 28ºC.

	Reference *(S.cerevisiae)*	**Fermol cryophile** *(S.cerevisiae)*	**CECT 12600** *(S.uvarum)*	**IFO 1802** *(S.kudriavzevii)*
Source	Wine (Alicante, Spain)	Wine (AEB, France)	Sweet wine (Alicante, Spain)	Decayed leaves (Japan)
12ºC				
days	21	17	17	11
Ethanol (%)	12.50 ± 1.45^{a}	12.50 ± 0.55^{a}	10.28 ± 0.16^{a}	10.17 ± 2.37^{a}
Glycerol (g/l)	4.58 ± 1.25^{a}	4.83 ± 1.13^{a}	10.77 ± 0.05^{b}	8.74 ± 0.14^{b}
Acetic acid (g/l)	0.14 ± 0.01^{b}	0.10 ± 0.01^{a}	0.13 ± 0.01^{ab}	0.10 ± 0.01^{a}
Acetaldehyde (mg/l)	6.98 ± 0.83^{a}	16.45 ± 0.64^{b}	29.90 ± 3.54^{c}	4.79 ± 1.44^{a}
28ºC				
days	6	3	4	11
Ethanol (%)	13.02 ± 0.57^{c}	9.79 ± 0.22^{a}	11.00 ± 0.08^{ab}	11.26 ± 0.29^{b}
Glycerol (g/l)	6.47 ± 0.54^{a}	7.04 ± 0.29^{a}	8.10 ± 0.22^{a}	7.04 ± 1.12^{a}
Acetic acid (g/l)	0.18 ± 0.03^{a}	0.16 ± 0.01^{a}	0.09 ± 0.01^{a}	0.73 ± 0.08^{b}
Acetaldehyde (mg/l)	4.35 ± 0.41^{a}	9.47 ± 1.88^{b}	21.00 ± 0.85^{c}	4.94 ± 1.24^{ab}

Table S2. ANOVA analysis of aroma profile of the different fermentations at 12°C and 28°C.

	12°C				28°C			
	Reference	**FCry***	**CECT 12600**	**IFO 1802**	**Reference**	**FCry***	**CECT 12600**	**IFO 1802**
Higher alcohols								
Isobutanol	33.20 ± 1.40^{b}	19.77 ± 0.36^{a}	48.82 ± 0.43^{c}	53.73 ± 0.58^{d}	29.09 ± 2.52^{ab}	42.51 ± 4.96^{b}	17.05 ± 0.14^{a}	31.80 ± 2.64^{ab}
Isoamyl alcohol	237.5 ± 24.9^{b}	114.3 ± 9.1^{a}	232.0 ± 19.5^{b}	201.0 ± 19.7^{ab}	189.7 ± 9.5^{ab}	225.0 ± 22.4^{b}	111.8 ± 16.8^{a}	114.6 ± 0.9^{a}
Benzyl alcohol	nd	nd	nd	nd	nd	nd	nd	nd
2-Phenylethanol	33.93 ± 0.86^{a}	27.18 ± 0.62^{a}	208.1 ± 20.4^{b}	83.45 ± 6.20^{a}	91.13 ± 0.41^{a}	96.00 ± 0.37^{c}	143.2 ± 1.26^{d}	31.49 ± 0.17^{a}
1-Hexanol	3.60 ± 0.09^{a}	2.84 ± 0.13^{a}	2.94 ± 0.19^{a}	4.56 ± 0.10^{b}	2.82 ± 0.18^{a}	2.27 ± 0.02^{a}	2.56 ± 0.28^{a}	3.85 ± 0.09^{b}
Total	308.2	164.1	491.9	342.7	312.7	365.8	274.6	181.7
Acetate esters								
Ethyl acetate	56.48 ± 2.75^{ab}	37.63 ± 0.32^{a}	72.93 ± 5.35^{b}	63.76 ± 3.01^{b}	72.39 ± 5.75^{a}	115.4 ± 4.0^{c}	46.19 ± 4.15^{a}	125.34 ± 2.21^{c}
Isobutyl acetate	nd^{a}	0.11 ± 0.01^{b}	0.11 ± 0.01^{b}	0.18 ± 0.01^{c}	0.13 ± 0.02^{a}	0.25 ± 0.02^{c}	0.11 ± 0.01^{a}	0.18 ± 0.00^{ab}
Isoamyl acetate	1.83 ± 0.09^{ao}	3.09 ± 0.07^{c}	1.28 ± 0.12^{a}	2.14 ± 0.22^{b}	3.89 ± 0.70^{ab}	7.03 ± 1.07^{b}	1.69 ± 0.03^{a}	1.76 ± 0.32^{a}
Hexyl acetate	0.09 ± 0.00^{ao}	0.11 ± 0.02^{b}	0.06 ± 0.00^{a}	0.08 ± 0.00^{ab}	0.07 ± 0.00^{ab}	0.08 ± 0.01^{b}	0.07 ± 0.00^{ab}	0.05 ± 0.00^{a}
Phenylethyl acetate	0.11 ± 0.01^{a}	0.26 ± 0.04^{a}	2.22 ± 0.23^{b}	0.63 ± 0.07^{a}	1.27 ± 0.12^{a}	2.06 ± 0.18^{bc}	2.36 ± 0.01^{c}	1.51 ± 0.04^{ab}
Benzyl acetate	nd	nd	nd	nd	nd	nd	nd	nd
Total	58.51	41.20	76.60	66.79	77.75	124.8	50.42	128.8
Ethyl esters								
Ethyl caproate	0.004 ± 0.000^{a}	0.006 ± 0.001^{a}	0.005 ± 0.000^{a}	0.006 ± 0.000^{a}	0.006 ± 0.000^{b}	0.003 ± 0.000^{a}	0.002 ± 0.000^{a}	0.003 ± 0.000^{a}
Ethyl caprylate	0.31 ± 0.05^{a}	1.31 ± 0.20^{c}	1.02 ± 0.04^{bc}	0.50 ± 0.05^{ab}	1.59 ± 0.11^{b}	0.53 ± 0.05^{a}	0.50 ± 0.08^{a}	0.20 ± 0.02^{a}
Ethyl caprate	0.04 ± 0.01^{a}	0.06 ± 0.01^{ab}	0.07 ± 0.00^{bc}	0.10 ± 0.00^{c}	0.20 ± 0.05^{bc}	0.06 ± 0.00^{ab}	0.25 ± 0.02^{c}	0.04 ± 0.00^{a}
Ethyl lactate	nd	nd	nd	nd	nd	nd	nd	nd
Total	0.35	1.38	1.10	0.61	1.80	0.59	0.75	0.24

*FCry: Fermol Cryophile.

Table S3. GO terms of the up and down regulated genes in the three species.

12ºC

up regulated genes: no significant GO terms.

down regulated genes

***S. cerevisiae* Fermol Cryophile**

3735	structural constituent of ribosome
3824	catalytic activity
5353	fructose transmembrane transporter activity
5355	glucose transmembrane transporter activity
15144	carbohydrate transmembrane transporter activity
15145	monosaccharide transmembrane transporter activity
15149	hexose transmembrane transporter activity
15578	mannose transmembrane transporter activity
16491	oxidoreductase activity
16614	oxidoreductase activity, acting on CH-OH group of donors
16616	oxidoreductase activity, acting on the CH-OH group of donors, NAD or NADP as acceptor
18456	aryl-alcohol dehydrogenase activity
22857	transmembrane transporter activity
22891	substrate-specific transmembrane transporter activity
22892	substrate-specific transporter activity
51119	sugar transmembrane transporter activity
70011	peptidase activity, acting on L-amino acid peptides

***S. bayanus* CECT 12600**

3824	catalytic activity
16491	oxidoreductase activity
16614	oxidoreductase activity, acting on CH-OH group of donors
16616	oxidoreductase activity, acting on the CH-OH group of donors, NAD or NADP as acceptor

S. kudriavzevii IFO 1802

3824	catalytic activity
4022	alcohol dehydrogenase (NAD) activity

16614 oxidoreductase activity, acting on CH-OH group of donors
oxidoreductase activity, acting on the CH-OH group of donors,
16616 NAD or NADP as acceptor
16491 oxidoreductase activity
18456 aryl-alcohol dehydrogenase activity

28°C

<u>up regulated genes</u>

***S. cerevisiae* Fermol Cryophile**

3735 structural constituent of ribosome
3743 translation initiation factor activity
5198 structural molecule activity
8135 translation factor activity, nucleic acid binding
15078 hydrogen ion transmembrane transporter activity
16491 oxidoreductase activity

***S. bayanus* CECT 12600**

3735 structural constituent of ribosome
3824 catalytic activity
5198 structural molecule activity
8135 translation factor activity, nucleic acid binding
16491 Oxidoreductase activity

***S. kudriavzevii* IFO 1802:** no significant GO terms.

<u>down regulated genes</u>: no significant GO terms.

Chapter 2

Correlation between wine aroma profile and gene expression in two hybrids between *Saccharomyces cerevisiae* and *S. kudriavzevii*

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Abstract

Aroma is one of the most important wine quality attributes in which yeasts play a crucial role, synthesizing aromatic compounds or releasing odourless conjugates. A present-day trend in winemaking consists of lowering fermentation temperature to achieve higher aroma production and retention. Recently isolated *S. cerevisiae* x *S. kudriavzevii* hybrids seem to have inherited beneficial traits from their parental species, like fermenting efficiently at low temperature or producing higher amounts of certain aromatic compounds. In the present research work we have tested the ability of two *S. cerevisiae* x *S. kudriavzevii* hybrids to produce secondary aroma compounds and other metabolites influencing wine quality, such as ethanol, acetaldehyde or acetic acid, at chemical and molecular level employing microarray technology. The hybrids used in this study showed different allele composition in several genes involved in aroma production, leading to different levels of expression and different oenological properties and aromatic profile in the resulting wines. Lalvin W27 pointed out in higher alcohol production at both temperatures, whereas VIN7 was the main acetate ester producer at both temperatures, and also yielded the higher ethyl ester amount at 12ºC. We did not find correlation between genome, transcriptome and chemical data in all cases. Temperature fermentation seems to have a crucial role in modulating aroma synthesis by yeasts, especially in hybrids because they may express the allele from one or other parental.

Keywords: *Saccharomyces* hybrids, gene expression, microarrays, alleles, aroma profile.

1. Introduction

Saccharomyces cerevisiae is the most common species used in fermentations of alcoholic beverages at industrial level due to this species possess high ability to impose on the other yeasts. Conversely, *S. kudriavzevii* species has not been related to industrial processes, having been isolated from decayed leaves in Japan (Naumov et al., 2000) and recently from oak barks in Portugal (Sampaio & Gonçalves, 2008) and Spain (Lopes et al., 2010). On the other hand, natural hybrids between *S. cerevisiae* and *S. kudriavzevii* conducting wine fermentations have recently been discovered and characterized by genetic approaches (Belloch et al., 2009; González et al., 2006, 2008). Hybridization process among *Saccharomyces* species has been proposed as an adaptation mechanism of yeasts to ferment at low temperatures (de Barros Lopes et al., 2002; Barrio et al., 2006; Sipiczki, 2008). Physiological data suggest that *Saccharomyces* hybrids might have inherited the ability to grow at high temperatures (30-37ºC) and ethanol tolerance from their *S. cerevisiae* parental and ability to grow at low temperatures (10-16ºC) from their *S. kudriavzevii* parental (Belloch et al., 2008). These physiological characteristics show *Saccharomyces* hybrids as better suited to produce wines in accordance with the new trends in winemaking, such as low temperature fermentations and wine aroma improvement (Lambrecht & Pretorius, 2000; Torija et al., 2003; Llauradó et al., 2002, 2005; Novo et al., 2003). Oenological characterization of hybrids *S. cerevisiae* x *S. kudriavzevii* has showed that they are able to ferment efficiently at low and intermediate temperatures, producing intermediate or higher amounts of glycerol,

less acetic acid and higher amounts of higher alcohols with regard to reference strains of *S. cerevisiae* and *S. kudriavzevii* (Gangl et al., 2009; González et al., 2007). Regardless of the limited studies on the fermentative potential of *Saccharomyces* hybrids, several strains, such as Lalvin W27 and Lalvin W46, are being commercialized to perform fermentations at low temperature enhancing varietal aromas (Lallemand Inc. WEB page).

Wine aroma profile is one of its most important quality attributes. Higher alcohols, acetate esters and ethyl esters are quantitatively the most important family of compounds forming secondary aroma. These compounds are synthesized by yeasts during alcoholic fermentation as a consequence of its secondary metabolism, a complex biochemical process in which a lot of interconnected reactions are involved. In the formation of these compounds numerous enzymes and other proteins participate, such as permeases, transaminases, decarboxylases, reductases, acetyltransferases and acyltransferases (Lambrechts & Pretorius, 2000; Swiegers et al., 2005; Swiegers & Pretorius, 2005). *Saccharomyces* yeasts also can participate in primary aroma release through glycosidases or carbon sulphur lyases. This enzymatic activity yields to the release of several aromatic compounds, such as monoterpenes or volatile thiols (Delcroix et al., 1994; Dubourdieu et al., 2006; Hernández et al., 2003; Mateo et al., 1997; Tominaga et al., 1995, 1998a,b).

In addition to desirable aroma compounds, other metabolites can be formed during winemaking, such as ethanol, glycerol, acetic

acid or acetaldehyde. Ethanol is one of the main compounds synthesized in wine fermentation and decreases flavour perception by increasing aromatic compounds solubility in wine and lowering the volatile fraction (Ferreira, 2007). One of the present-day trends in winemaking consists of lowering ethanol contents due to its negative effects on health (Pereira et al., in press; Zhang et al., 2011). Glycerol is involved in cryotolerance (Izawa et al., 2004) and osmoregulation (Ansell et al., 1997; Nevoigt & Stahl, 1997) and contributes to wine quality giving sweetness, smoothness and fullness, reducing wine astringency (Ishikawa & Noble, 1995; Llaudy, 2006; Remize et al., 2000). Acetic acid is the main compounds of volatile acidity of wine. High concentration of this compound gives an undesirable odour to wine. This can occurred as a consequence of stuck and sluggish fermentations (Zamora, 2009). Acetaldehyde is obtained by pyruvate decarboxylation and most of it is reduced to ethanol. However, a little quantity remains in wine, contributing to perception of wine oxidized if it appears in excessive amount (Zamora, 2009). As a consequence, aroma synthesis involves very complex processes where different pathways are interconnected and several genes participate.

After the *S. cerevisiae* genome was sequenced (Goffeau et al., 1996), transcriptomic, proteomic, metabolomic, and phenotypic analyses have been achieved. DNA microarray is one of the most powerful tools to analyze the transcriptome. However, all the studies using this technology to better understanding winemaking processes, temperature influence in growth or in aroma production and stress response have been done carrying out fermentations with *S.*

cerevisiae (Backhus et al., 2001; Beltrán et al., 2006; Erasmus et al., 2003; Marks et al., 2008; Pizarro et al., 2008; Rossignol et al., 2003; Rossouw et al., 2008; Varela et al., 2005) and comparing the expression of different species of the genus (Objective 4, Chapter 1). In addition, some studies about *S. pastorianus* (hybrid between *S. cerevisiae* and *S. bayanus*) applied on beer are available (Dunn et al., 2008; Horinouchi et al., 2010). However, there is no information in the case of *S. cerevisiae* x *S. kudriavzevii* hybrids. Furthermore, only in few of these studies, genomic expression data was correlated with phenotypical data.

In this research work, fermentations with natural Tempranillo must were carried out by two genetically different *S. cerevisiae* x *S. kudriavzevii* hybrids at low temperature (12ºC) and at standard red wine fermentation temperature (28ºC). Furthermore, a commercial non-cryotolerant *Saccharomyces cerevisiae* strain was used as a control. Flavour related compounds were studied at molecular level through DNA microarrays and correlated to chemical data.

2. Material and methods

2.1. Yeast strains

The yeasts strains used in this study were *S. cerevisiae* strain Lalvin T73 used as a reference strain and two hybrids between *S. cerevisiae* and *S. kudriavzevii*, Lalvin W27 and VIN7 (Supplementary material Table S1).

2.2. Total RNA extraction and cDNA labelling

Cells were collected by centrifugation (4000 rpm/min, 5 min) from two independent fermentations at 12ºC and 28ºC at the beginning of stationary phase, determined when 50% of reducing sugars were consumed. RNA extraction method was based on consecutive treatments with phenol-tris, phenol-chloroform (5:1) and chloroform-isoamyl alcohol (24:1), and a final precipitation with ethanol and sodium acetate (García-Martínez et al., 2004). RNA concentrations and purity were determined using a Nanodrop spectrophotometer ND-1000 (Nanodrop Technologies™, Wilmington, DE). RNA integrity was determined by electrophoresis in 1% agarose gel. 2-4 µg of total RNA from each sample was linearly amplified using the Low RNA Input Fluorescent Linear Amplification kit (Agilent Technologies™, Ca, USA). 2-3 ug of amplified cRNA was used as template for cDNA synthesis. cDNA was marked indirectly with "SuperScript™ Indirect cDNA Labeling System" (Invitrogen™, San Diego, CA). The fluorophores used were Cy3 and Cy5 mono-reactive Dye (Amersham GE Healthcare™, Amersham UK) and dye incorporation was monitored by a Nanodrop spectrophotometer.

2.3. cDNA hybridization

A mixture of 200 to 300 pmol of the two labeled samples was concentrated in a Concentrator Plus (Eppendorf™, Hamburg, Germany). Competitive hybridization was performed on a Yeast 6.4K Array, PCR-amplified ORFs of yeast S288c strain, (Microarray Centre,

UHN, Toronto, Ontario, Canada) in hybridization chambers AHC (ArrayIt Corporation, CA, USA) at 42ºC overnight. Pre-hybridization solution contained 3X SSC, 0.1% SDS and 0.1 mg/ml BSA; hybridization solution contained 5X SSC, 0.1% SDS and 0.1 mg/ml of salmon DNA. Microarrays were washed manually with different solutions containing different SSC 20X and SDS 10% concentrations (Sol.1: 2X SSC-0.1% SDS; Sol.2: 0.1X SSC-0.1% SDS; Sol.3: 0.1 SSC; Sol4: 0.01X SSC). Signal intensities of Cy3 and Cy5 were acquired with an Axon GenePix 4100A scanner (Molecular Devices, CA, USA) using GenePix Pro v.6.1 software, at a resolution of 10 µm.

2.4. DNA extraction and labelling

DNA extracted (Querol et al., 1992) was resuspended in 50 µl of de-ionized H_2O and digested with *Hin*f I (Roche Applied Science, Germany), according to the manufacturer's instructions, to an average length of 250 bp to 8 kb. The fragmented samples were purified using High Pure PCR Product Purification Kit (Roche Applied Science) and, 2 µg of the fragmented sample was labelled using BioPrime Array CGH Genomic Labelling System (Invitrogen, Carlsbad, CA). Cy5-dCTP and Cy3-dCTP dye-swap experiments were performed to reduce dye-specific bias. Unincorporated label was removed using MinElute PCR Purification Kit (Qiagen, Germany). Equal amounts of labelled DNA were used as probes for microarray hybridization.

2.5. DNA hybridization

Array competitive genomic hybridization (aCGH) was performed using Yeast 6.4K Array, PCR-amplified ORFs of yeast S288c strain, (Microarray Centre, UHN, Toronto, Ontario, Canada). The microarrays were pretreated for one hour at 65ºC with pre-hybridization solution (7.5ml 20X SSC, 0.5ml 10% SDS, 0.5ml 10mg/ml bovine aerum albumin in 50ml final volume). Pre-hybridization solution was washed during 15 seconds in mili-Q H_2O, 2 seconds in isopropanol, 2 seconds in mili-Q H_2O and dried by centrifugation at 1200 rpm/10min. Microarrays were treated with hybridization solution (15µl SSC, 0.6µl 10% SDS, 6µl 1mg/ml salmon DNA and DNA labelled in 60µl final volume) at 95ºC for 1min and at room temperature for 5 min before DNA hybridization. Signal intensities of Cy3 and Cy5 were acquired with an Axon GenePix 4100A scanner (Molecular Devices, CA, USA) using GenePix Pro v.6.1 software, at a resolution of 10 µm.

2.6. Microarray data analysis

Microarray data were derived from two and three independent experiments for DNA and RNA hybridization, respectively. Raw data with a global background subtraction were generated from GenePix pro 6.0. Analyses were done using Acuity 4.0 software (Molecular Devices, CA, USA). The individual data sets were normalized to a $\log_2$ ratio value of 1. After normalization, data were filtered to remove spots flagged as not found. Only spots with at least two replicates were considered. Finally, replicates were

combined and their medians were calculated. Genes with a two-fold log_2 ratio values were considered to be significantly expressed, in RNA hybridization. In the case of DNA hybridization, the log_2 ratio for each ORF was represented onto its corresponding chromosomal position using the sequenced reference strain S288c and caryoscopes were generated using ChARM v.1.1 (Myers et al., 2004). Hybrid strains chromosome gene composition was estimated using the RFLPs data, previously analyzed by González et al. (2008) and caryoscope data. Caryoscopes were carried out by diagramming the log_2 hybridization ratio for each gene mapped onto its corresponding chromosome of the reference strain of *S. cerevisiae*. The use of stringent conditions in the *S. cerevisiae*-based microarray (hybridization at 65ºC) did not allow the hybridization of *S. kudriavzevii* genes present in the hybrids. Differences in the log_2 ratio values observed in the caryoscopes revealed variations in the relative copy number of *S. cerevisiae* genes present in the hybrid strains in comparison with reference strain S288c.

3. Results

Microarray analysis was carried out with RNA extracted from cells harvested at the beginning of stationary phase from wine microfermentations at 12ºC and 28ºC in Tempranillo must. Oenological parameters appear in Table S1 (data extracted from Objective 3, Chapter 1). In the microvinifications at 12ºC, *S. cerevisiae* x *S. kudriavzevii* hybrid VIN7 pointed out in the production of acetaldehyde, acetate esters and ethyl esters whereas *S.*

cerevisiae x *S. kudriavzevii* hybrid Lalvin W27 pointed out in higher alcohols synthesis. Similarly, in the microvinifications at 28ºC, VIN7 also excelled in acetate ester production and Lalvin W27 in higher alcohol synthesis. However, the highest ethyl ester production was achieved by control strain Lalvin T73.

3.1. Global analysis of genes presenting changes in expression

Hybridization of cDNA from the two *Saccharomyces* hybrids was achieved using heterologous hybridization conditions on *S. cerevisiae* microarrays. Gene expression was determined at the beginning of stationary phase in fermentations carried out at 12ºC and 28ºC. Gene expression of the two cryophilic hybrids used in this study, Lalvin W27 and VIN7, was compared with gene expression of reference mesophilic strain, Lalvin T73. Only genes with a fold change in expression greater than 2 (positive or negative) regarding *S. cerevisiae* Lalvin T73 were taken into consideration for further analysis. Figure 1 shows the amount of up and down regulated genes found in each hybrid with respect to Lalvin T73. Comparison of up and down regulated genes between temperatures showed that there was a higher number of up and down regulated genes shared by both hybrids at 12ºC than at 28ºC, suggesting similar behaviour of the hybrids at both temperatures.

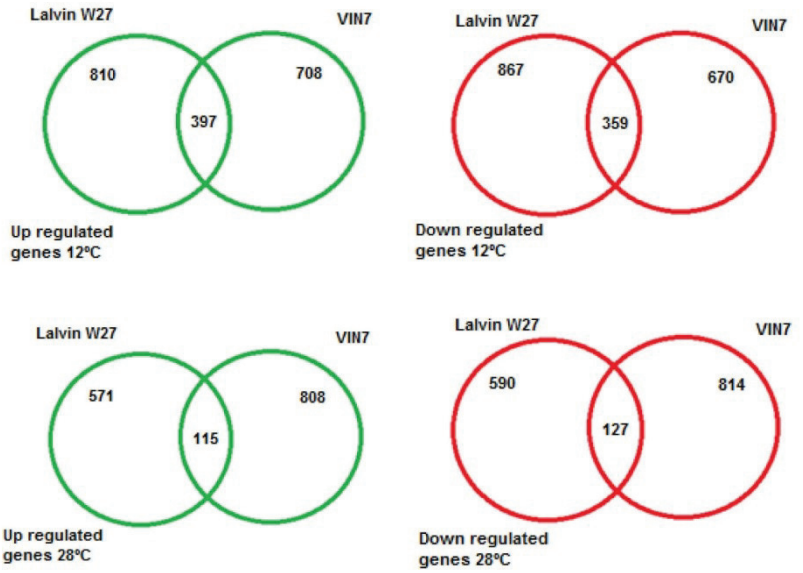

Figure 1. Global genetic expression analysis at 12ºC and 28ºC.

3.2. Analysis of the expression of the genes related to aroma production

Expression level of genes involved in aminoacids, higher alcohols, acetate esters, ethyl esters, ethanol, acetaldehyde, acetate metabolism and primary aroma release (glycosidases and glucanases) appear in Figure 2.

3.2.1. Genes related to aminoacid and higher alcohol metabolism

Higher alcohols are secondary aroma compounds produced by yeasts during the fermentation process. They contribute to wine flowery aromas, being desirable in amounts below 300 mg/l. Production of these compounds occurs throughout transamination

of branched-chain aminoacids (leucine, isoleucine, valine), aromatic amino acids (phenylalanine, tyrosine, tryptophan) and the sulfur-containing amino acid methionine to the corresponding α-ketoacid, followed by decarboxylation to aldehydes. Finally, those aldehydes are reduced to higher alcohols and NADH becomes NAD^+. These reactions are carried out by transaminases, decarboxylases and dehydrogenases.

The relative large higher alcohol production carried out by the hybrid Lalvin W27 (415.9 mg/l, Supplementary material Table S2) at 12ºC could not be explained with transcriptional data, since this strain did not present any gene related to higher alcohol production up regulated except for *ARO1* (aromatic aminoacid metabolism). Up regulation of *ARO1* would indicate a higher production of 2-phenylethanol, which was confirmed by chemical data. On the contrary, down regulation of *ILV2*, *LEU1* and *BAT1* would suggest less isobutanol and isoamyl alcohol amount, which was not confirmed by chemical data.

The relative low amounts of higher alcohols yielded by VIN7 could be attributed to down regulation of most genes related to branched-chain aminoacids biosynthesis (*ILV2*, *ILV5*), aminoacid permeases (*GAP1*) and alcohol dehydrogenases (*ADH1*, *ADH2*, *ADH5*). Down regulation of *ILV2* and *ILV5* confirmed the low isobutanol production of this hybrid with respect to the control strain. On the contrary, up regulation of *LEU2* did not lead to more isoamyl alcohol production. In addition, this strain presented up regulation of aromatic aminoacid metabolism gene *ARO1* which explained that

this hybrid produced higher 2-phenylethanol amount than the control strain.

Both hybrids produced superior amounts of higher alcohols than the control strain at 28ºC (Supplementary material Table S2), which could be explained by up regulation of alcohol dehydrogenase gene *ADH7* and decarboxylase gene *PDC6* in the case of Lalvin W27; and up regulation of leucine biosynthesis gene *LEU1,* alcohol dehydrogenases (*ADH4, ADH6*), decarboxylases (*PDC1*, *PDC6*) and transaminases (*BAT1*) in the case of VIN7.

Nevertheless, VIN7 also presented down regulation of *ARO1* gene confirming the low levels of 2-phenylethanol with respect to the control strain. On the other hand, VIN7 presented more active branched-chain aminoacids metabolism than the control strain (up regulation of *LEU1* and *BAT1*) showing a trend to produce higher amounts of isobutanol and isoamyl alcohol, which was confirmed by chemical data. Similarly, Lalvin W27 presented higher isobutanol and isoamyl alcohol amounts than the control strain, what could not be explained by gene expression.

3.2.2. Genes related to acetate ester production

Acetate esters are another group of aromatic compounds which constitutes secondary wine aroma. Acetate esters contribute to desirable fruity and floral wine aromas. They are synthesized by a condensation between higher alcohols and acetyl-CoA mediated by acetyltransferases.

Gene expression showed no correspondence between chromatographic data and transcriptome at 12ºC. The highest acetate ester production yielded by VIN7 (Supplementary material Table S2) was not possible to explain by transcriptional data, since there were no differences in the expression of the genes related to the production of these aromatic compounds between this strain and the control strain. Besides, up regulation of acetyltransferase gene *AYT1* in Lalvin W27 was also not in accordance with gas chromatopraphy data, since this strain presented slightly lower acetate ester amounts than the control strain.

Conversely, there was correspondence between chromatographic and transcriptional data at 28ºC. Both hybrid strains showed higher acetate ester production than the control strain (Supplementary material Table 2S), which can be explained by up regulation of acetyltransferase gene *ATF2*. Furthermore, Lalvin W27 presented down regulation in esterase gene *IAH1*, also contributing to reach high acetate ester levels.

3.2.3. Genes related to ethyl ester production

Ethyl esters are other of the main chemical compounds which constituted secondary aroma in wines. Likewise acetate esters, ethyl esters contribute to desirable fruity and flowery wine aroma. Ethyl esters are synthesized by condensation between ethanol and acyl-CoA, reaction mediated by acyltransferases.

There is no correspondence between chromatographic and transcriptional data at 12ºC. Both hybrid strains presented higher ethyl ester production than the control strain (Supplementary material Table 2S) what is not in accordance with gene expression data.

Conversely, at 28ºC, down regulation of acyltransferase gene *EEB1* in both hybrids could explain the lower ethyl ester amount compared with the control strain (Supplementary material Table S2). Nevertheless, both hybrids also presented up regulation of acyltransferase gene *EHT1*.

3.2.4. Genes related to ethanol, acetaldehyde and acetate metabolism

Ethanol, acetate and acetaldehyde levels are oenological parameters defining wine quality. Ethanol, a product of alcoholic fermentation made during wine production, can be oxidized to acetaldehyde by alcohol dehydrogenases. Acetaldehyde can be oxidized to acetate by aldehyde dehydrogenases. Acetate can react with CoA to form acetyl-CoA. Excessive acetaldehyde and acetate levels (>100 mg/l and >0.7-1.1 g/l, respectively) (Swiegers et al., 2005) are considered negative in wine. Besides, the current trend in oenology is to achieve wines with less ethanol content due to its negative health effects.

The significant high acetaldehyde levels reached by VIN7 at 12ºC (Supplementary material Table S1) can be explained by down

regulation of alcohol dehydrogenase genes *ADH1* and *ADH5* and aldehyde dehydrogenases genes *ALD3* and *ALD4*. In addition, VIN7 showed up regulation of decarboxylase genes *PDC2*, *PDC6*. Down regulation of aldehyde dehydrogenase genes also can explain that VIN7 showed lower acetic acid levels than the control strain (Supplementary material Table S1). On the contrary, down regulation of alcohol dehydrogenase genes did not explain that VIN7 yielded higher ethanol amount than the control strain. In addition, down regulation of *PDA1* lead to less acetyl-CoA available for acetate ester synthesis. In the case of Lalvin W27, down regulation of alcohol dehydrogenase genes *ADH1*, *ADH2* and *ADH5*, aldehyde dehydrogenase genes *ALD3* and *ALD4* and up regulation of decarboxylase genes *PDC2* and *PDC6* would lead to higher acetaldehyde levels than the control strain, but this seems to be compensated by down regulation in *PDC1*.

Down regulation in aldehyde dehydrogenase genes *ALD3* and *ALD4* by Lalvin W27 at 12ºC leads to lower acetic acid levels than the control strain, which is confirmed by chemical data (Supplementary material Table S1). Finally, down regulation of alcohol dehydrogenase genes would explain lower ethanol production by Lalvin W27 than by the control strain.

The significant high acetaldehyde levels yielded by hybrid VIN7 at 28ºC (Supplementary material Table S1) can be explained by up regulation of decarboxylase gene *PDC1* and down regulation of alcohol dehydrogenase genes *ADH1*, *ADH5* and aldehyde dehydrogenase genes *ALD3*, *ALD6*. On the other hand, up regulation

of alcohol dehydrogenase gene *ADH4* or down regulation of decarboxylase gene *PDC2* seemed to have less importance in acetaldehyde production. A combination between up and down regulation of alcohol dehydrogenases would lead to ethanol levels modulation, which explained the similarity between VIN7 and the control strain in the production of this compound (Supplementary material Table S1). Down regulation of aldehyde dehydrogenase genes *ALD3* and *ALD6* would lead to lower acetic acid levels than the control strain, which was not in accordance with our data (Supplementary material Table S1).

In the case of Lalvin W27 at 28ºC, down regulation of alcohol dehydrogenase genes *ADH1*, *ADH2*, *ADH5* and aldehyde dehydrogenase genes *ALD3*, *ALD6* and up regulation of decarboxylase gene *PDC6*, would not compensate for the down regulation of decarboxylases genes *PDC1* and *PDC5*, explaining the higher acetaldehyde levels produced by Lalvin W27 with respect to the reference strain. Down regulation of the alcohol and aldehyde dehydrogenase genes would lead to lower ethanol and acetic acid amounts produced by Lalvin W27 when comparing with the reference strain, which was not in accordance with chemical data (Supplementary material Table S1). Nevertheless, Lalvin W27 showed *ACS2* gene up regulated, which lead to acetic acid increase and acetyl-CoA decrease. Lalvin W27 also showed down regulation of other genes leading to acetyl-CoA decrease, *PDA1* and *LAT1*.

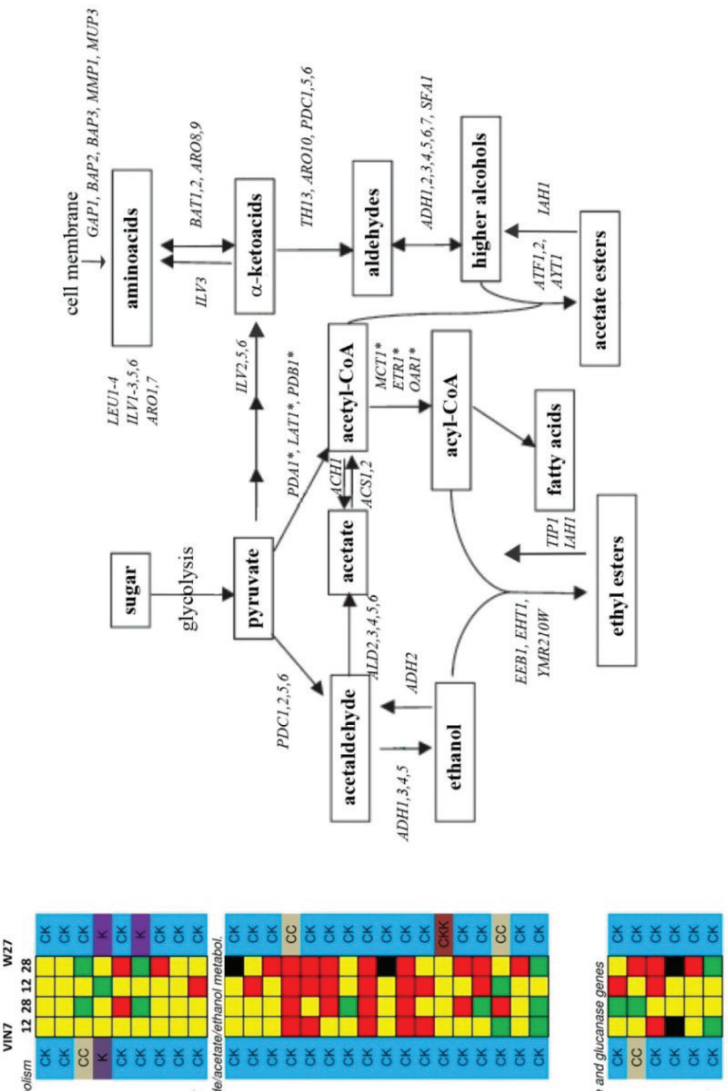

Figure 2. Expression of the genes involved in secondary aroma formation and other metabolites in yeasts. Red, down regulated; green, up regulated; yellow, no expression change; black, no hybridization; *mitochondrial genes.

3.2.5. Genes related to glycosidase and glucanase activities

Yeasts glycosidases, such β-D-glucosidases and α-L-arabinofuranosidades, are important to wine aroma since they are involved in breaking glycosidic linkage between aromatic compounds and sugars. The aromatic compounds released become volatile increasing wine primary aroma. Glucanases accelerate yeast autolysis and mannoproteins release at the end of fermentation (van Rensburg & Pretorius, 2000). The protein component of the mannoproteins is important for overall aroma stabilization, improving aroma intensity (Lubbers et al., 1994).

Both hybrids showed down regulation of *EXG1* (exoglucanase) at 12ºC. In addition, Lalvin W27 presented down regulation of *ABF2* (α-L-arabinofuranosidase). However, VIN7 presented up regulation of ORF *YIR007W* (glycosidase).

On the other hand, at 28ºC, Lalvin W27 presented ORF *YIR007W* up regulated, but also down regulation of *BGL2* (β-D-glucosidase), *EXG1* (exoglucanase) and *SPR1* (glycosidase). On the contrary, VIN7 showed up regulation in *ABF2* (α-L-arabinofuranosidase) and *BGL2* (β-D-glucosidase), pointing to more effectiveness in primary aroma release and stabilization.

3.3. Hybrid genomes

Relative gene copy number in the hybrid strains Lalvin W27 and VIN7 was determined by comparative genome hybridization

with respect to the *S. cerevisiae* homoploid strain S288C, whose complete genome sequence is known (Goffeau et al., 1996). Nucleotide divergences among the genomes of the parental species *S. cerevisiae* and *S. kudriavzevii* are on average ~30%. Due to the DNA hybridization stringency conditions used (<10% nucleotide divergence), most genes from the *S. kudriavzevii* genome fraction of the hybrids did not hybridize to the *S. cerevisiae* microarray, and hence, the hybridization differences correspond mainly to the hybrid genome fraction coming from the *S. cerevisiae* parental.

Genome composition of the hybrids used in this study, VIN7 and Lalvin W27 (Figure 2) showed that most of the genes related to aroma production are composed by one allele from each parental species (CK) although with some exceptions. The first exception consisted of genes in which the two alleles belong to *S. cerevisiae* species (CC). In the case of VIN7, several genes presenting two *S. cerevisiae* alleles are related to aminoacids and higher alcohol metabolism (*ILV6, LEU2, ADH6, ADH7*), acetate ester synthesis gene *ATF2* and glycosidase gene *BGL2*. In the case of Lalvin W27 the genes presenting two *S. cerevisiae* alleles are related to aminoacids and higher alcohol metabolism (*ARO1, BAP3, ADH1, ADH6, ARO10, THI3*) and *PDC2* gene involved in acetaldehyde production. It is remarkable that only *ADH6* presented the same modification in both hybrids. The second exception consisted of genes presenting three alleles, one from *S. cerevisiae* and two from *S. kudriavzevii* (CKK). This allele pattern could only be found in Lalvin W27 in one gene related to aminoacids metabolism (*ILV1*), one gene involved in acetate

formation from acetaldehyde (*ALD5*) and one gene related to acetyl-CoA synthesis (*PDA1*). The third exception consisted of genes presenting three alleles, two from *S. cerevisiae* and the other from *S. kudriavzevii* (CCK). This allele pattern could only be found in Lalvin W27 in one gene related to aminoacids metabolism (*LEU4*). Finally, only one allele of *S. kudriavzevii* (K) was observed in acetyltransferase gene *AYT1* in both hybrids and acyltransferase gene *EHT1* in the case of the hybrid Lalvin W27.

4. Discussion

Hybridization process in yeasts has been proposed as an adaptation mechanism to ferment at low temperatures (de Barros Lopes et al., 2002; Barrio et al., 2006; Sipiczki, 2008). Natural hybrids between *Saccharomyces cerevisiae* and *Saccharomyces kudriavzevii* have been isolated in wine fermentations at low temperature and genetically characterized (Belloch et al., 2009; González et al., 2006, 2008). In addition, oenological characterization of *S. cerevisiae* x *S. kudriavzevii* hybrids showed that these hybrids seems to have inherited advantageous traits from their parental species, such as efficiency to ferment at low and intermediate temperatures, producing intermediate or high amounts of glycerol, low acetic acid quantity and high amount of higher alcohols (Gangl et al., 2009; González et al., 2007).

In this study, low temperature fermentations were carried out by two genetically different *S. cerevisiae* x *S. kudriavzevii* hybrids,

Lalvin W27 and VIN7. These strains were selected due to the remarkable aroma production during wine microfermentations in Tempranillo must at 12ºC and 28ºC and their adaptation to ferment good at low temperatures. On the other hand, mesophilic Lalvin T.73 was used as reference. In addition to chemical determination of aroma profile of the wines yielded at 12ºC and 28ºC by the different hybrids, expression of the genes related to aroma production was determined using DNA microarrays. Gene expression was analysed at the beginning of the stationary phase, since the most active period of aroma compound accumulation appears to be in the earlier stages of fermentation (Bisson & Karpel, 2010; Rossouw et al., 2008). The genes involved in the synthesis of the main flavour metabolites were investigated at genomic and transcriptomic level to elucidate allele composition and differences in expression. A correlation between chemical data, genomic data and transcriptomic data was carried out.

The hybrids used in this study showed different allele composition in several genes involved in aroma production, what might lead to different levels of expression and different oenological properties and aromatic profile in the resulting wines.

Lalvin W27 excelled in higher alcohol production at both temperatures, whereas VIN7 was the best acetate ester producer at both temperatures, yielding the higher ethyl ester amount at 12ºC. Lalvin W27 produced excessive higher alcohol amounts, especially at 12ºC (around 381.8 mg/l at 28ºC and 415.9 mg/l at 12ºC). In wine, higher alcohol levels above 400 mg/l are undesirable according to

Rapp & Versini, 1991 as they can hide fruity aroma given by esters. Higher alcohol production by Lalvin W27 makes this strain an optimum candidate to hybridizate with other strains with high expression in acetyltransferases, in order to increase acetate ester levels and wine fruitiness.

The different higher alcohols are synthesized from different aminoacids, such as valine, leucine or phenylalanine. Genes *ILV1-6*, *LEU1-4* and *ARO1,7*, involved in valine, leucine and phenylalanine synthesis, are precursors of isobutanol, isoamyl alcohol and 2-phenylethanol, respectively. *BAT1* and *BAT2* genes codify the transaminases involved in branched-chain aminoacid metabolism (valine, leucine and isoleucine), whereas *ARO8* and *ARO9* genes codify the transaminases involved in aromatic aminoacids (phenylalanine) and methionine metabolism (Dickinson & Norte, 1993; Eden et al., 2001; Hazelwood et al., 2008; Lilly et al., 2006; Ugliano & Henschke, 2009). In this study, correspondence between chemical data and transcriptome was not possible in all cases. However, the levels of certain higher alcohols could be explained taking into account the expression of the genes involved in the synthesis of the aminoacid precursor or the specific transaminases related to the metabolism of those aminoacids.

In the case of ethyl ester production at 12ºC, gene expression (acyltransferase genes *EEB1*, *EHT1*, ORF *YMR210W* and esterase genes *IAH1*, *TIP1*) did not explain the relative high levels of these compounds detected in wine, as occurred in other compounds at any of the temperatures tested.

The lack of correspondence between chemical data and transcriptome might be due to the complexity of aroma formation comprising several interlinked pathways in which not all genes involved in a determinate pathway have the same importance. For instance, acyltransferase *EEB1* is thought to play a more important role in ethyl ester formation than acyltransferases *EHT1* or *YMR210W* (Rossouw et al., 2008; Saerens et al., 2006), in agreement with our chemical data. In addition, acetyltransferase *AYT1* (Alexander et al., 2002) seems not to be involved in acetate esters synthesis, also in accordance to our results.

Regarding acetate esters, it is difficult to correlate acetyltransferases (*ATF1*, *ATF2*, *AYT1*) expression with chemical data. Similar results were found by Beltrán et al. (2006) in wine fermentations carried out at 13ºC and 25ºC. These authors found that the high levels of acetate esters at 13ºC were not accompanied by up regulation of acetyltransferase genes. Furthermore, in a work correlating transcriptome with chemical data in different cryophilic *Saccharomyces* species (Objective 4, Chapter 1) similar results were observed. Substrate (higher alcohols) regulation might be a possible explanation for the lack of correlation between gene expression and acetate ester levels; however, this was not confirmed in this study. VIN7 hybrid was the main acetate ester producer at both temperatures, but not the main higher alcohol producer. No correlation between analytical data and transcriptome could be explained by temperature regulation. Different expression patterns depending on temperature were observed not only in the case of acetate esters but also in the other aromatic compounds analysed

in this study. Regulation by temperature might be acting at transcriptomic level or at post-translational level, affecting the enzyme kinetics. Further studies are needed to understand gene regulation in the case of hybrids due to differential expression of the alleles from one or other parental depending on the temperature. In the genomic analysis of the hybrids, one or two alleles from *S. kudriavzevii* constituted several genes. Presence of those alleles might affect gene expression in some cases since differences with respect to the control strain could be observed. Furthermore, alleles coming from *S. kudriavzevii* present in important flavour related genes such as *ARO1*, *ATF2*, *EEB1* and *BAT1* might be differently expressed depending on the fermentation temperature.

Aromatic compounds can also appear in wine must as odourless glycosilated conjugates (Baumes, 2009; Loscos et al., 2007; van Rensburg & Pretorius, 2000; Ugliano & Henschke, 2009). The breakage of this bond leads to the release of the aromatic compound, which becomes volatile and aromatic. This action can be carried out by yeast glycosidases during fermentation process (Hernández-Orte et al., 2008; Loscos et al., 2009; Ugliano et al., 2006; Zoecklein et al., 1997). In this background, the up regulation of some glycosidases presented by VIN7 was remarkable, indicating potential to improve wine primary aroma. It is also interesting to note that the gene codifying the recently described β-glucosidase codified by the ORF *YIR007W* (de Smidt et al., 2011) appeared up regulated in this hybrid at 12ºC, but without changes in expression at 28ºC, indicating a possible regulation by temperature at transcriptional level. On the

contrary, β-glucosidase codified by *YIR007W* appeared up regulated in Lalvin W27 at 28ºC.

Finally, correlation of allele composition of genes and transcriptome with chemical data in the genes presenting different allele patterns leads to the following observations:

a) In several cases, the allele/s from *S. kudriavzevii* does/do not seem to affect gene expression at any temperature because of the lack of changes in gene expression with respect to the reference strain. Examples: *ILV1*, *LEU4*, *BAP3*, *ALD5*.

b) In other cases, the expression of the allele/s from *S. kudriavzevii* seems to depend on the temperature. Examples: down regulation of *ARO1* in VIN7 at 28ºC (less 2-phenylethanol than Lalvin W27), up regulation of *ADH7* in Lalvin W27 at 28ºC (higher ethanol amount at this temperature than at 12ºC), up regulation of *EHT1* in both hybrids at 28ºC (higher ethyl esters amount at 28ºC than at 12ºC, only confirmed in the case of Lalvin W27) and down regulation of *BGL2* in Lalvin W27 at 28ºC.

In conclusion, the study at molecular level of aroma production carried out by yeasts is of undeniable complexity. Several interconnected pathways with large number of genes and enzymes are involved. We did not find correlation between genome, transcriptome and chemical data in all the cases, and further study is necessary to understand the regulation of aroma production pathways taking into account fermentation temperature, which

seems to be of crucial importance in modulating aroma production by yeasts.

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Supplementary material

Table S1. ANOVA analysis of oenological parameters of the fermentations at 12ºC and 28ºC.

	Control	Laivin W27	VIN7
Species	*S.cerevisiae*	*S.c x S.k*	*S.c x S.k*
Source	Wine (Spain)	Wine (Switzerland)	Wine (South Africa)
12ºC			
days	21	14	23
Ethanol (%)	12.51 ± 1.44^{a}	11.73 ± 0.21^{a}	14.71 ± 0.10^{a}
Glycerol (g/l)	4.58 ± 1.25^{a}	5.05 ± 1.18^{a}	5.48 ± 0.39^{a}
Acetic acid (g/l)	0.14 ± 0.01^{a}	0.08 ± 0.01^{a}	0.09 ± 0.03^{a}
Acetaldehyde (mg/l)	6.98 ± 0.83^{a}	5.38 ± 4.74^{a}	23.15 ± 1.48^{b}
28ºC			
days	6	5	6
Ethanol (%)	13.02 ± 0.57^{a}	13.43 ± 1.32^{a}	13.89 ± 0.37^{a}
Glycerol (g/l)	6.47 ± 0.54^{a}	6.30 ± 0.19^{a}	6.52 ± 0.04^{a}
Acetic acid (g/l)	0.18 ± 0.03^{a}	0.27 ± 0.03^{a}	0.23 ± 0.02^{a}
Acetaldehyde (mg/l)	4.35 ± 0.41^{a}	5.23 ± 0.29^{a}	24.20 ± 7.07^{b}

Table S2. ANOVA analysis of aroma profile of the different fermentations at 12ºC and 28ºC.

	12ºC			28ºC		
	Control	**Lalvin W27**	**VIN7**	**Control**	**Lalvin W27**	**VIN7**
Higher alcohols						
Isobutanol	33.20 ± 1.40^{a}	59.36 ± 3.59^{b}	30.74 ± 1.01^{a}	29.09 ± 2.52^{a}	55.90 ± 5.80^{b}	67.17 ± 1.83^{b}
Isoamyl alcohol	237.5 ± 24.9^{a}	300.1 ± 31.61^{a}	176.8 ± 11.0^{a}	189.7 ± 9.5^{a}	247.9 ± 0.43^{a}	240.1 ± 18.6^{a}
Benzyl alcohol	nd	nd	nd	nd	nd	nd
2-Phenylethanol	33.93 ± 0.86^{a}	51.85 ± 1.44^{b}	42.77 ± 2.32^{ab}	91.13 ± 0.41^{c}	75.72 ± 6.52^{ab}	55.68 ± 0.57^{a}
1-Hexanol	3.60 ± 0.09^{a}	4.62 ± 0.18^{c}	2.67 ± 0.03^{a}	2.82 ± 0.18^{a}	2.26 ± 0.13^{a}	2.36 ± 0.20^{a}
Total	308.2	415.9	253.0	312.7	381.8	365.31
Acetate esters						
Ethyl acetate	56.48 ± 2.75^{a}	51.43 ± 2.57^{a}	100.6 ± 10.8^{b}	72.39 ± 5.75^{a}	77.85 ± 0.00^{a}	108.7 ± 2.4^{b}
Isobutyl acetate	nd^{a}	0.088 ± 0.000^{b}	0.138 ± 0.010^{c}	0.130 ± 0.018^{a}	0.212 ± 0.002^{a}	0.177 ± 0.029^{a}
Isoamyl acetate	1.834 ± 0.090^{b}	0.629 ± 0.035^{a}	4.729 ± 0.306^{c}	3.89 ± 0.70^{a}	3.67 ± 0.49^{a}	1.83 ± 1.34^{a}
Hexyl acetate	0.085 ± 0.002^{c}	0.034 ± 0.002^{a}	0.096 ± 0.009^{b}	0.07 ± 0.00^{a}	0.069 ± 0.005^{a}	0.042 ± 0.010^{a}
Phenylethyl acetate	0.106 ± 0.007^{a}	0.136 ± 0.007^{a}	0.486 ± 0.016^{b}	1.269 ± 0.116^{b}	0.789 ± 0.064^{a}	0.361 ± 0.009^{a}
Benzyl acetate	nd	nd	nd	nd	nd	nd
Total	58.51	52.32	106.05	77.75	82.59	111.11
Ethyl esters						
Ethyl caproate	0.004 ± 0.000^{a}	0.002 ± 0.000^{a}	0.007 ± 0.001^{a}	0.006 ± 0.000^{a}	0.003 ± 0.000^{aa}	0.001 ± 0.000^{a}
Ethyl caprylate	0.308 ± 0.053^{a}	0.468 ± 0.066^{a}	1.489 ± 0.026^{b}	1.59 ± 0.11^{a}	0.639 ± 0.060^{a}	0.249 ± 0.072^{a}
Ethyl caprate	0.041 ± 0.007^{a}	0.093 ± 0.014^{a}	0.077 ± 0.009^{a}	0.20 ± 0.05^{a}	0.135 ± 0.006^{a}	0.095 ± 0.001^{a}
Ethyl lactate	nd	nd	nd	nd	nd	nd
Total	0.353	0.563	1.573	1.796	0.777	0.345

SUMMARIZED RESULTS

The results obtained in this doctoral thesis indicate large differences regarding aroma production among the different *Saccharomyces* species and hybrids.

S. cerevisiae, in spite of showing low β-D-glucosidase activity with respect to non-*Saccharomyces* strains, this activity was the highest among its genus. This fact was in accordance with the higher α-terpineol levels detected in Moscatel wines fermented by this species at 16ºC and the remarkable release of glycosilated terpenes in the fermentations carried out with aromatic precursor addition, when comparing with the other *Saccharomyces* species and hybrids. In accordance with the greater potential in releasing varietal aromas presented by *S. cerevisiae*, gene expression data at 12ºC showed up regulation of glycosidase gene *YIR007W* in the case of *S. cerevisiae* Fermol Cryophile. On the contrary, volatile thiols release seems to be favoured according to gene expression in *S. cerevisiae* Fermol Cryophile at 28ºC (up regulation of *IRC7*). Furthermore, *S. cerevisiae* species also presented *de novo* synthesis of lipid derivatives and terpenes and high percentages of geraniol bioconversion into other terpenes. On the other hand, *S. cerevisiae* species presented a relatively low geraniol retention inside the cell, which could lead to low sterol production and consequently, low cold adaptation. *S. cerevisiae* ferments very well at 28ºC, being remarkable in the production of all the groups constituting secondary aroma, higher alcohols, acetate esters and ethyl esters. The higher levels of higher alcohols yielded by *S. cerevisiae* are in accordance with the up regulation presented by *S. cerevisiae* Fermol Cryophile at 28ºC in genes related to aminoacids synthesis,

such as certain alcohol dehydrogenases, branched-chain aminoacid aminotransferases and one piruvate decarboxylase. *S. cerevisiae* Fermol Cryophile also showed up regulation in *ETR1*, gene involved in acyl-CoA formation, which could explain the high ethyl ester amounts synthesized at 28ºC. Nevertheless, down regulation in acetyltransferase gene *AYT1* of *S. cerevisiae* Fermol Cryophile would not explain the higher acetate ester production yielded by this species at 28ºC. However, the acetyltransferase codified by *AYT1* has a minor role in the synthesis of these aromatic compounds and it must be taken into account that gene expression data is from one strain solely. Finally, lowering fermentation temperature slightly affects aromatic profile of *S. cerevisiae*, since the wines produced by this species at 16ºC excelled only due to their ethyl ester levels, being the fruitiest.

Conversely, *S. uvarum*, the second most important species involved in wine fermentations, showed a very different behaviour. In spite of not being remarkable in β-D-glucosidase activity, *S. uvarum* showed greater release of varietal aromas than *S. cerevisiae* and also the highest levels of geraniol in Moscatel wines fermented at 16ºC. In accordance with the greater potential in releasing varietal aromas presented by *S. uvarum*, gene expression data at 12ºC showed up regulation of glycosidase gene *YIR007W* in the case of *S. uvarum* CECT 12600. On the contrary, volatile thiols release seems to be favoured according to gene expression in *S. cerevisiae* Fermol Cryophile at 28ºC. Differently to *S. cerevisiae*, terpene bioconversion was low, whereas, as in the case of *S. cerevisiae*, volatile thiols release seems to be favoured according to gene expression in *S.*

uvarum CECT 12600 at 28ºC (up regulation of *GLO1*). Nevertheless, *S. uvarum* was the most outstanding species at *de novo* synthesis (lipid derivatives, shikimic derivatives, nor-isoprenoids and terpenes).. On the other hand, *S. uvarum* presented high percentages of geraniol uptake. Maybe geraniol taken is used to sterol production, thus increasing cold resistance. This can be the explanation of the fact that secondary aroma profile of this species only was remarkable at low temperatures. *S. uvarum* pointed out in higher alcohols and acetate ester synthesis at 12ºC and 16ºC, especially in the production of 2-phenylethanol and its corresponding acetate as already seen by several authors (Antonelli et al., 1999; Di Stefano et al., 1981; Masneuf et al., 1998; Tosi et al., 2009). Up regulation of *ATF2* at 12ºC in the case *S. bayanus* CECT 12600 could explain the higher levels of acetate esters reached by this species. *S. bayanus* also showed up regulation in esterase TIP1, although this esterase only has been related to ethyl esters (Horsted et al., 1998). On the other hand, the high levels of higher alcohols yielded by this species are not so clearly explained observing gene expression data. Finally, sensory analysis of the wines produced by this species at 16ºC was favourable, since they revealed low fusel and sulfurous notes.

S. kudriavzevii has not been isolated from wine fermentations, but its origin is decayed leaves. However, this species did not find any trouble to reached dryness (< 4 g/l sugars) in all the fermentations carried out in this study (12ºC, 16ºC and 28ºC). Nevertheless, *S. kudriavzevii* showed a very limited potential to improve wine aroma, only pointing out in ethyl esters synthesis at

16ºC. As *S. uvarum*, presented low terpene bioconversion and high geraniol uptake. The latter maybe related to its cryotolerance.

Saccharomyces hybrids showed different behaviour depending on the species which hybridizate with *S. cerevisiae*. Hybrid between *S. cerevisiae* and *S. uvarum* presented outstanding *de novo* synthesis, whereas hybrids between *S. cerevisiae* and *S. kudriavzevii* and triple hybrid (*S. cerevisiae* x *S. uvarum* x S. *kudriavzevii*) were remarkable at releasing varietal aromas, especially triple hybrid, which excelled in the release of almost every group of primary aroma compounds. Nevertheless, gene expression data showed up regulation of some genes codifying glycosidases in *S. cerevisiae* x *S. kudriavzevii* hybrids Lalvin W27 and VIN7 at both temperatures studied (12ºC and 28ºC). As their parental species *S. cerevisiae*, all the hybrids presented high levels of α-terpineol in Moscatel wines fermented at 16ºC. Curiously, hybrids performed high geraniol uptake, as *S. uvarum* and *S. kudriavzevii*, and also high terpene bioconversion as *S. cerevisiae*. This combined behaviour could be the explanation for hybrids cryotolerance and terpene profile (high levels of α-terpineol). Regarding secondary aromas, hybrids pointed out mostly at 28ºC as their parental species *S. cerevisiae*. *S. cerevisiae* x *S. uvarum* hybrid excelled in acetate ester formation, whereas *S. cerevisiae* x *S. kudriavzevii* hybrids and triple hybrid excelled in higher alcohol synthesis. Gene expression data of *S. cerevisiae* x *S. kudriavzevii* hybrids Lalvin W27 and VIN7 at 28ºC does not clearly explain the higher yield of higher alcohols appearing in wines fermented by *S. cerevisiae* x *S. kudriavzevii* hybrids. In fermentations carried out at 16ºC with aromatic precursor

addition, triple hybrid also excelled in higher alcohol formation, which explain the high fusel notes detected in sensory analysis. This sensory analysis also revealed the wine produced by triple hybrid had high sulfurous notes. Both traits probably masked fruity nuances.

GENERAL DISCUSSION

Winemaking is an ancient process profusely studied through the years. *Saccharomyces cerevisiae* is the main species involved in alcoholic fermentation. Nevertheless, the role of *Saccharomyces uvarum* (*Saccharomyces bayanus var. uvarum*) and *Saccharomyces* hybrids between *S. cerevisiae*, *S. uvarum* and *S. kudriavzevii* have been recently described in winemaking (Demuyter et al., 2004; González et al., 2006; Masneuf et al., 1998; Massoutier et al., 1998; Naumov et al., 2000, 2001; Nguyen et al., 2000; Sipiczki, 2002, 2008). Hybrids seem to have inherited favorable traits from their parental species, such as a broad range of fermentation temperatures, high glycerol production, low synthesis of acetic acid and high levels of higher alcohols (Gangl et al., 2009; González et al., 2007). Moreover, cryotolerance of *Saccharomyces* hybrids makes them suitable to the new winemakers' tendencies, such as conducting wine fermentation at low temperatures (10-15ºC), that in the case of *S. cerevisiae* strains has been observed leads to a greater retention of varietal aromas, and an increase in the proportion of esters (Feuillat, 1997; Novo et al., 2003; Torija et al., 2003).

The aim of this Doctoral Thesis has been to analyse the potential of different *Saccharomyces* species and hybrids to produce wines at low temperature presenting desirable aromatic profiles.

This main objective has been fulfilled carrying out fermentations of several natural and artificial musts at different conditions and, using different chemical and molecular approaches,

determining the release, *de novo* synthesis and biotransformation of primary aromas and production of secondary aromas. Finally, correlation between aroma production and gene expression has been studied by microarray technology.

Primary aromas

Primary or "varietal aroma" is attributed to aromatic compounds whose origin is grapes. In case of "aromatic grapes" the elevated concentration of flavour-active precursor compounds practically ensures the presence of primary aromas in wines, whereas in case of "neutral grapes" the contribution of yeast to the release and *de novo* production of primary aromas has significant importance.

De novo synthesis of monoterpenes, lactones, shikimic derivatives and nor-isoprenoids carried out by *S. cerevisiae* have been already documented (Carrau et al., 2005; Bode and Birnbaum, 1981; Endrizzi et al., 1996; Hernández-Orte et al., 2008; Loscos et al., 2007). One of the most important findings of our study is the outstanding *de novo* formation of several primary aroma compounds at 16ºC by some of the *Saccharomyces* species and hybrids when comparing with *S. cerevisiae* production at the same or higher temperature (Hernández-Orte et al., 2008). Furthermore, the *Saccharomyces* species and hybrids were able to release higher amounts of several varietal aromas of different chemical groups from media supplemented with aroma precursors when compared with *S.*

cerevisiae at the same or higher temperature (Hernández-Orte et al., 2008; Loscos et al., 2007).

Release of varietal aromas is mediated by enzymatic activity of glycosidases. Several studies have shown that *S. cerevisiae* is not considered a good producer of β-D-glucosidases (Fia et al. 2005; Mateo and Di Stefano, 1997) and, similarly, all *Saccharomyces* species or hybrids in our study showed lower glucosidase activity than the *S. cerevisiae* control strain utilized. Moreover, correspondence between β-D-glucosidase activity and wine terpene profile was not found in the *Saccharomyces* species and hybrids tested. Different specificity in the β-D-glucosidases of *Saccharomyces* species and hybrids would account for some of the differences in the terpene profiles, although we found no studies supporting this hypothesis. Another explanation to the no correspondence between β-D-glucosidase activity and wine terpene profile might be the bioconversion of released primary aromas in other compounds by yeast metabolism (King and Dickinson, 2000; Zea et al., 1995). In our study three patterns of uptake and bioconversion of geraniol into linalool and α-terpineol were observed. The most interesting behavior was found in the case of several hybrid strains, since they showed high geraniol uptake, which could be related to sterol formation and cryotolerance increase (Zea et al., 1995), and high geraniol bioconversion percentage into other terpenes, giving higher aromatic complexity to wine.

Secondary aromas

Secondary or "fermentative aroma" comprises the aromatic compounds synthesized by yeasts during fermentation. Fermentation temperature has been demonstrated to highly affect yeast kinetics and wine quality. Several authors affirm that lowering fermentation temperature leads to a decrease in higher alcohol amounts and an increase in the proportion of ethyl and acetate esters in *S. cerevisiae* (Feuillat, 1997; Llauradó et al., 2002, 2005; Novo et al., 2003; Torija et al., 2003). In our study, differences in the production of higher alcohols and ethyl and acetate esters were not only dependent on the fermentation temperature but also on the yeast strain. Several *Saccharomyces* species and hybrids performed opposite to *S. cerevisiae* at low temperature. Higher alcohol increase at low fermentation temperature was observed in the case of *S. bayanus, S. kudriavzevii,* and some *S. cerevisiae* x *S. bayanus* and *S. cerevisiae* x *S. kudriavzevii* hybrids. Acetate esters decrease was observed in some *S. cerevisiae* and most of *S. cerevisiae* x *S. kudriavzevii* hybrid strains. Finally, ethyl esters decrease was observed in *S. cerevisiae*, *S. uvarum* and some *S. cerevisiae* x *S. kudriavzevii* hybrid strains. The differences found between our study and previous studies could be due to the use of cryotolerant *S. cerevisiae* strains and other cryotolerant *Saccharomyces* species and hybrids instead of non cryotolerant *S. cerevisiae* strains used by other authors. These differences would point to a particular fermentative metabolism and, hence, distinctive aroma production at low temperatures in case of cryotolerant *Saccharomyces* yeasts.

A comprehensive analysis of differences in the fermentative metabolism of *S. cerevisiae* and related cryotolerant *Saccharomyces* species and hybrids was carried out by DNA arrays. This technique allowed monitoring changes of gene level expression of whole genomes in a single experiment (Lashkari et al., 1997).

Comparison of gene expression among *S. cerevisiae*, *S. uvarum* and *S. kudriavzevii* and the hybrids between them showed that differences in higher alcohol levels could be explained by changes in gene expression at low (12ºC) and moderate (28ºC) fermentation temperatures. In general, changes in expression levels of *ADH1-5* genes involved in higher alcohol synthesis (Dickinson et al., 2003) does not agree with differences in higher alcohol concentration found among *S. cerevisiae*, *S. uvarum, S. kudriavzevii* and the hybrids at different temperatures. However, the concentration levels of several higher alcohols such as isoamyl alcohol, 2-phenylethanol and isobutanol could be explained taking into account the expression of genes involved in the synthesis of aminoacid precursors (*ILV1-6*, *LEU1-4* and *ARO1,7*) or particular transaminases related to the metabolism of those aminoacids (*BAT1,2* and *ARO8,9*) (Bisson et al., 2010).

Among fermentation metabolites, it is generally accepted that esters make the greatest contribution to the characteristic fruity odours of wine fermentation bouquet (Rapp and Mandery, 1986). Correlation between expression levels of genes involved in ethyl and acetate esters and the concentrations of these compounds chemically detected was not achieved. In the case of acetate

esters, the expression levels of acetyltransferases (*ATF1* and *ATF2*) were not in accordance with differences found in the concentrations of these compounds produced by the *Saccharomyces* species and hybrids at any temperature, as already observed in case of *S. cerevisiae* (Beltran et al., 2006). Neither the expression levels of acyltransferase genes *EEB1* (Rossouw et al., 2008; Saerens et al., 2006) were always in agreement with differences found in the concentrations of ethyl esters produced by the *Saccharomyces* species and hybrids at any temperature. Nevertheless, our results suggest that *EEB1* has more influence on the production of these aromatic compounds than *EHT1*, as already proposed by other authors (Rossouw et al., 2008; Saerens et al., 2006).

Correlation between transcriptome and exo-metabolome

In order to understand the lack of correlation between transcriptome and exo-metabolome, other factors affecting aroma synthesis must be taken into consideration. Among these factors might be interconnections between the different metabolic pathways and co-regulation by transcription factors, post-translational modifications of the enzymes, importance of the genes and enzymes involved in the same process or the presence of non-growing cells in the culture. These factors would make interpretation of individual gene and enzyme contributions problematic in the context of aroma compound production (Backhus et al., 2001; Rossouw et al., 2008). One hypothesis to explain the no-correlation in

the case of acetate esters yielded by the different *Saccharomyces* species tested could be that acetate esters synthesis could be influenced by higher alcohol availability in a significant way, being this factor sometimes more important than acetyltransferase levels of expression. Several authors observed that mutants and transformants overproducing certain higher alcohols also showed a clear increase in the synthesis of the respective acetate ester (Lee et al., 1995; Yoshimoto et al., 2001). Nevertheless, ester synthesis cannot be explained through higher alcohol availability solely. For instance, high oxygen and unsaturated fatty acid levels are known to increase fusel alcohol production, but to decrease ester levels (Hammond, 1993; Quain et al., 1985; Taylor et al., 1979).

Allele composition of hybrids´ genome

The hybrids between *S. cerevisiae* and *S. kudriavzevii* Lalvin W27 and VIN7 showed different allele composition in several genes involved in aroma production, different levels of expression and different aromatic profile in the resulting wines. However, there is no clear correspondence among genome, transcriptome and exo-metabolome, as commented before. In the genomic analysis of the hybrids analysed in this study (Lalvin W27 and VIN7), several genes were constituted by one or two alleles from the *S. kudriavzevii* parental. Some of those alleles seemed not to affect gene expression (*ILV5*, *LEU4*, *BAP3*, *ALD5*), since differences with respect to the reference strain could not be observed. Nevertheless, other alleles coming from *S. kudriavzevii* seemed to be differently

expressed depending on the fermentation temperature (*ARO1*, *ADH7*, *EHT1*, *BGL2*) in agreement with chemical data. Regulation by temperature might be acting at transcriptomic level or at post-translational level, affecting enzyme kinetics. Further study is needed to understand this regulation, especially in the case of hybrids, due to differential allele composition of the genes and differential expression depending on the fermentative conditions.

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CONCLUSIONS

• Release and generation of varietal (primary) aroma compounds from precursors was found to be strongly linked to the species or hybrid strain used. *De novo* synthesis was remarkable in the case of *Saccharomyces bayanus var. uvarum.* The triple hybrid (*S. cerevisiae* x *S. bayanus* x *S. kudriavzevii*) CBS 2834 and the double hybrid *S. cerevisiae* x *S. bayanus* S6U were the best aromas releasers from media supplemented with precursors. In addition, the presence of precursors in the fermenting media caused a surprising levelling effect on the fermentative (secondary) aroma composition.

• No relationship between β-D-glucosidase activity and terpenes profile in Muscat wines fermented with *Saccharomyces* species and hybrids was found. Bioconversion of geraniol into linalool and α-terpineol was observed in all *Saccharomyces* species and hybrids, being the percentages of geraniol uptake and bioconversion different among groups of strains. The most cryotolerant group of strains showed the highest geraniol uptake.

• Production of fermentative aroma is strongly influenced by fermentation temperature and yeast strain. Secondary aroma production at low temperature (12ºC) revealed an increase in ethyl esters production by non-*S. cerevisiae* cryotolerant strains. The increase in fermentation temperature (28ºC) produced an increase in acetate ester concentration in all strains. So lowering fermentation temperature not always means an increase in aromatic compounds as commonly supposed.

• No correspondence between the acetyltransferases expression levels and synthesis of acetate esters was found. In case of *Saccharomyces* species the concentration of acetate esters at 28ºC seem to be influenced by higher alcohol availability.

• The levels of certain higher alcohols could be explained taking into account the expression of the genes involved in the synthesis of the aminoacid precursor (*ILV1-6*, *LEU1-4* and *ARO1,7*) or the concrete transaminases related to the metabolism of those aminoacids (*BAT1,2* and *ARO8,9*).

• Ethyl esters synthesis at 28ºC and the expression levels of acyltransferases at the same temperature confirmed that *EEB1* has more influence on the production of these aromatic compounds than *EHT1*.

• In the genomic analysis of the hybrids analysed in this study, one or two alleles from *S. kudriavzevii* constituted several genes. Those alleles seemed not to affect gene expression in some cases (*ILV5*, *LEU4*, *BAP3*, *ALD5*) but to be differently expressed depending on the fermentation temperature in other cases (*ARO1*, *ADH7*, *EHT1*, *BGL2*), correlating with chemical data. Regulation by temperature might be acting at transcriptomic level or at post-translational level, affecting enzyme kinetics. Further study is needed to understand this regulation, especially in the case of hybrids, due to differential expression of the allele from one parental or from the other, depending on the temperature.

• Up regulation of recently described glycosidase codified by ORF *YIR007W* presented by VIN7 at 12ºC and Lalvin W27 at 28ºC was remarkable, indicating potential of these hybrids to primary aroma release from glycosilated aromatic precursors, as confirmed in fermentations with aromatic precursors addition.

• Hybridization seems to be a potential tool to achieve new yeast strains presenting favourable oenological traits, like increase in ester production and wine fruitiness. For instance, *S. cerevisiae* x *S. kudriavzevii* hybrid VIN7 was a good ester producer at both low and moderate temperatures, whereas Lalvin W27 could be a candidate to hybridizate with a strain presenting up regulation of acetyltransferases to achieve this goal.

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