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Differences in the regulation of the fermentative metabolism among species of the genus *Saccharomyces*.

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Title: Differences in the regulation of the fermentative metabolism among species of the genus *Saccharomyces*.

Abstract:

In recent years, there is a trend in the wine industry to produce aromatic wines with lower ethanol contents. *Saccharomyces cerevisiae* is the predominant yeast in wine fermentations because of its high fermentative capacity and ethanol resistance. However, the attributes provided by this species are not those demanded nowadays by the wine consumers. Therefore, new strategies are being introduced in the winemaking procedures to get wines with lower ethanol contents, more aromatic profile or with higher glycerol content. Wine fermentations conducted by other yeast species of the *Saccharomyces* such as cryotolerant *S. uvarum* and *S. kudriavzevii* are some of these new strategies. Indeed, it was observed that both species can reduce ethanol level in wine by a shift of the carbon flux toward the glycerol yield and aromatic compounds production, especially at low temperature. A sign that these species have developed different strategies in their regulation of the fermentative metabolism, like cold resistance mechanisms, that are different from the mechanisms displayed by wine strains of *S. cerevisiae*.

This report is part of the results of a global system biology project based on multi-'omic' experiments (metabolomic and transcriptomic) that will help us to understand and model the complex physiological and metabolic differences among *S. cerevisiae, S. uvarum and S. kudriavzevii* during a wine fermentation at low (12°c) and high temperature (25°c).

In this work, we focused on the first metabolic data obtained from several wine fermentations performed at 25°C with a set of *S. cerevisiae*, *S. uvarum* and *S. kudriavzevii* yeast strains. In batch cultures, mimicking wine fermentations, we quantified the metabolic differences among these *Saccharomyces* strains. Along the wine fermentations, in very well controlled bioreactors, we measured the concentrations of the most important extracellular compounds – glucose, fructose, glycerol, ethanol, organic acids (acetic acid, lactic acid, citric acid, tartaric acid, malic acid, succinic acid), and aromas (higher alcohols and esters) – gas production (CO_2 released during the fermentation process) and biomass parameters to identify signs of different metabolism strategies. The first results obtained from the fermentation performed at 25°C and presented below confirm that different fermentative mechanism exist among *Saccharomyces* species.

Key words: *S. uvarum*, *S.kudriavzevii*, metabolism, regulation, alcoholic fermentation

Título: Estudio de las diferencias en la regulación del metabolismo fermentativo dentro de las especies del género *Saccharomyces*.

Resumen:

Desde hace unos años, el sector vitivinícola está marcado por una demanda creciente para vinos con un contenido en etanol reducido y un perfil aromático más amplio. Hoy en día *Saccharomyces cerevisiae* es la especie de levadura más utilizada en enología dados su alto rendimiento fermentativo y su alta resistencia al etanol. Sin embargo, los consumidores están demandando nuevas características en los vinos, incluyendo vinos más aromáticos, que no proporciona esta especie. Esta es la razón por la que nuevas estrategias han sido introducidas en las bodegas, con finalidad de intentar reducir el contenido en alcohol de los vinos y aumentar su contenido en otros compuestos de interés tales como los compuestos aromáticos y/o el glicerol. La utilización de otras cepas de *Saccharomyces* capaces de fermentar a bajas temperaturas (12-15°C) como *S. uvarum* y *S. kudriavzevii* se ofrece como una solución a dicho problema. En efecto, se ha demostrado que estas dos especies pueden reducir el contenido en etanol mediante la desviación del flujo del carbono hacia la producción de glicerol y de compuestos aromáticos, particularmente a bajas temperaturas. Además, diversos estudios indican que estas dos especies han desarrollado varias estrategias a la hora de regular su metabolismo fermentativo de forma diferente a *S. cerevisiae*, como por ejemplo la capacidad de crecer a bajas temperaturas.

Los resultados expuestos en este trabajo son parte de los resultados de un proyecto de análisis global que se basa en el uso de varias herramientas "omicas" (metabolómica y transcriptómica) que nos servirán para entender las complejas diferencias que existen a nivel fisiológico y metabólico entre las especies *S. cerevisiae*, *S. uvarum* y *S. kudriavzevii* durante la fermentación alcohólica a baja (12°C) y alta (25°C) temperatura.

En este trabajo final de máster nos hemos centrado en los primeros resultados obtenidos a partir de diferentes fermentaciones realizadas a 25°C con un abanico de cepas de las especies *S. cerevisiae*, *S. uvarum* y *S. kudriavzevii*. En cultivos en batch, reproduciendo las condiciones de fermentación en bodega, cuantificamos las diferencias metabólicas que pudieran existir entre estas cepas. A lo largo de la fermentación, utilizando un sistema muy controlado de bioreactores, medimos el contenido de los compuestos más importantes en el mosto y procedentes de la fermentación alcohólica – azúcares residuales (glucosa, fructosa), etanol, glicerol, ácidos orgánicos (a. láctico, a. cítrico, a. succínico, a. tartárico, a. málico, a. acético) y aromas (ésteres y alcoholes superiores) – realizamos un seguimientos mediante la fermentación (el CO_2 producido), así como el contenido en biomasa, para identificar las diferencias metabólicas entre las tres especies. Estos primeros resultados obtenidos en las fermentaciones realizadas a 25°C y presentados a continuación nos dan una primera pista de los diferentes mecanismos respiro-fermentativo existentes dentro de las especies del género *Saccharomyces*.

Palabras claves: *S. uvarum*, *S. kudriavzevii*, metabolismo, regulación, fermentación alcohólica

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1. Introduction

1. Introduction

Winemaking is a conversion of grape juice made mainly by yeasts, leaded by *S. cerevisiae*, were a huge number of compounds are metabolically consumed and produced. In the modern wine production selected yeasts are used to inoculate grape must to control the fermentation, to reduce the risk of contamination, to increase the reproducibility and to generate specific characteristics in the wine. This technique allows wineries and oenologists to increase variety and quality of wines as consumers and market demand. Nevertheless, over the past two decades, the wine industry faces a series of thorny issues related to globalization, technological progress and climate change. In particular, the level of ethanol in wine has increased in most wine-producing regions, raising a number of issues related to consumer health, prevention policies, the effectiveness of the fermentation processes and wine sensorial quality. Thirty years ago, it was common to find wines at 12-13% (vol/vol) alcohol while now levels of 14% (vol/vol) are seen as normal, and it is not uncommon to find wine with ethanol concentrations over 15% (vol/vol). Today, wines with moderate ethanol levels are preferred, in accordance with health prevention policies. Different approaches to reduce alcohol levels in wines have been proposed at all stages of the winemaking process such as viticulture strategies, selection of new grape variety, dealcoholization treatments or microbial strategies. Among these, microbial strategies are less expensive and easier to implement. Maintaining moderate ethanol levels in wine, without residual sugars (unwanted in dry wines), can be solved by the development of strain yeasts with lower ethanol yield. Today, due to its high ethanol resistance and fermentative performance, most of commercial yeasts are *S. cerevisiae*, therefore being the most frequently used in wine fermentations, as well as the most studied species. Indeed, the yeast *S. cerevisiae* has been the subject of intensive research for metabolic engineering. However other species of the *Saccharomyces* genus such as *S. uvarum* and *S. kudriavzevii* and their interspecific hybrids or non-*Saccharomyces* species have shown their potential application to solve the new challenges of the winemaking industry, especially to produce more aromatic and less alcoholic wine.

1.1. Viticulture and climate change

The final alcoholic content of a wine and its aromatic profile mainly depend on the characteristic of the starting must, before the alcoholic fermentation. Among these characteristics, the balance between sugar content and acidity (industrial maturity), polyphenolic compounds concentration (phenolic ripeness) and primary aromas (aromatic ripeness) of the grapevine are relevant for the future wine since they respectively impact on its taste, color and bouquet. The enological maturity of the grapevine – which corresponds to the optimum moment for harvesting that will allow the best wine to be obtained in a given year and under given conditions – depends on the equilibrium that exist between those three factors: industrial maturity, aromatic ripeness and phenolic ripeness (Figure 1). Identifying the point of enological maturity requires a carefully surveillance throughout the growing season of grapevine by the winemaker but it results more and more difficult due to the ripeness perturbations caused by climate change.

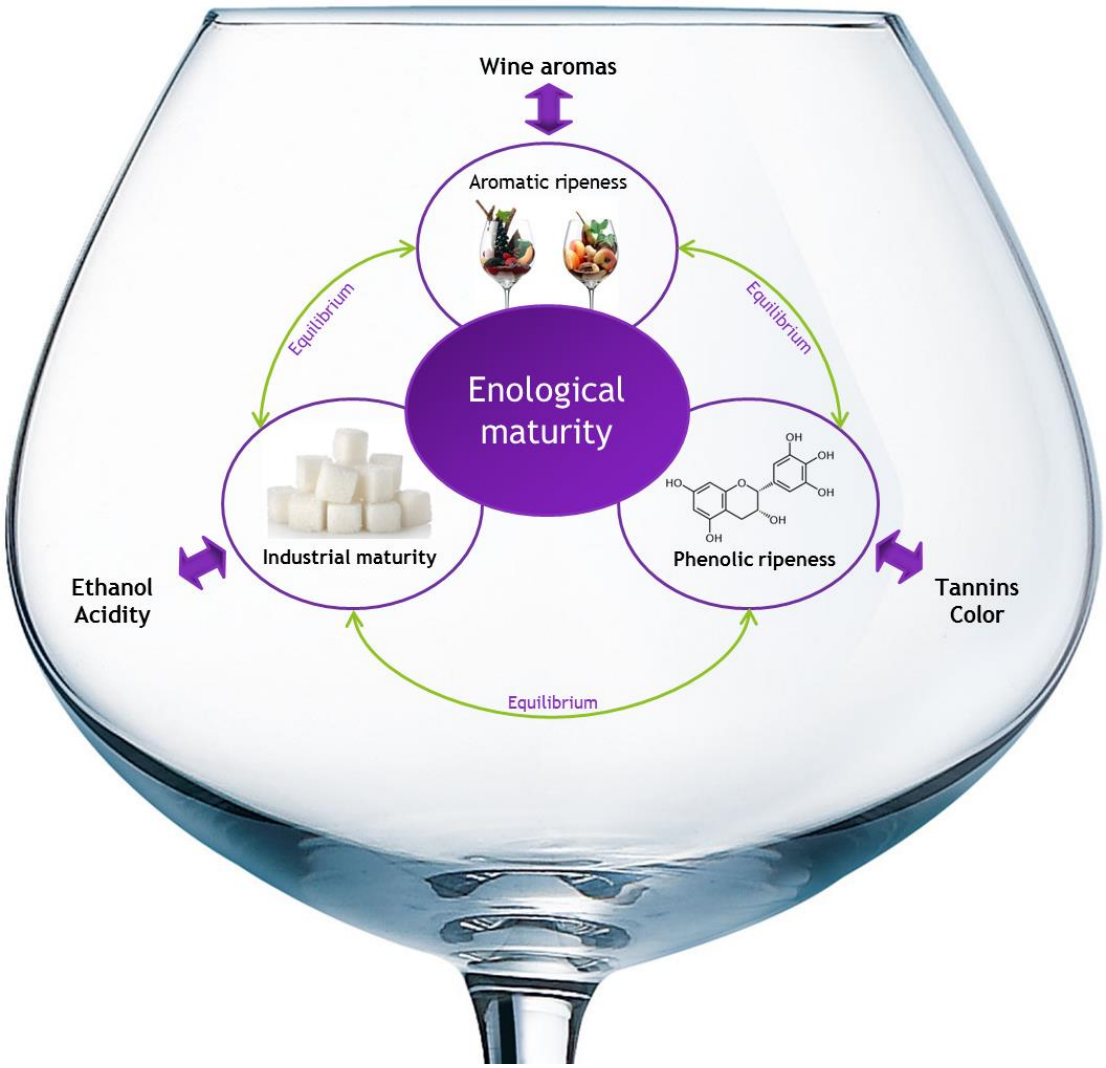


Figure 1.1: Diagram of the link between enological maturity, industrial maturity, aromatic ripeness and phenolic ripeness and their sensorial component in wine.

Nowadays, the reality of climate change, caused by human activities and in particular the emission of greenhouse gases, is admitted by the vast majority of the scientific community (Intergovernmental Panel on Climate Change, 2014). Among human activities, agriculture— and in particular viticulture—is highly dependent upon climatic conditions during the growing season. For instance, the annual increase of temperatures modifies the water status of soil and the vine phenology. Vine phenology—that is, the date on which bud break, flowering, and véraison (onset of ripening) occur—is driven by temperature. This relation is so strong that vine phenology can be predicted by models that are based only on temperature (Parker et al., 2011). Temperature also affects fruit ripening. Sugar accumulation increases with temperature (Coombe, 1987) while grape acidity, in particular the malic acid content, decreases in high temperature (Coombe, 1987). Relevant secondary metabolites involved in red wine color, like anthocyanins, are also negatively affected by high temperature. In a field study with an adapted experimental design, Spayd et al. (2002) showed that the amount of anthocyanin in grape skins increases with light but is negatively affected by high temperature. In summary, high temperatures leads the industrial maturity of grapevine to be reached before during the growing season while the phenolic ripeness is disrupted or stopped. As a consequence for the winemaker, a correct enological maturity point cannot be reached, accentuating the imbalance

between sugar content and phenolic maturity (Jones, 2007; Mira de Orduña, 2010). This high sugar content - converted in ethanol by yeast - low acidity and low phenolic compounds content lead wines to be more alcoholic, increasing the perception of heat, altering the perception of wine aromas complexity (Goldner et al., 2009; Pickering et al., 1998) and decreasing color intensity and stability.

1.2. Importance of the genus *Saccharomyces* and strain selection in winemaking

A reason why wine has become naturally higher in alcohol has to do with the higher sugar content of must, enhanced by high temperatures during growing season. This high sugar content (including glucose and fructose) is the principal metabolic source transformed into ethanol and carbon dioxide during the alcoholic fermentation, and the principal responsible for this transformation is the yeast. Therefore the reason why wine has become higher in alcohol has a lot to do with the yeast strain used during this process. Back in the 1950's the yeast would not survive in alcohol levels too much higher than 13.5%. In fact, it was common to get a "stuck fermentation" where yeasts would die before all the sugar in the grape juice had been converted into alcohol. Today however, we've developed and selected very resilient yeasts, strains that can survive in alcohol levels as high as 16.5%. Most of them belong to the *Saccharomyces* genus, and it´s not a surprising since all the species of the genus *Saccharomyces* diverged from an ancient common ancestor which acquired the ability to vigorously ferment sugars in both aerobic and anaerobic conditions (Crabtree effect) (Pronk JT, et al., 1996).

Very recently the species of the *Saccharomyces* genus (Figure 2) has been extent with the identification and characterization in South-East France of a ninth biologically distinct yeast species named *S. jurei* (Naseeb et al., 2017). The eight remaining species of the genus are *Saccharomyces cerevisiae*, *S. paradoxus*, *S. cariocanus*, *S. uvarum*, *S. mikatae*, *S. kudriavzevii*, *S. arboricola* and *S. eubayanus* (Martini AV and Martini A, 1987; Naumov et al., 2000; Libkind et al., 2011), and two natural hybrids, namely *S. pastorianus* (Masneuf et al., 1998; Querol et al., 2009) and *S. bayanus* (Nguyen et al., 2011).

Among these species, *S. cerevisiae* is the predominant one in most of the industrial fermentative processes such as dough production, winemaking, brewing, cider production, sake, cachaça, or also in traditional fermented beverages like pulque, masato, chicha, sorghum beer, palm wine, etc. (Ibáñez et al., 2014) and it has to do with its performances. It is the yeast best adapted to grow at high temperatures within the *Saccharomyces* genus, with the highest optimum (32.3°C) and maximum (45.4°C) growth temperatures (Salvadó et al., 2011). Also is the species with the highest ethanol resistance (Arroyo-López et al., 2010). Among the genus *Saccharomyces* species *S. cerevisiae* is also the most used for the production of bioethanol from hexoses, having had regularly employed highly fermenting strains (Greetham et al., 2014; Wimalasena et al., 2014). However, other species of the genus *Saccharomyces* have also been used as alternatives to the species *S. cerevisiae* in biotechnological applications, especially for their capacity to perform alcoholic fermentation at lower temperatures. Those species are defined as cold-adapted or cryophilic species. They are very useful for wineries in northern countries where fermentation takes place at low temperatures and also for winemakers to enhance the aromatic profiles of wines (Torija et al., 2003). In our study, we used two of these cryophilic species, *S. kudriavzevii* and *S. uvarum.*

- The species *S. kudriavzevii* is a cryophilic yeast exhibiting an optimal growth temperature of 25 °C and a growth temperature range between 6 and 32 °C (Arroyo-Lopez et al., 2009; Sampaio and Gonçalves, 2008; Salvadó et al., 2011), growing quite well at low temperatures (10-15ºC) (Belloch et al., 2008; Salvadó et al., 2011; Tronchoni et al., 2014). *S. kudriavzevii* has been mainly isolated in natural environments, like decaying leaves (Naumov et al., 2000) or oak barks (Lopes et al., 2010; Sampaio and Gonçalves, 2008). This species participates in hybrid formation with *S. cerevisiae* and *S. bayanus* species (figure 2). Those hybrids are present in industrial fermentations for cider-making or wine-making in central Europe (Belloch et al., 2009; González et al., 2008, 2007, 2006; Lopandic et al., 2007; Masneuf et al., 1998; Peris et al., 2012a, 2012b)

- The species *S. uvarum* is less common and appears mainly in fermentations at low temperatures (Demuyter et al., 2004; Masneuf-Pomarède et al., 2010) like in Hungary for the production of Tokaji wines. This species present several oenological characteristics that make it really interesting for wine-making. Comparison between *S. uvarum* and *S. cerevisiae* have shown that the species *S. uvarum* is more cryotolerant than *S. cerevisiae* and it 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, 2008). Also wines produced by *S. uvarum* have a higher aromatic intensity than those produced by *S. cerevisiae* (Coloretti et al., 2006).

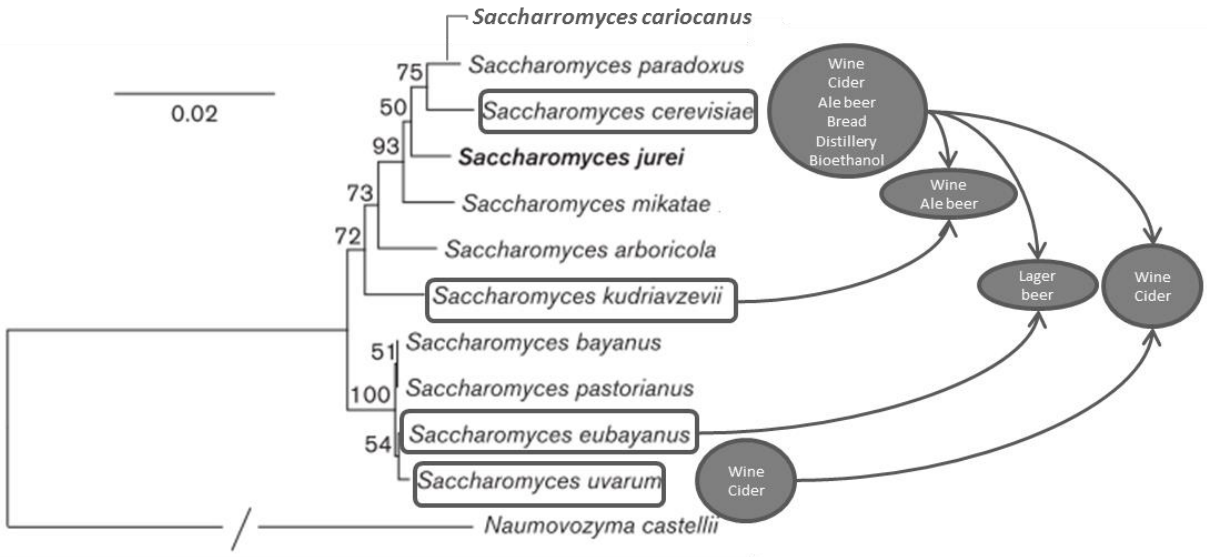


Figure 1.2: Adapted from Naseeb et al. 2017. Diagram of the phylogenetic relationships between the *Saccharomyces* species and their industrial specialization. The species involved in industrial processes and/or in hybrids are boxed in light grey. The products of industrial processes involving the hybrids and non-hybrids are boxed in dark grey. The arrows correspond to hybrids.

Also strain selection has been extended in recent years to non-*Saccharomyces* yeasts such as those belonging to genera *Candida, Kloeckera, Debaryomyces, Hanseniaspora, Hansenula, Pichia, Metschnikowia, Schizosaccharomyces, Saccharomycodes* or *Rhodotorula*. Although non-*Saccharomyces* species lack competitiveness under oenological conditions, mainly because they do not ferment so vigorously and display lower stress resistance than *Saccharomyces*, employing mixed starter cultures or sequential fermentations (e.g. *Candida cantarellii*/*S. cerevisiae*) to direct fermentations towards enhanced glycerol and reduced acetic acid production have proven successful

(Schuller and Casal, 2005). The use of co-cultures with *Metschnikowia pulcherrima* to reduce ethanol production has also been suggested (Morales P, et al., 2015; Contreras et al., 2015). Yeasts like *Candida zemplinina*, *Hanseniaspora vineae* and *Torulaspora delbrueckii* are considered positive contributors to overall organoleptic wine properties, while apiculate yeasts, such as *Kloeckera apiculata*, have a negative influence on wine quality because pronounced acetic acid and ethyl acetate formation associated with their low ethanol production (Schuller and Casal, 2005). Therefore, despite the recent interest in non-*Saccharomyces* yeasts to reduce the ethanol content of wines and to improve their aromas, most of these species are aerobic and are easily replaced by *S. cerevisiae* during wine fermentations. But probably their major disadvantage is the high production of acetic acid, an undesirable property in wines. That´s a reason why searchers have been focusing on other species of the genus *Saccharomyces* such as *S. uvarum* and *S. kudriavzevii* which seem to display better enological characteristics.

1.3. Overview of the metabolism strategies displayed by strains of the *Saccharomyces* genus during wine fermentation

Several studies have demonstrated that *S. uvarum* and *S. kudriavzevii* exhibit interesting physiological properties, such as adaptation to lower fermentation temperatures (Salvadó et al., 2011; Tronchoni et al., 2009, 2014), lower ethanol production and increased glycerol production without an increase in the acetic acid levels of wines (Oliveira et al., 2014; Pérez-Torrado et al., 2016). The study of the molecular basis of these differential properties suggests that these species show clear differences in regulating their respiro-fermentative metabolism.

During alcoholic fermentation in wine, approximately 92% of sugars (glucose and fructose) are used by yeast to produce ethanol and CO_2 (figure 3). The remaining fraction of sugar is used for the synthesis of biomass and of various byproducts, including glycerol, the most important byproduct after CO_2 and ethanol, organic acids (succinic acid, lactic acid, etc.) and aroma volatile compounds (esters and higher alcohols), which play a crucial role in wine organoleptic balance. On average, 16,83g of sugars is required to produce one degree of ethanol. That's why a decrease of the ethanol content in wine will result in the accumulation of alternative byproduct(s), provided that the carbon flux is diverted from ethanol production into other metabolic pathways.

The conversion of glucose to ethanol and CO_2 is redox neutral (Bloomberg and Adler, 1989). It means that this process maintains a redox balance in the cell which is a fundamental requirement for sustained metabolism and growth. Several redox couples are involved in the maintenance of this redox homeostasis but it should be pointed out the NADH/NAD+ couple, and to a lesser extent the NADPH/NADP+ couple. The NADH is mainly produced in the cytosol by glycolysis and in the mitochondria by the Krebs cycle. During alcoholic fermentation, the NADH produced during glycolysis is re-oxidized via alcohol dehydrogenase, producing ethanol and NAD+. The NAD+ regenerated is reused at the final step of glycolysis to maintain the carbon flux toward phosphoenolpyruvate and pyruvic acid production. However, an excess of NADH is produced during the formation of biomass - due to the requirement in fatty-acids for membrane construction - and of various byproducts such as acetic, succinic and pyruvic acids. This surplus has to be re-oxidized somewhere in the metabolism in order to maintain the redox balance. Under anaerobic conditions, this surplus of NADH formed as the result of biosynthetic reactions can be re-oxidized by the production of glycerol in yeast cytosol (glycerol – pyruvic redox balance).

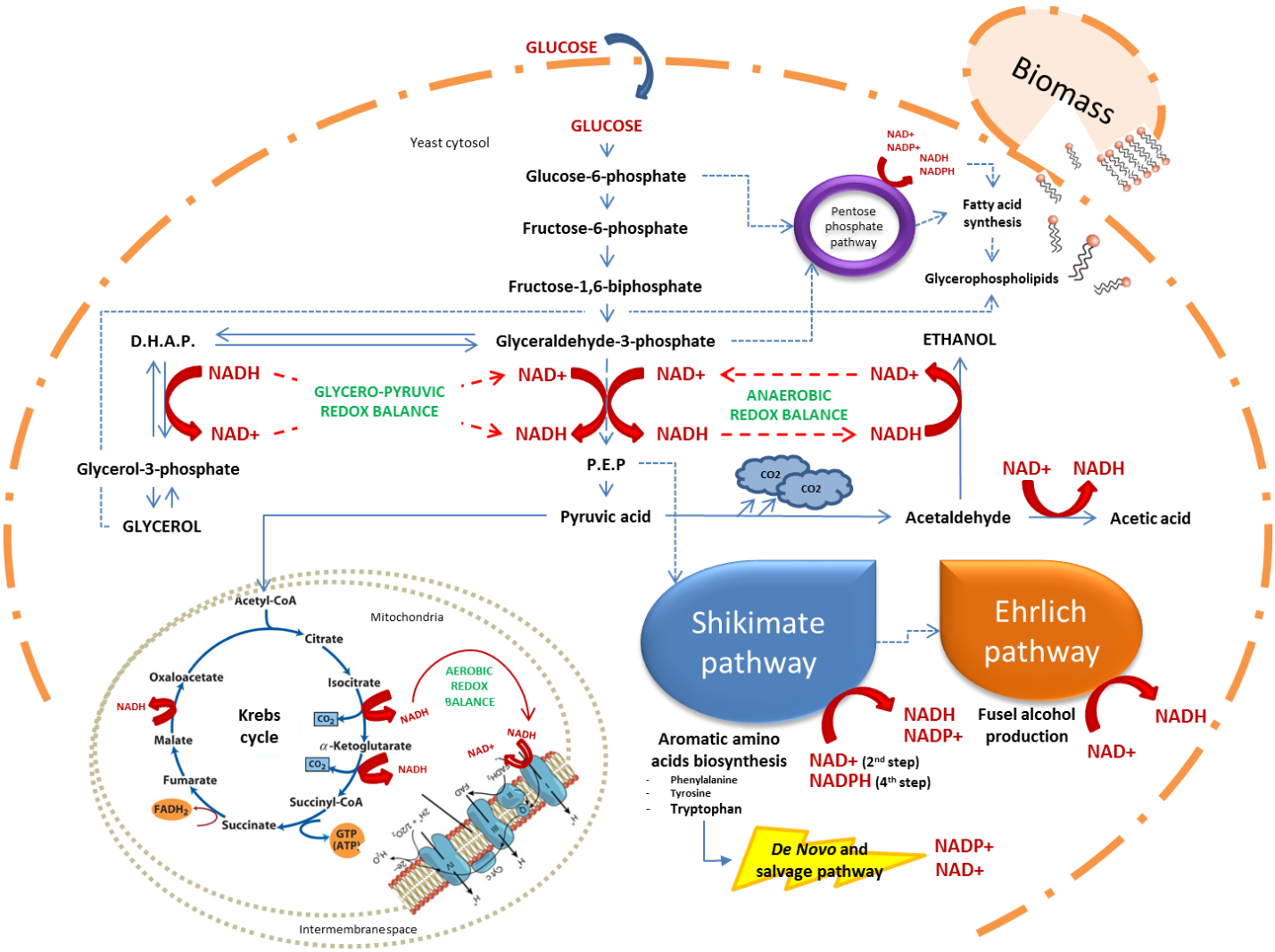


Figure 1.3: The glycolytic pathway and the main byproducts pathways involved in the maintenance of the redox homeostasis in yeast cell.

S. cerevisiae displays a high glycolytic activity. This metabolic activity in *S. cerevisiae*, reduces the production of cell biomass, but provides a tool, ethanol, to outcompete other microorganisms. Once all the carbohydrates are consumed, *S. cerevisiae* begins to consume ethanol by respiration after a metabolic shift, this life strategy is known as "make-accumulate-consume" (Thomson et al., 2005).

In a previous comparative study of the Crabtree effect in the *Saccharomyces* species (Oliveira et al., 2014), we confirmed in chemostat cultures at 12°C that *S. kudriavzevii* increases in 44.7% the glycerol extracellular content and produces more biomass than *S. cerevisiae*. Since glycerol is also involved in the maintenance of the redox balance during biomass production (glycerophoshpholipids are the main constituent of the plasmic membrane), our results suggest that the increased production of glycerol by *S. kudriavzevii* is also a consequence of its higher biomass production. To compensate this redox imbalance associated to biomass production, we postulated also an increase in amino acid biosynthesis, and consequently, in higher alcohols, as observed in wine fermentations performed by *S. kudriavzevii*, *S. uvarum* and also by non-wine strains of *S. cerevisiae*. By a global metabolic study in a batch culture and comparing the growth of the three species at 12 an 28ºC (López-Malo et al., 2013), we have also observed that the two cryotolerant species, *S. uvarum* and *S. kudriavzevii*, have developed different cold resistance strategies that are related with the higher amounts of glycerol

synthesis. *S. uvarum* showed great shikimate pathway activity (involved in biosynthesis of aromatic amino acids like phenylalanine, tryptophan and tyrosine), while *S. kudriavzevii* presented increased NAD+ synthesis (*De Novo* and salvage pathway). All this results suggest the presence of important differences in the regulation of the respire-fermentative metabolism in these species with respect to *S. cerevisiae* (López-Malo et al., 2013).

2. Justification and objectives

2. Justification and objectives

There is a constant dynamics of the biotech market relative to changing demand for new strains of yeast in order to meet the new needs and requirements of the fermentation industry. Nowadays, wine companies are looking for new fermenting yeast strains that are able to perform alcoholic fermentation at low temperatures to provide more aromatic wines. Other cryophilic species like *S. uvarum, S. kudriavzevii* and hybrids between *S. cerevisiae* and *S. kudriavzevii* are well adapted to ferment at low temperature, produce higher amounts of glycerol, less acetic acid and higher amounts of higher alcohols with regard to reference strains of *S. cerevisiae* (Arroyo-López et al., 2010, 2009; Gamero et al., 2013; González et al., 2007; Tronchoni et al., 2009).

In this work we want to identify the origin and evolution of the complex physiological and metabolic differences in the different *Saccharomyces* species of *S. cerevisiae*, *S. uvarum* and *S. kudriavzevii.* To do that, we proposed to use a global system biology approach based on multi-'omic' experiments. Bioinformatics and systems biology tools, particularly the integration of genome-scale metabolic reconstructions, metabolome/transcriptome data, measurements of metabolic fluxes as well as dynamic modelling techniques, will improve our understanding of the quantitative aspects of glycerol utilization efficiency and the regulation of fermentative metabolism between species of the genus *Saccharomyces* to be applied to solve the new challenges faced by wine industry due to global climate changes and new consumer's demands.

According to the background and justification presented, the objectives of this work were defined:

- To monitor, in very well controlled conditions, batch fermentation at 25°C in natural must of Merseguera with strains of *S. cerevisiae*, *S. uvarum* and *S. kudriavzevii* species, simulating wine-making process.
- To collect growth data and extracellular sample throughout the fermentation process
- To carry out the analysis of the first data collected from a multi-'omic' project among species of the genus *Saccharomyces*:
    - analysis of the main extracellular compounds consumed and produced during wine fermentation: sugars, ethanol, glycerol and organic acids
    - analysis of aroma compounds (esters and high alcohols)

3. Material and methods

3. Material and methods

3.1. Yeast strains

One *S. cerevisiae* (Sc) strain, a commercial wine strain (T 73, Lallemand, Montreal), originally isolated from wine in Alicante, Spain (Querol et al., 1992) as well as two *S. uvarum* (Su) strains (CECT12600 and BMW 58) and a *S. kudriavzevii* strain (Sk) (CR85), previously identified and differentiated in Lopes et al. (2010) were used in this study. The isolation source and geographical origin of the strains used herein are shown in Table 1. The day before the beginning of fermentation, a starter culture was cultivated in Erlenmeyer flasks containing 100 ml of YPD medium (2% glucose, 0,5% peptone, 0,5% yeast extract) at 25°C in an agitated incubator (Selecta, Barcelona, Spain). Strain inoculation was done at 10^6 cells/ml (OD_{600}=0,1).

Strain name	Species	Source	Isolation region
T 73	*S. cerevisiae*	Wine	Alicante, Spain
BMW 58	*S. uvarum*	Wine	
CECT 12600	*S. uvarum*	Sweet wine	Alicante, Spain
CR 85	*S. kudriavzevii*	*Quercus ilex* bark	Ciudad Real, Spain

Table 3.1: List of the *Saccharomyces* strains used in the study

3.2. Microvinification experiments

The must obtained from the Merseguera white grapes variety, collected in the vintage of 2015 in Titaguas (Spain) and stored in several small frozen volumes (4L, -20°C) was used for the microvinification assays. Dimethyl dicarbamate (DMDC) at 1 $ml.L^{-1}$ was added for sterilization purposes. Before use, must was clarified by sedimentation for 24 h at 4°C. After separation, initial must characteristics were measured. Merseguera must content was 166.25 $g.L^{-1}$ fermentable sugar (1,066$g.cm^{-3}$) with a probable alcoholic grade of 9,2%. Nitrogen level was 166,25 mg L^{-1} and pH 3,45. Sugars were measured by HPLC (see below) and yeast assimilable nitrogen was determined by the Formol Titration Technique (Gump, Zoecklein and Fugelsang, 2002). Before fermentation, the fermentable sugar level was increased by adding 47$g.L^{-1}$ of chemically pure sucrose (AppliChem Panreac, Darmstadt, Germany) to reach a probable alcoholic grade of 12%. (1,084 $g.^{cm-3}$). In addition, to avoid any stuck or sluggish fermentations, nitrogen content was adjusted by adding a nitrogen supplement, which consisted of 0,2 $g.L^{-1}$ of ammonium sulphate and 0,1 $mg.L^{-1}$ of thiamine hydrochloride (Sigma-Aldrich, Barcelona, Spain) as recommended by the International Organisation of Vine and Wine (O.I.V). All the fermentations were carried out in independent biological triplicates by using 470ml of must in sterile 500 ml very well controlled laboratory bioreactors (MiniBio, Applikon, Netherland). Data were integrated in the MyControl and BioExpert (Applikon, Netherland) softwares. Dynamic evolution of the fermentation was monitored using different probes and detectors to control and measure temperature, pH, dissolved O_2 and effluent gas content (CO_2, O_2 and ethanol; Multi-Gas Monitors INNOVA 1316, LumaSense Technologies). dCO2/dt graph were (Figure 4.1.1) obtained by calculating the average of gas data of the three bioreactors. Fermentation was monitored by density loss until a constant density was reached, considered to be the end of fermentation.

3.3. Sampling protocol

In this controlled environment, 10 samples were taken in 10 selected points of the fermentation using sterile syringe and 0.80 x 120mm Sterican needle (Braun, Germany). In those 10 time points (T1, T2, …, T10) distributed along the complete must fermentation, the collected volumes were used to determine extracellular metabolites, aromas and biomass parameters (OD_{600} and dry weight).

3.4. Biomass measurement

To determine the yeast population (cell/mL) at each sampling time, the optical density at 600 nm of a diluted volume of the sample was measured with an Eppendorf Biophotometer spectrophotometer (Eppendorf, Germany). Dry weight was obtained by mass difference between the weight of a pre-weighted 1.5mL Eppendorf tube and the weight of the same tube filled with 1mL of the sample and dried during 72h in a 65ºc heater (Selecta, Barcelona, Spain). Mass difference was determined using four decimals BP121S analytical balances (Sartorius, Sigma-Aldrich, Barcelona, Spain).

3.5. HPLC and GC analysis

The residual sugars (glucose and fructose), glycerol and ethanol from the microvinification samples were determined by HPLC (Thermo Fisher Scientific, Waltham, MA) using a refraction index detector and a HyperREZTM XP Carbohydrate Hþ 8 mm column (Thermo Fisher Scientific) equipped with a HyperREZTM XP Carbohydrate Guard (Thermo Fisher Scientific). Samples from T1 to T6 and from T7 to T10 were respectively diluted 10-fold and 3-fold, filtered through a 0.22-mm nylon filter (Symta, Madrid, Spain) and injected in duplicate. The analysis conditions were: eluent, 1.5 mM of H2SO4; 0.6 $ml.min^{-1}$ flux and oven temperature of 50°C. Volatile compounds extraction and gas chromatography were performed following the protocol of Rojas et al. (2001). The concentrations of higher alcohols and esters were determined using the 10 sample points along fermentation. Extraction was done using headspace solid phase-micro-extraction sampling (SPME) with polydimethylsiloxane (PDMS) fibers (Supelco, Sigma-Aldrich, Barcelona, Spain). Aroma compounds were separated by GC in a Thermo TRACE GC ULTRA chromatograph (Thermo Fisher Scientific, Waltham, MA) with a flame ionization detector (FID), using a HP-INNOWAX 30 m x 0.25 mm capillary column coated with a 0.25 mm layer of cross-linked polyethylene glycol (Agilent Technologies Inc.). Helium was the carrier gas used (flow 1 $ml.min^{-1}$). The oven temperature program was: 5 min at 60°C, 5 $°C.min^{-1}$ to 190°C, 20$°C.min^{-1}$ to 250°C and 2 min at 250°C. The detector temperature was 280 °C and the injector temperature was 220°C under splitless conditions. The internal standard was 2-heptanone (0.05% w/v). Volatile compound concentrations, in $mg.L^{-1}$, were determined using calibration curves of the corresponding standard volatile compounds and are given as the mean of two independent fermentations. 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), diethyl succinate, benzyl acetate, 2-phenyl-ethyl acetate, benzyl alcohol and 2-phenylethanol.

3.6. Statistical analysis

Data were analyzed with the SIGMAPLOT 13 and Microsoft Excel 2010 software package.

4. Results

4. Results

4.1. Must fermentation performance

4.1.1. Kinetic growth and gas emission of the *Saccharomyces* strains at 25°c

The kinetic growth curves from must fermentations Merseguera (white variety) at 25°C were inferred by following density loss (Figure 4.1.1)

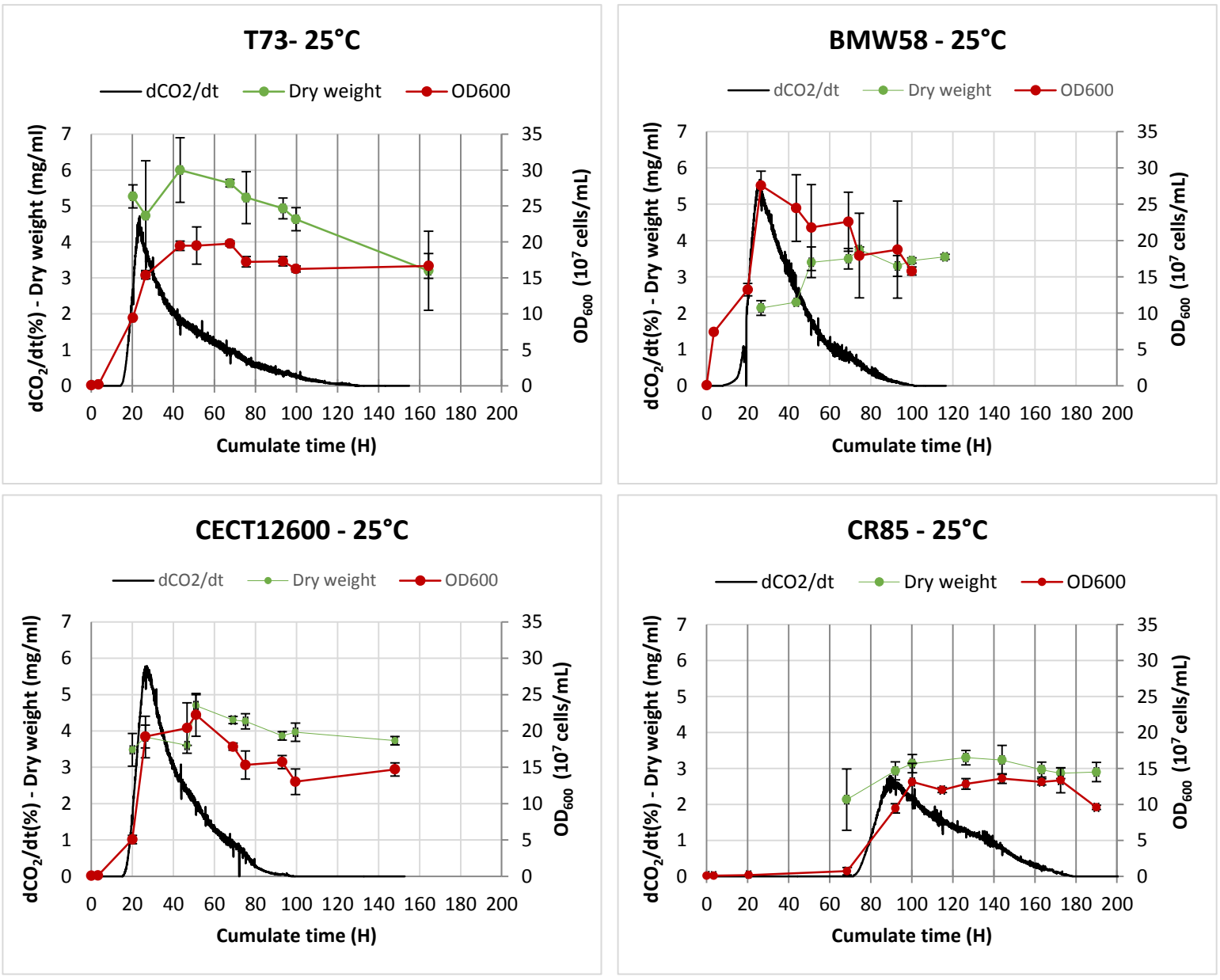


Figure 4.1.1: Kinetic growth curves and CO_2 gas emission of the four *Saccharomyces* yeast strains during fermentation process. CO_2 curves, OD curves and dry weight curves were obtained using the media and standard deviation of triplicates and drew with Excel 2010 software.

At 25°C, strain CR85 was the slowest to perform the alcoholic fermentation, with the largest lag phase, and start to ferment approximately 70 hours (≈3days) after inoculation. It is also the strain with the lowest CO_2 emission and the lowest optical density in stationary phase during the process. The Merseguera fermentation performed by the two *S.u* strains CECT12600 and BMW58 were quite

similar. They were the two strains with the highest CO_2 emission (≈5,5%) by comparison with *S. k* (≈2,7%) and *S. c* (≈4,6%) strains. BMW58 was the strain with the highest biomass production (OD_{600}) during the fermentation process among the four strains. T73 showed intermediate CO_2 emission rate between *S. k* and *S. u* strains and a maximum cell concentration similar to BMW58 and CECT12600 (≈20 OD_{600}) but higher than CR85 strain. T73 was also the strain with the highest dry weight along the fermentation process, followed respectively by CECT12600, BMW58 and CR85.

4.1.2. Main metabolites in wine

Remaining sugars (Glucose and Fructose), glycerol and ethanol were determined at the 10 sample points throughout the fermentation process by H.P.L.C coupled with a R.I detector.

Figure 4.1.2 A and figure 4.1.2 B show the consumption of glucose and fructose during fermentation at 25ºC. For the four strains used, we observed that glucose is consumed preferably to fructose. Strains T73, BMW58 and CECT12600 show a very similar consumption rate of glucose and fructose throughout the fermentation process, while strain CR85 began to ferment sugars later in relation with its extended lag phase in comparison with other strains. Residual concentration of glucose was below 3g/L for *S. u* and *S. k* strains and below 1g/L for *S.c* strain (Table 4.1.2). For fructose, from 100 hours cumulate time, the fructose consumption rate seemed to slow down for *S.u* strains), to reach a residual content in fructose between 20-30 g/L (especially for CECT12600 strain, but for BMW58 strain, I should have wait more time to really confirm that hypothesis).

Comparison of glycerol production rate between individual strains at 25ºC (Figure 4.1.2 C) revealed significant differences. From 0 to 25 cumulated hours, *S. c* and *S. u* strains behave identically. But after 25 hours of fermentation, the glycerol rate curve of *S.c* strain bend over to reach a final glycerol content of 5.94g/L (Table 4.1.2) while CECT12600 and BMW58 *S. u* strains maintain their glycerol production to respectively reach a 7.21 g/L and 6.95 g/L glycerol concentration, more than 1 point higher than *S. c* strain. At 25ºc, in relation with its extended lag phase, the *S. k* CR85 strain was the last strain in producing glycerol. Nevertheless it was also the highest producer of glycerol with a final content of 7.88g/L. This metabolic difference during the exponential phase (at about 25 cumulated hours) suggests the existence of a shift in *S. c* metabolism unlike others strains of *S. u* and *S. k*.

As for the ethanol production, T73 strain was the higher producer respectively followed by *S. k* and *S. u* strains (Table 4.1.2). After 90 hours of fermentations, *S. u* strains seemed to slow their ethanol production. *S. k* strain began to produce ethanol later (lag phase) with a final alcoholic content between *S. c* and *S. u* strains.

Table 4.1.2: Final content of the main compounds analyzed in wine samples. Media of triplicates with their respective standard deviation are indicated in the table.

Final content - Merseguera must 25°c				
	Glucose	**Fructose**	**Glycerol**	**Ethanol**
T73	0,94 ± 0,07	2,55 ± 0,10	5,93 ± 0,04	12,68 ± 0,12
CECT12600	2,94 ± 0,48	27,00 ± 2,81	7,21 ± 0,54	10,67 ± 0,39
BMW58	2,17 ± 1,04	21,10 ± 8,66	6,95 ± 0,68	10,10 ± 0,94
CR85	2,42 ± 0,24	20,82 ± 1,73	7,88 ± 0,30	11,25 ± 0,59

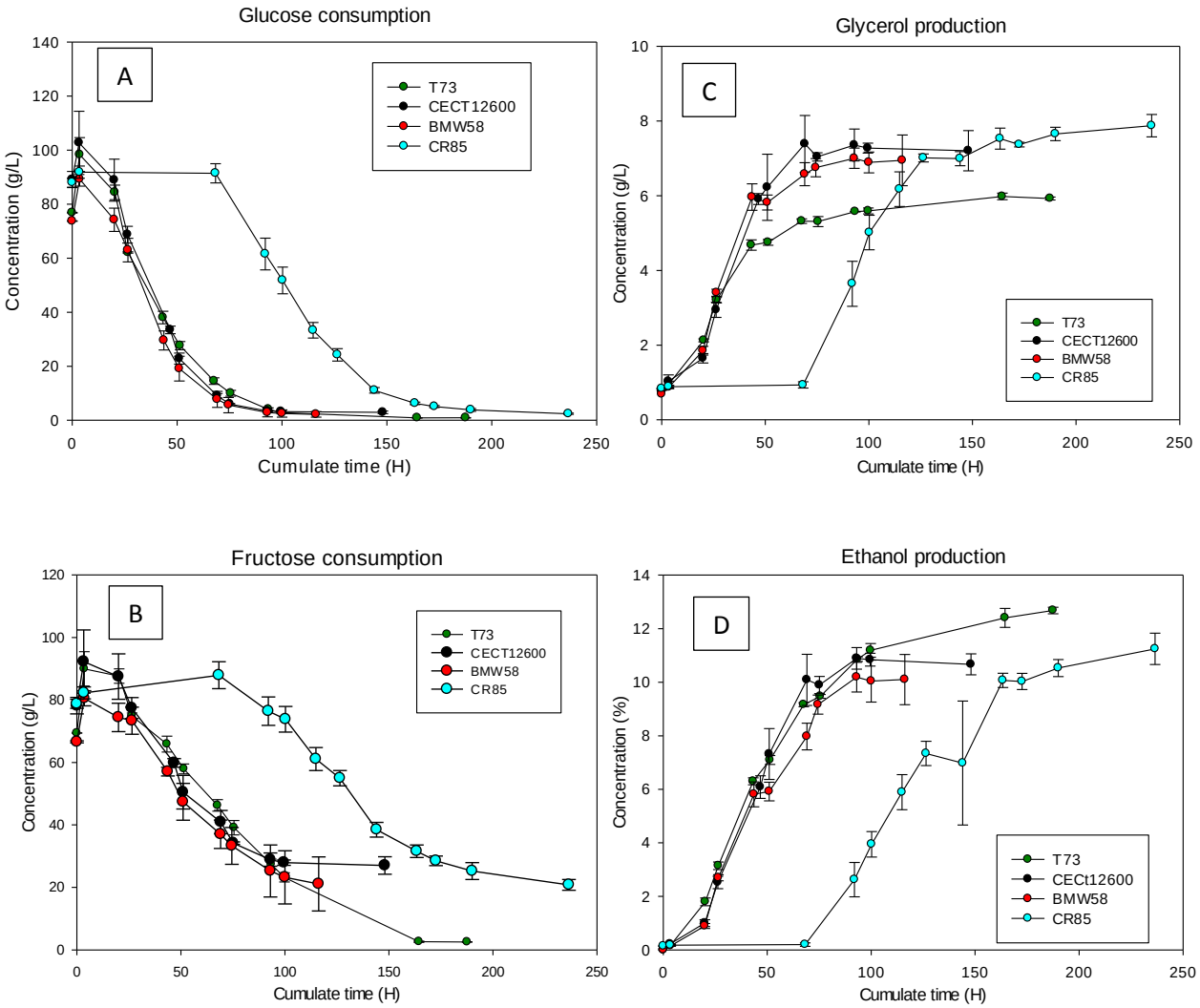


Figure 4.1.2: Curves of the main metabolites during the fermentation process

4.1.3. Organic acids

Organic acid (lactic acid, succinic acid, acetic acid, tartaric acid, malic acid and citric acid) were also determined at the 10 sample points throughout the fermentation process by H.P.L.C coupled with a UV (210nm) detector. Nevertheless, due to their little content in wine samples, co-elution with unidentified compounds and the lower resolution of the UV detector, the correct detection and quantification of organic acids tended to be more difficult, especially for acetic acid. In figure 4.1.3 are presented the four organic acids involved in the fermentation process, malic acid and tartaric acid were not added since they are not metabolized by *Saccharomyces* yeasts.

Comparison of lactic acid produced by the strains demonstrated that strain CECT12600 was the highest producer, followed in order by the BMW58 then T73 and CR85 strains. The kinetic of the lactic content proportionally increase with the cell concentration in the media to reach a stationary value for the all strains used. Comparison of succinic acid rate revealed that BMW58 was the highest producer. For CR85 and CECT12600 strains, the succinic acid content slightly increase while for the *S. c* strain it remains almost equal along the total fermentation time. Since succinic acid is one of the intermediate of the aerobic Krebs cycle, it is a sign that a part of the carbon flux was consumed by respiration at the beginning of fermentation for strains BMW58, CECT12600 and CR85, while the *S. c*

strain choose the fermentative way. Comparison of citric content did not reveal significant difference between the four strains whereas for acetic acid, CR85 was the higher producer. T73 was the second higher producer, and *S. u* strains the lowest. In addition, for *S. u* and *S. k* strains, the comparison of acetic acid rate with biomass evolution showed an increase in acetic acid level until the stationary growth phase was reached, after what acetic acid decreased. Contrary to *S. u* and *S. k* strains, acetic acid content in T73 wine remained constant. In all case, the final acetic concentrations in wines were below the upper limit of the sensory threshold of acetic acid (0.7 – 1.2 g/L) and below the residual concentration accepted by the O.I.V (20meq/L = 1,2g/L).

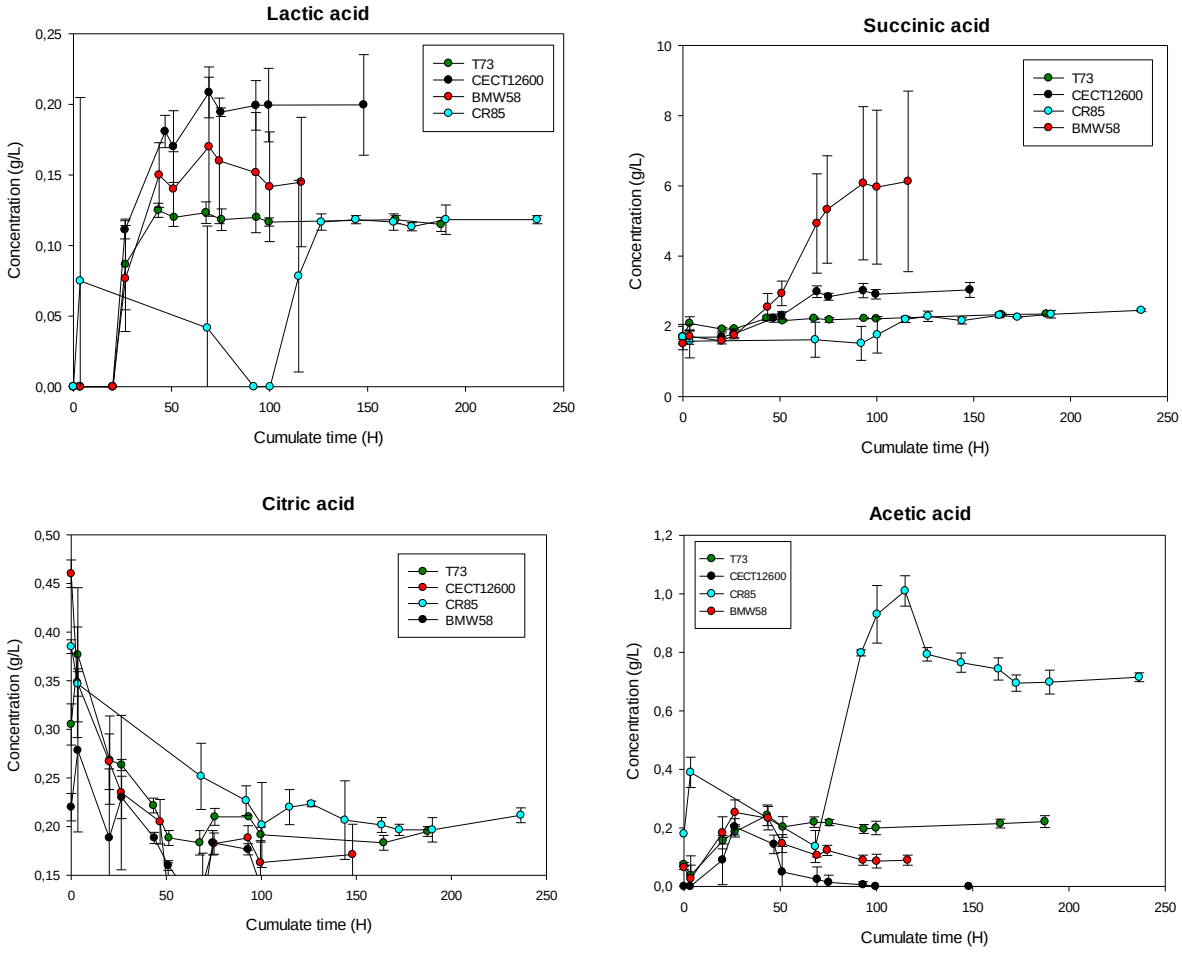


Figure 4.1.3: Evolution of the organic acids rate during wine fermentation performed at 25ºc. Media of triplicates or duplicates are indicated with their standard deviation.

4.2. Aromas compounds

Table 4.2 shows the final content of the different acetate esters, ethyl esters and high alcohols produced by the *Saccharomyces* strains during must fermentation at 25ºc. Ethyl lactate, diethyl succinate and benzyl acetate were not detected in wine sample during GC analysis. *S. uvarum* wines had the highest content in total esters and total high alcohols.

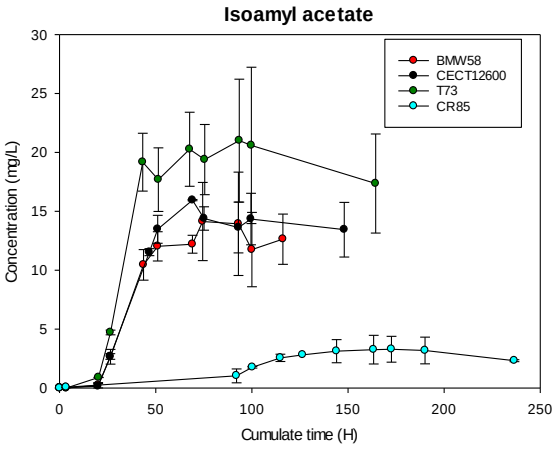
Table 4.2: Production of aroma compounds by *Saccharomyces cerevisiae*, *Saccharomyces uvarum* and *Saccharomyces kudriavzevii* at 25ºc. Media of duplicates are indicated with their standard deviation below. Esters are signaled in grey. Numbers in bold indicate that the compound concentration is higher than the threshold perception values in wine (Swiegers and Pretorius, 2005). ND = no detection.

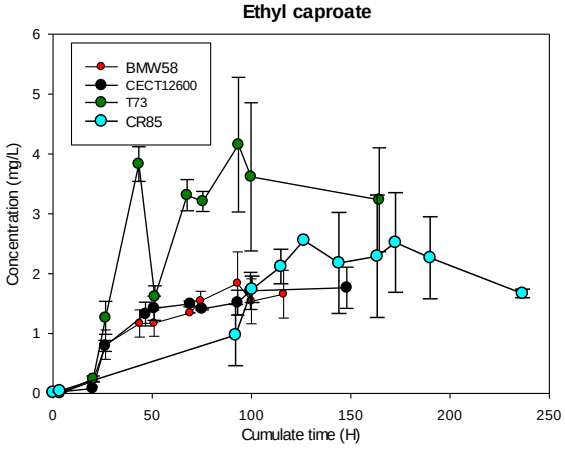
	Ethyl acetate	Isobutyl acetate	Isobutanol	Isoamyl acetate	Isoamyl alcohol	Ethyl caproate	Hexyl acetate	Ethyl lactate	1-hexanol	Ethyl caprylate	Ethyl caprate	Diethyl succinate	Benzyl acetate	2-phenyl-ethyl acetate	Benzyl alcohol	2-phenyl-ethanol	Σ Total Esters	Σ Total Alcohols
BMW58	142,273 ± 22,273	0,447 ± 0,235	62,14 ± 12,68	**12,642** ± 2,128	413,686 ± 74,797	1,657 ± 0,40	**1,062** ± 0,260	ND	2,853 ± 1,543	1,362 ± 0,676	**12,915** ± 3,589	ND	ND	**13,579** ± 3,755	6,206 ± 8,776	729,908 ± 85,431	185,937	1214,793
CECT12600	148,066 ± 24,989	0,387 ± 0,037	53,792 ± 6,821	**13,447** ± 2,324	318,63 ± 44,464	1,765 ± 0,345	**1,280** ± 0,230	ND	1,544 ± 0,175	1,534 ± 0,164	**9,879** ± 1,665	ND	ND	**9,759** ± 0,733	23,457 ± 6,954	567,105 ± 58,237	186,117	964,528
T73	96,246 ± 15,373	0,423 ± 0,122	69,443 ± 14,929	**17,365** ± 4,211	569,103 ± 105,377	3,236 ± 0,868	**1,138** ± 0,199	ND	1,366 ± 0,145	2,044 ± 1,201	**3,465** ± 3,014	ND	ND	**2,953** ± 0,320	19,611 ± 0,169	166,796 ± 11,157	126,870	826,319
CR85	108,371 ± 4,042	0,138 ± 0,018	39,060 ± 0,525	**2,328** ± 0,081	235,412 ± 6,954	1,668 ±0,071	0,530 ± 0,037	ND	1,805 ± 0,019	1,097 ± 0,638	**3,595** ± 3,51	ND	ND	1,516 ± 0,066	24,336 ± 0,593	111,176 ± 9,446	119,243	411,789

4.2.1. Acetate and ethyl esters

For five of the eleven esters analyzed we observed significant differences in the production rate during alcoholic fermentation, namely isoamyl acetate, isobutyl acetate, 2-phenyl-ethyl acetate, hexyl acetate and ethyl caprate. Final concentrations of these volatile compounds were compared with their respective threshold perception values in wine (Swiegers and Pretorius, 2005). Except for isobutyl acetate (the four strains) and hexyl acetate (only CR85 strain), final concentrations were higher than their respective perception threshold value.

At 25ºc, CR85 strain displayed the lowest levels of isoamyl acetate, isobutyl acetate, 2-phenyl-ethyl acetate and hexyl acetate. For isoamyl acetate, isobutyl acetate and hexyl acetate, strains BMW58, CECT12600 and T73 showed a similar and higher production rate than the *S.k* strain. Especially for 2-phenyl-ethyl acetate, *S.u* strains are the highest producers and rapidly exceed the *S.c* strain which displayed an intermediate production rate between *S.u* strains and the *S.k* strain. BMW58 and CECT12600 strains were also the highest ethyl caprate producers. For ethyl caproate, strain T73 was the highest producer.





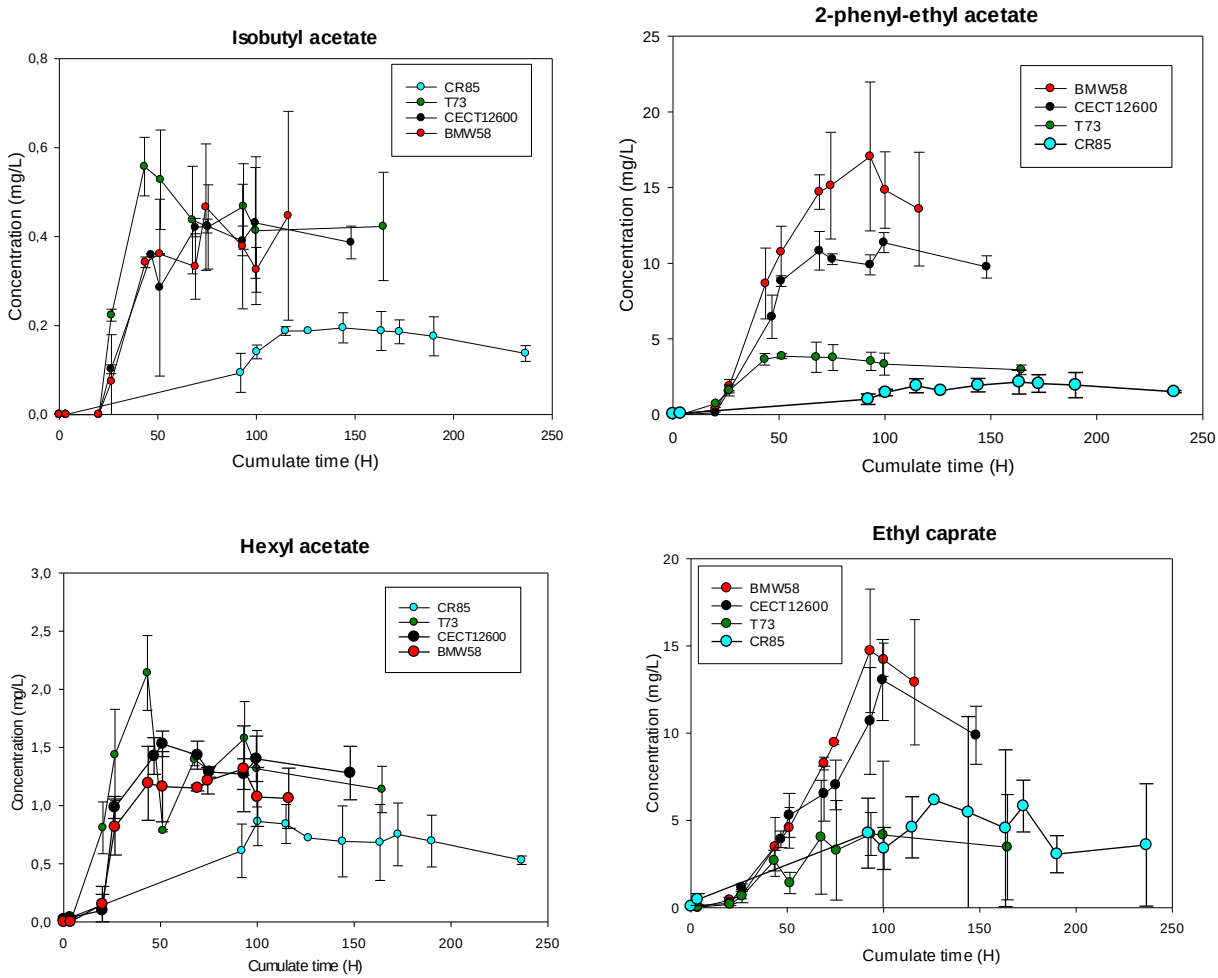


Figure 4.2.1: Among the species *S. cerevisiae*, *S. uvarum* and *S. kudriavzevii*, five of the eleven esters analyzed by gas chromatography showed significant difference in their production rate during the fermentation process at 25ºc.

4.2.2. Differences in high alcohols production rate

Figure 4.2.2 shows the concentration rate of the high alcohols for which significant differences were observed between the *Saccharomyces* strains during the merseguera must fermentation at 25ºc. For 2 of the 5 higher alcohols measured by gas chromatography clear differences were observed. Similarly to 2-phenyl-ethyl acetate, *S.u* strains were the highest 2-phenyl-ethanol producers whereas T73 and CR85 strains showed lower production rate. And for isoamyl alcohol, a similar evolution to isoamyl acetate was observed with BMW58, CECT12600 and T73 that had a similar and higher production rate than the *S.k* strain.

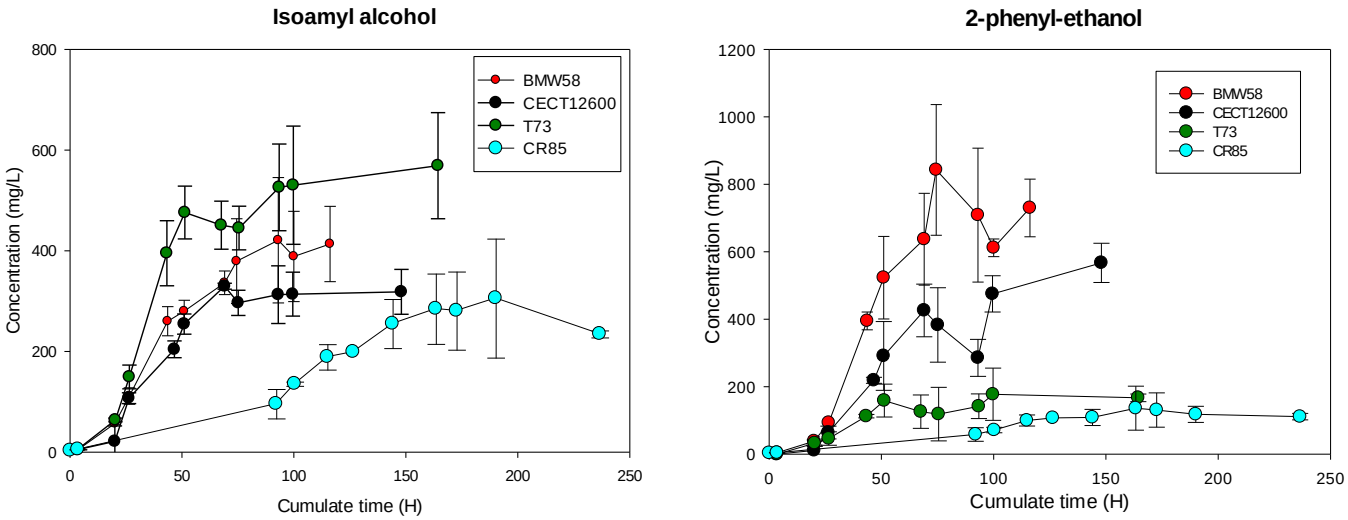


Figure 4.2.2: Among the species *S. cerevisiae*, *S. uvarum* and *S. kudriavzevii*, three of the five high alcohols analyzed by gas chromatography displayed significant difference in their production rate during the fermentation process at 25ºc.

5. Discussion

5. Discussion

In this study we have confirmed differences in the fermentative characteristics of very different species of the genus *Saccharomyces*. The two strains of species *S. uvarum* (CECT12600 and BMW58), and the strain of species *S. kudriavzevii* (CR85) are well cold-adapted species, while the *S. cerevisiae* strain (T73) is a commercial yeast strain currently used in winemaking process. We showed here that during alcoholic fermentation, species of *S. uvarum*, *S. kudriavzevii* and *S. cerevisiae* differs one from another in their biomass yield, carbon metabolism and aromas production.

5.1. *S. cerevisiae* directs carbon flux through ethanol production while cryotolerant strain through glycerol yield

Our results showed that at 25°C *S. uvarum* and *S. kudriavzevii* produced a higher glycerol content than *S. cerevisiae*. Glycerol is a positive contributor to wine quality since it reduces wine astringency (Ishikawa and Noble, 1995; Remize et al., 2000) and increase unctuosity, providing slight sweetness, smoothness and fullness. In vivo, glycerol is involved in adaptation of yeast to low temperature (Izawa et al., 2004), and it is important for osmoregulation (Ansell et al., 1997; Nevoigt and Stahl, 1997). Similar studies comparing glycerol production by *S. cerevisiae*, *S. kudriavzevii* and *S. uvarum* support our findings (Kishimoto 1994; Bertolini et al. 1996; Zambonelli et al. 1997; Gonzalez et al. 2007; Arroyo-Lopez et al. 2010). This increase in glycerol production and decreased in ethanol content by BMW58, CECT12600 and CR85 strains confirms that *S. uvarum* and *S. kudriavzevii* species have the ability to direct part of carbon flux from glycolysis to the production of glycerol during alcoholic fermentation, maintaining the redox balance by a switch between anaerobic redox balance and glycerol-pyruvic redox balance (Figure 1.3).

Moreover, comparison of organic acids kinetics revealed that succinic acid level in BMW58, CECT12600 and CR85 increased during fermentation process while it was constant for T73 strain. BMW58 was the highest producer of succinic, followed by CECT12600 and CR85 strains. Succinic acid is one of the intermediate of the aerobic Krebs cycle, and Krebs cycle produced a series of cofactors that are essential for the electron transport chain in mitochondria to perform respiration. Thus, increase in succinic acid content reveals that a part of the carbon flux was consumed by respiration in parallel to fermentation at the beginning of the process for strains BMW58, CECT12600 and CR85, while T73 strain did not respire. Since respiration is the most efficient way to produce ATP energy necessary for all metabolism and physiological events – 1 glucose leads to 38ATP in aerobic and 2ATP in anaerobic - cell division is faster in aerobic than anaerobic conditions. And in environmental medium, a way to compete for yeast is to grow more rapidly or to inhibit the growth of other microorganism, like by producing ethanol. Therefore, our results suggest that *S. uvarum* strains, in addition to produce ethanol, use the initial remaining oxygen present in must and in the upper space of the bioreactor vessel, to grow faster and to be more competitive at the first step of fermentation.

On the other hand, our *S. cerevisiae* behaved very differently. The constant succinic acid content in T73 wine associate with the high production rate of ethanol confirm the Crabtree effect observed in most of *S. cerevisiae* wine strains (Pronk JT, et al., 1996). In presence of residual oxygen (upper space and dissolved oxygen), T73 rather consume the carbon source by way of the fermentation pathway

than by aerobic respiration, in order to rapidly produce ethanol and to outcompete ethanol sensitive microorganisms.

5.2. Redox imbalance at 25°C in CR85 wine

Acetic acid is another very important organic acid and byproduct produced during alcoholic fermentation. It is associated with vinegar flavor and its maximum concentration in wine was fixed to 1,2g/L by the International Code of Oenological Practices of the O.I.V but it is commonly detected above below this range (Guth, 1997; Swiegers et al., 2005). In the context of wine making, acetification is an undesirable change in the balance of the acids in the wine generally due to spoilage of the wine by acetic bacteria that convert the alcohol in the wine into acetic acid and ethyl acetate. Acetification occurs when the naturally occurring acetic bacteria begin to multiply in the wine, typically due to an error in the winemaking process. Nevertheless, yeast are also producers of acetic acid. In presence of an excess of NAD+, acetaldehyde can be oxidized to acetic acid, its carboxylic form, instead of being reused in the anaerobic redox balance that leads to phosphoenolpyruvate (Figure 1.3). Our results (Figure 4.1.3) showed how the *S. k* strain began to produce and then slightly consumed acetic to reach a final concentration higher than in *S. u* and *S. c* wines. Since NAD+ is necessary to oxidized acetaldehyde, it suggests that at 25°C, the intracellular redox content (NADH/NAD+) in *S. k* is unbalanced and that carbon flux tends to an excess of NAD+ in the cytoplasmic space. This hypothesis is consistent with the lower cellular growth and lower aromas content observed in *S. kudriavzevii* wine as the pentose phosphate pathway (production of fatty acids for plamic membrane) and Ehlrich pathway (production of high alcohols) lead to a consume of NAD+ (Figure 1.3).

5.3. *S. uvarum* strains conferred more aromas to wines

Several studies have assessed the influence of fermentation parameters (principally temperature and nitrogen addition) on the final fermentative aroma content of the wine, focusing mostly on higher alcohols and esters, responsible for various floral and fruity notes (Swiegers et al., 2005). In particular the choice of yeast strain is determinant in the final concentration of these volatile compounds in wine and has already been compared between wine strains of *S. cerevisiae* (Camarasa et al., 2011; Torija et al., 2003). Nevertheless, there is little information about the kinetic production of aromas during wine fermentation between other species of the genus *Saccharomyces,* especially if we consider non-industrial strain. Here we provide one of the first comparative study of aromas kinetics among species of *S. uvarum, S. kudriavzevii* and *S. cerevisiae,* with industrial (T73, BMW58, CECT12600) and non-industrial (CR85) isolate strains. In our work, analysis of the aromas compounds produced along the fermentation process indicated that the highest contents in esters and high alcohols were obtained using *S. uvarum* BMW58 and CECT12600 strains. On the one hand, comparison between *S. uvarum* and *S. cerevisiae* confirm that the species of *S. uvarum* produce smaller acetic acid quantities, lower amounts of isoamyl alcohol and isoamyl acetate but higher amounts of 2-phenyl-ethanol, 2-phenyl-acetate, ethyl caprate and numerous secondary compounds as described in Sipiczki, 2008. It is consistent with previous results which described that wines produced by *S. uvarum* strains have a higher aromatic intensity than those produced by *S. cerevisiae* (Coloretti et al., 2006).

Moreover, comparison between aromas and biomass curves show how the increase in aromas production is simultaneous to the increase in yeast biomass in the medium. *S. c* and *S. u* grew well in our white grape must while *S. k* had the lowest biomass production among the four species. Indeed, comparison between natural strain of *S. kudriavzevii* and industrial strains of *S. cerevisiae* and *S. uvarum* revealed how reduced is the aromatic content in wine from CR85 at 25°C. A cause of this lower content in *S. k* wine has to do with its lower biomass concentration, and as consequences may lead to the excess of NAD+ which is not reduced to NADH in Ehlrich and pentose phosphate pathways as commented before. A reason of this lower growth is consistent with the nature origin of the strain. *S. kudriavzevii* has been mainly isolated in natural environments, like decaying leaves (Naumov et al., 2000). This natural origin is a reason why *S. kudriavzevii* is well adapted to ferment in range of low temperature (12-15°C) as described by Arroyo-López et al., (2010, 2009) and Gamero et al., (2013). At 25°C and in bioreactor, we mimicked industrial conditions that are far from original environment conditions of CR85. Higher temperature and osmotic stress seems to be a brake on the growth of this strain.

However, according to the industrial origin of the *S. u* and *S. c* strains used in this study, our experimental conditions did not affect their growth and they rapidly reach a normal cell concentration in stationary phase (Figure 4.1.1) in comparison with data from other enological characterization of *S. c* and *S. u* species (Rossignol et al., 2003). In addition, our *S. u* strains BMW58 and CECT12600 behaved similarly in their biomass production, carbon flux use and aromas production. Thus, the significant difference observed between species of *S. u* and *S. c* in some of aromas production kinetics (e.g. isoamyl acetate, 2-phenyl ethanol, 2-phenyl ethyl acetate) proves that each species has favored the production of one or another aroma. This specific feature must have arisen from environment pressure and different genetic evolution that have affected their respective ancestors after differentiation of the two species. To confirm our hypothesis, it would be interesting to perform fermentation in the same experimental conditions with another wine strain of *S. cerevisiae.* Also, transcriptomic analysis and identification of the metabolic reactions up or down regulated during aromas biosynthesis will help us to better understand these strategies.

5.4. Further perspectives of the study

Several metabolic pathways of known or suspected importance in wine fermentation displayed high levels of remarkably coordinated upregulation during the growth phase. Genetic variation among species of *Saccharomyces* may account for some of the observed differences in terms of biomass, wine aromas, ethanol production and byproducts production. Nevertheless, further work will be needed to dissect the genetic basis for the metabolic differentiation we observed between *S. uvarum*, *S. kudriavzevii* and *S. cerevisiae*. Indeed, the information provided by this study represents a starting point for deciphering the complex regulatory circuits during wine fermentation and should help us to understand the metabolic strategies of wine yeasts. To completely describe the strategies involved, other tools like transcriptomic, metabolomic, informatics models and further investigation are necessary.

6. Conclusions

The main conclusions of this study are listed below:

- *S. cerevisiae* wine strain mainly directs carbon flux through ethanol production. A way to outcompete with other microorganism and to maintain the NADH/NAD+ redox balance.

- *S. uvarum* and *S. kudriavzevii* species shunt part of the carbon flux of the glycolysis to glycerol yield.

- *S. uvarum* species, and to a lower extent *S. kudriavzevii,* use the remaining oxygen to respire at the first step of the fermentation process.

- The use of species of *S. uvarum* is a good alternative to respond to the trend of the wine market as *S. uvarum* reduces the ethanol content in wine and increase aromatic compounds level.

- At 25ºC, *S. kudriavzevii* produce more glycerol and less ethanol than *S. cerevisiae* but also produce lower aromatic compounds. Results obtained from further fermentation realized at lower temperature (12ºc) will helps us to confirm that *S. kudriavzevii* is also a good alternative to *S. cerevisiae* in winemaking.

- More tools like transcriptomic and informatics models are necessary to completely understand the complex mechanisms involved in the regulation of the fermentative metabolism among species of *Saccharomyces*

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